Recent Methodologies for Brominated Flame Retardant Determinations by Means of Liquid Chromatography–Mass Spectrometry

P. Guerra, A. Covaci, E. Eljarrat, and D. Barceló

Abstract In this chapter, an overview of current analytical methods, including different sample preparation techniques as well as the different instrumental approaches, is presented. The strategy literature search for the preparation of this chapter was based on the recent analytical reviews published on brominated flame retardants (BFRs), with emphasis on hexabromocyclododecane (HBCD) and tetrabromobisphenol-A (TBBPA). Additionally, we have included all new articles published in peer-reviewed scientific journals, conference proceedings, or official reports found on the internet. The analytical procedures based on the use of liquid chromatography, used for the determination of some BFRs such as TBBPA, HBCD, as well as some metabolites and transformation products are presented. Analytical performances of the different approaches (ESI, APCI, and APPI as ionization modes) are reported and compared with those obtained by gas chromatographic techniques. Conclusions and future perspectives are outlined.

Keywords BFR metabolites, Brominated flame retardants, Hexabromocyclododecane, Liquid chromatography, Tandem mass spectrometry, Tetrabromobisphenol A

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Abbreviations

APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
BEHTBP	Bis(2-ethyl-1hexyl)tetrabromophthalate
BFR	Brominated flame retardant
BPA	Bisphenol A
Br_4Cl_2-DBP	1,1′-dimethyl-3,3′,4,4′-tetrabromo-5,5′-dichloro-2,2′-bipyrrole
BRP	Brominated phenols
Cl_7-MBP	2,3,3′,4,4′,5,5′-heptachloro-1′-methyl-1,2′-bipyrrole
CRM	Certified reference material
DBDPE	Decabromodiphenylethane
DCM	Dichloromethane
DiBBPA	Dibromobisphenol A
ECNI	Electron capture negative ionization
EF	Enantiomeric fraction
EHTeBB	2-ethylhexyl-2,3,4,5-tetrabromobenzoate
EI	Electron ionization
EPI	Enhanced product ion
ESI	Electrospray ionization
GC	Gas chromatography
GPC	Gel permeation chromatography
HBCD	Hexabromocyclododecane
HCH	Hexachlorocyclohexane
HRMS	High resolution mass spectrometry
IDL	Instrumental limit of detection
IS	Internal standard

ISP	Ion spray
K_{OW}	Octanol–water partition coefficient
LC	Liquid chromatography
LLE	Liquid–liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LRMS	Low resolution mass spectrometry
MAE	Microwave assisted extraction
MeO	Methoxylated
MonoBBPA	Monobromobisphenol A
MS	Mass spectrometry
MS–MS	Tandem mass spectrometry
NCI	Negative chemical ionization
OH	Hydroxylated
PBB	Polybrominated biphenyls
PBCD	Pentabromocyclododecenes
PBDE	Polybrominated diphenyl ether
PLE	Pressurized liquid extraction
POP	Persistent organic pollutant
ppt	Parts per trillion
QqLIT	Quadrupole linear ion trap
QqQ	Triple quadrupole
QqTOF	Quadrupole time of flight
RF	Response factor
S/N	Signal-to-noise
SD	Standard deviation
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
T4	Thyroxin
TBBPA	Tetrabromobisphenol-A
TBCD	Tetrabromocyclododecadienes
TBECH	1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane
TriBBPA	Tribromobisphenol A
UAE	Ultrasonic-assisted extraction
UPLC	Ultra pressure liquid chromatography
USDP	Ultrasonic-supported dissolution and precipitation
WHO	World Health Organization

1 Introduction

Traditionally, the analysis of persistent organic pollutants (POPs) has been based upon gas chromatography (GC) as the principal separation technique due to the volatility of these compounds. The analysis of polybrominated diphenyl ethers (PBDEs), one

of the main brominated flame retardant (BFR) family, has been focused almost exclusively on GC coupled to mass spectrometry (GC–MS). More detailed information of PBDE determinations by means of GC–MS is presented in Chapter 3 [1].

During the last years, scientific interest for other BFRs, such as hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA), has emerged, and consequently, new analytical methods have been developed. At the beginning, similar GC–MS approaches used for PBDE analysis were used for HBCD and TBBPA. HBCD has been determined using GC–electron capture negative ionization (ECNI)–MS. However, this technique has some limitations: interconversion of HBCD diastereoisomers above 160°C, decomposition of HBCDs at temperatures above 240°C, and partial breakdown in dirty GC systems [2]. On the other hand, acidification and derivatization steps are compulsory in the GC–MS analysis of more polar BFRs such as TBBPA [3].

The difficulties encountered in the GC analysis of HBCD and TBBPA created the need for alternative methods. Despite the relatively limited chromatographic resolving power of liquid chromatography (LC), methods employing LC–MS and LC–MS–MS offer good results for HBCD and TBBPA. LC allows the isomer-specific determination of HBCD, in contrast with the total HBCD analysis obtained by GC–MS. The identification and quantification of the three HBCD isomers is important because the isomeric patterns differ under different production condition and between matrices. Besides, HBCD enantiomers can be separated and determined using chiral LC–MS methods [4]. Since TBBPA is a phenolic compound, its determination by LC is the simplest and the most attractive option [4].

However, it is known that GC–MS has the advantage to have higher sensitivity compared with LC–MS methods [5]. To improve sensitivity and specificity of LC–MS methods in the field of environmental analysis, approaches based on LC–tandem MS (MS–MS) have been developed. First studies were focused on the use of triple quadrupole instruments (QqQ), but more recently, other instrumental configurations were developed and applied to BFR determinations. New works applied LC–hybrid MS techniques such as quadrupole time of flight (QqTOF) and quadrupole linear ion trap (QqLIT) instruments [6]. Different ionization techniques were also tested for LC–MS–MS methodologies, but the most used is electrospray ionization (ESI). It is well known that ESI is subjected to sample matrix effects that can cause enhancement or suppression in the signal of the target analytes [7, 8]. For this reason, the possibility to use isotopic-labeled standards such as ^{13}C- or d_{18}-α, β and γ-HBCD, and ^{13}C-TBBPA is very useful in order to compensate the matrix effects.

It is also important to note that LC methods may be useful in the analysis of BFR metabolites and transformation products. For example, the nonpolar nature of the diphenyl ether structure in PBDEs and the introduction of polar hydroxyl functional groups upon its metabolization provide a new molecule with different properties, such as nonvolatile nature that does not allow the direct analysis of PBDEs metabolites by GC–MS. Recent papers are focused on the development of LC–MS approaches for this purpose [9].

The development of analytical methods for BFRs is difficult due to the complexity of the environmental matrices and the usually low concentrations of target compounds [10]. Sample preparation techniques must also be developed and optimized to obtain sensitive and selective methodologies. In this chapter, extraction methods applied to a wide range of matrices are reviewed as well as the different purification and fractionation strategies.

2 Sample Preparation

For HBCD and TBBPA, sample treatment procedures have typically been based on protocols previously developed and used for the determination of PBDEs. Because of the complexity of environmental matrices and the low levels at which these compounds are present, such sample treatments include a number of steps for exhaustive extraction and preconcentration of the target compounds, followed by purification and fractionation before final chromatographic separation and detection. Below are relevant data on selected analytical procedures used for the determination of HBCD and TBBPA in a wide variety of abiotic and biotic samples. Furthermore, due to particular physicochemical properties, the determination of individual HBCD diastereomers and TBBPA may require specific analytical approaches, including additional fractionation.

2.1 Extraction

For both abiotic and biotic samples, the selection of the extraction technique depends on the nature of the matrix investigated. Different procedures are used for solid and liquid samples. The amount of sample required varies largely depending on the contamination level anticipated in the sample and on the sensitivity provided by the detection technique. Similar to abiotic samples, only drying and homogenization is usually carried out before extraction of biological samples. Except for serum and plasma, (semi) liquid (e.g., eggs) samples are usually freeze-dried and then treated as any other solid biotic sample. In general, similar extraction techniques and solvents are used for BFR analysis in abiotic and fat-containing matrices, and the main differences between both sets of analytical protocols refer only to the subsequent cleanup steps.

