Determination of benzothiazoles, benzotriazoles and benzenesulfonamides in seafood using Quick, Easy, Cheap, Effective, Rugged and Safe extraction followed by gas chromatography - tandem mass spectrometry: method development and risk assessment

Óscar Castro, Sílvia Borrull, Eva Pocurull*, Francesc Borrull

Universitat Rovira i Virgili, Department of Analytical Chemistry and Organic Chemistry, Sescelades Campus, Marcel·lí Domigo s/n, 43007 Tarragona, Spain

*corresponding author: eva.pocurull@urv.cat +34 977558492

Abstract

The common use of benzothiazoles, benzotriazoles and benzenesulfonamides has led to widespread ubiquity in several environmental matrices. Their occurrence in edible fish could represent an additional exposure route for the population. The present study aims to develop a method for the simultaneous determination of these three compound families in seafood samples. Based on QuEChERS extraction, different salt combinations and clean-up strategies have been evaluated to achieve the highest recoveries while reducing the matrix effect in low and high lipidic content species. The best results were obtained with the original method salts and the lipid-selective push-through clean-up, which combined with gas chromatography-tandem mass spectrometry led to recoveries between 50-112% with negligible matrix effects and method detection limits between 0.15 – 9.50 ng g^{-1} d.w. The application of the method to commercially available samples confirmed the presence of BTs as well as BSAs, with the latter being determined in seafood for the first time. Exposure and risk assessment calculations indicated a minor risk for the population when consuming fish.

Keywords

QuEChERS, benzothiazoles, benzotriazoles, benzenesulfonamides, seafood

1. Introduction

The population has adapted to a way of life where daily commodities are fundamental. From furniture to electronics, all the surrounding objects have been produced using chemicals and also contain many chemical compounds. Some of them are produced in massive amounts every year, leading to a widespread ubiquity of chemicals that nowadays have been indicated as potential contaminants [1]. It is by these negative effects that organisations such as the OECD (Organisation for Economic Co-operation and Development) and the EPA (Environmental Protection Agency) have listed some of them as High Production Volume chemicals (HPVs) [2]. This list is intended to prioritize the research on compounds with huge production over the year to collect enough data to evaluate consumer, occupational and environmental exposure. The list contains compounds from different families including benzothiazoles (BHTs), benzotriazoles (BTRs), and benzenesulfonamides (BSAs). These compounds are usually found on their own, i.e., 1-H-benzothiazole, 1-H-benzotriazole, and benzenesulfonamide, or as derivatives of the previously stated (usually methylated and halogenated variants). Both benzothiazoles and benzotriazoles share a heterocyclic structure with a 1,3-thiazole or a 3 nitrogen five-membered respectively [3,4]. As for benzenesulfonamides, these are formed by a benzene or toluene ring with a sulfonamide moiety where the derivates are formed. As high production volume chemicals, these are used in industrial as well as consumer products with applications including vulcanization acceleration, antifreeze formulations, UV light stabilizers, or dye synthesis precursors, among others [5]. Thus, their extensive use in many everyday commodities has become a major source of these reaching the environment and becoming a potential threat. The presence of these benzocompounds in environmental matrices such as air [6–8], water, soil, and even biota [9,10] has been described by several authors. The pathway followed by these compounds

to reach the aquatic environment starts upon their release from household and industrial runoffs. Their removal in wastewater treatment plants (WWTP) is mostly ineffective, especially for some benzenesulfonamides, which concentrations become 4-6 times higher in the effluents due to biodegradation/bioconversion processes [11]. Because of their high mobility, their path reaches the aquatic environment, where benzothiazoles, benzotriazoles and benzensulfonamides become available to aquatic organisms [5]. Their possible accumulation in seafood becomes a problem as these could then become accessible to the population through dietary intake, thus turning seafood consumption into an alternative exposure route.

With seafood species being considered high-complexity samples due to the huge number of interferences and coextracted compounds found when analysing them, it represents an analytical challenge to determine compounds. Regarding the methodologies used for the determination of these compounds in seafood samples, scarce information is found in the literature. Whereas benzotriazoles have been determined using different extraction techniques with good results such as ultrasonic-assisted extraction (USE) [12,13], QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) [10,14,15] or pressurized liquid extraction (PLE) [16], methods involving the determination of benzothiazoles in seafood samples are limited in literature (use of USE [12] and PLE [9]) and no method exists for the determination of benzenesulfonamides. After the extraction, the use of an efficient clean-up of the obtained extract is mandatory for this type of sample. For this purpose, authors have used strategies such as solid-liquid extraction (SPE) or dispersive solid-liquid extraction (d-SPE) using sorbents like florisil, PSA or C_{18} [10,12,14,15,17]. Regarding the determination techniques used for the analysis of these samples, chromatography, especially liquid chromatography, is the preferred technique, mostly coupled with tandem mass spectrometry to reach the desirable levels [9,14,18].

To the best of our knowledge, this is the first method for the simultaneous determination of benzotriazoles, benzothiazoles and benzenesulfonamides in fish samples. Moreover, the study aims to find an effective clean-up to diminish the matrix effect to a level where it can be disregarded, thus enabling the use of a single external calibration in the analysis of several fish and seafood species. The application of the following method to commercially available fish specimens is intended to provide reliable data for the calculation of exposure and risk assessment related to the ingestion of these compounds via dietary intake.

