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Extraction, isolation, and purification of analytes from samples of marine origin – A multivariate task☆

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ABSTRACT

The development of a multivariate study for a quantitative analysis of six different polybrominated diphenyl ethers (PBDEs) in tissue of *Atlantic Salmo salar* L. is reported. An extraction, isolation, and purification process based on an accelerated solvent extraction system was designed, investigated, and optimized by means of statistical experimental design and multivariate data analysis and regression. An accompanying gas chromatography–mass spectrometry analytical method was developed for the identification and quantification of the analytes, BDE 28, BDE 47, BDE 99, BDE 100, BDE 153, and BDE 154. These PBDEs have been used in commercial blends that were used as flame-retardants for a variety of materials, including electronic devices, synthetic polymers and textiles. The present study revealed that an extracting solvent mixture composed of hexane and CH_2Cl_2 (10:90) provided excellent recoveries of all of the six PBDEs studied herein. A somewhat lower polarity in the extracting solvent, hexane and CH_2Cl_2 (40:60) decreased the analyte %-recoveries, which still remain acceptable and satisfactory. The study demonstrates the necessity to perform an intimately investigation of the extraction and purification process in order to achieve quantitative isolation of the analytes from the specific matrix.

1. Introduction

1.1. Safety control of food and feed

Fresh farmed bread salmon is an important export product of Norway. Last years figures show an export volume of 683 000 tons that correspond to a value of 21.8 billion NOK [1]. This necessitates an accurate and reliable safety control of the fish products. Safety control of foodstuffs includes chemical analysis of environmental pollutants such as polybrominated diphenyl ethers (PBDE), poly-aromatic hydrocarbons (PAH), and polychlorinated biphenyls (PCB). This class of compounds can be identified and quantified by means of chromatography and mass spectrometry. Prior to the instrumental analysis, the analytes are extracted from the specific matrixes and subsequently submitted for various purification steps in order to concomitant isolate the pollutants in a pure and quantitatively fashion.

Extraction, isolation and purification of specific analytes from samples of marine origin may be a laborious task. Such process involves usually numerous laboratory operations and the utilization of series of solvents, heating, and treatment with strong acids and other various harsh reagents.

Despite several research groups have identified and quantified brominated diphenyl ethers in a variety of samples, including sediments [2], air [3–8], indoor dust [9], bird eggs [10–12], water [13–15], fish [16–18], marine mammals [19–22], human blood [23] and serum [24], adipose tissue [25–28], human liver [28] and milk [29], none of the these studies involved a systematic investigation of the experimental variables that influence the extractability of the thermal unstable PBDEs.

1.2. The analytes – polybrominated diphenyl ethers (PBDE)

Commercial available technical PBDEs have been offered as blends of penta-, octa- and decabromodiphenyl ethers, which also contain significant quantities of both tetra- and heptabromodipehnyl ethers. This class of compounds were widely used as flame retardants for a variety of products, for example on coatings on plastic housings, electrical wires, and printed circuit boards of electrical and electronic equipment, on textiles, paints, and polyurethane foam filling utilized in furniture. The annual (mid 1990s) world production of technical PBDE was approximately

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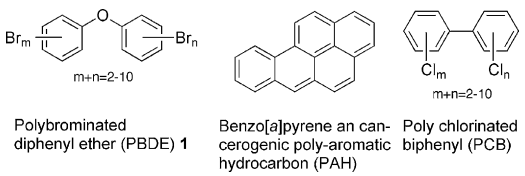

Fig. 1. Polybrominated diphenyl ether, polyaromatic hydrocarbone, and polychlorinated biphenyl.

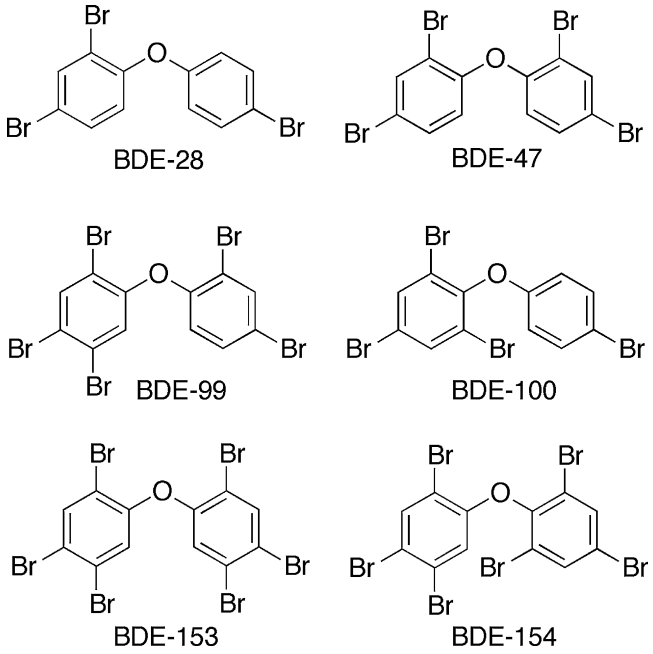

Fig. 2. Structures for the brominates diphenyl ethers BDE-28, BDE-47, BDE-99, BDE-100, BDE-153 and BDE-154.

50 k metric tons [30]. The widespread use of the PBDEs as flame retardants during the last 30 years has resulted in an unanticipated consequence, namely, a continuous and gradually significant migration of PBDEs from the flame retardant treated products to the environment. PBDEs show relatively low reactivity at ambient temperature and a high hydrophobicity, which make several of the PBDEs to environmental persistent organic pollutants (POPs). Moreover, some of the PBDE congeners, especially the tetra-, penta-, and hexabrominated ones exhibit bioaccumulation and toxicological potentials [31–33] (Fig. 1).

