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High throughput sample preparation in combination with gas chromatography coupled to triple quadrupole tandem mass spectrometry (GC–MS/MS): A smart procedure for (ultra)trace analysis of brominated flame retardants in fish

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ABSTRACT

In this study, gas chromatography (GC) coupled to triple quadrupole tandem mass spectrometry (MS/MS) operated in electron ionisation mode (EI) has been shown to be an effective tool for the (ultra)trace analysis of several representative brominated flame retardants (BFRs) including polybrominated diphenyl ethers (PBDEs), pentabromotoluene (PBT), pentabromoethylbenzene (PBEB), etc. in complex food and environmental matrices. Using this type of instrumentation, improved selectivity and sensitivity of the instrumental analysis was achieved. In addition to GC–MS/MS (EI), a GC–MS method employing QqQ as a single quadrupole in negative chemical ionisation (NCI) mode was also developed, as this technique might be preferred for those compounds where EI did not provide suitable (intensive enough) mass transitions (e.g., decabromodiphenyl ethane). Following the development of the GC–MS/MS method, a substantial simplification of the sample preparation method was achieved by employing an ethyl acetate QuEChERS-based extraction followed by silica minicolumn clean-up. Using this novel approach, six samples may be prepared in approx. one hour, thus significant time savings were achieved compared to routinely used methods. In addition, the method employs the reduced amounts of organic solvent and other chemicals. Under the optimised conditions, recoveries of all target analytes using both GC–MS/MS (EI) and GC–MS (NCI) were within the range of 70–119% and repeatabilities of the analytical procedure were $\leq$16% at all three spiking levels (0.1, 1 and 5 μg kg^{-1}). Regarding quantification limits (LOQs), as expected, a single quadruple operated in NCI provided significantly lower LOQs compared to EI. However, using the triple quadrupole mass analyser, comparable LOQs were achieved for both methods (0.005–1 μg kg^{-1} and 0.005–0.1 μg kg^{-1} for GC–MS/MS (EI) and GC–MS (NCI), respectively). Moreover, when highly selective mass transitions in GC–MS/MS (EI) were used for identification and quantification, a significant decrease of problematic interferences was observed compared to NCI where most of the compounds were quantified according to the less selective *m*/*z* 79 corresponding to a bromine atom.

1. Introduction

Clear evidence exists that fish consumption provides health benefits for the cardiovascular system and is suitable for secondary prevention in coronary heart disease. Being an important source of long chain *n*-3 polyunsaturated fatty acids, fatty fish, in particular, may significantly contribute to consumers' dietary exposure to several classes of contaminants. In addition to persistent organochlorine compounds, recent market basket studies have detected polybrominated diphenyl ethers (PBDEs) as ubiquitous contaminants of this important commodity and fish along with seafood are classified as the main food commodities responsible for their dietary intake [1]. With the exception of three commercial PBDEs mixtures, the usage of BFRs has until now mainly comprised of tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD) [1]. As a result of their potential to bioaccumulate in the environment, the goods containing more than 0.1% of PentaBDE (brominated diphenyl ether) and OctaBDE technical mixtures have been prohibited in the EU since August 2004, and the ban was further extended to electrical and electronic goods with DecaBDE in July 2008 [2,3]. In response to these legislation acts, the 'alternative' BFRs suitable for commercial applications as an option

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to PBDEs have been introduced to the market. As might be assumed several of them such as bis(2,4,6-tribromphenoxy)ethane (BTBPE) and decabromodiphenyl ethane (DBDPE) have been already detected in the environment [4]. Moreover, the continuous release of PBDEs in to the environment from products that remain in use or from land fill sites cannot be avoided [5].

Based on the composition of PBDE technical mixtures and occurrence in the environment, the majority of studies to date have been mainly focused on eight PBDE congeners of primary interest (BDE 28, 47, 99, 100, 153, 154, 183 and 209), which were, together with HBCD and brominated biphenyl (BB) 153, included by the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM Panel) into the core group of BFRs, that should be monitored and which are relevant for dietary exposure [6,7].

Taking into account all the above mentioned facts, a simple, inexpensive, rapid and highly sensitive analytical method, which enables collection of a large set of reliable data in a short time, is needed to fulfil the effectiveness for the control of food contamination.

As regards common laboratory practices, gas chromatography (GC) coupled to mass spectrometry (MS) represents the 'gold standard' determinative step for the analysis of BFRs in biotic matrices. Considering a poor tolerance of this technique to non-volatile matrix impurities, attention has to be paid to the proper choice of sample preparation strategy. In many cases, time consuming multi-step procedures including (i) non-selective isolation of lipids followed by (ii) various clean-up steps and fractionation are used. Typically, extraction in a Soxhlet apparatus with large volumes of non-polar or semi-polar organic solvents is carried out to isolate target analytes from the biotic matrices. Lipids and other co-extracts are further removed using gel permeation chromatography (GPC) and/or solid-phase extraction (SPE) with different sorbents [8–12]. A destructive clean-up technique such as sulphuric acid treatment is also applied in some laboratories. Alternatively, semi-automated techniques including microwave assisted extraction (MAE), pressurised liquid extraction (PLE) or super critical fluid extraction (SFE) are used [8–12]. Most recently, another novel approach derived from the QuEChERS (quick, easy, cheap, effective, rugged and safe) procedure [13,14] (originally developed for the analysis of pesticide residues in low fat-high moisture matrices), has been successfully adopted for the analysis of PBDEs and other organic pollutants in fish using GC–MS in electron ionisation (EI) mode with a time-of-flight (TOF) ion analyser [15].