2. Materials and methods

2.1. Chemicals and reagents

The present study focuses on the determination of three different compound families: benzothiazoles, benzotriazoles, and benzenesulfonamides. The studied benzothiazoles included 1-*H*-benzothiazole (BT), 2-aminobenzothiazole (NH_2BT), chlorobenzothiazole (ClBT), 2-hydroxybenzothiazole (OHBT), and 2-(methylthio)-benzothiazole (MeSBT). Benzotriazoles comprised 1-*H*-benzotriazole (BTR), 4-methyl-1-*H*-benzotriazole (4TTR), 5-methyl-1-*H*-benzotriazole (5TTR), and 5,6-dimethyl-1-*H*-benzotriazole (XTR). Finally, benzenesulfonamides included benzenesulfonamide (BSA), *para*-toluenesulfonamide (p-TSA), *N*-methyl-*para*-toluenesulfonamide (Me-p-TSA), *N*-ethyl-*para*-toluenesulfonamide (Et-p-TSA), and *ortho*-toluenesulfonamide (o-TSA). All compounds were purchased at Sigma-Aldrich (Darmstadt, Germany). Deuterated compounds supplied by LGC Standards (Teddington, UK) were used as internal standards: d_4-benzothiazole (d_4-BT) for benzothiazoles, d_4-benzotriazole (d_4-BTR) for benzotriazoles, and d_4-p-toluenesulfonamide (d_4-p-TSA) for benzenesulfonamides.

Individual stock solutions of 1000 mg L^{-1} were prepared using ethyl acetate as solvent. Working mixture solutions of 100 mg L^{-1} of each of the compound families (benzothiazoles, benzotriazoles, and benzenesulfonamides) were prepared also with ethyl acetate and stored at -20 °C until further dilution. Solvents used throughout the study were all GC grade with purity higher than 99%. Ethyl acetate, acetone, and acetonitrile were purchased from Scharlab (Barcelona, Spain), hexane from JT Baker (Deventer, The Netherlands). Ultrapure water was obtained from a Synergy water purification system from Millipore (Massachusetts, USA). Helium and nitrogen used for the chromatographic and mass spectrometer systems were both of 99.999% purity and were supplied by Carburos Metálicos (Tarragona, Spain). Materials such as QuEChERS salt packets (Original, AOAC, and EN15662), disposable syringes, and PTFE filters were purchased from Scharlab (Barcelona Spain) while LipiFiltr cartridges were supplied by Carlo Erba (Barcelona, Spain).

2.2. Sample preparation

Fish samples used for the method optimization process as well as for the application on real samples were purchased at different local stores. Specimens of cod (*Gadus morhua*), mackerel (*Scomber vincialis*), squid (*Loligo vulgaris*) and tuna (*Thunnus thynnus*) were purchased and divided into two groups depending on the lipid content: low lipid content (1% for cod and 6% for squid) and high lipid content (17% for mackerel and 16% for tuna). Samples were covered with aluminium foil once purchased to prevent cross-contamination and kept at -24 ºC until further treatment. Due to the study being focused on the ability to obtain data for its posterior use on ingestion risk, only the edible parts of the specimens were selected. Therefore, muscle fillets were dissected from each specimen and then freeze-dried using a miVac Duo freeze-drying system from Genevac (Ipswich,

United Kingdom). Moisture content (%) of the species was calculated as the weight difference before and after lyophilization. Once completely dried, samples were ground and homogenised. Finally, samples were sieved through a 500 µm mesh to obtain a homogenised particle size and kept in a glass container until their analysis. As for spiked samples, these were prepared by adding the compound mixture at the desired concentration to the freeze-dried fish samples previously weighted and covered in GC-grade acetone. Samples were mixed to facilitate the contact between the matrix and the compounds and then left to dry overnight.

2.3. QuEChERS extraction method

The followed procedure for the determination of benzothiazole, benzotriazole, and benzenesulfonamide in fish samples was adapted from a previous study for which organophosphate esters were determined in the same matrix [19]. The procedure was carried out using 100 mg of lyophilized sample weighted in a 50 mL centrifuge tube. Next, 10 mL of ultrapure water and 10 mL of acetonitrile were added to the tube, and it was vortex mixed for 1 minute. A packet containing the salts corresponding to the original method for QuEChERS extraction (4g of magnesium sulfate and 1 g of anhydrous sodium acetate) was added to the tube and it was subsequently vortex mixed for 3 min. Tubes were then centrifuged at 4000 rpm (1789 xg) for 5 min using a Hettich Universal 32R centrifuge (Tuttlingen, Germany) and the supernatant (acetonitrile layer) was collected and passed through a LipiFiltr push-through cartridge. Extracts were then evaporated to circa 0.5 mL under a gentle nitrogen stream. The extracts were filtrated with a 0.22 µm PTFE syringe filter and spiked with the internal standard at 50 µg L^{-1}. Finally, these were reconstituted to 2 mL using ethyl acetate and 1 mL was aliquoted into injection vials and injected into the GC-MS/MS system.

