Communication

Determination of Hexabromocyclododecanes in Fish Using Modified QuEChERS Method with Efficient Extract Clean-Up Prior to Liquid Chromatography–Tandem Mass Spectrometry

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Abstract: A modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) sample preparation method coupled with liquid chromatography–tandem mass spectrometry (LC–MS/MS) was proposed for the determination of α-, β-, and γ-hexabromocyclododecane (HBCD) diastereomers in whole-fish homogenate samples. The main modification of the QuEChERS method concerned a clean-up step in which the combination of pH-tuned dispersive liquid–liquid microextraction (DLLME) with 18.4 M H₂SO₄ digestion allowed to successfully eliminate the matrix substances from the final extract. For the target HBCDs, good response linearity was obtained with coefficients of determination \((R^2) > 0.998\) for the concentration range corresponding to 0.1–50 ng of analyte per g of sample. Limits of quantifications (LOQs) were 0.15–0.25 ng g⁻¹ ww (wet weight), and the recoveries from samples spiked at levels of 0.5 and 5 ng g⁻¹ ranged from 89% to 102% with relative standard deviations <7.5%. The accuracy of the method was verified by analysis of the NIST standard reference material SRM 1947, and a good agreement (90%) was obtained with the certified value for the α-HBCD present in the sample. Finally, the method was applied to the analysis of 293 fish samples collected in water bodies from all over Slovakia, in which the highest concentrations were determined for α-HBCD with the maximum value of 31 ng g⁻¹ ww.

Keywords: QuEChERS; dispersive liquid–liquid extraction; sulfuric acid treatment; liquid chromatography; tandem mass spectrometry; hexabromocyclododecanes; fish samples

1. Introduction

Hexabromocyclododecane (HBCD) is a group of synthetic organobromines, which is generally used as flame retardant [1]. Due to persistent, bioaccumulative, and toxic properties, it was added to the Stockholm Convention’s persistent organic pollutants (POPs) list in 2013, with specific exemptions for production and use in expanded polystyrene and extruded polystyrene in buildings [2].

HBCD belongs to very hydrophobic substances (\(\log K_{ow} > 5\)) with low solubility in water, which tends to accumulate in biota. Directive 2013/39/EU of the European Parliament and of the Council [3], amending the European Water Framework Directive (WFD) [4], classifies HBCD as a priority substance and requires its monitoring in biota (fish) samples for which environmental quality standard (EQS) was set at 167 ng g⁻¹ ww (wet weight). The HBCD concentrations determined are one of the indicators, indicating the long-term status of water quality as well as the suitability of fish for human consumption.

Generally, HBCD analysis involves the determination of three major diastereomers forming technical mixtures: α-HBCD (10–13%), β-HBCD (1–12%), and γ-HBCD (75–89%) [1]. Because of the
complexity and high lipid content of fish samples, great emphasis must be placed on the preparation of extracts with the lowest possible content of co-extractives in order to prevent analytical interferences and to avoid build-up of matrix residues in the analysis system. Among the conventional methods used for sample preparation for the determination of HBCDs are Soxhlet extraction (SE), accelerated solvent extraction (ASE), gel permeation chromatography (GPC), sulfuric acid digestion, clean-up, and fractionation on solid-phase extractions (SPE) columns packed with Florisil, silica, or acidified silica, etc. [5–12]. The disadvantages of these methods are long extraction periods (SE: 6–12 h), large solvent consumption (SE, GPC: up to hundreds of mL), laboriousness, or high cost of equipment setup (ASE).