1.3. Chemical behaviour of the PBDEs

Brominated flame-retardants (Fig. 2) are designed to lose bromine radicals at elevated temperatures [34] (Fig. 3). The liberated bromine radical will subsequently interfere with the radical processes taking place under the combustion processes, and thus strangle the evolving fire. Due to this behaviour of the PBDEs, we believed that two aspects could be of paramount importance for the extraction process, namely, (1) exact determination (fine tuning) of experimental conditions that afford complete recovery of the analytes (the various PBDEs), (2) extraction conditions that do not exceed the chemical- and thermal stability of the BDEs present in the sample and thus avoid degradation processes (Fig. 3).

For example, a loss of one or more bromine atom(s) from a PBDE can provide an erroneously high level of some of the less brominated BDEs. A few examples are: BDE 47 – Br$^\bullet$ → BDE 28, BDE 100 – Br$^\bullet$ → BDE 28, BDE 153 – Br$^\bullet$ → BDE 99, BDE 154 – Br$^\bullet$ → BDE 99. The BDE congers that lose the bromine atom on the other hand will be detected at too low amounts. One of the major objectives was thus to develop and optimize a solvent extraction and purification protocol that combined a minimization of the chemical- and thermal stress towards the BDEs, but concurrently maintained sufficient strong conditions in order to obtain a complete recovery of the analytes.

1.4. Overall method improvement by optimizing the extraction and purification protocol

Even though PBDEs previously have been detected and quantified by ingenious and accurate analytical methods in such a large selection of samples, it has never, to the best of our knowledge, been conducted any in-dept investigation of the experimental variables that influencing the extractability of the various PBDEs. This spurred us to undertake the present study, where we investigated such features by means of statistical experimental design, multivariate data analysis, and multivariate regression. Tissue samples Atlantic salmon (*Salmo salar* L.) bred in a fish farm was used as the biological matrix. Such samples were submitted for our telescoped extraction and purification process using accelerated solvent extraction (*ASE*) with the goal to establish a robust and reliable extraction, purification, and GC–MS method.

The PBDEs 1 vary with respect both to the number of bromine atoms (1–10) as well as to the substitution pattern on the two phenyl rings. These variations furnish the various PBDE congeners different physical and chemical properties. In this context, the lipophilic dispersion was particularly interesting. On the basis of this we realized that an accurate investigation of the recovery of the BDEs during the solvent extraction process employed for the biological material was mandatory. Without an extraction that yields an optimum recovery of the analytes, in this case the PBDEs 1, the subsequent step, the quantification by means of GC–MS analysis will provide erroneously low results. Moreover, harsh conditions throughout the solvent extraction process may also lead to erroneous results since the BDEs may undergo debromination and will thus be identified and quantified as other congers or within another class of compounds.

Investigation of brominated diphenyl ethers 1 in samples of biological matrices can be divided into three discrete independent steps, namely (1) solvent extraction, (2) isolation and purification of the analytes 1, and (3) identification and quantification by means of instrumental technique. Each of the single items (1)–(3) constitutes a crucial step in the development of an overall robust and reliable analytical method. In the present work, we have focused the investigation on the extraction and analysis of the six BDE congeners shown in Fig. 2. These BDEs are found in the commercially

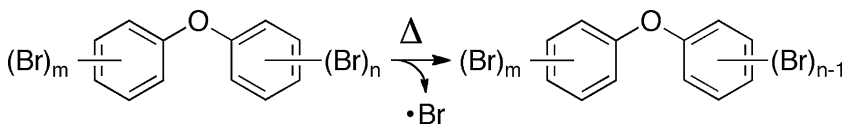

Fig. 3. Thermal degradation of brominates diphenyl ether releasing bromine radical.

Table 1
Variables and experimental levels.

	Variable	Experimental levels		
		−1	0	+1
x_1	Static time	1 min	5 min 30 s	10 min
x_2	Flush %	60%	80%	100%
x_3	Purge time	60 s	105 s	150 s
x_4	Cycles	1	3	5
x_5	Temperature	20 °C (rat.)	–	40 °C
x_6	Solvent hexane:CH_2Cl_2	80:20	–	20:80
x_7	Design of column (silica, g)	19.0 g	22.0 g	25.0 g
x_8	Number of extractions	1	2	3

available technical penta-BDE formulation and are thus some of the most environmentally dispersed and for that reason embody an important class of molecules for environmentally monitoring.

2. Experimental

2.1. Chemicals and reagents

Solvents, acids, bases and other reagents were of analytical quality and purchased from Sigma–Aldrich (Oslo, Norway). The brominated diphenyl ether standards were purchased from Chiron (Trondheim, Norway).

In order to determine the detection limits of the trace DSQ for BDE-28, BDE-47, BDE-99, BDE-100, BDE-153 and BDE-154, two sets of five standard solutions (10 samples) with concentration of 25 fg μL^{-1} and 50 fg μL^{-1}, respectively, were prepared. The solutions were analysed using the GC–MS method reported above.

BDE-28 and BDE-47 shows a detection limit of 25 fg μL^{-1} (S/N ≥ 10), whereas BDE99, BDE-100, BDE-153, and BDE-154 reveals a detection limit of 50 fg μL^{-1} (S/N ≥ 10).