2.4. Gas chromatography-tandem mass spectrometry

The extracts obtained by the extraction process were analysed using an Agilent 8890 GC system coupled to an Agilent 7000D triple quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). Samples were automatically injected by a PAL RSI 120 automatic injector from CTC Analytics (Zwingen, Switzerland). The chromatographic system worked with two HP-50 capillary columns (15 m x 0.25 mm i.d. and 0.25 µm film thickness) from Agilent connected by a pneumatic switching device (PSD) which permitted the use of backflush mode (concurrent backflush, flow at -1.0192 mL/min at 19.331 min until the end). Target analytes were separated using an oven temperature program as follows: the temperature was initially set at 75 ºC (held for 2.87 min) and then raised at a rate of 15 ºC/min to 300 ºC (held for 5 min) with a total run time of 22.87 min and a 6-min solvent delay. The carrier gas used was helium at a constant rate of 1.2 mL/min in the first column and 1.4 mL/min in the second column. A volume of 25 µL was injected using an Agilent Multi-Mode Inlet (MMI) in solvent vent mode with an initial temperature of 75 ºC held for 0.37 min and subsequently raised at 600 ºC/min rate to 325 ºC (held for 5 min). Solvent vent mode operated at a vent flow of 120 mL/min and 5 psi for 0.37 min and purge to split vent was set at 60 mL/min at 2.87 min. The triple quadrupole mass spectrometer system operated in electron ionisation mode at 70 eV with temperatures of 230 ºC, 150 ºC, and 150 ºC for ion source, quadrupole 1 and quadrupole 2, respectively. For the quantification, multiple reaction monitoring mode was used with one quantifier transition (Q) and two qualifier transitions (q) to identify the target compounds. Data analysis was performed using Agilent MassHunter Workstation (Qualitative and Quantitative Analysis) version 10.0. Table S1 gathers information regarding the parameters used for the GC-MS/MS(QqQ) system.

193

194 *2.5.Exposure assessment and risk characterization*

195 Values of exposure associated with the dietary intake of benzothiazoles, benzotriazoles,
196 and benzenesulfonamides by the population of Tarragona were assessed by using a
197 methodology that combines the concentrations found of the target compounds in the fish
198 samples along with the fish consumption data. Exposure values (E_t) were obtained as the
199 result of applying equation 1 to the obtained dataset [20]. Thus, the global human
200 exposure for contaminant *t* appears as the sum of the contribution of each fish species,
201 considering the mean consumption of the individual species *f* (C_f, g kg bw^{-1} day^{-1})
202 previously normalised by dividing by the mean body weight for each gender/age
203 population subgroup, and the concentration of each of the analysed contaminants *t* for the
204 species *f* ($X_{t,f}$, ng g^{-1}) expressed in wet weight basis (w.w.).

205 $$E_t = \sum_{f=1}^{p} C_f X_{t,f} \qquad \text{Eq. 1}$$

206 Based on the method detection limit (LOD) and method quantification limit (LOQ), three
207 scenarios were estimated for below-LOQ and non-detected compounds. An upper-bound
208 scenario (UB) was assessed assuming LOQ and LOD concentration values for below-
209 LOQ and non-detected compounds, respectively. The middle-bound scenario was
210 estimated as half of the concentration of the LOQ and LOD for below-LOQ and non-
211 detected compounds respectively. Finally, for the lower-bound scenario (LB), compounds
212 under the LOQ were estimated as the LOD value and non-detected compounds
213 concentrations were reduced to zero. Calculations regarding the risk assessment were
214 performed using the methodology suggested by the European Safety Authority (EFSA)
215 and the Environmental Protection Agency (EPA) [21,22]. Non-genotoxic and non-
216 carcinogenic compounds were evaluated using NOAEL (*non-observed-adverse-effect-*

level) values. These compound-dependant values were used to calculate the acceptable daily intake (ADI_t), which can be easily obtained by dividing the oral NOAEL value by an uncertainty factor. The risk associated with the intake of the compound *t* can be then calculated using equation 2 where ADI_t, E_t, and R_t are the acceptable daily intake, the global dietary exposure, and the risk factor, respectively.

$$R_t = (E_t / ADI_t)*100 \quad \text{Eq. 2}$$

2.6. Study of population and data collection

The data concerning the consumption of seafood by people living in Tarragona was gathered from a survey conducted in Catalonia over the year comprised between 2002 and 2003 (ENCAT, 2003) [23]. The data is segregated by population groups depending on age and gender. A total of 2160 individuals with ages ranging between 10-80 (1164 women and 996 men) participated in the study which consisted of a food frequency questionnaire and two 24-hour recalls along with anthropometric measurements (height, body weight, circumferences, etc.) and a socioeconomic general questionnaire. Moreover, participants also administered data about food habits, chronic diseases, physical activity, smoking, and nutrition knowledge. All the data obtained was collected by trained dietitians via personal interviews at the volunteers' homes. The mean consumption of the species analysed in the present study by the population of Tarragona is summarized in Table S2.