An alternative to the conventional sample preparation methods is the QuEChERS (stands for quick, easy, cheap, effective, rugged, and safe) method, which was originally designed for fruit and vegetable analysis [13]. This method comprises of two parts: an acetonitrile (MeCN) salting-out extraction of a sample homogenate in an aqueous medium, followed by dispersive solid-phase extraction (dSPE) to remove most of the matrix interference. Recently, two QuEChERS-based methods have been published to determine brominated flame retardants (BFRs), including HBCD isomers in fish. In the first study [14], the original method employing dSPE clean-up was optimized for the determination of 12 BFRs in fish samples. In the second study [15], the QuEChERS-like method was reported, in which the dSPE purification step was replaced by the extract clean-up using a double Si-SPE columns assembly (one column acidified with H2SO4) and GPC. It was mentioned that GPC was necessary to reduce matrix effect and to achieve the established limit of quantification (LOQ) of 10 pg g⁻¹. Similarly, as in the second study, the necessity of applying more efficient extract purification when analyzing high-fat matrices has led to many modifications of the clean-up step of the original QuEChERS method (use of freezing technique, dual dSPE, GPC, silica minicolumn, EMR [enhanced matrix removal/lipid sorbent] [16–21].

Recently, a new method combining pH-tuned dispersive liquid–liquid microextraction (DLLME) with acid digestion using 18.4 M H2SO4 for clean-up of fatty acetonitrile (MeCN) extracts (after QuEChERS extraction) has been developed in our laboratory [22]. In this method, in the DLLME part, a ternary component solvent extraction system consisting of the MeCN extract (dispersive water-miscible solvent), water-immiscible extraction solvent (chloroform), and alkaline (0.5 M CH3COONa) water solution is used. After DLLME, the whole obtained extract is treated with about 10-fold greater volume of H2SO4 (in our case 1 mL), and, finally, hexane is used for the back extraction of target analytes from the H2SO4. This clean-up provides many advantages, such as high lipid removal efficiency, rapidity, low cost (cheap chemicals, no need for expensive sorbents), low chemical and glassware usage, no need for special instrumentation, or less bench space. The lipid removal comprises of complete removal of fatty acids, which are partitioned from the organic phase into the basic aqueous phase in the DLLME clean-up step. The disadvantage of the method is that it is only suitable for the analysis of organic compounds stable in H2SO4. Its applicability for the analysis of complex biological samples has been demonstrated in our previous study for the determination of polybrominated diphenyl ethers and organochlorine pesticides in fish [23].

Regarding the chromatographic analysis of individual α-, β-, and γ-HBCD diastereomers, the preferred choice is the reversed-phase liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) [24].

The main goal of this study was to apply and optimize the clean-up method combining pH-tuned DLLME with H2SO4 digestion for purification of the QuEChERS extract prior to LC–MS/MS determination of HBCD diastereomers in fish.

2. Materials and Methods

2.1. Standards and Reagents

α-, β-, and γ-1,2,5,6,9,10-HBCD were obtained as neat compounds from Sigma–Aldrich (Steinheim, Germany), while their corresponding C13-labeled standards, each at a concentration of 50 µg mL⁻¹ in toluene (purity ≥99%), were from Wellington Laboratories (Guelph, ON, Canada).
Anhydrous magnesium sulfate, sodium chloride, anhydrous sodium acetate, H$_2$SO$_4$ (18.4 M), and chloroform—all Emsure grade—and methanol and MeCN (both LiChrosolv), were produced by Merck (Darmstadt, Germany). Water Optima LC–MS grade was from Fisher Scientific (Hampton, NH, USA), and glacial acetic acid (≥99.9%) HiPerSolv Chromanorm for LC–MS was from VWR (Radnor, PA, USA).

A 0.5 M sodium acetate solution was prepared by dissolving CH$_3$COONa in Milli-Q water produced by a Direct-Q 3 water purification system (Millipore, Molsheim, France).

Stock solutions of each HBCD isomer obtained as a neat material were prepared in methanol at a concentration of 1 mg mL$^{-1}$. A standard working mixture of HBCD isomers at a concentration of 1 and 0.1 µg mL$^{-1}$ was prepared by dilution of their stock solutions with methanol. Similarly, a standard working mixture of isotopically labeled standards at a concentration of 0.5 µg mL$^{-1}$ was prepared from the purchased standard solutions.

