GC-MS/MS LARGE VOLUME INJECTION ANALYSIS OF FIFTEEN POLYBROMINATED DIPHENYL ETHERS IN MUSSELS

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Introduction

Brominated flame retardants are compounds added to inhibit or slow down the ignition of combustible materials. Polybrominated diphenyl ethers (PBDEs) are one of the classes of substances used worldwide as flame retardants in electronic appliances, textiles, furnishings and various other consumer products. There are theoretically 209 PBDE congeners, although the commercial products predominantly consist of: penta-BDEs (mainly the congeners 47, 85, 99, 100, 153, 154), octa-BDEs (mainly the congeners 153, 183, 196, 197, 206, 207, 209) deca-BDEs (almost exclusively 209) [1].

Since the last decade, the occurrence of PBDEs in the environment was widely exploited in several European and Asiatic countries and in USA. In Italy very little has been done on this topic and therefore only few information have been collected on the environmental levels, dietary exposure and toxicological effects related to these compounds.[2] In the Scientific Opinion on Polybrominated Diphenyl Ethers (PBDEs) in Food issued by EFSA, no data were submitted from Italy. [3] Therefore, there is the need to improve the knowledge on PBDEs contamination patterns and levels in order to evaluate human and wildlife exposure and to assess the related risk which could, at the end, bring to the definition of residue limits by the European Commission. In March 2014 the European Commission issued a recommendation in which member states are requested to monitor brominated flame retardants in food, namely PBDEs, hexabromocyclodecanes (HBCDDs), tetrabromobisphenol A and derivatives, brominated phenols and derivatives and the emerging brominated flame retardants.[4]

In 2013 our institution started a Project founded by the Italian Ministry of Health with the aim to provide comprehensive information on PBDEs spread in the Italian aquatic environment through the investigation of mussels contamination. In this work the development and the preliminary validation results of an analytical method able to determine fifteen PBDE congeners at trace levels in mussel tissues are reported.

Materials and methods

Chemicals and materials

Individual PBDE congeners 49, 66, 77, 85, 138, 197, 206 and the 77- and 138- $^{13}C_{12}$-labelled mixture (declared chemical purity > 98%) were supplied by Wellington Laboratories (Guelph, Ontario, Canada). The Method 1614 Labeled Surrogate Stock Solution (LSSS) (28, 47, 99, 100, 153, 154, 183, 209, $^{13}C_{12}$-labelled, declared chemical purity > 98%) and Method 1614 Native Par Stock Solution (28, 47, 99, 100, 153, 154, 183, 209, declared chemical purity > 98%) were purchased by Cambridge Isotope Laboratories (Andover, MA, USA).

Pesticide grade *c*-hexane and dichloromethane and analytical grade ethyl acetate, *n*-hexane were supplied by Carlo Erba Reagents (Rodano, Milano, Italia). Fluka pesticide grade isooctane was from Sigma–Aldrich (Steinheim, Germany). Extrelut-NT3 columns were supplied by Merck (Darmstadt, Germany) and the Isolute silica 2g/6mL columns were from Biotage (Uppsala, Sweden). Magnesium sulphate and sodium chloride for the QuEChERS-like extraction were delivered from Sigma–Aldrich (Steinheim, Germany) and the Bio-Beads SX-3 polystyrene resin was purchased by R-Biopharm (Darmstadt, Germany).

Analytical method

The sample preparation was inspired to already published procedures[5]. Briefly, 20 grams of mussel tissue spiked with 1 ng of the Labeled Surrogate Standards were mixed with 5 mL of ultrapure water and shaken vigorously with 15 mL of ethyl acetate in a polypropylene centrifuge tube for 1 min. Subsequently, 6 g of anhydrous magnesium sulphate and 3 g of sodium chloride were added to the mixture. The tube was shaken for another 1 min, centrifuged and an aliquot of 10 mL was removed from the upper organic layer. The solvent was eliminated using the Genevac EZ-2 centrifuge (SP Scientific, Ipswich, Suffolk, UK). The residue was redissolved in 3x1mL of hexane and loaded on top of an Extrelut NT-3 column, acidified with 3 ml of