Currently, GC–MS with unit resolution mass analysers operated both in EI or negative chemical ionisation (NCI) mode are most often employed for identification and quantification of PBDEs and other BFRs in complex food and environmental matrices [12,16,20]. When higher brominated compounds are included in the list of target analytes, NCI mode which enables monitoring of bromine ions $[Br]^-$, isotopes m/z 79 and 81, is the preferred option providing higher sensitivity compared to EI [10,12,16,20]. However, this detection approach is not selective enough, other co-eluting thermodegradable highly brominated compounds might interfere [16,18,19]. There is an increased risk of interferences occurring whenever relatively short capillary columns (10–15 m) and fast oven temperature programs intended for a 'gentler' GC separation are employed [12]. On the other hand, while operating in EI, more specific $[M]^{+\bullet}$ and $[M–Br_2]^+$ serve as identification ions, nevertheless, potential interferences with chlorinated compounds might occur [10,12,18,19]. In any case, the use of EI low-resolution (LR) MS is a good tool for the determination of brominated compounds only at relatively high concentration levels; on the other hand, it allows very accurate quantification, as ^{13}C-labelled standards might be used [10,12,16]. High-resolution (HR) instruments represent another reliable option in the analysis of brominated compounds offering higher sensitivity compared to 'traditional' LRMS. The detection limits (LODs) obtained by HRMS strongly depend on the type of ion analyser, for example, common HRTOF–MS instruments offering the possibility of retrospective data mining (as full spectral information is available), might not enable better LOD compared to conventional single quadrupoles (Q) [12,16,18,19].

In order to overcome the limitations discussed above, the use of tandem MS (MS/MS) using ion trap (IT) or triple Q (QqQ) analysers should be considered as the best alternative, minimising interferences by improved selectivity based on selection of appropriate precursor and product ions. Moreover, a significant decrease of chemical noise in the chromatogram is obtained, thus, thanks to the improved sensitivity, reliable determination of even (ultra)trace levels of BFRs required e.g., for human exposure studies, is feasible [16–19,21]. Several previous studies have been reported on the application of IT for the trace analysis of PBDE in different matrices [18,19,22]. On the contrary, the power of the QqQ up until now has been mainly demonstrated in pesticide residues analysis and/or organic contaminants other than BFRs [17,23–27]. There has been a very limited number of publications produces on BFRs, focusing mainly on water [28,29], human breast adipose tissue [17,19,21] and fish [18,30]. However, in the latter case, only PBDEs were included in the list of target analytes.

In the presented study, the application potential and suitability of GC–QqQ–MS/MS (EI) for the (ultra)trace analysis of BFRs in fish muscle tissue was evaluated and compared with GC–MS employing QqQ as a single quadrupole both in EI and NCI mode. A large number of BFRs including not only the above mentioned priority PBDEs (BDE 28, 47, 99, 100, 153, 154, 183 and 209), but also additional PBDE congeners (BDE 49, 66, 85, 196, 197, 203, 206 and 207) and alternative BFRs were included in the target analyte list. Integration of QqQ detection technique into BFRs analysis was expected to further improve an overall performance of the procedure based on a high throughput sample preparation approach originally developed for the multi-class analysis of persistent organic pollutants (POPs) and polycyclic aromatic hydrocarbons (PAHs) in fish [15].

2. Experimental

2.1. Standards

Certified standards of individual PBDE congeners (No. 28, 37, 47, 49, 66, 77, 85, 99, 100, 153, 154, 183, 196, 197, 203, 206, 207, 209), ^{13}C-BDE 209, hexabromobenzene (HBB), pentabromotoluene (PBT), pentabromoethylbenzene (PBEB), bis(2,4,6-tribromophenoxy)ethane (BTBPE), octabromo-1-phenyl-1,3,3-trimethylindane (OBIND) and decabromodiphenyl ethane (DBDPE) (all with declared purity $>98\%$) were supplied by Wellington Laboratories (Guelph, Ontario, Canada). Calibration solutions prepared in isooctane containing BDE 28–203, HBB, PBT, PBEB and BTBPE at concentration levels 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 and 500 ng mL^{-1} and BDE 206, 207, 209, OBIND and DBDPE at 0.25, 0.5, 1, 5, 10, 50, 100, 500 and 1000 ng mL^{-1} were stored at 5 °C. Each calibration level contained surrogate standard BDE 37 at 10 ng mL^{-1} and syringe standards BDE 77 and ^{13}C-BDE 209 at 5 and 50 ng mL^{-1}, respectively. For the acquisition of full scan spectra and further MS/MS transition optimisation, individual standards of all compounds were prepared in isooctane (10,000 ng mL^{-1}) and stored as stated above. The standard reference material Lake Michigan Fish Tissue, SRM 1947 ($10.4 \pm 0.5\%$ (w/w) of fat) was supplied by National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

2.2. Chemicals, reagents and other material

n-Hexane, dichloromethane and isooctane were supplied by Merck (Darmstadt, Germany). Ethyl acetate was purchased from Sigma-Aldrich (Steinheim, Germany). All solvents were of analytical grade. Silica (0.063–0.200 mm) supplied by Merck was activated by heating at 180 °C for 5 h than deactivated by adding 2% of deionised water, shaking for 3 h and finally, stored in a desiccator for 16 h before use. Magnesium sulphate and sodium chloride needed for the QuEChERS-like extraction were delivered from Sigma-Aldrich and Lach-ner (Neratovice, Czech Republic), respectively. Pasteur pipettes (D812, 230 mm length) and glass wool were obtained from Poulten & Graf GmbH (Wertheim, Germany) and Merck, respectively.