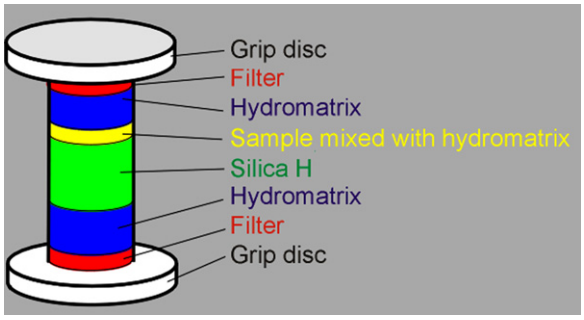

Fig. 4. Outline for the compressed column. For the ASE instrument: the *pressure* is automatically set to $p = 1500$ psi, the *heat time* is a function of the extraction temperature, so it will be constant for a certain range of temperatures, for the *type of cell*, only discrete variations are possible, small, medium and large. In this study, it was decided to use the small one. The *volume* will also be constant because it is function of the *cell type*. The ASE has sensors that regulate the volume extraction in relation of the dimension of the cell. The *compressed column* will be the same in all the experiments.

2.2. Design and preparation of a telescoped extraction and purification process

A telescoped extraction and purification process for the analytes was designed for the application with an accelerated solvent extraction (ASE) system. The ASE technology offers a rapid and convenient way for extraction of POPs from biological samples. Fig. 4 shows a sketch of the column that was designed and utilized throughout the extraction and purification of the biological sample matrices.

Accelerated solvent extraction equipment possesses a number of experimental variables that may be adjusted independently of each other. For the present work, an ASE® 300 instrument was utilized. The following instrumental variables were available for tuning the extraction process:

Table 2
Experimental design matrix. Fractional factorial design 2^{8-4}.[a]

#	Experimental variables[a]								Responses – recoveries of the BDEs					
	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8	BDE-28	BDE-47	BDE-99	BDE-100	BDE-153	BDE-154
1	−1	−1	−1	−1	−1	−1	−1	−1	103.0	91.5	60.8	52.5	46.0	41.9
2	+1	−1	−1	−1	+1	+1	+1	−1	46.0	66.5	31.2	31.8	22.3	26.7
3	−1	+1	−1	−1	+1	+1	−1	+1	116.7	112.7	89.7	89.4	66.8	73.7
4	+1	+1	−1	−1	−1	−1	+1	+1	104.7	135.1	105.7	74.6	81.2	59.3
5	−1	−1	+1	−1	+1	−1	+1	+1	99.7	119.8	91.1	67.7	66.1	53.2
6	+1	−1	+1	−1	−1	+1	−1	+1	86.5	99.4	81.7	78.7	53.4	55.5
7	−1	+1	+1	−1	−1	+1	+1	−1	60.1	73.5	68.6	64.2	40.5	40.2
8	+1	+1	+1	−1	+1	−1	−1	−1	51.3	59.9	57.9	51.7	32.8	33.0
9	−1	−1	−1	+1	−1	+1	+1	+1	104.0	93.1	59.3	52.6	42.7	40.0
10	+1	−1	−1	+1	+1	−1	−1	+1	107.2	92.1	54.1	48.4	60.0	61.6
11	−1	+1	−1	+1	+1	−1	+1	−1	110.0	93.4	58.8	50.7	38.8	36.8
12	+1	+1	−1	+1	−1	+1	−1	−1	99.0	72.4	41.3	37.6	32.3	32.2
13	−1	−1	+1	+1	+1	+1	−1	−1	45.6	48.0	57.3	43.8	58.8	64.5
14	+1	−1	+1	+1	−1	−1	+1	−1	153.8	88.9	32.5	31.7	19.9	21.5
15	−1	+1	+1	+1	−1	−1	−1	+1	122.2	90.1	36.5	33.2	22.0	23.5
16	+1	+1	+1	+1	+1	+1	+1	+1	110.3	73.2	32.6	29.7	26.7	26.6
17	0	0	0	0	−1	0	0	0	120.6	78.4	40.9	35.8	38.4	36.0
18	0	0	0	0	−1	0	0	0	143.5	97.7	29.5	30.2	15.0	16.4
19	0	0	0	0	−1	0	0	0	66.4	61.6	32.4	29.2	24.0	24.3
20	0	0	0	0	+1	0	0	0	65.0	78.2	68.2	58.9	52.0	43.1
21	0	0	0	0	+1	0	0	0	91.3	95.6	80.6	69.2	74.3	63.4
22	0	0	0	0	+1	0	0	0	88.4	83.0	70.9	58.7	63.2	55.8

[a] Generators for the experimental variables x_5–x_8 are defined by $x_5 = x_1 \times x_2 \times x_3$, $x_6 = x_1 \times x_2 \times x_4$, $x_7 = x_1 \times x_3 \times x_4$, and $x_8 = x_2 \times x_3 \times x_4$. Confounding pattern (aliases) for the two-variable interactions for this fraction factorial design are: $\beta_{12} + \beta_{35} + \beta_{48} + \beta_{67}$, $\beta_{13} + \beta_{25} + \beta_{47} + \beta_{68}$, $\beta_{23} + \beta_{15} + \beta_{46} + \beta_{78}$, $\beta_{14} + \beta_{28} + \beta_{37} + \beta_{56}$, $\beta_{24} + \beta_{18} + \beta_{36} + \beta_{57}$, $\beta_{34} + \beta_{17} + \beta_{26} + \beta_{58}$, $\beta_{18} + \beta_{27} + \beta_{38} + \beta_{45}$.
x_1 Static time [1 min, 5 min 30 s, 10 min], x_2 Flush % [60%, 80%, 100%], x_3 Purge time [60 s, 105 s, 150 s], x_4 Cycles [1,3,5], x_5 Temperature [20 °C (rat.) – 40 °C], x_6 Solvent Hexane:CH_2Cl_2 [80:20 – 20:80], x_7 Design of column (silica, g) [19.0 g, 22.0 g, 25.0 g], x_8 number of extractions [1–3].