concentrated sulphuric acid, connected on top of a silica cartridge 2g/6 ml. After loading, the analytes were directly eluted with 7 mL of hexane. The Extrelut column was then disconnected and the silica further eluted with 10 mL of a mixture of hexane/dichloromethane (3/1 v/v). The solvent was carefully reduced to 0.5 mL under a gentle stream of nitrogen and the sample sumbitted to Gel Permeation Chromatography (GPC) on a Gilson Aspec XL coupled to a pump 307 and a UV-VIS detector 152 (Middleton, USA). The collected fraction was again evaporated under gentle stream of nitrogen and finally dissolved in 250 µL of a 2 ng/mL mixture of the two syringe standards (77- and 138- $^{13}C_{12}$). The instrumental analysis was performed on an Agilent 7890A GC (Agilent technologies, Santa Clara, CA, USA) coupled to a triple quadrupole mass spectrometer Agilent 7000 MS system equipped with a 7693 series automatic injector. In the programmable temperature vaporisation (PTV) inlet, 10 µL of the final sample extract were injected in solvent vent mode (vent flow 50 mL/min, pressure 5 psi, time 0.5 min, purge flow to split vent 50 mL/min at 2 min) and the injector temperature was programmed as follows: 0.5 min at 50 °C, increased to 325 °C at 600 °C/min. The chromatographic separation was achieved in temperature programmed mode using a DB-5HT (15m x 250µm x 0.25µm) Agilent-Technologies as follows: 50 °C, ramp to 80 °C at 15 °C /min, ramp to 220 °C at 60 °C /min, ramp to 250 °C at 30 °C /min, ramp to 270 °C at 5 °C /min, ramp to 325 °C at 10 °C /min and hold for 5 min. The MS/MS parameters and the chosen transitions as target and qualifiers are summarised in Table 1.

Method validation

To date the European Commission did not set maximum limits for PBDEs in food, therefore the natural contamination levels in mussels were taken as reference concentrations to plan a suitable validation study. A bulk sample obtained carefully mixing and homogenizing several mussel samples was used to assess method performances. Replicated analysis (n=7) at five concentrations (20, 100, 200, 400 and 600 pg/g) were performed in intralaboratory reproducibility conditions (different time). Almost all the analytes were detected in the bulk blank sample and the concentrations of some of them were not negligible (congeners 47, 49, 99, 100). The mean levels of incurred congeners were subtracted to each spiked level. Therefore, for each analyte, the method performance evaluation was undergone only when the spiked concentration was at least equal to the incurred level. The isotope dilution multipoint calibration technique was used to determine the linear response interval of the detector. Standard mixtures of the 15 PBDE congeners (0.1, 0.5, 1.0, 5.0, 10, 50, 100 ng/mL in *iso*-octane) and of the 8 labelled surrogate standards (all at 2 ng/mL in *iso*-octane) were injected in the GC-MS/MS in three different days to study the instrument linearity interval. Good determination coefficients r^2 (>0.995) were obtained for each congener applying the least square method to two subintervals of concentrations (0.1-5.0 ng/mL and 5.0-100 ng/mL).

Results and discussion

Preliminary experiments on the method were carried out following the procedure proposed by Kalachova et al.[5] for PBDEs in fish muscle. In that work, after the sample extraction, only a single purification step using manually packed Si-colums was foreseen. The results were, at the beginning, cheering, but the chromatograms obtained from the mussels analysis were non sufficiently clean and the whole method showed to be not enough robust to be applicable to a complex matrix like mussels: further purification steps were needed. Moreover the amount of matrix to be anaysed was doubled respect to the Kalachova et al. procedure in order to improve the limits of quantification (LOQs): the goal was to reach about 2-5 pg/g for all the analytes, except for BDE-206 and BDE-209 for which higher LOQs were expected (ca 200 pg/g). Firstly an H_2SO_4 fat hydrolisis on Extrelut-NT-3 was added to the initial Si-SPE (2g/6mL) clean-up and improvements in recovery and precision were observed. Nevertheless some shifts in retention times were still noticed indicating the need of further improvements. A final step of Gel Permeation Chromatography allowed to achieve the necessary method robustness also for the more troublesome heavy congeners (BDE-206 and -209). With regards to instrumental determination, the detection/quantification steps were carried out selecting for each analyte two of the more selective and abundant ion transitions (Table 1).