2.3. Instruments

A tissue grinder was supplied by Retsch (Haan, Germany). A rotary vacuum evaporator Buchi Rotavapor R-114 and R-200 with a heating bath were obtained from Buchi Rotavapor (Flawil, Switzerland). A centrifugal machine Rotina 35R was supplied by Hettich Zentrifugen (Tuttlingen, Germany).

2.4. Tested material

For the quality assurance/quality control (QA/QC) of the entire method, trout (2.1% (w/w) of fat) was used, which was obtained from the Czech retail market and has been previously tested for the presence of BFRs.

2.5. Extraction and clean-up

The fish samples were prepared by a method described by Kalachova et al. [15] which was originally designed for the analysis of PAHs, polychlorinated biphenyls (PCBs) and selected lower brominated PBDEs. In this study, a sample preparation method was adjusted for the analysis of a wide range of brominated compounds including those highly brominated. Briefly, 10 g of fish tissue homogenate (with surrogate BDE 37–10 ng absolute) was mixed with 5 mL of distilled water and shaken vigorously with 10 mL of ethyl acetate in a polypropylene centrifuge tube for 1 min. Subsequently, 4 g of anhydrous magnesium sulphate and 2 g of sodium chloride were added to the mixture. The tube was shaken for another 1 min, centrifuged, and an aliquot of 5 mL was removed from the upper organic layer. The solvent (5 mL) was carefully eliminated to the last drop under the gentle stream of nitrogen.

The evaporated extract was re-dissolved in 1 mL of *n*-hexane and purified using a handmade silica minicolumn. The fat determination and the choice of the silica minicolumn size according to the fish muscle fat content are described elsewhere [15]. Collected eluate was carefully evaporated using a vacuum rotary evaporator and the residual solvent was removed under a gentle stream of nitrogen. Residues were finally re-dissolved in 0.5 mL of isooctane (the final concentration of matrix was 10 g mL^{-1}) containing BDE 77 (5 ng mL^{-1}) and ^{13}C-BDE 209 (50 ng mL^{-1}) used as syringe standards.

2.6. Instrumental analysis

All GC–MS experiments were performed using a gas chromatograph Agilent 7890A GC (Agilent Technologies, Santa Clara, CA, USA) coupled to a triple quadrupole mass spectrometer Agilent 7000B MS (Agilent Technologies) operated both in EI and NCI mode. The GC system was equipped with an Agilent 7693A autosampler (Agilent Technologies), a carbon dioxide cooled multimode inlet (MMI) and a pneumatics control module (PCM). For the separation, a DB-XLB capillary column (15 m × 0.18 mm i.d. × 0.07 μm film thickness; Agilent Technologies) was used. Optimised conditions of different GC set-ups tested within the experiments are summarised in Table 1.

For methods (i) and (iii), the triple quadrupole was operated in multiple reaction monitoring (MRM) mode detecting two transitions per analyte as listed in Table S-1 (Supplementary data). The temperatures of the transfer line, ion source, 1st and 2nd quadrupole were 300, 280, 150 and 150 °C, respectively. The collision cell gases were nitrogen (1.5 mL min^{-1}) and helium (2.25 mL min^{-1}). The electron multiplier (EM) gain set-up is shown in Table S-1 (Supplementary data). Both MS resolutions were 1.2 amu full width at half maximum. The dwell times were adjusted to 20–80 ms depending on the number of transitions per time window to achieve 5 cycles s^{-1} (Hz).

For methods (ii) and (iv), the QqQ was operated as a single Q in selected ion monitoring (SIM) mode in EI and NCI monitoring two and four ions per analyte, respectively. The ions monitored in method (ii) were identical with precursor ions in the method (i) and (iii) as well as the EM gains and dwell times. For method (iv), the quantification and confirmation ions along with EM gains are summarised in the Table S-2 (Supplementary data). The dwell times were adjusted to 9–65 ms depending on the number of ions per time window to achieve 5 cycles s^{-1} (Hz). In the NCI method, methane was used as ionisation gas at 40% flow rate. The temperatures of the transfer line, ion source and 1st quadrupole were 300, 150 and 150 °C, respectively.

Table 1
Optimised conditions of several GC set-ups tested within the experiments.

System	(i) GC–MS/MS (EI) (ii) GC–MS (EI)	(iii) GC–MS (EI)	(iv) GC–MS/MS (NCI)
Injection mode	Cold pulsed splitless	Solvent vent PTV	Cold pulsed splitless
Injector temperature program	80 °C (0.20 min), 600 °C min^{-1} to 285 °C	50 °C (0.17 min), 600 °C min^{-1} to 325 °C	80 °C (0.20 min), 600 °C min^{-1} to 285 °C
Vent time, flow and pressure	–	0.17 min, 50 mL min^{-1}, 5 psi	–
Injection pulse pressure	50 psi	–	50 psi
Splitless period	1.5 min	2.67 min	1.5 min
Carries gas flow (He)	1.5 mL min^{-1} (11 min), 15 mL min^{-1} to 3 mL min^{-1}	1.5 mL min^{-1} (11 min), 15 mL min^{-1} to 3 mL min^{-1}	1.5 mL min^{-1} (14.5 min), 15 mL min^{-1} to 3 mL min^{-1}
Injection volume	2 μL	8 μL	2 μL
Oven temperature program	110 °C (1.5 min), 30 °C min^{-1} to 320 °C, (3.5 min)	110 °C (2.67 min), 30 °C min^{-1} to 320 °C, (5.5 min)	110 °C (1.5 min), 15 °C min^{-1} to 180 °C, 5 °C min^{-1} to 200 °C, 30 °C min^{-1} to 320 °C (5 min)
Run time	12 min (13.5 min including DBDPE)	15.2 min (16.8 min including DBDPE)	19.2 min

MassHunter quantitative analysis software (v. B.04.04) (Agilent Technologies) was used for data processing.