2.1.1 Water

Because of their hydrophobic character and thus low concentrations in water, large volumes (up to 1,000 mL) are typically required to ensure detectability. Suzuki et al. [11] reported recoveries above 77% for α-, β-, and γ-HBCD, after two

sequential liquid–liquid extractions (LLE) with dichloromethane (DCM) of a spiked landfill leachate. However, solid-phase extraction (SPE) on Abselut Nexus cartridges was suggested as a faster alternative allowing the simultaneous determination of TBBPA (recovery 103 ± 16%) and a significant reduction in the organic solvent consumption (from 50 mL DCM to 5 mL acetone) that still provided acceptable recoveries (54–85%) of the three HBCD diastereomers. To the best of our knowledge, no other techniques have been described for the measurement of HBCD and TBBPA in water samples.

2.1.2 Air and Dust

For abiotic solid samples, Soxhlet is widely accepted as a robust, efficient, and low cost solid–liquid extraction technique. Soxhlet has been used for the determination of HBCDs in indoor air (after preconcentration on polyurethane foam) [12, 13]. Typical solvents include *n*-hexane, DCM, acetone, and their binary mixtures. The main drawbacks of the Soxhlet extraction, i.e., long extraction times (typically >8 h) and large solvent consumption, can be at least partially avoided by pressurized liquid extraction (PLE). Abdallah et al. [14] included an *in-cell* purification using 1.5 g Florisil and Hydromatrix in the extraction cell under the sample. Even using this approach, the extracts had to be further purified by LLE extraction with concentrated H_2SO_4 followed by column chromatography on Florisil before instrumental analysis.

2.1.3 Soil, Sediment, and Sewage Sludge

Soxhlet extraction is the most used extraction technique for the determination of HBCDs and TBBPA in soils and sediments. In general, mixtures of acetone and *n*-hexane in different proportions (1:1 or 1:3, v/v) have been found to provide the best recoveries for HBCDs and TBBPA [15, 16]. PLE has also been evaluated for the analysis of BFRs in dried soils, sediments, and sewage sludge, and mixtures of DCM and *n*-hexane at 100°C have been used [17]. Recently, ultrasonic-assisted extraction (UAE) has been used for the extraction of HBCD isomers from sewage sludge using a mixture of DCM-acetonitrile (1:1) [18]. Microwave-assisted extraction (MAE) with acetone/*n*-hexane (1/3, v/v) at 90°C for 12 min has been employed for the extraction of HBCD isomers from marine sediments [19].

2.1.4 Biological Tissues

BFRs are usually extracted from serum by successive treatment with solvents of different polarity. In some cases, a treatment with a HCl:2-propanol mixture is carried out for protein denaturation before LLE [20]. However, direct solvent shaking with ethyl acetate and acetonitrile has also been demonstrated to

provide low but reproducible recoveries for γ-HBCD and TBBPA. One of the main limitations of these LLE-based procedures is the long waiting time or centrifugation required for phase separation. Alternatively, an SPE-based method using Abselut Nexus sorbents have been proposed for the determination of TBBPA in serum [21]. After serum or milk denaturation with formic acid and isopropanol, Thomsen et al. [22, 23] have employed SPE on OASIS HLB cartridges to extract HBCD from human serum or milk. Furthermore, the SPE-based methods proved to be less laborious and allowed reduced solvent consumption and processing time, possibility of miniaturization, and parallel sample preparation, which increases throughput.

A comprehensive method for the determination of major BFRs, including HBCD isomers and TBBPA, has been reported in human samples (serum, adipose tissue, and freeze-dried milk) [24]. For serum samples, a first LLE with ethyl acetate was performed. For freeze-dried milk samples, a first SLE with acetone: DCM 1:1 (v/v) was realized. Further, for all samples, an LLE with simultaneous partitioning with acetonitrile and hexane was applied on dried extracts (serum, milk) or directly on liquid fat samples.

For fat and oil samples, the first sample treatment is to dissolve the lipids in an appropriate solvent. Typically, sample intake was between 0.5 and 1 g, and quantitative recoveries >60% have been reported for HBCDs [25]. Column extraction using a multilayer column containing appropriate sorbents for a preliminary purification has been widely used for biological tissues. This technique has a number of advantages, such as minimum sample pretreatment required, simplicity, and high recoveries for HBCDs and TBBPA (>80%) [26].

Alternative enhanced extraction techniques, such as PLE or MAE, have also been used. Eljarrat et al. [27] adapted a selective PLE for the simultaneous analysis of total HBCDs in fish tissue. Ready-to-analyze extracts were obtained also here, but slightly higher recoveries (52–103%) were reported as compared to those found for sediments. A similar method has been used by Eljarrat et al. [28] for the extraction of HBCD isomers from lyophilized human milk. Fredriksen et al. [29] have also used PLE for the extraction of HBCD and TBBPA from marine biological samples.

2.1.5 Consumer Products

BFRs, such as HBCD and TBBPA, have been extracted from various polymers or from consumer products based on these polymers. The extraction efficiency of several methods, such as PLE, MAE, and UAE, were compared for the recovery of HBCD and TBBPA from styrenic polymeric plastics [30]. PLE resulted in complete extraction of TBBPA and HBCD (recovery >95%), while MAE gave comparable performance to PLE for HBCD but lower extraction yields for TBBPA (~80%). UAE, finally, offered relatively low extraction recoveries (10–50%) for both BFRs. In another study, BFRs, including HBCD and TBBPA, were extracted in a short step by ultrasonic-supported dissolution and precipitation (USDP) from styrenic polymers [31]. To analyze HBCD in flame-retarded textiles (e.g., curtains), three

different extraction methods (Soxhlet, UAE, and soaking extractions with toluene and DCM) were compared [32]. During Soxhlet extraction using toluene, the percent contribution of α-HBCD to total HBCDs increased slightly and that of γ-HBCD decreased, indicating that γ-HBCD was isomerized to some extent at the boiling point of toluene (around 120°C). For UAE, the temperature of the water bath can easily increase over time during the extraction, which might lead to undesirable effects. Soaking extraction with DCM was chosen as the most facile procedure to extract HBCD diastereomers from textiles.

2.2 Cleanup and Fractionation

The nonselective nature of the exhaustive extraction procedures and the complexity of the sample matrices result in complex extracts that require further purification. For abiotic samples (sediment, soil, and sewage sludge), the cleanup should ensure sulfur removal, while for biotic samples, lipid elimination should be accomplished before chromatographic analysis. Lipid elimination can be accomplished by destructive or nondestructive methods. Otherwise, similar protocols can be used for purification of the extracts almost irrespective of the matrix nature.

2.2.1 Sulfur Removal

Sediment, soil, and sewage sludge extracts often contain relatively large amounts of elemental sulfur, which may hamper the determination of BFRs even if selective separation and detection techniques are used. Treatments with Cu powder [33] or by gel permeation chromatography (GPC) [16, 32] are efficient approaches for sulfur elimination.

2.2.2 Nondestructive Methods for Lipid Removal

GPC [20] and adsorption chromatography on selected sorbents [17] are nondestructive treatments applied for lipid elimination. Silica gel, alumina, and Florisil with different degrees of activation have been widely used for lipid removal by adsorption chromatography under atmospheric conditions. Because of its limited capacity for retention of lipids, silica has been used in combination with alumina [33]. Alumina and Florisil have been preferred as fat retainers because of their higher lipid-retaining capacity in procedures involving *in-cell* PLE [14]. For obvious reasons, when extraction and cleanup are combined in a single step, the total lipid content determination should be carried out separately.