2.2. Fish Samples

European chub (Squalius cephalus), selected as a bioindicator of water pollution, was used as a source of testing fish matrix for the method development. They were collected by electrofishing (deep-frozen after catch) during a fish survey carried out in water bodies (rivers, streams, reservoirs) from all over Slovakia (from about 280 sampling points) in 2015 and 2018 within the project: Monitoring and assessment of water body status (see the "Funding" section). To prepare whole-fish composite homogenate samples, several pieces (2–7) of the whole fish were homogenized using a knife mill Grindomix GM (Retsch, Haan, Germany) to give a wet weight of about 600 g. The samples were stored at −20 °C until extraction and analysis.

The accuracy of the method was studied by the analysis of the standard reference material SRM 1947 (Lake Michigan Fish Tissue), which was prepared from fillets from adult lake trout (Salvelinus namaycush) by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). This SRM was a frozen fish tissue homogenate with 10.4% of extractable fat and 73% of water.

2.3. Lipid and Moisture Determination

Gravimetric methods, according to our work [25], were used to determine the lipid content and moisture in fish homogenates. For the determination of total lipids, 5 g of fish homogenate was extracted with 5 mL of a 6:4 (v/v) acetone/ethyl acetate solvent mixture on a vortex mixer (Stuart SA8, Bibby Scientific, Stone, UK) for 3 min. Next, 0.5 g of NaCl and 2 g of MgSO$_4$ were added, the mixture was shaken for 3 min, and the organic phase was separated using centrifugation (centrifuge Rotina 380, Hettich, Tuttlingen, Germany). A 1 mL aliquot of the upper organic phase was air-dried and then oven-dried (at 103 °C) to constant weight, and the percent lipid content was calculated from the mass of the residue and the intake mass. The moisture was determined based on the mass difference of 2–3 g portions of fish homogenate before and after drying at 60 °C for 24 h. In general, the lipid content was determined in triplicates and moisture in duplicates.

2.4. Sample Preparation

Sample extraction was performed according to the QuEChERS method modified by Lankova et al. [14]. The 20 µL of a mixture of isotopically labeled internal standards and 10 mL of Milli-Q water were added to 5 g of fish homogenate weighed in a 50 mL polypropylene (PP) centrifuge tube (Corning, CentriStar, Sigma–Aldrich, Steinheim, Germany). The tube was mixed and allowed to stand for 30 min. Then, 10 mL of MeCN was added, and the tube was shaken using a vortex mixer at 800 rpm for 1 min. Next, 1 g of NaCl and 4 g of anhydrous MgSO$_4$ were added, and again, the tube was shaken vigorously for 1 min. Then, it was centrifuged at 5000 rpm for 5 min.

For the clean-up part, a 2 mL aliquot of the upper organic phase was transferred to a 15 mL PP centrifuge tube containing 8 mL of 0.5 M CH$_3$COONa solution. Then, 100 µL of chloroform was added, the tube was shortly shaken and centrifuged at 5000 rpm for 5 min.
In the H\textsubscript{2}SO\textsubscript{4} clean-up step, the whole sedimented phase was transferred to a 1.7 mL click seal microcentrifuge tube (GoldenGate Bioscience, Claremont, CA, USA), and 1 mL of concentrated H\textsubscript{2}SO\textsubscript{4} was added slowly. Next, 200 \(\mu\)L of hexane was added, the tube was shaken briefly by hand and then centrifuged in a microcentrifuge (Mikro 220R, Hettich, Tuttlingen, Germany) at 10,000 rpm for 5 min. The upper phase was transferred into a 2 mL vial with insert style conical well (Chrom4, Suhl-Wichtshausen, Germany) and evaporated to dryness in a stream of nitrogen. Finally, the residue was re-dissolved in 100 \(\mu\)L of methanol to obtain extract for LC analysis. The workflow of sample preparation is presented in Figure 1.