3. Results and discussion

3.1. GC-MS conditions

Benzotriazoles, benzothiazoles, and benzenesulfonamides are polar compounds not chromatographically easy to determine, especially when using GC. Their low signal response along with its usually tailed peaks turns this group of compounds, benzotriazoles specifically, into an analytical challenge. Tailing peaks can be associated with instrumental-related issues, however, for this group of benzocompounds, specifically benzotriazoles, tailing can be linked to the higher polarity of the compounds, which may cause secondary interactions with active sites present in the liner and column. The low signal response can be typically addressed with strategies such as sample treatment enrichment, higher injection volumes and/or sample weights, or even derivatization. However, one of the disadvantages of using QuEChERS is its low enrichment factors, especially since a minimum weight of the sample is used to prevent higher matrix effects. Thus, experiments regarding injection volume and different sample weights were performed. In this case, the use of derivatization agents was avoided to simplify the procedure. The use of the Multi-Mode Inlet (MMI) present in the instrument allowed the injection of 25 µL of the sample extract using the Solvent vent mode. Parameters such as oven initial temperature, injector initial temperature, vent time, vent pressure, vent flow and purge time and flow were optimised using the Solvent Elimination Wizard present in the Agilent MassHunter software. As regards the increase of sample weight, further explanation of its optimisation can be found in the following section regarding extraction optimisation. Backflush conditions were also optimised using the backflush wizard integrated with the Agilent MassHunter Software. The optimisation of the solvent vent mode and the application of higher injection volumes produced peaks of a higher area at lower concentrations, thus leading to lower instrumental limits (low µg L^{-1}) and counteracting the dilution of the extraction process.

3.2. Extraction procedure optimisation

When working with fish or seafood samples, techniques such as solid-liquid (SLE) or ultrasonic-assisted extraction (USE) may require large solvent volumes and long extraction times to achieve effective extractions. Additionally, using techniques such as pressurized liquid extraction (PLE) reduces the time and solvent needed, but highly rises the amount of matrix components present in the extract. For these reasons, QuEChERS are believed to be a good alternative. This extraction technique, as its acronym states, enables quick, easy, cheap, effective, rugged, and safe determinations. Its' use has widely expanded from its initial purpose. Since its first application for the determination of pesticides, QuEChERS are nowadays being used for the extraction of many other compound families [24–26].

For the present work, the QuEChERS extraction efficiency of the target compounds in fish samples was studied. The extraction procedure was optimised using two different fish species with different lipid contents. Thus, cod and mackerel were used to perform the optimisation procedure representing low and high lipid content species, respectively. The differentiation of lipid content was based on the analytical differences that may be found when analysing fish species with a higher lipid content, as fat present in some of the species may interfere with the determination process concerning extraction efficiency and enhanced or suppressed signal (matrix effect).

The optimisation process for the present method relied on the selection of the salts used for the extraction as well as the selection of the most convenient clean-up strategy in terms of apparent recovery and matrix effect reduction for the studied compounds. Previous method optimisation procedures started with the selection of the QuEChERS extraction salts, comparing the apparent recoveries of raw extracts directly injected after the salt addition step [19]. However, in the present study, the injection of raw extracts (no clean-

289 up) resulted in the apparition of high levels of interferences, thus hindering the
290 determination of some of the compounds and making it impossible to obtain useful data.
291 The appearance of these interferences may be a direct cause of the lipid content present
292 in the samples combined with the use of a higher injection volume. Therefore, it was
293 decided to start the optimisation process by selecting the most efficient clean-up
294 procedure to reduce the interferences found in the raw extracts and enable the obtention
295 of useful data.

296

297 *3.2.1. Clean-up selection*

298 Fish samples are considered of high complexity due to the elevated content of compounds
299 present in the matrix. Among them, lipids stand out as one of the biggest challenges to
300 overcome. Fat content may act as a compound retainer, thus hindering the extraction
301 process and reducing its efficiency and it could also affect the instrumental signal,
302 enhancing or suppressing it [27,28]. It is, therefore, necessary to assess convenient
303 strategies to minimize its effect when extracting fish samples. Following QuEChERS
304 extraction, several clean-up methodologies have been described in the literature amongst
305 which dispersive solid-phase extraction (d-SPE) is one of the most common due to its
306 simplicity. Other strategies include liquid-liquid extraction, solid-phase extraction, or the
307 use of novel devices such as LipiFiltr [29]. Dispersive-solid phase extraction is based on
308 the use of sorbents to absorb interferences that might be present in the QuEChERS extract
309 once the extraction is performed. The obtained extract is transferred to a tube containing
310 the sorbent and is then thoroughly mixed. The variety of sorbents susceptible to being
311 used for d-SPE is large and can be easily accessed commercially. Nevertheless, as the
312 present study focuses on the reduction of lipid content from the extract, only sorbents
313 susceptible to achieving that aim were tested. Thus, sorbents such as primary secondary

amine (PSA) and a combination of the same sorbent with C_{18} were tested. On the other hand, liquid-liquid extraction using hexane and LipiFiltr cartridges was also tested as an alternative to d-SPE. The use of hexane as a cleaning solvent has been described by several authors to extract lipids from matrices with high-fat content [20,30–32], therefore, the use of hexane LLE after QuEChERS extraction may be an easy-to-handle alternative to d-SPE. Moreover, devices such as LipiFiltr have recently appeared as new alternatives to the previously mentioned ones. In this case, LipiFiltr is intended to selectively subtract lipids from QuEChERS extracts using a sorbent contained in a push-through cartridge.