A cleanup based on dispersive solid phase extraction with primary–secondary amine was used recently for sewage sludge extracts [18]. Yet, it has been shown

that such cleanup is not sufficient for complete matrix removal as matrix effects showed high ion suppression up to 50% for all three HBCD diastereoisomers. Method recoveries ranged between 80 and 113% (standard deviation (SD) <9%).

2.2.3 Destructive Methods for Lipid Removal

Similarly to PBDEs, HBCDs and TBBPA are stable under strong acid conditions [2, 33]. The simplest approach consists of direct addition of the acid (e.g., concentrated sulphuric acid) to the sample extract dissolved in *n*-hexane. However, this treatment requires several sequential LLE and centrifugation steps, which result in a multistep and time-consuming procedure. The dispersion of sulphuric acid onto the surface of activated silica gel results in a sorbent, which can be easily loaded into a column. The use of acidified silica avoids the emulsion problems of the LLE approach, reduces the sample handling and solvent consumption, and increases sample throughput [33]. Although in many applications, the use of acidified silica is enough to yield sufficiently clean extracts, several studies have described the use of acidified silica in combination with silica, Florisil, or alumina in multilayer columns for improved purification [33]. All approaches provide similar satisfactory results concerning recovery and reproducibility. Although not thoroughly investigated, the use of silica gel modified with alcoholic NaOH or KOH may cause losses of bromine atoms from HBCD [33].

2.2.4 Fractionation

For specific applications, isolation of the target analytes from other organohalogenated compounds present in the extract can be mandatory to avoid interferences during final determination. Deactivated silica gel has also been successfully applied for the quantitative isolation of PBDEs from HBCD diasteroisomers and TBBPA. In this case, *iso*-octane was used for the elution of PBDEs, while a more polar solvent, i.e., 15% diethyl ether:*iso*-octane (v/v), was required to elute HBCDs and TBBPA [16, 26]. The use of the semipolar diethyl ether was necessary to recover the late eluting β-HBCD isomer [16].

Florisil (activated at 450°C for 12 h and subsequently deactivated with 0.5% H_2O, w/w) has been successfully used to separate neutral organohalogenated compounds from phenolic analytes, including TBBPA [34]. In this case, neutral compounds (e.g., PBDEs) were firstly eluted with mixtures of DCM and *n*-hexane (1:3, v/v), while polar mixtures of acetone and *n*-hexane (15:85, v/v) and methanol and DCM (12:88, v/v) were needed to elute phenolic analytes.

Polystyrene divinyl benzene-based sorbents, such as Oasis HLB®, are a valuable alternative for the fast separation of HBCD diasteroisomers from TBBPA. Only 7 mL of a mixture DCM:*n*-hexane (1:1, v/v) was required to elute HBCDs from the SPE cartridge, while 8 mL of DCM sufficed for subsequent quantitative elution of TBBPA [24]. The two resulting fractions containing HBCDs and TBBPA,

respectively, were further purified onto a silica cartridge using *n*-hexane–dichloromethane for elution.

The retention behavior of individual HBCD isomers on silica gel and Florisil was investigated using diverse mobile phase solvents and accounting for matrix effects. The β-HBCD diastereomer is substantially retained on both Florisil and silica regardless of the solvent used, and therefore, it undergoes selective loss during cleanup [35]. This sequence is counterintuitive to sequences based on reverse-phase chromatography with a C_{18}-column, in which the α- (and not the β-) isomer is eluted first when using a polar solvent. These results indicate that care should be taken when isolating HBCDs and other molecular diastereomers from environmental and biological samples and that reported concentrations of β-HBCD in the literature may be negatively biased.

3 Shortcomings of Gas Chromatography

Instrumental analysis of HBCDs and TBBPA are generally performed by means of GC−MS and/or LC−MS. The analytical methodology for the determination of HBCDs and TBBPA has been previously reviewed by Covaci et al. [2, 36]. The present section builds on the previous review and highlights advances in their separation and detection post-2007.

3.1 HBCD

Traditionally, HBCD has been analyzed using GC–MS, usually operated in ECNI mode for which the monitoring of the $[Br]^-$ ions allows a higher sensitivity. Detailed information regarding the GC–MS analysis of HBCDs can be found in recent reviews by Covaci et al. [2, 36]. However, the GC technique is more problematic for HBCD than for most PBDEs and has a number of serious limitations:

Technical HBCD consists of three diastereoisomers: α-, β-, and γ-HBCD, the latter being predominant. Interconversion of the HBCD diastereoisomers occurs when technical HBCD is exposed to temperatures above ca. 160°C [37], and therefore, diastereoisomer-specific analysis of HBCDs by GC–MS is not possible.

Close inspection of chromatograms shows that the HBCD peak is always somewhat broader than the near eluting PBDE peaks. Because HBCD elutes from the GC column at temperatures higher than 160°C, and due to thermal isomerization, a broad, unresolved peak is observed [2]. Since the response factors of the three diastereoisomers do not apparently differ very much [38], HBCDs can be quantified as total HBCDs by GC–MS. However, uncertainties are larger compared to those obtained for a number of PBDEs, which is shown by a larger relative SD in quality charts (ca. 25–30%).

At higher temperatures (around 240°C), as well as in dirty injection systems, HBCDs further degrade to lower brominated analogs (Fig. 1) [39]. Also, it has been shown that pure HBCD undergoes decomposition by elimination of HBr at temperatures above 240°C [37]. Not surprisingly, partial breakdown and even complete loss of HBCD have been reported in GC systems. When analyzing HBCDs by GC–MS, the cleanliness of extracts and the liner are essential.

In ECNI–MS, the dominant ions for HBCD are the $[Br]^-$ ions (m/z 79 and 81), while the larger fragment ions have low abundances [39]. In this case, structural confirmation of HBCD, for which the formation of larger fragment ions is necessary, is not possible in ECNI–MS.

Brominated compounds, which have been used as internal standards (e.g., PBDE or polybrominated biphenyls (PBB) congeners), have a better thermal stability and so cannot be used to compensate for the breakdown of HBCD during GC separation. Furthermore, since isotopically labeled HBCD standards cannot be used when monitoring only Br^- ions, the quantification of HBCD by GC–ECNI–MS is problematic.

Due to its lack of stereoisomer specificity, the use of GC in the analysis of HBCDs should be discouraged. If GC is the only alternative, thermal degradation of HBCDs should be minimized through cold on-column injection, short narrow-bore GC columns, thin film stationary phases, and high carrier gas flow rates. Cold on-column injection, short GC columns, thin film stationary phases, and high flow rates are several measures to minimize the risk of thermal degradation and to reduce the elution temperature of HBCD. Such approach has been recently applied for the identification and quantification of BFRs, including HBCD and TBBPA in styrenic polymers. Short run times (<10min) were employed for the separation of all BFRs using 15 m DB-5 type capillary columns (0.32 mm ID) combined with high oven

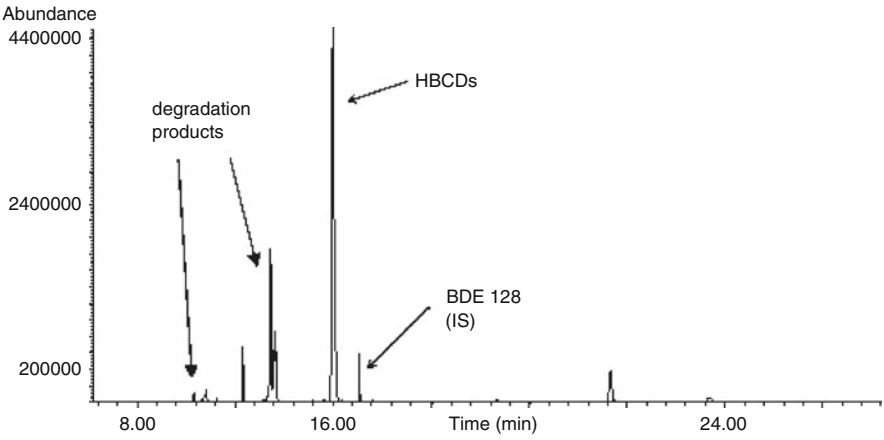

Fig. 1 GC–ECNI–MS chromatogram in selected ion monitoring mode of ions m/z 79 for a dust sample. Degradation products (corresponding to PBCDes and TBCDes) are also indicated. Reproduced from [39]

ramping. Retention time for HBCD was 4 min, and there was minimal degradation seen in the chromatograms [31].