3. Results and discussion

As mentioned in the Introduction, BFRs are ubiquitous contaminants occurring widely in all areas of the environment including the aquatic ecosystem. 'Cocktails' of various BFRs both obsolete and those in current use might be accumulated in fish. It is worth pointing out that this otherwise healthy food may represent to humans the main source of exposure to BFRs (similarly to other POPs). In order to comprehensively assess the contamination patterns of a large series of fish samples, a new analytical procedure, meeting high-end criteria in terms of performance characteristics, labour demands/sample throughput as well as cost effectiveness, has been implemented in our study.

Attention was paid both to the selection of a suitable sample preparation procedure and optimisation of GC–MS determination step. Altogether, 22 BFRs representing various structure classes were included in the list of target analytes. In the first phase, detection/quantification based on MS employing a QqQ mass analyser was carefully optimised. In order to cover all the possible alternatives for fish extract analysis, QqQ operated as a single Q, using both EI and NCI, was also tested. With regards to the aim to reliably determine even (ultra)trace levels of target BFRs, large volume (LV) programmable temperature vaporisation (PTV) injection technique as a tool to further decrease of limits of quantification (LOQs) was also employed for sample introduction. Once the determination step was optimised, the sample preparation procedure based on (i) partition of analytes (induced by added inorganic salts) from the sample homogenate into the ethyl acetate organic and (ii) silica mini-column clean-up, was validated. This fast and simple QuEChERS-like approach was developed in our earlier study that concerned the isolation of multiple-classes of contaminants [15]. Since BFRs were represented only by seven major tri- to hepta-BDEs, a detailed description of method implementation for other BFRs is provided in the paragraphs below.

3.1. GC–MS/MS (EI) optimisation

Within the GC–MS/MS (EI) experiments, appropriate GC conditions together with suitable mass transitions for all target compounds had to be determined. The optimisation of the MS/MS method consisted of (i) acquisition of respective MS spectra in full scan mode (m/z 100–1000 mass range); (ii) selection of precursor ions; (iii) product ion scans at different collision energies (CEs) 5, 10, 15, 20, 25, 30, 35 and 50 eV; and (iv) fine tuning of CE in MRM mode. For each compound, two MS/MS transitions were chosen to fulfil the generally applied identification criteria: according to the SANCO document [31], one precursor ion with two product ions or two precursors with one product ion should be employed. It should be noted that the set of quality control (QC) requirements to which we refer here was originally designed for pesticide residue analysis, but is now also commonly applied to other organic food contaminants. An overview of quantitative and confirmation MS/MS transitions and CE selected for each compound in EI mode is given in Table S1 (Supplementary data). Using full scan mode, the major (the most intensive) ions for most compounds were chosen either from $[M]^{+}\bullet$ or $[M–Br_2]^{+}$ isotopic pattern, and identified as suitable precursors. However, several exceptions were observed. For decabrominated BDE 209 and its ^{13}C-labelled analogue, ions in $[M–Br_4]^{+}$ pattern were also highly intensive. In the case of OBIND, ions $[M–CH_3]^{+}$ and $[M–C_{11}H_9Br_4]^{+}$ were formed, and $[M–C_6H_2Br_3O]^{+}$ was observed as the most intensive ion in the mass spectrum of BTBPE. For DBDPE, isotopic pattern corresponding to $[M–C_7H_2Br_5]^{+}$ was detected. The fragmentation of the selected precursor ion led subsequently to the formations of product ions. For PBDEs, the most selective and sensitive MS/MS transitions applicable for quantification and confirmation purposes were obtained by the loss of losing Br_2 and COBr from the respective precursor ion. In the case of 'alternative' BFRs (BTBPE, HBB, PBT, PBEB and OBIND), the loss of Br, CH_3, and CH_3CH_2 was also observed; for more details see Table S1. To obtain the best precursor to product ion transition signal, different CEs (between 5 and 50 eV) were tested. Higher brominated compounds (with more than seven bromine atoms in their molecule) typically required higher CEs and the optimal values reached up to 49 eV for BDE 209. As the next step, the dwell time parameter was optimised to obtain at least 10 data points per peak, thus providing a good peak shape, and, at the same time, maintain sufficient sensitivity [32]. As a result, depending on the number of MS/MS transitions in each particular time segment, values in the range of 20 and 80 ms were chosen. Moreover, to intensify the signal of higher BFRs that provided less intensive signal compared to lower BFRs, a higher EM gain was set, as shown in Table S1. However, for DBDPE, despite using EM gain 100, very poor signal belonging to this BFR was observed. As a consequence, due to the unsatisfactory sensitivity of this analyte, which was unsuitable for the (ultra)trace analysis of the environmental samples, DBDPE was not included in the GC–MS/MS method employing EI (nor in the method employing GC–MS in EI). On the other hand, it was possible to detect it with good sensitivity using GC–MS in NCI.