Experiments comparing the previously stated strategies (d-SPE with PSA and PSA+C_{18}, hexane LLE and LipiFiltr) were conducted using spiked samples of fish species from both lipid contents (cod and mackerel). Therefore, 0.1 g of fish were weighed in a glass tube and subsequently spiked with the target compounds following the protocol for spiked samples described in the previous section. QuEChERS extraction step was performed with the original method salts packet for all the experiments. Once the extracts were obtained, they were treated differently for each of the procedures. (a) For d-SPE clean-up, the extracts were transferred to a tube containing 200 mg PSA or 200 mg PSA + C_{18} (1:1) and vortexed for 3 min. The extracts were then centrifuged at 4000 rpm for 5 min and the supernatant (liquid layer) was transferred to a 20 mL glass vial. (b) For LLE, the extracts were transferred to a separatory funnel and 10 mL of hexane were added. The funnel was thoroughly mixed for 3 min and then left for 5 min to stabilize. The bottom layer (ACN) was collected in a 20 mL glass vial. (c) For LipiFiltr clean-up, the extracts were passed through a LipiFiltr push-trough cartridge using a 5 mL syringe and then collected in a 20 mL glass vial. All the extracts obtained from the different clean-up strategies were then evaporated under a nitrogen stream until circa 0.5 mL and were subsequently filtrated with a 0.22 µm PTFE syringe filter and reconstituted with EtAc to

2 mL after including the I.S. mixture. The same procedure was performed for blank samples to subtract their signal from the spiked ones. The different clean-up strategies were evaluated in terms of apparent recoveries (%R, sample spiked prior extraction) and matrix effect (%M.E., extract spiked prior reconstitution). The apparent recoveries (%R, Eq. 1) and the matrix effects (%M.E., Eq.2) were calculated based on the following equations:

$$\text{Apparent recovery (R, \%)} = [(A-n)/B)] *100 \quad \text{Eq. 1}$$

$$\text{Matrix effect (ME, \%)} = ([(C-n)-B]/B) *100 \quad \text{Eq. 2}$$

where A is the peak area of the spiked cod/salmon compound before the extraction, B is the peak area of the same compound from an analytical standard directly injected in the system and C is the peak area of the spiked cod/salmon compound after the clean-up procedure. In both cases, n is the peak area that may be found in the blank sample. Accordingly, M.E. > 0 indicates ion enhancement and M.E. < 0 indicates ion suppression. Figures 1 and 2 gather the results obtained for the studied clean-ups.

Observing the results, benzenesulfonamides signal is mostly enhanced for all the studied congeners while, on the other hand, benzothiazoles tend to suppress the signal when in presence of the matrix. As for benzotriazoles, mild signal enhancement and suppression are observed, being 5TTR the most affected compound. All in all, matrix effect values were comprised between -45 and 20% for the low lipid content species and between -43% and 30% for the high lipid content species. The effect of lipid content is notable in special for benzenesulfonamides, where a higher tendency to enhance the matrix is observed. The use of LipiFiltr diminishes the matrix effect for all the target compounds, thus being the best option to use for clean-up. Regarding the apparent recoveries when including the clean-up step, better recoveries are obtained when using LipiFiltr. This could be explained

363 due to the reduction of the matrix effects along with an easy-to-handle procedure, as no
364 additional transfers are needed when using a push-trough cartridge, whereas d-SPE and
365 LLE require an additional transfer and phase separation step. For these reasons, LipiFiltr
366 was selected as the optimal clean-up strategy.

367

368 *3.2.2. QuEChERS salts packet optimisation*

369 Once the clean-up strategy was selected, the content of the salt packet used for the
370 QuEChERS extraction was evaluated. Three different mixtures were tested corresponding
371 to three different official methods described for QuEChERS extraction: the original
372 method (OR), the Association of Official Agricultural Chemists method (AOAC) [33],
373 and the European Committee for Standardization EN 15662 method (EN) [34]. The
374 methods mainly differ on the type and number of salts present in the mixture. Thus, the
375 OR method first described by Anastassiades et al. [35] contains 4 g of magnesium sulfate
376 and 1 g of sodium chloride. On the other side, the AOAC and the EN methods also include
377 a buffer to regulate pH, in the case of the AOAC method the sachet contains 6 g of
378 magnesium sulfate and 1.5 g of anhydrous sodium acetate. As for the EN method, the
379 mixture is composed of 4 g of magnesium sulfate, 1 g of sodium chloride, 0.5 g of sodium
380 hydrogen sesquihydrate, and 1 g of sodium citrate. Extraction of cod and mackerel
381 samples was performed per triplicate for each of the salt combinations and the apparent
382 recoveries of the method were evaluated. The results obtained can be observed in Figure
383 3. Differences were mostly negligible for most of the compounds in both fish species,
384 being 5TTR and p-TSA the compounds with the most notable recovery differences. The
385 use of the original salts resulted in a higher recovery value for these compounds in both
386 low and high lipid content species. ClBT also depicted better recoveries when working
387 with the original method in presence of the low lipid matrix. All in all, the results obtained

388 for these three method alternatives were similar. Thus, the original method was selected
389 due to its slightly better results for some of the compounds.