![Figure 1. Workflow of sample preparation.](image)

2.5. Instrumental Analysis

Analyses were performed on an LC–MS/MS system comprising a 1290 Infinity LC (Agilent Technologies, Palo Alto, CA, USA) and a Thermo Scientific TSQ Endura triple quadrupole MS (Thermo Fisher Scientific, San Jose, CA, USA). After the injection of 5 \(\mu\)L sample extract, the analytes were separated on a 100 mm Poroshell 120 EC-C18 column (2.1 mm i.d.; 2.7 \(\mu\)m particle size, Agilent Technologies) maintained at 45 °C. To avoid interferences from the HPLC system, a 50 mm Poroshell 120 EC-C18 column (3.0 mm i.d.; 2.7 \(\mu\)m particle size) was inserted between the mixer and the injection valve. The mobile phase consisted of water (component A) and a mixture of methanol and MeCN with 0.01% of acetic acid in the ratio of 7:3 (v/v) (component B). The LC time program was as shown in Table 1.
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Table 1. LC (liquid chromatography) time program.

| Step | Time (min) | Mobile Phase A (%) | Mobile Phase B (%) | Flow Rate (mL min\(^{-1}\)) |
|------|------------|--------------------|--------------------|-----------------------------|
| 1    | 0          | 90                 | 10                 | 0.3                         |
| 2    | 0.5        | 50                 | 50                 | 0.3                         |
| 3    | 7.5        | 0                  | 100                | 0.4                         |
| 4    | 10.0       | 0                  | 100                | 0.5                         |
| 5    | 10.4       | 0                  | 100                | 0.5                         |
| 6    | 10.5       | 90                 | 10                 | 0.5                         |
| 7    | 13.0       | 90                 | 10                 | 0.3                         |
| 8    | 13.5       | 90                 | 10                 | 0.3                         |

The MS was operated in electrospray ionization negative mode with the following ion source settings: spray voltage, −3500 V; sheath gas, 40 arb (arbitrary units); sweep gas, 0 arb; auxiliary gas, 7 arb; ion transfer tube temperature, 333 °C; vaporizer temperature, 317 °C. Settings used for multiple reaction monitoring (MRM) detection were as presented in Table 2.

Table 2. MS/MS transitions, collision energies (CE), and radio frequency (RF) lens voltages for hexabromocyclododecane (HBCD) determination.

| Analytes          | Precursor Ion (m/z) | Transition 1^a/CE (m/z)/(V) | Transition 2/CE (m/z)/(V) | RF Lens (V) |
|-------------------|---------------------|------------------------------|---------------------------|-------------|
| α-, β-, γ-HBCD    | 641                 | 81/10                        | 79/10                     | 115         |
| C13: α-, β-, γ-HBCD | 653               | 81/10                        | 79/10                     | 127         |

^a Transition 1 was used as quantifier.

3. Results and Discussion

3.1. Sample Preparation

In the extraction part of sample preparation for fish analysis, the QuEChERS extraction modified by Lankova et al. [14] was applied. A single dSPE clean-up of crude extract using C18 and PSA (primary-secondary amine) sorbents, according to this study, was also tested, but the resulting extract purity was insufficient for subsequent LC–MS/MS analysis. This was demonstrated by the high content of fatty matrix co-extractives (remaining after evaporating a solvent) having negative effects on the instrumental analysis (deterioration of LC-system, an increase of LOQs, etc.). Thus, for the purification of the fatty MeCN extract after QuEChERS extraction, the clean-up method involving pH-tuned DLLME with H\(_2\)SO\(_4\) digestion from our previous study [22] was used and optimized.

To obtain the amount of extracted analytes corresponding to 1 g of fish homogenate, the volume of the crude MeCN extract (2 mL) and the volumes of 0.5 M CH\(_3\)COONa solution (8 mL) and chloroform (100 µL) for DLLME were doubled in comparison with the original method.

For the back extraction of HBCDs from the H\(_2\)SO\(_4\) medium, the volume of hexane was increased to 200 µL (compared to the original volume of 80 µL). Due to the use of an LC method for the extract analysis, a solvent exchange step was included in the procedure. The hexane extract was first evaporated to dryness using a stream of nitrogen, and the residue thus obtained was then re-dissolved in 100 µL of methanol.