(1) *static time* – the static solvent extraction time, (2) *flush %* – the amount of solvent flushed through the extraction cell following the static heating step, (3) *purge time* – the period nitrogen gas purges the extraction cell, (4) *cycles* – the number of times the static heating and the flushing steps is performed, (5) *temperature* – the temperature to which the extraction cell is heated, (6) *type of solvent* – the solvents that are used for the extraction, e.g. hexane, dichloromethane, acetone, ethyl acetate, or mixtures of several solvents, (7) *column design* – the height of the various layers that constitute the column package material (see Fig. 4), (8) *number of extractions* – the number of extractions that is performed, (9) *volume* – the solvent volume used during the extraction process, (10) *pressure* – the fluid pressure in the extraction cell used during the extraction process, and finally, (11) *cell type* – the dimension/volume of the extraction cells (available volumes were 34 mL, 66 mL and 100 mL), (12) *column compress* – the type of packaging material used for the extraction cell (hydromatrix, silica (H^+), Na_2SO_4, etc.), (13) *heating time* – the time the sample was heated during the extraction process.

An evaluation of these experimental variables (1–13) ended up with the variables numbered 1–8. This short list of experimental variables along with the selected experimental levels is shown in Table 1.

By means of statistical experimental design [35] using a fractional factorial design with centre experiments ($2^{8-4}+3$), of resolution R = (IV) the variables were comprehensively investigated and an entire optimization was also carried out using multivariate regression [36] and principal component analysis [37,38]. The general form of each model η_{28}, η_{47}, ..., η_{154} explaining the recovery of the analytes, is given by Eq. (1):

$$\left.\begin{aligned} y_k &= \beta_0 + \sum_{i=1}^{8}\beta_i x_i + \sum_i\sum_j \beta_{ij}x_i x_j \\ \eta_k &= \sqrt{y_k} \end{aligned}\right\} \quad k=28,\ 47,\ 99,\ 100,\ 153,\ 154 \tag{1}$$

Table 2 shows the experimental design provided in coded units along with the experimental results achieved for each of the experiments. The confounding pattern of the R = IV resolution design permit one to estimate the main factors confounded with eventually three factor interactions. The two factor interactions are confounded with other two factor interactions. A detailed scheme of the two-factor confounding pattern is given in the table footnote of Table 2.

The scaling of the experimental variables was performed according to Eq. (2):

$$x_i = \frac{z_i - \{z_{i,L} + \frac{1}{2}\times(z_{l,H} - z_{i,L})\}}{z_{i,H} - \{z_{i,L} + \frac{1}{2}\times(z_{l,H} - z_{i,L})\}}, \quad i = 1, \ldots, 8 \tag{2}$$

This scaling was performed in order to facilitate the estimation of the regression coefficients. The x_i of the equation above is the experimental variable i (i = 1, ..., 8) given in scaled units, z_i is the experimental variable i given in real units, $z_{i,L}$ and $z_{i,H}$ are the selected low (−1) and high (+1) experimental values (real unit, values given in Table 1), respectively, of the experimental variable i. The measured data (y_k) that is the %-recovery of each of the six BDEs was determined by GC–MS using calibration curves with internal standard. The root-square transformation shown in the second line of equation (1), was found to afford a better fit [$\eta = f(x_1, x_2, \ldots, x_8)$] during the regression analysis.

Table 3
Reproducibility of GC–MS method for quantification of PBDEs.

#	BDE	$\bar{c}$ [ng mL^{-1}][a]	s[b]	$Stv\%$[c]
1	28	9.64	0.27	2.7
2	47	9.77	0.25	2.5
3	99	9.90	0.29	2.9
4	100	9.83	0.31	3.2
5	153	9.60	0.66	6.9
6	154	10.02	0.36	3.6

[a] Mean value of ten runs of ten standard solutions that contain all the six PBDEs in a quantity of 10 ng mL^{-1} each.
[b] Standard deviation estimated for the ten runs of ten various samples.
[c] $Stv\% = (s \times 100) \times \bar{c}^{-1}$.

2.3. Multivariate modelling and graphical representation of the models

Computation and the graphical illustration of the multivariate models were carried out using procedures developed in-house for MATLAB version 6.1 or later [39,40]. These procedures have previously been validated by comparison with results achieved from several commercial computer programs for statistics and mathematical model building [41].

The SAS software was used to estimate regression coefficients and the adjacent product statistics for the various multivariate regression models describing each of the PBDEs outlined in Eq. (1).

2.4. The extraction–purification step

A sample (25 g of salmon) is freeze-dried for two days. From the obtained powder, a sample (2.8 g) is mixed with hydromatrix (2.2 g). Each column (for the experiments #1–22, Table 2) was constructed in the following way: a steel column with a volume of 66 mL was used. A filter was placed in the bottom of the column followed by a layer of hydromatrix (1.0 g), a layer of silica (H^+) (19.0 g, 25.0 g or 22.0 g), the dry biological sample, the BDEs solution (50 ng, 200 μL of a 250 ng mL^{-1} solution), a new layer of hydromatrix (7.0 g), and then finally a filter. The extractions were performed with an ASE® 300 accelerated solvent extraction system.

In addition to the samples, #1–22, Table 2, that were added PBDE, several blank samples were analyzed in order to determine the prior pollution. In the cases PBDEs were detected, the determined values were adjusted.