390

391 *3.2.3. Sample weight*

392 Once the clean-up and the salts were selected, some experiments were performed to
393 observe the extraction and clean-up potential of the developed method. Thus, extractions
394 of different sample weights were carried out to analyse the effect of a higher sample
395 weight on the apparent recoveries and the method efficiency [36]. The increase in sample
396 weight could lead to a higher response, thus reducing the method limits of detection.
397 Sample weights of 0.1, 0.25, and 0.5 g were used to perform the analysis with the same
398 conditions previously described. Figure 4 comprises the apparent recoveries found when
399 the sample weight was increased. As observed in Figure 4, most of the compounds were
400 affected by the variation of sample amount for both species, reducing its apparent
401 recovery as the quantity of sample to be extracted increased. This can be related to the
402 sample/solvent ratio. Increasing the amount of sample without raising the volume of
403 extraction solvent could cause the compounds not to be extracted enough from the matrix,
404 as the contact between the sample and the solvent is reduced. Moreover, the matrix effects
405 were also evaluated in this case, and no significant differences were observed when
406 compared with the matrix effects previously achieved, thus reinforcing the theory of the
407 extraction sample/solvent ratio not being correct. Even though a higher volume of
408 extraction solvent could have been tested to enhance efficiency when extracting higher
409 sample volumes, the experiments were eluded as a higher acetonitrile volume would drive
410 to longer evaporation times and could also affect the capacity of the LipiFiltr cartridge
411 clean-up efficiency.

412 *3.3. Method performance parameters*

413 To test the efficiency of the developed method, parameters such as apparent recoveries
414 ($\%R_{app}$), linearity, method detection limits (LOD), method quantification limits (LOQ),
415 repeatability (intra-day), and reproducibility (inter-day) were evaluated under the optimal
416 conditions. Cod and mackerel samples, being species with low and high lipid content,
417 respectively, were used to perform the evaluation.

418 Matrix effects previously calculated were found between -10 and 10% for most of the
419 compounds in both species, indicating that the matrix effect was negligible. Thus, the
420 quantification of the analytes was performed with internal standard calibration using
421 solvent standards directly injected into the system along with the apparent recoveries
422 depending on the lipid content of the analysed species.

423 Method limits of detection (MDL) and quantification (MQL) were estimated for each of
424 the target compounds based on their instrumental detection (direct injection, S/N = 3) and
425 quantification limits (first concentration on the instrumental linear range), the application
426 of the apparent recovery values (depending on its lipid content) and the dilution
427 calculations from the extraction procedure. For compounds present in the matrix (BT,
428 MeSBT, BSA, OHBT and Et-p-TSA), MDL were estimated as the blanks' average signal
429 plus three times the standard deviation of the blank. Thus, MDLs were comprised between
430 0.15 – 0.40 ng g^{-1} d.w. for BTs, 0.60 – 9.50 ng g^{-1} d.w. for BTRs and 0.15 – 6.85 ng g^{-1}
431 d.w. for BSAs. Regarding the MQLs, those were set between 0.30 – 1.00 ng g^{-1} for BTs,
432 5.00 – 19.50 ng g^{-1} for BTRs and 0.60 – 13.70 ng g^{-1} for BSAs. Apparent recoveries of
433 the method ranged between 50 – 105 % and 64 – 112% for BT in low and high lipid
434 content species, respectively; between 70 – 102% and 75 – 107% for BTRs in low and
435 high lipid content species, respectively; and between 71 – 85% and 67 – 84% for BSAs
436 in low and high lipid content species, respectively. Repeatability and reproducibility were

437 tested at 10 and 50 ng g^{-1} d.w. with values of %RSD below 14 and 12% (repeatability)
438 and below 19 and 15% (reproducibility). The quality parameters of the method can be
439 found in Table 1.

440

441 *3.4. Application to commercial fish samples*

442 The developed method was applied to samples of four different fish species bought in
443 local markets from Tarragona, Spain. Thus, triplicates of cod (*Gadus morhua*), mackerel
444 (*Scomber vincialis*), squid (*Loligo vulgaris*) and tuna (*Thunnus thynnus*) were analysed
445 using the developed method. To confirm the presence of the target compounds in the
446 samples, parameters such as retention time (min) and the presence of the qualifier
447 transitions as well as their ratios were compared with the data obtained from the analytical
448 standard. Table 2 comprises the target compounds found in at least one of the samples.
449 Concentrations are expressed on wet weight basis to enable easier comparison between
450 values found in the literature and the present study as well as to perform the exposure and
451 risk calculations. Figure 5 shows an example of the obtained chromatograms.