A comparison of the developed sample preparation method with other QuEChERS-based methods for the determination of HBCDs in fish is shown in Table 3. In the proposed method, unlike other methods, only common cheap chemicals were used. The most complicated purification procedure was employed in the method by Tavoloni et al. [15], in which special SPE cartridges and GPC were used. By this method, a high extract clean-up efficiency was achieved, but it was also associated with high consumption of toxic solvents (hexane, dichloromethane). The high purity of the QuEChERS extract was also obtained by the proposed method in a simpler, cheaper, and more environmentally friendly way.
Table 3. Comparison of the developed sample preparation method with other QuEChERS (quick, easy, cheap, effective, rugged, and safe)-based methods for the determination of HBCDs in fish.

| Ref. | Fish Homogenate Mass | Water Addition | Extractant | Added Salts | Crude Extract Volume | Clean-Up |
|------|----------------------|----------------|------------|-------------|----------------------|----------|
| [14] | 5 g                  | 10 mL          | 10 mL MeCN + 0.2 mL formic acid | 1 g NaCl + 4 g MgSO₄ | 2 mL | dSPE: 0.1 g C18 + 0.02 g PSA + 0.3 g MgSO₄ 0.2-µm nylon filter |
| [15] | 20 g                 | 5 mL           | 15 mL ethyl acetate | 3 g NaCl + 6 g MgSO₄ | 10 mL | SPE: Extrelut NT-3 (+H₂SO₄), Isolute silica columns GPC |
| This work | 5 g                  | 10 mL          | 10 mL MeCN | 1 g NaCl + 4 g MgSO₄ | 2 mL | DLLME: 8 mL 0.5 M CH₃COONa + 100 µL CHCl₃ Acid digestion: 1 mL 18.4 M H₂SO₄ |

Abbreviations: MeCN—acetonitrile; dSPE—dispersive solid-phase extraction; C18—octadecyl silica; PSA—primary-secondary amine; SPE—solid-phase extraction cartridge; GPC—gel permeation chromatography; DLLME—dispersive liquid–liquid microextraction.

3.2. Method Performance

Due to the unavailability of a suitable HBCD-free fish matrix, the calibration curves were prepared using MeCN calibration solutions, which went through the entire purification process as all samples to compensate for losses. Response linearity for the target HBCD isomers was evaluated by analyzing calibration solutions at concentrations ranging from 0.05 ng mL⁻¹ to 25 ng mL⁻¹ (corresponds to 0.1–50 ng per g of homogenate). To compensate for matrix effects and instrument variability, all measurements were related to the ratio of the target analyte response to the respective C13-labeled internal standard (isotope internal calibration) at a concentration of 2 ng g⁻¹. The coefficients of determination (R²) for the individual HBCD isomers and for the range studied were >0.998 (see Table 4), showing good linearity.

Table 4. Method performance characteristics for the determination of HBCDs in chub fish homogenate.

| Analyte | Linear Range (ng g⁻¹) | LOD (ng g⁻¹) | LOQ (ng g⁻¹) | Recovery (RSD) a |
|---------|------------------------|--------------|--------------|------------------|
| α-HBCD  | 0.1–50                 | 0.075        | 0.25         | 89 (5.8)         | 98 (3.0) |
| β-HBCD  | 0.1–50                 | 0.045        | 0.15         | 100 (7.4)        | 99 (2.0) |
| γ-HBCD  | 0.1–50                 | 0.076        | 0.25         | 102 (7.1)        | 98 (3.4) |

Abbreviations: LOD—limit of detection; LOQ—limit of quantification; RSD—relative standard deviation. a n = 6.