Analytical protocols for PBDEs are usually based on using a ^{13}C-PBDEs [42] or the less expensive perfluorinated PBDEs [43,44] as a standard. We decided to avoid using such a standard in the present study since one of our hypotheses imply that degradation of the PBDEs can take place under thermal and chemical stress of strong acids, and that the extrability varies significantly depending on the degree of bromination. Thus, an analytical protocol was developed using PCB-207 as internal standard containing 30 μL of a 5.0 μg mL^{-1} PCB-207 solution in ethyl acetate that was added to the extracted solution. The solvent was evaporated and adjusted to 10.0 mL with hexane:CH_2Cl_2 (50:50) in a graduated flask. An aliquot of 5.0 mL of each of the final solutions was further purified by treatment with concentrated sulphuric acid (1.0 mL, 97%), centrifuged (2500 rpm × 15 min), separated from the acidic phase, evaporated to a volume of approximately 0.5 mL and then used directly for the GC–MS analysis.

2.5. Equipment and chromatographic conditions

A Thermo Finnigan Trace GC coupled to a Trace DSQ mass spectrometer was used. Splitless with surge injections of 1 μL were made by a Thermo Finnigan AI/AS 3000 autosampler. Surge pressure was 379 kPa with a surge duration time of 1.5 min. The

Table 4
Multivariate models describing recovery of the six BDEs, BDE 28, BDE 47, BDE 99, BDE 100, BDE 153, and BDE 154, along with the scores (t_1 and t_2) and the loadings (p_1 and p_2) obtained through the principal component analysis of the regression coefficients.

Reg.	Regression models for the various brominates diphenyl ether (BDE)						Results from PCA	
Coef.	BDE 28	BDE 47	BDE 99	BDE100	BDE153	BDE154	p_1	p_2
β_0	9.5997	9.1962	7.4918	7.0404	6.5142	6.4071	−0.9922	0.0528
β_1	0.0777	−0.0007	−0.4125	−0.3495	−0.3333	−0.3448	0.0291	0.1973
β_2	0.0121	−0.0765	0.0713	0.0920	−0.1314	−0.1661	−0.0020	0.0456
β_3	−0.1320	−0.2280	−0.1762	−0.1824	−0.3727	−0.3303	0.0337	0.0542
β_4	0.6693	−0.2063	−0.8775	−0.8252	−0.5504	−0.4503	0.0413	0.5371
β_5	−0.6706	−0.2669	0.3701	0.3124	0.5748	0.5878	−0.0127	−0.4628
β_6	−0.6830	−0.5240	−0.1528	0.0484	−0.0566	0.1827	0.0270	−0.2650
β_7	0.4332	0.5101	−0.0516	−0.1614	−0.2300	−0.4141	0.0063	0.2475
β_8	0.5404	0.5875	0.5529	0.4711	0.6214	0.5098	−0.0740	−0.0247
β_{12}	−0.1290	−0.0072	0.2371	−0.0494	0.3122	0.0555	−0.0005	−0.1534
β_{13}	0.3727	−0.0279	−0.0333	0.1531	−0.2220	−0.1229	−0.0114	0.1873
β_{23}	−0.2439	−0.2643	−0.6265	−0.4819	−0.6052	−0.5407	0.0592	0.1742
β_{14}	0.4780	0.0421	−0.0865	0.0053	0.0572	0.0859	−0.0206	0.1804
β_{24}	0.2579	0.1493	−0.3978	−0.3576	−0.4769	−0.5108	0.0252	0.3202
β_{34}	0.0985	−0.1247	−0.3677	−0.3494	−0.1841	−0.1020	0.0132	0.1768
β_{18}	−0.0576	0.1055	0.3780	0.2354	0.5196	0.4117	−0.0227	−0.2514
R^2	0.772	0.793	0.747	0.813	0.702	0.811		
							Explained variance	
$t_1{}^T$	−9.6107	−9.2549	−7.6074	−7.1307	−6.6370	−6.5034	98.2%	
$t_2{}^T$	1.6064	0.5475	−0.7373	−0.5960	−0.8331	−0.7870		1.6%

injector temperature was set at 275 °C. The helium carrier gas operated in constant flow at 1.2 mL min^{-1}. The GC column was a 30 m × 0.25 mm i.d. (0.25-μm film thickness) RTX-5MS capillary column (Restek, Bellefonte, PA, USA). The GC oven program was as follows: isothermal at 110 °C for 2 min, 8 °C min^{-1} to 180 °C for 1 min, 2 °C min^{-1} to 240 °C for 5 min, 2 °C min^{-1} to 270 °C for 1 min, and 10 °C min^{-1} to 325 °C for 3 min. The GC–MS transfer line was held at 300 °C. The mass spectrometer was operating in the negative chemical ionization mode (NCI) using methane (2 mL min^{-1}) as the reagent gas and the ion-source temperature was 250 °C. Selective ion monitoring of the two bromide ions at m/z 79 and 81 was used to detect the BDEs. Dwell time and scan rate were 80 ms and 500 ms, respectively. The acquisition started after 15 min. The response factors for all the compounds were determined using quantification standards (Cambridge Isotope Laboratories, Andover, MA, USA) with known amounts of target compounds and internal standards.

2.6. Precision of the GC analytical quantification method

GC–MS analyses of the samples that contained the six various PBDEs were repeated ten times each in order to determine the accuracy and the precision of the method. The mean values of the determined concentrations, $\bar{c}_{\mathrm{BDE\,28}}, \ldots, \bar{c}_{\mathrm{BDE\,154}}$, the adjacent estimated standard deviations, $s_{\mathrm{BDE\,28}}, \ldots, s_{\mathrm{BDE\,154}}$, and the percentage standard deviation are all given in Table 3.

Table 3 displays $\%Stv \in [2.5\ 3.6]$ for five of the analytes. The BDE153 shows a somewhat larger $\%Stv = 6.9$. This variation is most probably due to the molecular difference of the analytes that results in varied responses in the GC–MS.