3.2 TBBPA

Although TBBPA is the most widely used BFR, this compound is not frequently measured, probably due to its presence at lower concentrations in biota compared to PBDEs and HBCDs and due to its lower bioaccumulation potential. Among the predominant BFRs, TBBPA is the most polar molecule, which demands therefore more complicated methods for a proper determination. Acidification and derivatization are compulsory before GC analysis, while LC has the advantage that no derivatization step is required [4]. Both the derivatization and acidification step can introduce errors and/or losses [4]. In terms of sensitivity, LC–ESI–MS–MS can be competitive with published GC–EI–MS–MS techniques, with LODs in the ppt-range [21].

A GC–HRMS method requiring derivatization with methyl-chloroformate was developed by Berger et al. [34]. However, this method suffered from a rather restricted linear range and incomplete derivatization, leading to lower recoveries. This might also be explained by the presence of bulky bromine substituents adjacent to the two hydroxyl groups, resulting in an incomplete double derivatization. Derivatization that can lead to insertion of even larger groups on the TBBPA molecule is even more difficult.

4 Analysis by Liquid Chromatography

4.1 TBBPA

Due to polar characteristic of TBBPA molecule, LC–MS method appears to be the method of choice for their analysis because no derivatization of the phenolic group is required [3, 36, 40]. Another advantage of the LC–MS determination of TBBPA is the possibility to use the ^{13}C-labeled TBBPA as surrogate standard. This greatly enhances the quality of the analytical data obtained by compensating for matrix-related effects that can affect analyte ion intensity [3].

Tollbäck et al. [41] reported that the most suitable LC–MS interface for TBBPA analysis is ESI operating in negative ionization mode finding LODs 30–40 times lower compared to atmospheric pressure chemical ionization (APCI). In addition, it permits monitoring of the intact TBBPA molecule through the soft ionization of ESI resulting in improved method selectivity and accuracy. Similar results were found by Morris et al. [26].

The LC behavior of TBBPA depends greatly on the mobile phase. Chu et al. (2005) found that by using methanol as mobile phase, the response of target compounds was about one third greater than when using acetonitrile. The use of methanol as mobile phase resulted in better limit of quantification (LOQ) due to the more stable detector baseline obtained. In general, a more advantageous analysis was obtained using methanol and water as mobile phase [3, 42]. It is important to note that in terms of sensitivity, LC–ESI–MS can be competitive with published GC–EI–MS techniques with LODs in the ppt range [21].

Ion-trap MS was also reported for the determination of TBBPA in sediment and sewage sludge after LC separation [43]. The ion-trap scan range was set from m/z 145–543. On the other hand, Guerra et al. [42] proposed a methodology based in the use of LC–QqLIT–MS in order to analyze TBBPA and related compounds bisphenol A (BPA), monobromobisphenol A (MonoBBPA), dibromobisphenol A (DiBBPA), and tribromobisphenol A (TriBBPA) in sewage sludge and sediment samples. Figure 2 showed the chromatographic separation of TBBPA and related

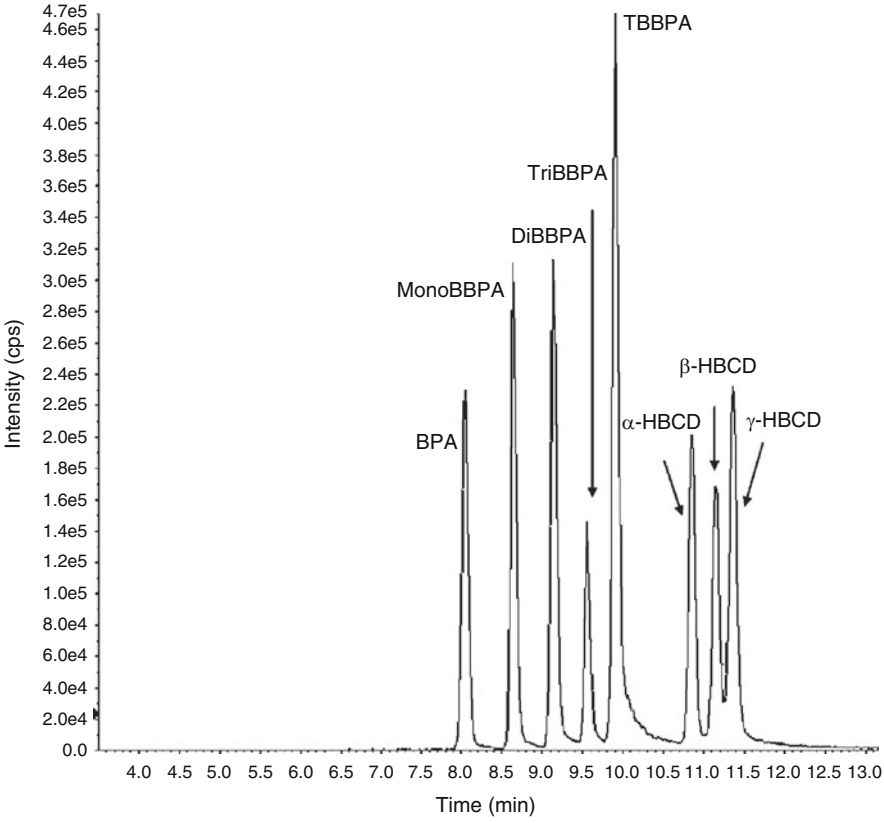

Fig. 2 Total ion chromatogram (TIC) obtained for a standard mixture containing BPA, MonoBBPA, DiBBPA, TriBBPA, TBBPA, α-, β-, and γ-HBCD at 500 pg/μL

compounds together with the diastereoisomeric HBCDs. On the other hand, ultra performance liquid chromatography (UPLC)–ESI–MS–MS was reported to analyze TBBPA in soil and food samples [44, 45]. This technique combines all the advantages of LC–MS–MS in addition to shorter residence time of the analyte on-column. The short analysis time (4 min) can double the efficiency of the analytical method [3].

4.2 HBCD

4.2.1 Diastereoisomer Determination

Regarding detection systems, MS is the preferred analytical technique, in terms of selectivity and sensitivity. Among different MS analysers, the QqQ is the most used [42, 46]. However, the increasing robustness and widespread use of ion trap instruments, due to their high versatility and relatively low cost, led to the development of methodologies based on this technique for HBCD detection and quantification [16, 42, 47–50].

There has been some concern due to the apparent differences in the stability of HBCD diastereoisomers in different solvents. This was explained by the relatively lower solubility of γ-HBCD in acetonitrile compared to methanol. Thus, Tomy et al. [50] suggested the use of methanol for final extracts to inject in LC–MS analysis.

Regarding diastereoisomers separation, any ratio of methanol/acetonitrile as mobile phase allows the separation of all three diastereoisomers. However, an increase in the percentage of acetonitrile in the mobile phase resulted in a slightly better separation of HBCDs, mainly between β- and γ-HBCD [46]. In order to enhance the sensitivity, mobile phase modifiers have been used, commonly ammonium acetate [7, 11, 26, 42, 51] and acetic acid [24]. It is well documented and experimentally confirmed that HBCD tend to associate with several anions forming different adducts that can affect the sensitivity and the accuracy of the determinations [16, 47, 52]. The addition of different ammonium salts to the mobile phase, i.e., ammonium chloride or ammonium acetate, in order to encourage (Cl method) or try to inhibit (Ac method), respectively, the formation of the chlorine adducts of the molecular ion were carried out. The Cl method showed higher sensitivity, and the limits of detection (0.23–0.41 pg on column) and quantification (0.77–1.35 pg on column) were up to 14 times lower than those obtained applying the Ac method.