Following the development of the MS/MS detection strategy, the injection and chromatographic parameters were optimised. The extracts and standards in isooctane were injected using 2 μL in cold pulsed splitless mode (see Table 1) which is specially suitable when thermally labile compounds (such as BDE 209) are to be analysed. Regarding chromatographic separation when analysing PBDEs, not only resolution of target analytes but also potential co-elutions with other non-target compounds have to be taken into consideration since many isomers may be present in real-life samples. Considering this issue, 30–60 m long capillary columns may seem to be the best option. However, when highly brominated thermally degradable compounds have to be analysed, shorter columns (10–15 m) are often required. Unfortunately, due to the faster GC run, the risk of co-elutions may arise [8–10]. Using tandem mass spectrometry in EI mode, the problem of co-elution with other brominated compounds, which might be encountered especially in NCI (m/z 79 used as a quantification ion for all analytes), could be easily resolved as highly selective MS/MS transitions are used. When PBDE isomers with the same number of bromine atoms has to be analysed, chromatographic separation is the only mean by which they may be distinguished. In our case, a 15 m long DB-XLB capillary column with non-polar stationary phase was used for the analyte separation. At the same time, a short as possible oven temperature program was employed to minimise the thermodegradation of BDE 209 and at the same time maintaining good chromatographic separation of early eluting PBDEs. An example of the chromatogram of the fish tissue spiked with the mixture of all target BFRs at the concentration 1 μg kg^{-1} is shown in Fig. 1. Baseline separation was achieved for all compounds except for BDE 28 & PBT, BDE 37 & PBEB, BDE 49 & HBB, and BDE 196 & 203. However, compounds in first three pairs had different MS/MS transitions, thus quantification could be easily achieved. In the case of isomeric BDE 196 & 203, the chromatographic resolution of 0.72 was achieved, and even though the peaks were not baseline separated, their quantification was feasible.

Using the same oven temperature program (differing only in the time period for which the initial oven temperature was held) the large volume PTV injection was tested for extracts/standards introduction. Finally, the injection volume of 8 μL was chosen as

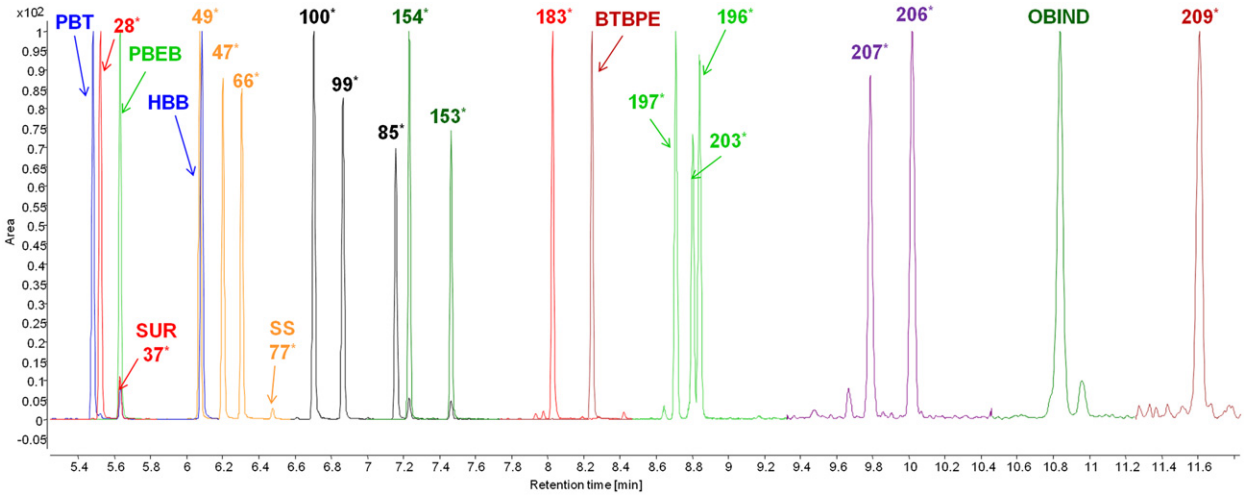

Fig. 1. An example of chromatogram (quantification MS/MS transitions) of the fish tissue spiked with the mixture of all target BFRs at a concentration of 1 μg kg^{-1} analysed using method (i) GC–MS/MS (EI). (Note: SUR—surrogate, SS—syringe standard, *—indicates BDE congener).

Table 2
Comparison of method quantification limits (MQLs) as obtained by GC–MS/MS (EI), GC–MS (EI) and GC–MS (NCI) using cold pulsed splitless (CPS) and PTV injection technique.

Analyte	MQL (μg kg^{-1})			
	GC–MS/MS (EI)		GC–MS (EI)	GC–MS (NCI)
	CPS 2 μL	PTV 8 μL	CPS 2 μL	CPS 2 μL
BDE 28	0.005	0.005	0.05	0.005*
BDE 49	0.005	0.005	0.05	0.005
BDE 47	0.005	0.005	0.05	0.005
BDE 66	0.005	0.005	0.05	0.005
BDE 100	0.005	0.005	0.05	0.005
BDE 99	0.005	0.005	0.05	0.005
BDE 85	0.005	0.005	0.05	0.005
BDE 154	0.005	0.005	0.05	0.005
BDE 153	0.05	0.005	0.05	0.005
BDE 183	0.05	0.005	0.05	0.005
BDE 197	0.1	0.05	0.5	0.05
BDE 203	0.1	0.05	0.5	0.05
BDE 196	0.1	0.05	0.5	0.05
BDE 207	0.5	0.05	1	0.05
BDE 206	1	0.1	5	0.05
BDE 209	1	0.5	5	0.1
PBT	0.05	0.005	0.1	*
PBEB	0.01	0.005	0.1	0.005
HBB	0.05	0.005	0.1	0.005
BTBPE	0.01	0.005	1	0.005
OBIND	1	0.5	5	0.1
DBDPE	5	1	5	0.1

N/A—not available

* PBT co-eluted with BDE 28 and quantified as a sum of these two analytes.