3. Results and discussion

3.1. Experimental results

Table 2 shows the experimental design with adjacent measured responses, the percent recoveries for the six PBDEs that was investigated. High recovery values 122% (entry #15) and 154% (entry# 14), respectively are obtained for both BDE-28 and BDE-47 120% (entry #5) and 135% (entry #4), while the higher BDEs often show modest-poor recoveries (BDE-153, 15–22%, entries #18, #14and #15). This apparently anomalous phenomenon can be justified by the BDEs instability and their tendency to lose bromine atoms furnishing lower congeners, as explained in Section 1.3 (PBDE reactivity).

The centre points experiment (entries #17–22) display acceptable reproducibility for the recoveries in entries #21–22 (performed at high temperature, $T = +1$), and entries# 17–18 (performed at low temperature $T = -1$). Entries #19 and #20 show a somewhat poor reproducibility within each under-set.

3.2. Computation results – multivariate predictive and explorative models

Multiple linear regression (MLR) [45,46] and partial least-squares regression (PLSR) [37] were utilized in order to establish the six discrete multivariate predictive models, Eq. (1), one for each of the BDE congers described in Fig. 2.

Table 4 provides the estimated numerical values of the regression coefficient β_0, β_1, β_2, …, β_{18} $(+\beta_{27} + \beta_{38} + \beta_{45})$ for each of the multivariate models. The numerical values provided in Table 4 embody the values as estimated through least-squares fit.

In order to simplify the comparison of these models, the regression parameters were formed into a 16 × 6 element matrix that in transposed form[1] was submitted to principal component analysis (PCA). The goal of this PCA was two-fold, namely, (1) to determine the similarity or dissimilarities between the six various models, and (2) to identify which of the experimental variables that shows significant influence on the recovery of the various BDEs in the extracted samples. The principal component analysis provided a model composed of two principal components, which explained ≈99.7% of the total variance in the data matrix. Principal component #1 (PC#1) accounted for 98.2% of the variance and 1.6% was

[1] Table 4 provides the regression coefficients in a form where the columns represent a model, and each row a regression coefficient. In order to analyze this data table by means of principal component analysis, the table must be transposed, that is converting the column into rows and rows into columns. In this way, each empirical equation becomes an object and the regression coefficients become the variables.

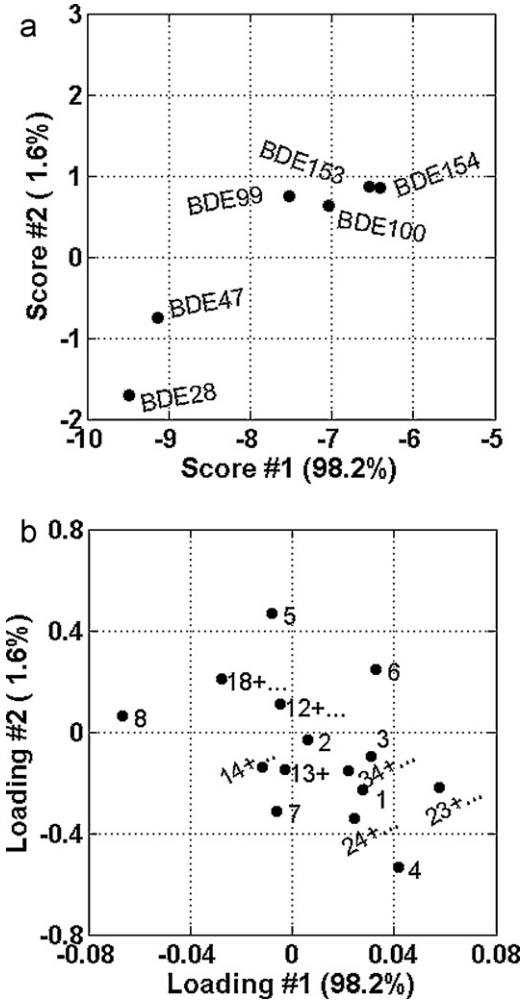

Fig. 5. (a) 2D score plot of score PC#1 versus score PC#2 achieved from an principal component analysis (PCA) of the regression coefficients of multivariate predictive models estimated for the recovery of the brominated biphenyl ethers BDE28, BDE47, BDE99, BDE100, BDE153, and BPD154 from matrixes of salmon tissue. The PCA explains ≈99.7% of the total variance in two (a = 2) principal components (98.17% + 1.57%). (b) The corresponding 2D loading plot shows the importance of the various variables expressed in the PC loading #1 that is spanned by experimental variable x_8 and the interaction $x_2x_3 + x_1x_5 + x_4x_6 + x_7x_8$ and the PC loading #2 that is spanned by x_4 and x_5.

described by PC#2. Even though the PC#2 only account for 1.6% of the explained variance, we selected to include this and interpret this as an significant factor. Fig. 5 shows the 2D score plot of PC score #1 versus PC score #2. The scoreplot 5(a) shows structure in both direction spanning the various PBDEs along a diagonal from lower left corner up to upper right corner, whereof the less substituted PBD is found in the lower left corner and most substituted PBDEs in the upper right corner.

This plot points out that BDE99, BDE100, BDE153, and BPD154 are similar due to their assembling in the same region of the 2D-score plot (the upper right corner). BDE47 and BDE28, are different from the four other BDEs, since these two are located in another part of the 2D-score plot, namely on the left hand side in the lower left corner. In addition to being separated along the principal component #1 axis, the two groups of BDEs are also separated along principal component #2. Examining the 2D-loading plot, the PC loading #1 versus the PC loading #2 reveals that the coefficients β_8 (−) and $\beta_{23} + \beta_{15} + \beta_{46} + \beta_{78}$ (+) span the principal component #1, although with minor contribution of β_1, β_3 and β_4, while principal component #2 is spanned by the coefficients β_4 (+) and β_5 (−) with minor contributions of the coefficients β_6, β_7, $(\beta_{34} + \beta_{17} + \beta_{26} + \beta_{58})$, and $(\beta_{18} + \beta_{27} + \beta_{38} + \beta_{45})$.