LC–MS or LC–MS–MS using ESI or APCI are versatile tools for the isomeric-specific determination of HBCDs trace levels. Budakowsky and Tomy [53] have shown that APCI have lower intensities compared with a similar experiment with ESI. Consequently, ESI mode is the preferred one for determining diastereoisomer HBCDs by several studies [7, 42, 48, 53]. However, Suzuki and Hasegawa [11]

reported signal to noise (S/N) ratio values 2–5 times higher compared to ESI when APCI was applied on the HBCD analysis in leachate.

Different methods for the analysis of diastereoisomeric HBCD using LC–ESI–MS–MS and the selective reaction monitoring (SRM) for the transition $[M–H]^-$ (m/z 640.6) → $[Br]^-$ (m/z 79 and 81) have been developed obtaining LODs between 0.5 and 6 pg on-column [46, 53]. Morris et al. observed differences in the sensitivity of different HBCDs between two MS instrument: α-HBCD proved to be the most sensitive of the three stereoisomers with the single quadrupole instrument, while γ-HBCD was the most sensitive on the ion-trap [16].

Matrix-related effects, either signal enhancement or more commonly signal suppression, can have a pronounced effect on quantitative measurements. Based on these observations, the use of isotopic-labeled standards are helpful to achieve accurate analytical measurement data on the diastereoisomers. For this reason, several methodologies include the use of both ^{13}C- and d_{18}-labeled surrogates as recovery and/or instrument standards [7, 42, 46, 53].

It is important to note that some other LC–ESI–MS–MS methods have been developed for the simultaneous analysis of diastereoisomeric HBCDs and TBBPA [11, 42]. Two different LC–QqLIT–MS methods were developed by Guerra et al. comparing an SRM experiment with an enhanced product ion (EPI) experiment to analyze α-, β-, and γ-HBCD together with TBBPA and related compounds (BPA, MonoBBPA, DiBBPA, and TriBBPA). The developed methods display excellent LODs in SRM mode (0.1–1.8 pg), but even better results are obtained in EPI mode (0.01–0.5 pg) [42].

4.2.2 GC Versus LC for HBCD Analysis

A few studies have investigated the comparability of results issued using GC or LC methods. However, the results are not conclusive due to a large variation in the methodologies employed in each studies and the type of samples for which the comparison was achieved. In a study by Van Leeuwen and de Boer [54], total HBCD determined by GC–ECNI–MS was measured in 22 out of the 44 fish samples in concentrations between 0.20 and 230 ng/g wet weight (ww). The sum of the three HBCD diastereomers as obtained by LC–ESI–MS–MS was found to differ from the total HBCD by GC, with the GC–ECNI–MS results being in average 4.4 times higher, according to the regression analysis. The authors concluded that the LC–ESI–MS–MS data are more accurate than those obtained by GC–ECNI–MS.

In a similar study, Abdallah et al. [39] have analyzed HBCDs in indoor dust samples ($n = 37$) using GC–ECNI–MS and LC–ESI–MS–MS. This study has investigated also the suitability of using different internal standards (IS), including ^{13}C-labeled HBCD during the GC analysis and using the response factors (RF) of individual α-HBCD and γ-HBCD isomers to calculate the total HBCD concentrations by GC. Statistical comparison showed that concentrations obtained via GC–ECNI–MS were statistically indistinguishable ($p > 0.05$) from those obtained

using LC–ESI–MS–MS. The closest match between the two techniques was obtained using ^{13}C-α-HBCD as IS and the average RF for α-HBCD and γ-HBCD. Significant correlations (Pearson coefficient values $r > 0.9$, $p < 0.01$) were obtained between the GC–ECNI–MS and LC–ESI–MS–MS results obtained for the investigated dust samples, with slopes ranging from 0.76 to 1.36 for the various IS and RF calculations. Although the conclusion was that LC–ESI–MS–MS results in reliable data and, most important, in individual isomeric concentrations, it was acknowledged that GC–ECNI–MS can give a good estimation of the total HBCDs, if the appropriate ISs and calculation methods are used.

4.2.3 Enantiomeric Determination

Since α-, β- and γ-HBCD are chiral, each diastereoisomer has a pair of enantiomers: (+)α, (−)α, (+)β, (−)β, (+)γ, and (−)γ, respectively [36]. The enantiomers have identical physicochemical properties and abiotic degradation rates but may have different biological and toxicological properties and therefore different biotransformation rates. These transformations may result in nonracemic mixtures of the enantiomers that were industrially synthesized as racemates [8, 49, 53, 55, 56]. The rates of metabolization process of the enantiomers of environmental pollutants may be significantly different. For example, there are some cases where only one enantiomer is being decomposed while the second is being accumulated in the environment, such as for hexachlorohexane (HCH) [15]. Because of that, an understanding of the environmental and biological fate of the HBCD enantiomers is required.

In order to separate the enantiomers of (+/−)α, (+/−)β and (+/−)γ-HBCD, permethylated β-cyclodextrin columns has been successfully used [7, 28, 49]. The separation is achieved in a single analysis with LODs between 10 and 20 injected pg on-column. It is well known that ESI is subject to sample matrix effects that can cause enhancement or suppression of the target analytes signal and can adversely affect their quantification [8, 50]. In order to avoid this effect that can affect enantiomeric fraction (EF) calculations, Marvin et al. [8] introduced the corrected EF values. This correction is based on the use of isotopic-labeled standards (d_{18}-HBCDs) since d_{18}-labeled enantiomeric analogs behave in an identical manner to their native counterparts. Two other factors influencing the obtained EF values were observed by Marvin et al. The first one is the organic solvent content in the mobile phase; an EF decrease with increasing organic fraction content in the mobile phase was observed. The second factor is the column bleed that would compete with the enantiomers during the ion charge process in the ion spray source [8].

Different enantiomeric behavior studies were done by Jànak et al. [46, 56] and Guerra et al. [49, 57]. The first author [46] studied the EF in different fish species from the Western Scheldt Estuary, where a strong enrichment of (+)-α-HBCD was observed for bib and whiting liver. In the case of sole, the EFs were similar for liver and muscle, with a slight enrichment of (−)-α-HBCD. In 2008, the same author

[56] found clear differences between α-HBCD enantiomeric fractions in the different species analyzed, indicating species-specific stereoselective mechanism for uptake and metabolism of α-HBCD enantiomers. In Spanish sediment samples [49], EF obtained suggested a higher presence of (+)-α-HBCD and (+)-γ-HBCD as compared to technical mixtures. Finally, EF values in human breast milk from Spain have been analyzed [57], where an enrichment of (+)-γ-HBCD in the human body have been found. Regarding α-enantiomer, it was possible to determine EF value only in two samples, showing enrichment of (−)-α-HBCD. Figure 3 shows the chromatogram obtained for a standard solution and for two human breast milk samples.