optimal and related PTV parameters were calculated by means of the MassHunter software based on the selected volume and solvent type (for more details see Table 1). The comparison of method quantification limits (MQLs) when using cold pulsed splitless and large volume PTV injection is summarised in Table 2. For the lower brominated PBDEs, MQLs employing both injection techniques were identical and reached the lowest calibration point 0.05 ng/mL which corresponds to 0.005 μg kg^{-1} in fish tissue. Although there is the possibility to detect even lower concentrations using the PTV injection, the MQLs for these compounds were finally set at 0.005 μg kg^{-1}, as the results below this level would suffer from quite high method uncertainty which would increase with decreasing levels of sample contamination. The improvement of MQLs using PTV injection was demonstrated for the BDE 183–209 and 'alternative' BFRs, as they were decreased by circa one order of magnitude. Under these conditions, MQLs were almost identical to those obtained using GC–MS in NCI. However, as the use of PTV injection led to increased maintenance of the GC system and prolonged the time of the analysis by at least 5 min needed for the cooling of PTV injection port between two consequent analyses, we finally decided to validate the method employing cold splitless injection.

3.2. Comparison of GC–MS/MS (EI) with GC–MS (EI)

For the GC–MS (EI) method, the QqQ was operated as a single Q (the first Q used in SIM, collision cell switched off and the second Q served as a transfer ion channel). The GC conditions were adopted from the GC–MS/MS method with cold pulsed splitless injection and precursor ions selected from the full scan spectra obtained during the MS/MS transition optimisation were used as quantification/confirmation ions. Due to a lower selectivity of such type of an ion analyser (already mentioned in the Introduction), a slight increase of background noise was observed. The comparison of MQLs using single and tandem MS, when both were operated in EI mode, is shown in Table 2. The deterioration of MQLs using single MS was observed, as expected, and was significant mainly for higher brominated compounds; this led to an increase of MQLs by approx. one order of magnitude. The decline of selectivity and thus worsening of MQLs might especially cause problems in the analysis of complex matrices such as food and environmental matrices where the occurrence of a high amount of interferences is to be expected. However, when analysing highly contaminated samples, GC–MS (EI) with a single Q might be used.

3.3. GC–MS (NCI) optimisation

As already presented in the scientific literature [17,19,28], the development of GC–MS/MS method in NCI mode for PBDEs was not possible. Using this type of ionisation, fragments from $[Br]^-$ and $[HBr_2]^-$ isotopic patterns were observed in full scan spectra (m/z 50–1000 mass range) for PBDEs with seven or less bromine atoms in a molecule and only one transition forming bromine atom from $[HBr_2]^-$ was observed. Unfortunately, this type of MS/MS transition suffered from low sensitivity and selectivity; thus was not suitable for identification (quantification) purposes.

Moreover, no confirmation was feasible. Because of that, the optimisation of MS/MS transitions in NCI mode was not pursued and, on this account, a method in the SIM mode was developed. The most abundant ion corresponding to bromine atom at *m/z* 79 was chosen as a quantification mass for all compounds except for BDE 209 (discussed more in detail further in this section). For tri- to -heptaBDE, *m/z* 81 and $[HBr_2]^-$ at *m/z* 159 and 161 served as confirmation ions. In the case of highly brominated PBDEs (octaBDE 196, 197, 203, nonaBDE 206, 207 and decaBDE 209), intensity of $[HBr_2]^-$ in spectra declined and other abundant ions formed via cleavage of the C–O bond were present. This phenomenon allowed using ^{13}C-labelled BDE 209 as a syringe standard even in NCI mode, as the $[C_6Br_5O]^-$ corresponding to *m/z* 489 (BDE 209) and 494 (^{13}C-BDE 209) were intensive enough, thus might be used as quantification masses instead of *m/z* 79. Moreover, for BDE 196, 203 and 206, ions corresponding to $[M–Br_3]^-$ and $[M–Br_4]^-$ were observed in their mass spectra and chosen as a confirmation for these three compounds. For PBT, PBEB and HBB, ions from molecular patterns were chosen for confirmation purposes and for BTBPE, OBIND and DBDPE, $[C_6H_2Br_3O]^-$, $[HBr_2]^-$ and $[C_7H_2Br_5]^-$ were monitored, respectively. Table S-2 (Supplementary data) shows a summary of quantification and confirmation ions for all compounds included in this study. A dwell time parameter was optimised, as in the MS/MS (EI) method, to obtain at least 10 data points across each peak. Increased EM gain was used for BDE 209, ^{13}C-BDE 209 and DBDPE; see Table S-2 (Supplementary data).

Regarding the GC method used with MS in NCI mode, at first, identical parameters were used as for GC–MS/MS (EI). However, since *m/z* 79 was used as a quantification mass for all compounds (except for BDE 209); co-elution of BDE 37 & PBEB and BDE 49 & HBB occurred. The oven temperature program rate was reduced and after 1.5 min hold at 110 °C, increased by 15 °C min^{-1} up to 180 °C followed by even slower programming of 5 °C min^{-1} up to 200 °C and finally 320 °C was reached at the rate of 30 °C min^{-1}. Using this oven temperature program, all compounds (up to BDE 209) were eluted in 17 min compared to 12 min of GC–MS/MS (EI). When DBDPE was included into the method, the time needed for the analysis extended to 19 min. Unfortunately, a slowdown of oven temperature programming at the beginning of the analyses caused co-elution of BDE 28 & PBT and no additional adjustment of the GC method led to the separation of all target analytes in one run. BDE 28 and PBT were therefore left unresolved and quantified as a sum of these two compounds. An example of the chromatogram of the fish tissue spiked with the mixture of all target BFRs at the concentration of 1 μg kg^{-1} obtained by GC–MS (NCI) is shown in Fig. 2.