Table 1: Selected ions for quantification and confirmation of target PBDEs and their ^{13}C-labelled analogues

Ion Source	**Ion Source T (°C)**		**Quadrupole T (°C)**			**Interface T (°C)**	
EI	230		150			320	
Time segment	**Analyte**	**Molecular Weight (Da)**	**Precursor Ion (m/z)**		**Product Ion (m/z)**		**CE(V)**
1	**BDE 28**	406.9	**405.9**	$[M]^+$	**246**	$[M\text{-}Br_2]^+$	20
			407.8	$[M]^+$	**248**	$[M\text{-}Br_2]^+$	21
	BDE28-L	418.9	**419.8**	$[M]^+$	**260**	$[M\text{-}Br_2]^+$	16
2	**BDE 49**	485.8	**486**	$[M]^+$	**326**	$[M\text{-}Br_2]^+$	22
			488	$[M]^+$	**328**	$[M\text{-}Br_2]^+$	34
	BDE 47	485.8	**486**	$[M]^+$	**326**	$[M\text{-}Br_2]^+$	24
			484	$[M]^+$	**324**	$[M\text{-}Br_2]^+$	24
	BDE47-L	497.8	**497.8**	$[M]^+$	**338**	$[M\text{-}Br_2]^+$	20
	BDE 66	485.8	**326**	$[M\text{-}Br_2]^+$	**219**	$[M\text{-}CBr_3O]^+$	34
			486	$[M]^+$	**326**	$[M\text{-}Br_2]^+$	24
	BDE 77	485.8	**485.8**	$[M]^+$	**326**	$[M\text{-}Br_2]^+$	24
			483.7	$[M]^+$	**376.7**	$[M\text{-}CBrO]^+$	17
	BDE77-L	497.8	**497.7**	$[M]^+$	**338**	$[M\text{-}Br_2]^+$	24
3	**BDE 100**	564.7	**565.6**	$[M]^+$	**406**	$[M\text{-}Br_2]^+$	17
			403.8	$[M\text{-}Br_2]^+$	**297**	$[M\text{-}CBr_3O]^+$	34
	BDE100-L	576.7	**575.7**	$[M]^+$	**416**	$[M\text{-}Br_2]^+$	38
	BDE 99	564.7	**565.7**	$[M]^+$	**405.8**	$[M\text{-}Br_2]^+$	28
			403.8	$[M\text{-}Br_2]^+$	**297**	$[M\text{-}CBr_3O]^+$	35
	BDE99-L	576.7	**577.7**	$[M]^+$	**418**	$[M\text{-}Br_2]^+$	26
	BDE 85	564.7	**565.7**	$[M]^+$	**406**	$[M\text{-}Br_2]^+$	28
			403.8	$[M\text{-}Br_2]^+$	**297**	$[M\text{-}CBr_3O]^+$	34
4	**BDE 154**	643.6	**643.6**	$[M]^+$	**483.8**	$[M\text{-}Br_2]^+$	20
			481.7	$[M\text{-}Br_2]^+$	**375**	$[M\text{-}CBr_3O]^+$	39
	BDE154-L	655.6	**655.6**	$[M]^+$	**496**	$[M\text{-}Br_2]^+$	44
	BDE 153	643.6	**643.6**	$[M]^+$	**483.9**	$[M\text{-}Br_2]^+$	20
			483.7	$[M\text{-}Br_2]^+$	**374.9**	$[M\text{-}CBr_3O]^+$	40
	BDE153-L	655.6	**655.7**	$[M]^+$	**496**	$[M\text{-}Br_2]^+$	26
	BDE 138	643.6	**643.7**	$[M]^+$	**484**	$[M\text{-}Br_2]^+$	26
			483.7	$[M]^+$	**374.9**	$[M\text{-}Br_2]^+$	40
	BDE138-L	655.6	**655.7**	$[M]^+$	**496**	$[M\text{-}Br_2]^+$	26
5	**BDE 183**	722.5	**721.6**	$[M]^+$	**721.6**	$[M]^+$	5
			721.6	$[M]^+$	**561.8**	$[M\text{-}Br_2]^+$	17
			561.8	$[M\text{-}Br_2]^+$	**454.8**	$[M\text{-}CBr_3O]^+$	35
	BDE183-L	734.5	**733.7**	$[M]^+$	**733.7**	$[M]^+$	5
6	**BDE 197**	801.4	**801.7**	$[M]^+$	**641.7**	$[M\text{-}Br_2]^+$	14
			641.7	$[M\text{-}Br_2]^+$	**641.7**	$[M\text{-}Br_2]^+$	10
			639.6	$[M\text{-}Br_2]^+$	**639.6**	$[M\text{-}Br_2]^+$	15
7	**BDE 206**	880.3	**719.5**	$[M\text{-}Br_2]^+$	**719.5**	$[M\text{-}Br_2]^+$	0
			641.6	$[M\text{-}Br_3]^+$	**641.6**	$[M\text{-}Br_3]^+$	0
			719.8	$[M\text{-}Br_2]^+$	**612.7**	$[M\text{-}CBr_3O]^+$	47
			641.6	$[M\text{-}Br_3]^+$	**482**	$[M\text{-}Br_5]^+$	47
8	**BDE 209**	959.2	**799.4**	$[M\text{-}Br_2]^+$	**799.4**	$[M\text{-}Br_2]^+$	0
			639.5	$[M\text{-}Br_4]^+$	**532.3**	$[M\text{-}CBr_5O]^+$	30
	BDE209-L	971.2	**811.5**	$[M\text{-}Br_2]^+$	**811.5**	$[M\text{-}Br_2]^+$	0
			811.5	$[M\text{-}Br_2]^+$	**651.3**	$[M\text{-}Br_4]^+$	54