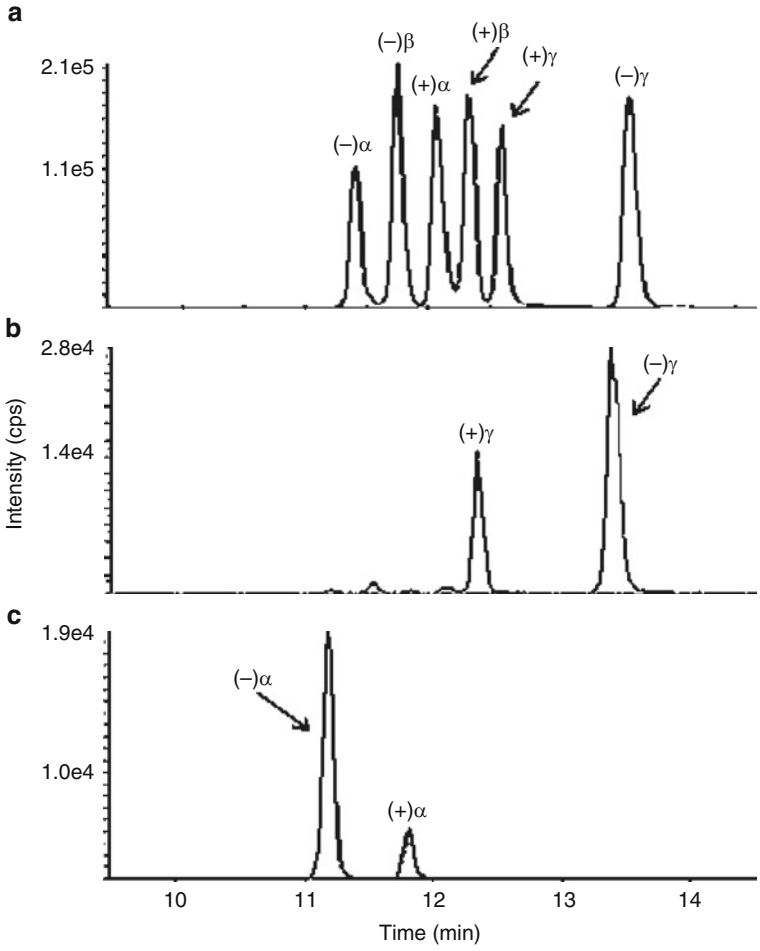

Fig. 3 Enantiomeric HBCD analysis by LC–ESI–MS–MS for (**a**) standard solution at 1,000 pg/μL, and (**b**, **c**) human breast milk samples from Spain

4.3 Other BFRs

ESI, APCI, and atmospheric pressure photoionization (APPI) are the ionization techniques commonly used in LC–MS analyses. These techniques are suitable for the ionization of polar, less polar, and slightly polar to nonpolar compounds, respectively. Although ESI efficiently ionizes HBCD isomers and TBBPA [42, 43, 50, 51, 53], the use of this ionization mode for other emerging BFRs have failed. APCI ionization has been also applied only for the analysis of TBBPA and HBCDs [11]. APPI is the most used ionization mode for the analysis of PBDEs, but the limited availability of APPI in most analytical laboratories has made this mode of ionization less appealing [58]. Abdallah et al. [59] performed a ^{13}C-labeled isotope

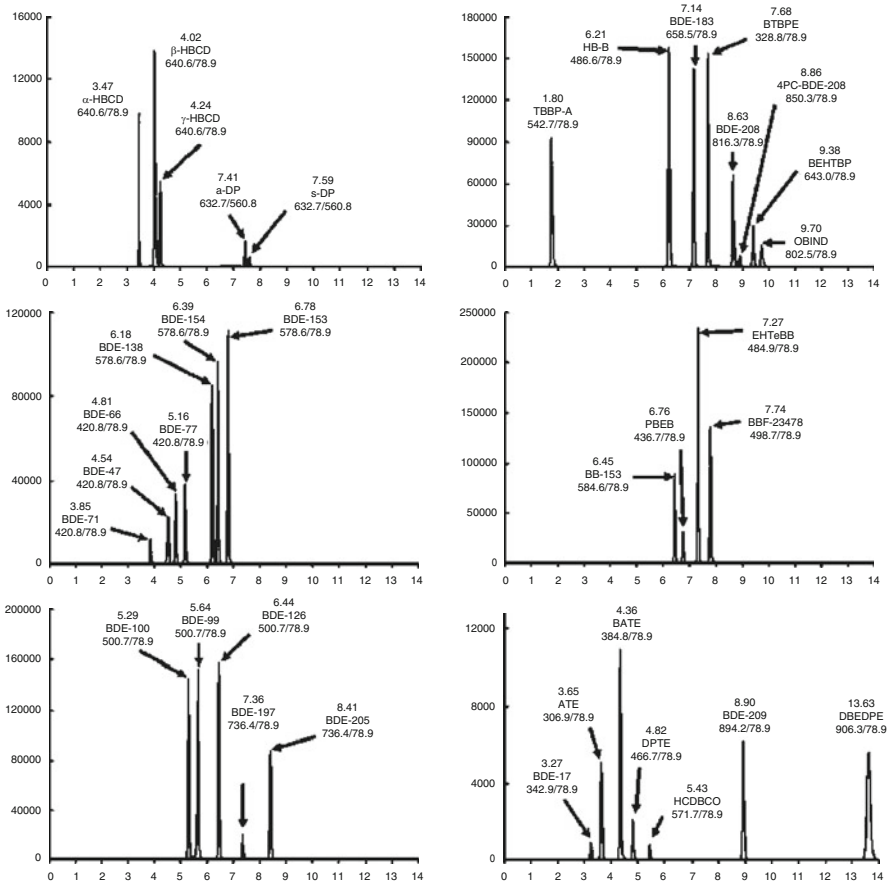

Fig. 4 Reconstructed MRM chromatograms obtained from 400 pg on-column injection of various BFRs with the peak intensity (cps) of *y*-axis and retention time (min) of *x*-axis by LC–APPI–MS–MS. Reproduced from [58]

dilution LC–APPI–MS–MS method to analyze 14 different PBDEs, between tetra- to deca-BDE congeners in house hold dust.

Another method was developed to analyze 36 halogenated flame retardants (HFR) in fish by LC–APPI–MS–MS. Some of the quantified compounds were 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EHTeBB), bis(2-ethyl-1hexyl)tetrabromophthalate (BEHTBP), HBCD isomers, and BDE-47, -66, -71, -77, -100, -99, -126, -138, -154, -153, -183, -197, -206, and -209. Moreover, LC–APCI–MS–MS was performed for the analysis of 38 HFRs in wastewater, finding EHTeBB, decabromodiphenylethane (DBDPE), BEHTBP, and TBBPA at pg/mL levels (Fig. 4) [58]. Different LC columns were evaluated, and a C18 column was selected due to the adequate selectivity for the 38 compounds, using a mixture of water and methanol as the optimal mobile phase. The average of on-column instrumental limit of detection (IDLs) was 6.1 pg, showing better sensitivity compared with some literature values [58].

A new emerging BFR recently included in some environmental studies is the 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane (TBECH). This compound can exist as four diastereoisomers are thermally sensitive, and they can interconvert at temperatures of 123°C or higher. That is because the development of a LC–MS method may be useful in order to obtain the separation of the four isomers. Arsenault et al. [60] developed a LC–ESI–MS method for the four TBECH isomers. They could not detect the molecular ion; however, analytes were detected monitoring the bromide ion (Br^-) using SIM mode. They also observed that, using APCI conditions, a very weak molecular ion was detected.

4.4 BFR Metabolites and Transformation Products

When organic compounds are into the environment, they could be widely distributed and subjected to different processes that contribute to their elimination and/or transformation [61]. Depending on the compartment in which the transformation occurs, the products could be a metabolite, when the organism metabolism is involved or, a transformation product if it reacts in the environment. Therefore, in exposed organism, metabolism is an important factor in determining the bioaccumulation, fate, pharmacokinetics (or toxicokinetics), and toxicity of contaminants.

In order to analyze metabolites and transformation products of BFRs, LC–MS is a useful technique due to the higher polarity of these compounds compared with the parent compounds.

4.4.1 HBCD Metabolites and Transformation Products

Abdallah et al. [39] found HBCD transformation/degradation products in dust samples via LC–MS–MS using a C18 analytical column and water/methanol as mobile phase in presence of ammonium acetate. These products were identified as

four pentabromocyclododecene (PBCDe) isomers. Moreover, two additional peaks were observed in chromatograms and corresponded to two congeners of tetrabromocyclododecadienes (TBCDe). The monitoring of m/z 560.8→79.0 and 480.4→79.0 was done for PBCDs and TBCDs, respectively. The elution of all compounds is obtained in less than 15 min. Different results were observed by Davis et al. [62] when analyzed in waste water sludge and freshwater sediments by LC–APPI–MS. In this study, only one TBCD isomer as a degradation product of HBCD was detected. It was proposed that TBCD is the result of a dihaloelimination reaction of HBCD resulting in the loss of two bromines from vicinal carbons with the further formation of a double bond between the adjacent carbon atoms.