3.4. Sample preparation

The sample preparation procedure applied in this study was based on the transfer of hydrophobic target analytes from aqueous sample suspension (partition supported by inorganic salts) into ethyl acetate followed by a clean-up (fat removal) of organic phase on a silica minicolumn, which was earlier developed for the multi-class analysis of POPs and PAHs in fish within the frame of the 7FP European project CON*ff*IDENCE [15,33]. The number of analytes from our previous work was extended to include also highly brominated PBDE and other 'alternative' BFRs. Since the clean-up procedure might become the most critical step, elution profiles of the newly included analytes together with recoveries on silica minicolumns were tested first. Since all 'new' analytes were eluted using 10 mL of elution mixture (no change against original sample preparation method was needed) and recoveries in the range of 82–110% were obtained, the clean-up procedure was confirmed as suitable for the extending scope of the method. In the next step, the extraction efficiency was tested for the new compounds added to the analyte list. Considering the acceptable recoveries obtained by this sample preparation during the preliminary experiments, the validation study of the entire method followed and the results are discussed in detailed below in Section 3.5.

3.5. Method validation

The combination of the novel sample preparation strategy and the optimised instrumental determination step provided either by GC–MS/MS (EI) or GC–MS (NCI) was evaluated within the validation study conducted on blank fresh fish muscle tissue spiked at 0.1, 1 and 5 μg kg^{-1} with all target compounds (six repeated analyses for each concentration level). Samples were spiked and left to equilibrate over night before the analysis. Tables 3 and 4 provide mean recoveries (expressed as average percentage of recovery not corrected to the recovery of the internal standard; REC, %) and repeatabilities (expressed as relative standard deviation; RSD, %) obtained by the analysis of spiked fish tissue employing GC–MS/MS (EI) or GC–MS (NCI), respectively. Using these two instrumental approaches, recoveries in the range of

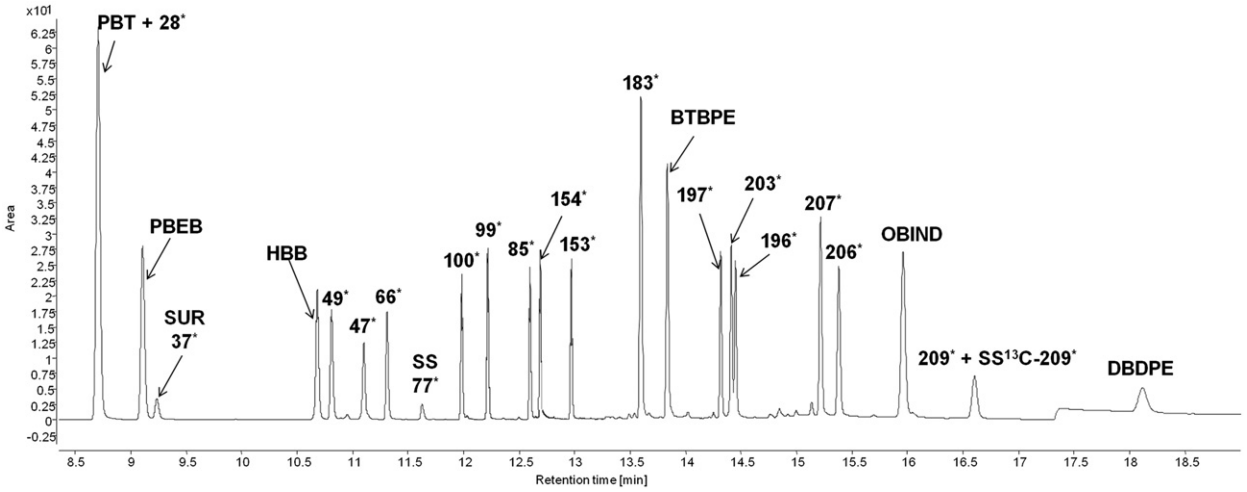

Fig. 2. An example of chromatogram (*m/z* 79) of the fish tissue spiked with the mixture of all target BFRs at the concentration 1 μg kg^{-1} analysed using method (iv) GC–MS (NCI). (Note: SUR—surrogate, SS—syringe standard, *—indicates BDE congener).

Table 3
Recoveries (REC, %) and repeatabilities (RSD, %) calculated from the results of repeated analyses ($n=6$) of fish tissue (fat 2.1%, w/w) spiked with target analytes at three concentration levels 0.1, 1 and 5 μg kg^{-1}. GC–MS/MS (EI), i.e., method (i), was used for analyses.

Analyte	0.1 μg kg^{-1}		1 μg kg^{-1}		5 μg kg^{-1}	
	REC (%)	RSD (%)	REC (%)	RSD (%)	REC (%)	RSD (%)
BDE 28	81	5	89	2	92	6
BDE 49	89	5	100	6	97	4
BDE 47	89	7	78	7	83	5
BDE 66	95	4	100	6	96	5
BDE 100	98	3	102	8	98	5
BDE 99	105	4	106	8	100	5
BDE 85	99	6	107	7	106	6
BDE 154	91	5	99	10	93	6
BDE 153	77	6	107	10	101	7
BDE 183	83	16	100	8	104	10
BDE 197	98	7	86	12	93	12
BDE 203	87	9	79	12	81	12
BDE 196	95	6	83	14	81	8
BDE 207	N/A	N/A	85	12	89	14
BDE 206	N/A	N/A	79	10	86	13
BDE 209	N/A	N/A	81	8	79	11
PBT	82	8	115	10	114	5
PBEB	85	5	105	7	105	4
HBB	106	13	102	12	103	3
BTBPE	119	14	113	13	113	12
OBIND	N/A	N/A	104	11	107	10

N/A—not available.