452 Four benzothiazoles and two benzenesulfonamides were quantified in the analysed
453 samples, whereas no BTRs were found. ΣBTs highest values were found for squid (49.8
454 ng g^{-1} w.w.) followed by cod (25.9 ng g^{-1} w.w.), tuna (11.7 ng g^{-1} w.w.) and finally
455 mackerel (6.4 ng g^{-1} w.w.). BT appeared in all the analysed samples with the highest
456 concentrations ranging between 42.7 ng g^{-1} w.w. (squid) and 1.4 ng g^{-1} w.w. (tuna).
457 Benzothiazole concentrations were followed by NH_2BT (2 – 5,9 ng g^{-1} w.w.), OHBT (1,6
458 – 3,1 ng g^{-1} w.w.) and MeSBT (1,1 – 1,9 ng g^{-1} w.w.). Regarding the distribution of the
459 compounds based on the lipid content, species with higher lipidic content such as tuna
460 and mackerel appear to have lower levels of benzothiazoles. For ΣBSAs, cod presented

461 the highest values (43.8 ng g^{-1} w.w.), followed by tuna (9.4 ng g^{-1} w.w.) and squid (3.3
462 ng g^{-1} w.w.). Samples of cod and tuna presented BSA concentrations of 15 and 9.4 ng g^{-1}
463 w.w., whereas Et-p-TSA was detected in cod and squid at 28.8 and 3.3 ng g^{-1} w.w.,
464 respectively. Opposite to benzothiazoles, no tendency is observed for their distribution in
465 different lipid content species.

466 To date, few studies have proven the presence of benzothiazoles in seafood samples.
467 Trabalón et al. [9] and Jia et al. [12] reported the presence of BT, NH_2BT, and MeSBT in
468 samples of fish (species from local markets of Tarragona, Spain) and molluscs (from the
469 Bohai Sea, China), respectively. Both studies agreed on BT as the benzothiazole with the
470 highest detection frequency as well as with the highest concentrations. Regarding the
471 concentrations, substantial differences were found in both studies, as per the fish from
472 Tarragona, BT concentrations ranged between 13-82 ng g^{-1} d.w., whereas molluscs from
473 the Bohai Sea reached concentrations up to 13400 ng g^{-1} d.w. (132 – 13400 ng g^{-1},
474 geometric mean: 595 ng g^{-1}). Squid appeared as the species with the highest
475 concentrations in both the Trabalón et al. [9] study and the present study. NH_2BT and
476 MeSBT concentrations were comprised between 11-70/ 0.165 ng g^{-1} d.w. and 11-24/ 14.2
477 ng g^{-1} d.w. for both studies, respectively. Other benzothiazoles were also detected in some
478 of the studies, Trabalón et al. [9] reported the presence of ClBT at 6-38 ng g^{-1} d.w.,
479 whereas Jia et al. [12] along with another study conducted by Chen et al. [37] on the
480 presence of benzothiazoles in fish specimens from Chung-li City (Taiwan) reported the
481 presence of OHBT at 20.1 ng g^{-1} d.w. and 15.4 – 26.1 ng g^{-1} d.w., respectively. Related
482 to the literature, the concentrations found in the analysed samples are similar to the ones
483 found in fish samples from the other studies. It is necessary to point out that the
484 concentrations obtained from the other studies are given on a dry weight basis, thus being
485 slightly higher than the ones from the present study. The detected congeners (BT, NH_2BT,

MeSBT and OHBT), their distribution (BT with the highest concentrations) and their concentrations agree with what the literature has reported to date about the presence of benzothiazoles in fish samples.

Regarding benzenesulfonamides, no data is available on their presence in seafood as this is the first method developed for their determination in this matrix. The presence of these compounds in water is also scarcely found in the literature. Hence, some studies have reported their presence in superficial waters and wastewater influent and effluent samples. Richter et al. [38] studies in Berlin's WWTP showed the presence of BSA in both influent and effluent water samples with values of 0.05 µg L^{-1} and 0.35 µg L^{-1}, respectively. Further studies by the same authors also reported higher levels of BSA in effluent samples than in influent samples, thus reporting a possible biodegradation/bioconversion of higher molecular weight sulfonamides to BSA during the WWTP process. A high removal efficiency of p-TSA at WWTP (~90%) was also denoted by the authors [11]. Regarding other compounds such as Et-p-TSA, Jover et al. [39] and Herrero et al. [40] reported concentrations of 0.086 and 36 -70 µg L^{-1} for influent and 0.122-0.831 and 0.026-0.074 for effluent, respectively. A similar correlation as with BSA appears for Et-p-TSA, with higher concentrations also found in effluent waters, suggesting again a possible bioconversion. BSA and Et-p-TSA have also been detected in superficial waters. Samples from Spanish rivers such as Besós, Ebre, Llobregat or Turia presented concentrations between 0.006 – 0.011 ng L^{-1} for BSA and 0.002 – 0.155 ng L^{-1} for Et-p-TSA [39,40]. Other authors such as Ajibola et al. [41], have also reported the presence of p-TSA, o-TSA and BSA in influents and effluents from WWTPs in Athens, Greece. In this case, BSA and p-TSA were mostly removed, while o-TSA was formed during the process. Even though in disagreement with the previous commented studies, authors point out the site-specific differences in the type of treatments used in each WWTP (sludge retention

time, pH value, temperature, etc.), which could then lead to these removal efficiency variations. On account of the previously reported data, the migration of these compounds towards aquatic environments and further incorporation in seafood seems a possible alternative. The concentrations found in the present study are in agreement with the said theory, as the major detected benzenesulfonamides in the analysed samples, BSA and Et-p-TSA, coincide with the benzenesulfonamides with the higher release from WWTP. Moreover, the lack of p-TSA detection could also be linked to the high removal efficiency.