A small number of studies have been carried out in order to determine HBCD metabolism. In a study with dolphins, three HBCD metabolites were detected using an LC–ESI–MS instrument [63]. Two metabolites were identified as monohydroxy-HBCD and the third metabolite could not be identified. Hydroxylated metabolites of β- and γ-HBCD were found; however, no significant decrease in α-HBCD concentration was observed during the incubation with rat and harbor seal microsomes. However, in vitro experiments with microsomes of dab and flounder showed that α-HBCD was also biotransformed resulting in two monohydroxy-HBCD metabolites. Huhtala et al. [64] confirmed this result by finding a monohydroxy-HBCD after an in vitro study in rainbow trout liver microsomes.

Brandsma et al. [65] studied the presence of hydroxylated metabolites of HBCD in three wildlife species (tern egg, seal, and flounder) as well as Wistar rats exposed to HBCD for 28 days. A total of four different types of metabolites were found using LC–MS–MS: the monohydroxy metabolites of TBCDe, PBCDe, and HBCD, and a dihydroxy-HBCD (Fig. 5). Another metabolic pathway in the rat is debromination of HBCD to PBCDe and TBCDe. In this study, all the hydroxylated metabolites found with LC–MS–MS were confirmed by GC–MS, but an additional metabolite was found with GC–MS, the dihydroxy-PBCDe [65].

4.4.2 PBDE Metabolites

The nonpolar nature of de diphenyl ether structure in PBDEs and the introduction of polar hydroxyl functional groups in the molecule upon its metabolism provide a special challenge in the analysis of PBDEs metabolites. Due to the structural similarity of some hydroxylated (OH)-PBDE compounds to thyroid hormones such as thyroxin (T4), these metabolites are suspected to mimic hormones in the body, making them more deleterious than the parent compounds [9].

Direct analysis of OH-PBDEs by GC–MS is not amenable due to their nonvolatile nature. All analytical methods applying GC–MS for the analysis of OH-PBDE metabolites must include a derivatization step. Usually, diazomethane was used as derivatizing agent. This compound needs to be handled with extreme care due to its explosive characteristics. On the other hand, the efficiency of the derivatization varies from sample to sample since the reaction may give less than 100% yield.

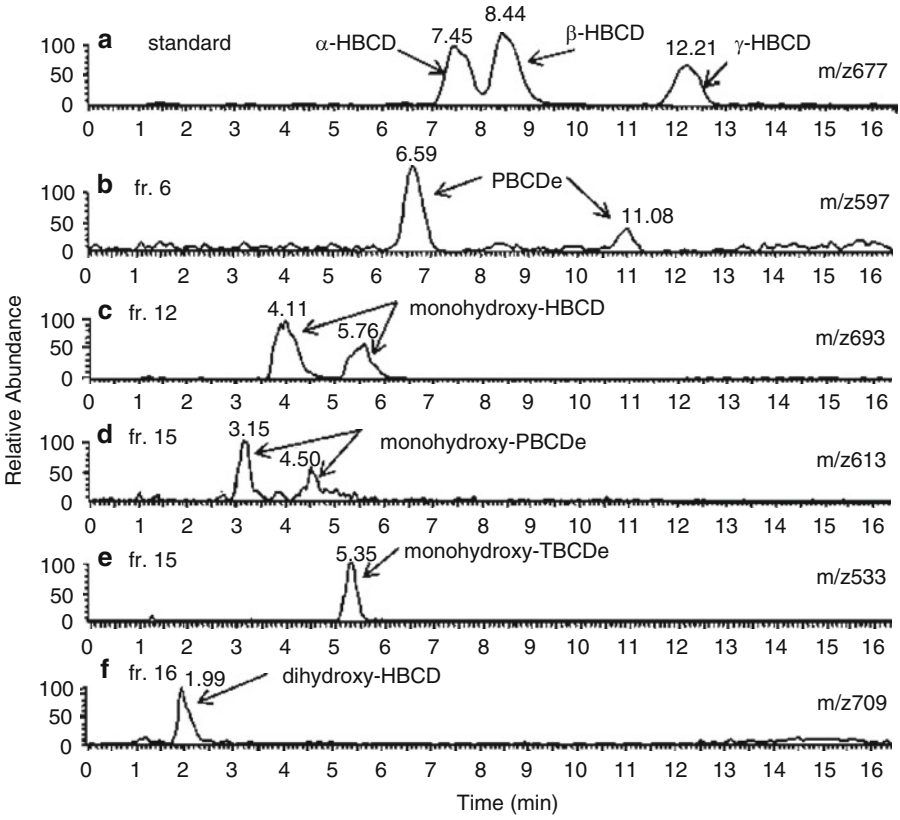

Fig. 5 (**a**) Selected ion LC–MS chromatogram of α-, β-, and γ-HBCD standard extract. Selected ion LC–MS chromatograms in adipose tissue of a male rat sample: (**b**) PBCDe in fraction 6, monohydroxy-HBCD in fraction 12, (**c**) monohydroxy-HBCD in fraction 12, (**d**) monohydroxy-PBCDe in fraction 15, (**e**) monohydroxy-TBCDe fraction 15, (**f**) dihydroxy-HBCD in fraction 16. Reproduced from [65]

Finally, additional sample preparation/cleanup steps could introduce errors and long analysis time. For all these reasons, the analysis of OH-PBDE metabolites by LC–MS seams interesting. However, these metabolites are poorly ionized by LC–ESI–MS and consequently are not easily detected at low levels [9, 66].

In 2007, Mas et al. [66] proposed the analysis of eight underivatized OH-di to OH-tetraBDEs by negative ion spray ionization (ISP) tandem mass spectrometry (LC–ISP–MS–MS) in soil fish, sludge, and particular matter. This method was shown to be efficient, robust, sensitive, and selective with LOQ at the high pg/g dry weight level. Another LC–APCI–MS–MS method was developed for the separation and detection of nine OH-PBDEs, ranging from tri- to hexa-brominated, as well as MS–MS fragmentation information [9]. As mobile phase, water and acetonitrile were used and the reversed-phased separation was completed on a C18 analytical

column, resulting in a 35 min separation of the nine congeners followed by a 10 min reequilibration.

Methoxylated (MeO)-PBDEs have been reported in a few studies and the current knowledge indicates that the MeO-PBDEs found in wildlife are mostly a consequence of accumulation via natural sources in marine environments such as in sponges and green algae [67]. Kato et al. [68] have analyzed OH- and MeO-tetraBDEs and hydroxylated and methoxylated analogs of tetrabromobiphenyl (diOH-tetraBB and diMeO-tetraBB) using LC–APCI–MS–MS in marine biota. A good advantage of the proposed APCI method is that the simultaneous determination of OH- and MeO-brominated analogs could be completed within 22 min after a single cleanup GPC procedure.

A selective and sensitive method using negative LC–APCI–MS–MS [69] was also developed to enable analysis of selected natural persistent organohalogens accumulated in marine biota. Selected analytes were three MeO-tetraBDEs, a diMeO-tetraBDE and two halogenated methyl bipyrroles (Cl_7-MBP and Br_4Cl_2-DBP). The method only required 10 min to allow the separation of selected analytes. In this method, the fragmentation pathways of MeO-BDEs produced characteristic SRM transitions needed to resolve isomeric compounds, $[M–Br+O]^-$ and Br^- ions, for MeO–BDE analogs and $[M–Cl+O]^-$ and Br^- or degradation product ions for 1,1′-dimethyl-3,3′,4,4′-tetrabromo-5,5′-dichloro-2,2′-bipyrrole (Br_4Cl_2-DBP) and 2,3,3′,4,4′,5,5′-heptachloro-1′-methyl-1,2′-bipyrrole (Cl_7-MBP), respectively.