Table 4
Recoveries (REC, %) and repeatabilities (RSD, %) calculated from the repeated analyses ($n=6$) of fish tissue (fat 2.1%, w/w) spiked with target analytes at three concentration levels 0.1, 1 and 5 μg kg^{-1}. GC–MS (NCI), i.e., method (iv), was used for analyses.

Analyte	0.1 μg kg^{-1}		1 μg kg^{-1}		5 μg kg^{-1}	
	REC (%)	RSD (%)	REC (%)	RSD (%)	REC (%)	RSD (%)
BDE 28	81*	6*	77*	2*	70*	4*
BDE 49	102	9	114	8	96	4
BDE 47	93	10	99	8	80	5
BDE 66	92	7	118	8	97	4
BDE 100	103	3	109	8	100	3
BDE 99	108	7	113	9	112	4
BDE 85	103	9	110	8	107	3
BDE 154	98	9	109	8	103	3
BDE 153	97	12	96	8	105	3
BDE 183	89	10	86	8	102	6
BDE 197	93	8	103	9	101	8
BDE 203	112	4	118	9	97	10
BDE 196	109	10	119	9	99	10
BDE 207	94	7	100	10	104	12
BDE 206	98	6	96	10	115	12
BDE 209	109	13	113	12	109	10
PBT	*	*	*	*	*	*
PBEB	97	9	100	9	79	11
HBB	104	6	108	8	90	6
BTBPE	114	8	103	9	84	8
OBIND	109	12	102	10	108	13
DBDPE	103	16	103	6	99	9

* PBT co-eluted with BDE 28 and quantified as a sum of these two analytes.

70–119% were achieved, and repeatabilities were equal or less than 16% even at the lowest spiking level. At each spiking concentration, REC and RSD were calculated only for analytes of which MQLs were lower than the particular tested level. The key performance characteristics documented through the above outlined validation protocol met the criteria applied in the EU in food contaminants control (SANCO document No. 12495/2011 [31] originally designed for pesticide residue analysis but commonly applied also for other organic food contaminants), i.e., recoveries were in the range of 70–120% and repeatabilities lower than 20%. To control the entire sample preparation for real-life samples, the recovery of the surrogate standard (BDE 37 in this particular case) added to the sample prior the extraction was monitored.

The trueness of the method was demonstrated through the analysis of standard reference material 1947 and the determined concentrations for all analytes were in accordance with certified/reference values.

The MQLs were defined as the lowest concentration of each analyte in matrix at which the quantification and identity confirmation transitions/ions provided signal to noise ratio (S/N) > 6 for the quantitative transition/ion and S/N > 3 for at least one confirmation transition/ion. Based on preliminary measurements using matrix samples contaminated at low concentrations, the MQLs of selected BFRs in fish muscle tissue were in the following ranges: 0.005–1 μg kg^{-1} for GC–MS/MS (EI) and 0.005–0.1 μg kg^{-1} for GC–MS (NCI); for more details see Table 2. Higher values were, as expected (regardless the detection mode), obtained for higher brominated compounds, especially in the case of EI mode.

With regards to a wide concentration range of target analytes, it was necessary to use an extensive scale of working standard solutions for calibration 0.05–500 ng mL^{-1} (0.25–1000 ng mL^{-1} in the case of BDE 206, 207, 209, OBIND and DBDPE). Weighted linear regression ($1/x$) was used and regression coefficient (R^2) was calculated for the calibration curves from the MQL up to the highest calibration point (500 or 1000 ng mL^{-1}). R^2 values higher than 0.99 were obtained for all tested calibration ranges what meant that the linearity criterion was met. To eliminate potential injection inaccuracies, the peak area ratio of the target analyte and particular syringe standard was applied for the final quantification. Syringe standards were used as follows: BDE 77 for BDE 28–203, PBT, PBEB, HBB and BTBPE, and ^{13}C-BDE 209 for BDE 206–209, OBIND and DBDPE.

4. Conclusions

The novel approach for determination of 22 representative BFRs in fish muscle tissue using GC–MS/MS has been developed. The most important features of this analytical method and the benefits from its application can be summarised as follows:

- Triple quadrupole MS operated in EI single reaction monitoring (SRM) mode was shown to be an effective tool for the analysis of BFRs in complex matrices providing distinctly improved selectivity and sensitivity compared to a routinely used single quadrupole MS. Using this type of ion analyser, accurate determination of even (ultra)trace levels of BFRs, which might be important under certain conditions, e.g., within total diet studies, is feasible.
- Selection of specific mass transitions in EI allowed resolution of chromatographically co-eluting compounds, which would be impossible to separate using rather non-selective NCI. However, in specific cases (e.g., DBDPE), when no mass transitions intensive enough were present in EI mass spectra, NCI was the preferred option.
- The recoveries of all target analytes were within the acceptable range of 70–119% and repeatabilities of the analytical procedure were ≤16% at all three spiking levels (0.1, 1 and 5 μg kg^{-1}). Under the optimised conditions MQLs were in the

range of 0.005–1 μg kg^{-1} and 0.005–0.1 μg kg^{-1} using GC–MS/MS (EI) and GC–MS (NCI), respectively.

Disclaimer

Mention of brand or company names in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the Institute of Chemical Technology, Prague.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.11.073.

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