Finally, in order to further improve method limits also the injection technique was optimized: ten microliters of sample were analysed in GC-MS/MS using a programmable temperature vaporisation injector (PTV). Satisfactory peak shapes were obtained togheter with lower LOQs. Experiments are in progress to conclude an

interlaboratory validation study. Accuracies studied in intra-laboratory reproducibility conditions were satisfactory for all the congeners: apparent recoveries (R%) were between 73 and 116% and precisions between 0.8 and 24% (Table 2). Real recoveries of the surrogate labelled standards were generally higher than 60% except for the labelled BDE-209.

Table 2: Preliminary validation results in mussel tissue (R: apparent recovery, R% surrogate std: real recoveries of the labelled standards)

Spiking Level (pg/g)	BDE	RSD_r%	R%	R% surrogate std	BDE	RSD_r%	R%	R% surrogate std	BDE	RSD_r%	R%	R% surrogate std
20		4.9	97	57		NE*	NE*	NE*		NE*	NE*	NE*
100		6.4	100	52		4.8	105	64		NE*	NE*	NE*
200	**28**	9.7	99	66	**49**	5.3	98	73	**47**	7.2	79	73
400		1.4	88	77		3.1	101	85		6.1	87	85
600		1.7	91	74		4.1	107	79		11	85	79
20		8.0	109	70		15	92	70		20	73	69
100		14	99	64		23	105	64		20	92	62
200	**66**	17	94	73	**77**	9.7	95	73	**100**	16	94	70
400		3.4	74	85		2.2	81	85		3.7	88	84
600		12	89	79		7.0	95	79		6.5	88	80
20						13	99	71		23	94	58
100		20	82	65		13	104	65		7.9	96	55
200	**99**	16	87	73	**85**	4.0	98	73	**154**	15	87	60
400		2.9	79	86		2.0	105	86		9.1	86	64
600		4.6	84	82		9.3	101	82		3.6	89	68
20		15	94	63		14	89	63		14	84	66
100		8.3	96	60		12	96	60		5.6	98	61
200	**153**	14	101	64	**138**	18	101	64	**183**	8.6	88	68
400		2.4	88	72		2.2	90	72		0.8	81	82
600		6.5	93	70		3.0	98	70		6.4	83	80
20		13	90	66		12	96	41		7.5	106	41
100		6.7	104	61		3.6	106	44		7.0	99	44
200	**197**	11	92	68	**206**	2.9	110	52	**209**	8.0	106	52
400		2.1	85	82		3.5	115	81		2.2	110	81
600		7.9	83	80		12	116	74		7.1	116	74

*NE: not evaluated (high background level)

Conclusions

In the best of our knowledge only few methods for PBDEs include all the congeners here determined. The procedure meets the required criteria in terms of performance characteristics, labour demands/sample throughput as well as cost effectiveness, enabling the analysis of a batch of twelve samples in 8-10 work-hours. The method seems suitable to perform an extensive monitoring of the background levels of PBDE in mussels from Italian coasts.

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