The limits of the method were determined from ten replicate analyses of the blank chub composite sample (lipid content, 3.6%; moisture, 77%) spiked at a concentration of 0.1 ng g⁻¹. The limits of detection (LOD) and quantification (LOQ) were calculated based on the standard deviation (SD) of determination as 3 × SD and 10 × SD, respectively. Table 4 shows that the LOQ for α- and γ-HBCD was 0.25 ng g⁻¹ ww, and for β-HBCD was 0.15 ng g⁻¹ ww, respectively. These values are three orders of magnitude lower than the requirement for the EQS (167 ng g⁻¹ ww), set by the WFD, and at the same level as obtained in the study by Lankova et al. [14] (0.1–1 ng g⁻¹ ww). For illustration, Figure 2 presents total MRM chromatograms from the analysis of the blank chub composite sample spiked with the studied HBCDs at LOQ level of 0.25 ng g⁻¹. Presented chromatograms showed the advantages of MRM detection, such as great sensitivity and high specificity, which are particularly helpful in the
The baseline separation of the analytes with no interferences from the matrix can be seen.

![Chromatogram from the analysis of a real chub sample](image)

**Figure 2.** Total ion MRM chromatograms from the LC–MS/MS analysis of the blank chub composite sample spiked with the test analytes at 0.25 ng g\(^{-1}\) (LOQ) level (a) and internal standards at 2 ng g\(^{-1}\) (b). MRM—multiple reaction monitoring; LOQ—limit of quantification.

The recovery and repeatability (expressed as relative standard deviation, RSD) of the method were evaluated from repeated analyses of the blank composite sample spiked at 0.5 and 5 ng g\(^{-1}\) \((n = 6)\). From the results presented in Table 4, it can be seen that the recovery of three HBCD isomers was in the range of 89–102% with RSD <7.5% and fell within the acceptable recovery range of 70–120% with RSD ≤20% [26].

The method's accuracy was verified by the analysis of the NIST standard reference material SRM 1947 prepared from lake trout. For \(\alpha\)-HBCD, the certified concentration of 3.39 ng g\(^{-1}\) ww was provided in the SRM's certificate of analysis with no uncertainty because of the lack of sufficient information to assess adequately the uncertainty associated with this value. The determined value for \(\alpha\)-HBCD was 3.04 ng g\(^{-1}\) ww \((n = 3)\) with RSD of 5.4%, showing good accuracy (90%) of the method.

The applicability of the method was confirmed by analyzing 293 real fish samples. The samples were analyzed in duplicate, and in case of positive results, the average value was reported. The determined HBCD isomer concentrations were in the following ranges: 0.25–31 ng g\(^{-1}\) ww for \(\alpha\)-HBCD, 0.15–1.6 ng g\(^{-1}\) ww for \(\beta\)-HBCD, and 0.25–4.8 ng g\(^{-1}\) ww for \(\gamma\)-HBCD, respectively. Thus, all results obtained for the monitoring samples were far from exceeding the EQS value for HBCD in fish. In Figure 3, the chromatogram from the analysis of a real sample of chub fish is presented.

![Chromatogram from the analysis of a real chub sample](image)

**Figure 3.** Chromatogram from the analysis of a real chub sample; \(\alpha\)-HBCD: 19 ng g\(^{-1}\), \(\beta\)-HBCD: 0.99 ng g\(^{-1}\), \(\gamma\)-HBCD: 2.7 ng g\(^{-1}\) (lipid content: 13%, moisture: 62%). HBCD—hexabromocyclododecane.
4. Conclusions

The clean-up method combining pH-tuned DLLME with H₂SO₄ digestion provided a QuEChERS extract with a purity suitable for subsequent LC–MS/MS analysis of HBCDs in whole-fish homogenate samples. The resulting sample preparation was cheap, not laborious, and allowed to prepare ten samples in two hours. However, simple and inexpensive sample preparation instrumentation was in contrast to complex and expensive LC–MS/MS equipment employed. The LOQs for α-, β-, and γ-HBCD below 0.3 ng g⁻¹ ww were much lower than the EQS established by the WFD and were fully sufficient to control fish contamination. This was confirmed by the analysis of SRM and real samples.

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