On the basis of the assumption that the six preliminary models provided in Table 4 constitute two discrete groups that each exhibit different behaviours; one model from each of the two groups was selected for further interpretation.

The preliminary models for the recovery of BDE28 and BDE154 (η_{28} and η_{154}) were hence subjected for thorough interpretation and variable pruning. The interpretation of model η_{28} (that thus also includes BDE 47), was proposed to include the following coefficients in the final model: β_0 (mean value), β_4 (cycles), β_5 (temperature), β_6 (solvent), β_8 (number of extractions), and the interactions: $\beta_{13} + \beta_{25} + \beta_{47} + \beta_{68}^*$, $\beta_{23} + \beta_{15} + \beta_{46}^* + \beta_{78}$, and $\beta_{14} + \beta_{28} + \beta_{37} + \beta_{56}^*$. The cross terms of the three confounded various two-variable interactions are the proposals for the actual interaction. Among the possible two-factor interactions, terms emphasized in bold face were expected to be the actual ones. A new multivariate regression using the PLSR method provided the following model using a = 3 principal components to explain 99.7% of the variance in the response (that is $\sqrt{y_{28}}$). The estimated regression model is given in Eq. (3):

$$y_{28} = (9.651 + 0.5963 \times x_4 - 0.5739 \times x_5 - 0.6109 \times x_6 + 0.6867 \times x_8 + 0.5088 \times x_6 \times x_8 - 0.3722 \times x_4 \times x_6 + 0.6205 \times x_5 \times x_6)^2 \quad (3)$$

The product statistics indicate a model with reasonably good predictive ability: R^2 = 0.855, Q^2 = 0.586, Press = 18.511, RMSEP = 0.569, RSD = 0.600.

In a similar way, further analysis was performed on the model describing the recovery of BDE 154 (and thus also BDE 99, BDE 100, and BDE 153). The interpretation of the preliminary model suggested to include the following coefficients in the final model: β_0 (mean value), β_1 (static time), β_3 (purge time), β_4 (cycles), β_5 (temperature), β_7 (design of column (silica, g)), β_8 (number of extractions), $\beta_{23} + \beta_{15}^* + \beta_{46} + \beta_{78}^*$, $\beta_{24} + \beta_{18}^* + \beta_{36} + \beta_{57}^*$, $\beta_{18}^* + \beta_{27} + \beta_{38}^* + \beta_{45}^*$, where the starred$^{(*)}$ coefficients are the ones we thought were the most likely, and decided to look closer at these.

The variable pruning was carried out and a new model was established using the PLSR method. The model achieved explained 99.5% of the variance with a = 5 PLS components. The estimated numerical values of the regression coefficients are provided in

Table 5
Optimizing extraction experiments.

#	Experimental variables[a]								Measured responses[b] – the recoveries of the BDEs					
	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8	BDE 28	BDE 47	BDE 99	BDE100	BDE153	BDE154
1	5′30	80	105	5	48	40:60	22	1	85	81	87	88	92	96
2	5′30	80	105	3	50	10:90	22	1	104	105	105	103	103	99

[a] x_1 Static time [min], x_2 Flush % [0%], x_3 Purge time [s], x_4 Cycles [number of cycles], x_5 Temperature [°C], x_6 Solvent Hexane:CH_2Cl_2 [vol:vol], x_7 Design of column [silica, g], x_8 Number of extractions.
[b] % – recovery of the various brominated diphenylethers of spiked samples.