3.5. Dietary intake and risk assessment

The dietary exposure of BTs, BSAs and BTRs was calculated for six subgroups (boys/girls, adult men/women and senior men/women) based on lower-, middle- and upper-bound scenarios. Exposure values ranged between 3.96 – 11.07 ng kg^{-1} bw day^{-1} for ΣBTs, 1.73 – 6.68 ng kg^{-1} bw day^{-1} for ΣBSAs and 1.06 – 3.37 ng kg^{-1} bw day^{-1} for ΣBTRs. The highest exposure values for ΣBTs and ΣBTRs were observed for the girls' subgroup in all the studied scenarios, whereas ΣBSAs exposure reached the highest level for senior men. On the other side, adult men and senior women are the subgroups with the lowest exposure values. Table S3 compiles the obtained values for all the studied subgroups and scenarios. Benzothiazole is the only compound in the present study with an associated NOAEL value corresponding to an oral repeated dose. Thus, a pTDI was estimated from the oral NOAEL of 5.1 mg kg^{-1} bw day^{-1} [9] by dividing the present value by an uncertainty value of 100 to compensate for the species difference, the human variation and the use of a subchronic study for the obtention of the value. As BT appeared in all the analysed samples, only one scenario was evaluated. Calculated dietary risk values (R_t) were comprised between $6.9x10^{-3}$(senior women) and 1.86x10-2 (girls), being

536 the later group with the higher value (Figure S1). Regarding the contribution of each of
537 the species, squid and cod are responsible for up to 94-99% of the risk of each of the
538 subgroups. Even though no threshold value to compare has been established for BT to
539 date, the obtained values ($<1.8x10^{-3}$) suggest that minor risk is expected from the
540 ingestion of the studied HPVs via dietary intake.

541

542 **4. Conclusions**

543 The use of QuEChERS extraction combined with a novel clean-up device such as
544 LipiFiltr followed by GC-QqQ-MS/MS has been successfully developed for the
545 determination of benzothiazoles, benzotriazoles and benzenesulfonamides in seafood
546 samples. Problematic analytical issues like matrix effect caused by a high complexity
547 matrix such as seafood or low instrumental signal have been solved using a simple
548 dilution of the extract combined with an effective clean-up step whereas the instrumental
549 signal was effectively enhanced; thanks to large volume injection. All in all, the proposed
550 method enables the analysis of a wide range of seafood species while avoiding the
551 necessity of using matrix-matched calibrations due to the negligible matrix effects. Its
552 easy procedure and reliable quality parameters turn this method into a useful tool to
553 generate data from both the aquatic environment contamination and the populations'
554 exposure and risk associated with seafood intake. On this behalf, the method has been
555 successfully applied to different local seafood samples, obtaining data on its
556 benzothiazole, benzotriazole and benzenesulfonamides contamination. The analysed
557 samples reported the presence of low levels (<42.7 ng g^{-1} w.w.) of some benzothiazoles
558 (BT, MeSBT, NH_2BT and OHBT) along with benzenesulfonamide and ethyl-p-
559 toluenesulfoamide, which, to the best of our knowledge, have been determined in seafood

samples for the first time. Studies on the exposure and risk associated with the dietary intake of the analysed samples by the population of Tarragona suggested values between 3.96 – 11.07 ng kg^{-1} bw day^{-1} for ΣBTs, 1.73 – 6.68 ng kg^{-1} bw day^{-1} for ΣBSAs and 1.06 – 3.37 ng kg^{-1} bw day^{-1} for ΣBTRs, being girls the population subgroup with the highest exposure. Regarding the risk assessment, BT showed a risk factor of 0.18%, which indicates a minor risk of developing chronic adverse effects due to the ingestion of this compound through the diet. Future research should be focused on the development of novel methodologies including more target compounds of these high production volume chemical families. Moreover, assays aiming to elucidate NOAEL values of the compounds of the present study are urgent, as to date, only benzothiazole has an assigned value.

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Figure 1. Apparent recoveries (R%) (50 ng g^{-1} d.w. n=3, %RSD < 19%) obtained for the different clean-up strategies: LipiFiltr, PSA, PSA+C_{18} and hexane.

Figure 2. Matrix effects (M.E. %) (50 ng g^{-1} d.w. n=3, %RSD < 22%) obtained for the different clean-up strategies: LipiFiltr, PSA, PSA+C_{18} and hexane.

Figure 3. Apparent recoveries (Rapp% 50 ng g^{-1} d.w. n=3, %RSD < 16%) when using different extraction salts packets corresponding to three different official methods.

Figure 4. Effect on apparent recoveries (Rapp % 50 ng g^{-1} d.w. n=3, %RSD < 21%) of the amount of fish extracted.

Figure 5. Chromatograms obtained from the analysed samples.