Finally, a LC–ESI–MS–MS method with greater sensitivity and better separation efficiency [70] was established for the simultaneous analysis of four estrogens, BPA, 10 OH-PBDEs, and 15 brominated phenols (BRPs) in blood plasma sample, using dansyl chloride as derivatization agent that reacts, which provides a new method to derivatize phenolic compounds prior to LC–MS analysis. The ionization and fragmentation of the isolated dansyl derivatives ESI–MS–MS resulted in protonated molecular ions $[MH]^+$ or $[MH]^+$-2 of their dansyl derivatives, producing the same major product ions at m/z 171 and 156. Thus, 30 analytes belonging to four classes of phenolic compounds could be analyzed within 22 min after sample treatment (Fig. 6). However, the sample preparation is longer due to the use of dansyl chloride.

5 Interlaboratory Studies

An important gap is the lack of harmonized methods for the determination of HBCDs and TBBPA in environmental matrices. Regular intercalibration studies and participation in laboratory proficiency studies (when available) are important so as to maintain high quality of analytical data. Up to now, only a limited number of intercalibration studies have been performed with a rather limited number of participating laboratories. Moreover, these interlaboratory studies referred to HBCDs, but none of them reported intercomparison for TBBPA determination.

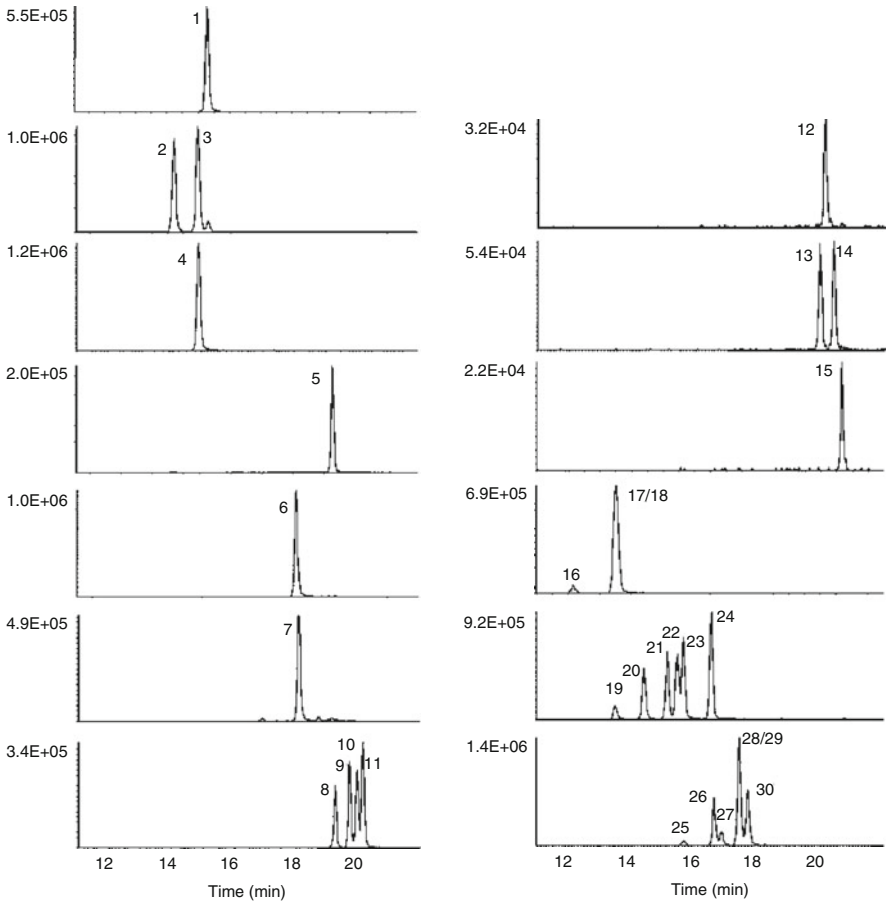

Fig. 6 LC–MS–MS MRM chromatographic profiles of 30 analytes in a standard mixture of 10 ng/mL: E1 (1), βE2 (2), αE2 (3), EE (4), BPA (5), 2′-OH-6′-Cl-BDE-7 (6), 6′-OH-BDE-17 (7), 3-OH-BDE-47 (8), 5-OH-BDE-47 (9), 6-OH-BDE-47 (10), 4′-OH-BDE-49 (11), 2′-OH-6′-Cl-BDE-68 (12), 6-OH-BDE-90 (13), 2-OH-BDE-123 (14), 6-OH-BDE-137 (15), 3-BRP (16), 2/4-BRP (17/18), 2,6-diBRP (19), 2,3-diBRP (20), 2,5-diBRP (21), 2,4-diBRP (22), 3,4-diBRP (23), 3,5-diBRP (24), 2,3,6-triBRP (25), 2,3,4-triBRP (26), 2,4,6-triBRP (27), 2,3,5/2,4,5-triBRP (28/29), 3,4,5-triBRP (30), analyzed on a 100 × 2.1 mm^2, i.d.; Waters XBridge column. E1: estrone; βE2: 17β-estradiol; αE2: 17α-estradiol; EE2: Ethinyl estradiol. Reproduced from [70]

In order to assess the quality of HBCD determinations, the Norwegian Institute of Public Health organized interlaboratory comparison studies in 2005 and 2007. The purposes of these studies were (a) to assess the comparability of results obtained using different analytical techniques, (b) to provide a quality assurance instrument for the participating laboratories, and (c) to assess the readiness of expert laboratories to determine HBCD in biological samples [71]. Up to 13 laboratories determined either the total HBCD concentration, or concentrations of individual HBCD isomers, or both in cod liver oil, herring filet, salmon filet, butter,

and chicken meat. The laboratories were able to determine total HBCD concentrations in the marine samples with satisfying quality (RSD <35%). However, the analysis of samples with low HBCD contamination (<about 2 ng/g lipid weight) should be improved. No statistically significant differences were found between total HBCD concentrations obtained by LC–MS and GC–MS.

An additional factor is that there are currently no certified reference materials (CRMs) for HBCDs that can be used for method validation. Up to now, indicative values for HBCDs have been issued for several reference materials (e.g., lake trout from Cambridge Isotope Laboratories or indoor dust from the US National Institute for Standards and Technology), but the certification of HBCDs and TBBPA in a wider range of environmentally relevant materials is needed.

6 Conclusions and Perspectives

PBDEs have typically been analyzed using GC–MS, but the high injection port temperatures required to transfer these analytes to the GC column can result in degradation of the desired compounds. These could occur in the case of highly PBDEs and other BFRs such as HBCDs. In the case of TBBPA or metabolites (OH-PBDES), a derivatization step is needed, given more possibilities for errors because it is not always reproducible and quantitative.

The LC–MS and LC–MS–MS methods appear to be methodologies of choice to analyze TBBPA, HBCD-isomers, and HBCD-enantiomers because no derivatization is needed, and the isomeric separation is obtained. The ESI, APCI, and APPI are the atmospheric pressure ionization modes commonly used in LC–MS analyses. On the other hand, the use of ^{13}C-TBBPA and ^{13}C- and d_{18}-HBCD-isomer-labeled standards as internal standards enhances the quality of the analytical data through compensation for matrix-related effects that can affect analyte ion intensity, reproducibility, and trueness.

Also important is the recent development of methods for the identification of metabolites of PBDEs and HBCD by LC–MS–MS, thereby avoiding the problems of derivatization. The future challenge is to adapt these LC–MS techniques for the analysis of emerging flame retardants in different sample matrices as well as their potential metabolites and transformation products.

On the other hand, in order to improve the quality of these methodologies, interlaboratories comparison studies should be done, especially in the case of HBCDs and TBPPA where more experience and interest have been obtained in the last years. Finally, another relevant point is the lack of CRMs. While effective analytical quality control requires the availability of CRMs, there is a need to certify HBCD and TBBPA in various appropriate CRMs.

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