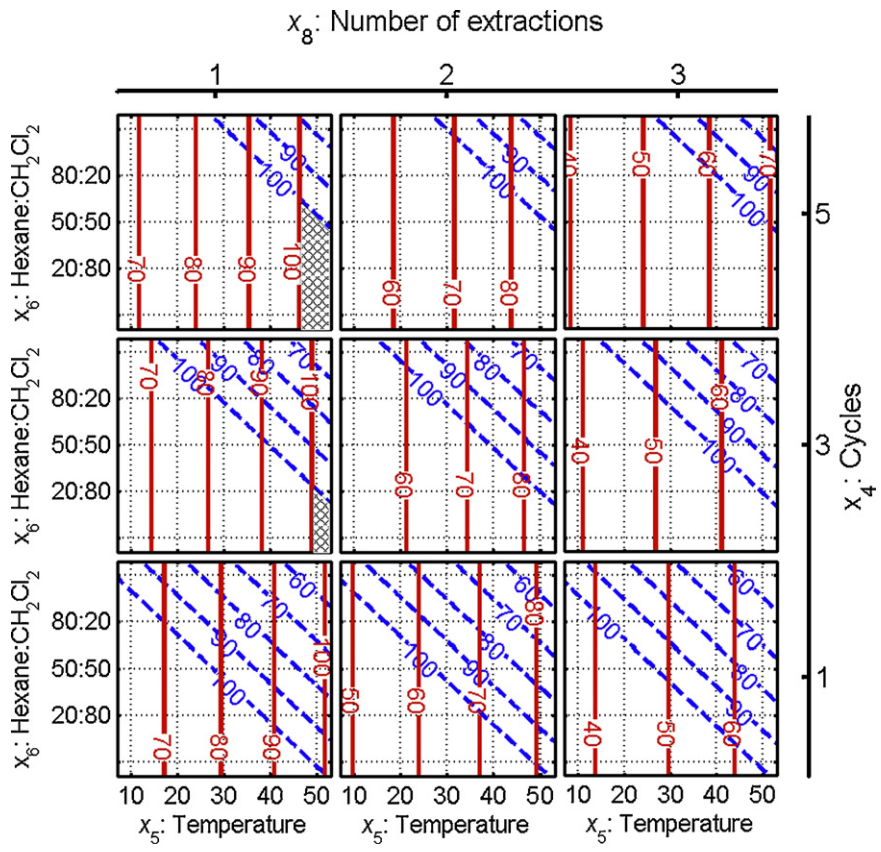

Fig. 6. The is-contour plot of the response surface shows the predicted %-recovered BDE 28 and BDE 154 as a function of four experimental variables, cycles (x_4), temperature (x_5), type of solvent blend (x_6), and number of extractions (x_8). The dashed (blue coloured) contour lines show the %-recovered BDE 28 and the solid lines (red coloured) illustrate the %-recovered of BDP 154. The two experimental variables x_4 and x_8 are varied at three discrete levels each, while the two variables x_5 and x_6 are continuously varied over the given range. The other experimental variables were set at fixed levels, namely, static time (x_1) = −1 (1 min), flush % (x_2) = +1 (100%), purge time (x_3) = −1 (60 s), design of column, that is the amount of silica [g] (x_7) = −1 (19 g). The hatched areas that are curtailed by the contour lines and the axes of the plot represent the areas where an optimized recovery of all of the analytes is predicted to be found. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Eq. (4):

$$y_{154} = (6.510 - 0.3430 \times x_1 - 0.3293 \times x_3 - 0.4423 \times x_4 + 0.4726 \times x_5 - 0.4561 \times x_7 + 0.4983 \times x_8 - 0.5274 \times x_2 \times x_3 - 0.4993 \times x_2 \times x_4 + 0.3691 \times x_1 \times x_8)^2 \quad (4)$$

The product statistics indicate a model with reasonably good predictive ability: $R^2 = 0.852$, $Q^2 = 0.538$, Press = 13.190, RMSEP = 0.449, RSD = 0.472.

The two models, Eqs. (3) and (4), were used to produce the multivariate response surfaces that explain the effect of the four experimental variables: x_4, x_5, x_6, and x_8 on the two responses that explain %-recoveries of the analytes BDE 28 (+BDE 47) and BDE 154 (+BDE 99, BDE 100, BDE 153), see Fig. 5.

The experimental variables, x_1, x_2, x_3, and x_7 that were identified as not making any significant contribution to explain the variation on the recovery of the analytes, can be considered to be kept at the fixed level corresponding to the centre values of the experimental domain (the constrains that constitute the experimental domain are provided in Table 1).

The experimental variables, x_4, x_5, x_6, and x_8 taken together with the two responses y_{28} and y_{154}, curtail only small regions of the experimental domain where a concurrent and complete (optimized) recovery of the two analytes BDE 28 and BDE 154 is expected to be found. Furthermore, taking this together with the result of the principal component analysis described above, it is to be expected that the optimized recovery of the four analytes BDE 47, BDE 99, BDE 100, and BDE 153 will be found in the same experimental region. The solid contour lines represent the predicted recovery of BDE 154, and the dashed contour lines represent the predicted recovery of BDE 28. The hatched areas that are curtailed by the contour lines and the axes of the plot represent the areas where an optimized recovery of all of the analytes is expected to be found.

Table 5 shows results from experiments conducted in order to evaluate the predictions of the developed models (3) and (4). The extraction conditions provided in entry #1 and #2 of Table 5 are both taken from the hatched areas of the contour plot provided in Fig. 6.

According to the mathematical models obtained, a complete recovery is expected (predicted) for each of the two experiments. Found recovery values were approximately 100% for all of the analytes 1 for the experiment reported in entry #2, Table 5. For the experiment reported in entry #1 of Table 5, the recoveries were determined to be in the range 81–96%.

The differences between the two experiment, entry #1 and #2 of Table 5, reveals that the more polar extracting solvent mixture (10:90 hexane:CH_2C_2) ensures excellent recoveries of all of the six BDEs studied herein. Some of the practical results of this study are: higher polarity of the extracting solvent mixture allows to (1) reduce the number of cycles (x_4), (2) the extracting procedure becomes faster, and the overall analytical time is thus reduced, and (3) the method becomes more suitable for routine analysis.

Even though the models predict a complete recovery for the extraction conditions reported in entry #1, Table 5, somewhat

lower, but acceptable and satisfactory, recovery-% values of the analytes were achieved. An explanation of this can be due to the fact that a major polarity of the extracting solvent discriminate in a minor extent the different brominated compounds on the base of the number of present bromine atoms, ensuring a complete extraction. With a lower polarity of the solvents, a less efficient extraction of the BDE congeners is achieved. In this way only BDE congeners with several bromine atoms present are favoured in the extraction procedure. In fact, results of entry #1 reveal in general that increased recoveries could be related to the number of bromine atoms present in the congeners.

4. Conclusion

A multivariate study for the quantitative analysis of six different brominated diphenyl ethers (BDEs) in salmon tissue is disclosed. It was found that the more polar extracting solvent mixture (10:90 hexane:CH_2C_2) combined with a reduced number of extraction cycles ensured excellent recoveries of all of the six BDEs studied herein. Moreover, the control experiments conducted utilizing the provided settings confirmed the predictability of the two models, which furnishes an essential basic tool for the development of extraction and purification protocols for environmental monitoring of many other biological matrices.

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