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Research Article

Quantitative Headspace Solid-Phase Microextraction Gas Chromatography Mass Spectrometry (HS-SPME-GC-MS/MS) Method for the Determination of Tributyltin in Sediment: Validation according to EU Directive Requirement

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Abbreviations EQS: Environmental Quality Standard

Abstract

The Directive 2013/39/EU has reviewed the list of priority substances under the European Water Framework Directive (WFD; Directive 2000/60/EC) and has strengthened the principle of flexibility for Member States in applying the Environmental Quality Standard (EQS) for alternative matrices. Coherently, it has established new EQS for biota and it has invited Member States to set EQS for more opportune matrices, able to advantage the monitoring strategy and offer the same level of protection, such as sediment. The present work developed a (HS)SPME-GC-MS/MS method for the analysis of Tributyltin (TBT) in sediment samples and, through an in depth validation process, it assessed the compliance with the technical specification required by the EU Directive 2009/90/EC for chemical status analysis under the scope of the WFD. The EQS established for TBT in sediment by the Italian Environmental Ministry was used as reference. The method fulfils the minimum performance criteria required by the EU Directive 2009/90/EC (Method Detection Limit (MDL) and Minimum Level Of Quantitation (ML), expressed as ng Sn g^{-1} d.w., were 0.2 and 0.5, respectively). More generally, the analytical figures of merit achieved, were satisfying for the target TBT concentration range (recovery: 90-111%, intermediate precision range: 6-12%).

Introduction

The EU Directive 2013/39/EU [1] has amended the Water Framework Directive (WFD, Directive 2000/60/EC [2]) and the Directive 2008/105/EC [3], by considering the latest knowledge on emerging pollutants, their toxicological impact and environmental fate. It has reviewed the list of priority pollutants in surface water bodies (now counting 45 substances) and the associated Environmental Quality Standards (EQS). In the revision process, it has strengthened the principle of assessing the chemical water quality by focusing on the aquatic compartments where the target substances primarily concentrate and so their levels are more likely to be measurable. Accordingly, it established the EQS in biota for 10 lipophylic priority toxicants and it invited Member States to set their own EQS for matrices able to offer the same level of protection as well as advantaging the monitoring strategy.

The EU Directive 2013/39/EU has not reviewed the EQS of Tributyltin (TBT) for water, which are still set at 0.2 ng TBT^{+} L^{-1} (Annual Average Concentration, AA-EQS) and 1.5 ng TBT^{+} L^{-1} (Maximum Allowable Concentration, MAC-EQS). To monitor these levels is very challenging for routine laboratories. In fact, the legislation requires the use of analytical methodologies which fulfil strict Minimum Performance Criteria in order to ensure the quality and the comparability of data generated by all the laboratories in charge. More specifically, the Commission Directive 2009/90/EC of 31 July 2009 [4], laying down technical specifications for chemical analysis and monitoring of water status within the scope of the Directive 2000/60/EC, requests the use of methods featured by 1) a limit of quantification equal or below a value of 30% of the relevant EQS, and 2) an uncertainty of measurement of 50% or below (k = 2) estimated at the level of relevant EQS.

To overcome this analytical problem, which is common to other priority substances, Member States can take the initiative and establish new EQS for alternative matrices which can be detected without the need of high resolution instruments. In the case of TBT, the Italian Government set the

EQS for sediments at 5 μg TBT^{+} kg^{-1} (Ministerial Decree 260/2010 [5], Legislative Decree 172/2015 [6]), corresponding to 2 μg Sn kg^{-1}. The choice of sediment as preferential compartment for assessing TBT water quality is due to the strong tendency of TBT to associate with natural sorbents, and its persistence under anoxic conditions [7].

Several methods have been published for the analyses of TBT in sediment and soil-like materials [8-9], including the ISO 23161:2009 [10] which, unfortunately, is not suitable to assess the EQS set by the Italian government because it is featured by a quantification limit of 10 μg TBT^{+} kg^{-1}.

The analysis of TBT in sediments is not exempt of analytical challenges, because of its trace level presence (ng g^{-1}), strong adsorption onto the sediment and susceptibility to degradation during sample preparation steps. The Solid Phase Microextraction (SPME) technique represents a valuable technique to extract TBT from environmental samples in a quick and efficient way. As other modern microextraction techniques, SPME minimizes the use of organic solvents and simplifies the sample preparation by reducing into a single step the derivatization, concentration and isolation of the analytes. It relies on the use of a thin film of a suitable stationary phase, coated to a silica fibre, which adsorbs the target analytes once immersed into the sample itself or its gaseous phase (headspace mode, HS). Compared to the common extraction techniques, it is competitive in terms of high pre-concentration power and low interaction with matrix.

Despite several valuable SPME–GC–MS methods for TBT in sediment have been already published in the literature [11-21], they cannot be straightforward used in institutional monitoring because their validation does not fulfil the legislation requirements. Taking advantages from SPME methods published in the literature, in the present work we developed a simple (HS)SPME–GC–MS/MS methodology for TBT in sediment and we critically evaluated its possible use for monitoring within the scope of WFD. The EQS established for sediments by the Italian Environmental Ministry was used as reference. An in-depth uncertainty study was performed to assess the accomplishment of the analytical criteria required by the Commission Directive 2009/90/EC for monitoring methods under the WFD.

The proposed method relies on the use of the ion trap mass spectrometer operating in MS/MS mode as detection system. Compared to the single quadrupole mass filter, this detection technology provides higher sensitivity and selectivity, resulting in a good option for the analysis of trace pollutants in complex matrix samples. The matrix match signal ratio external calibration was used for quantification purpose. This approach allows to quantify different samples by the use of one external calibration curve and it is suitable for ion trap MS, as well as any other kind of mass spectrometer detector. Differently from most of the methods reported in the literature, the two mostly used quantification approaches for SPME applications, i.e. the standard addition [11-15] and isotopic dilution [22, 23] methods, were discarded. In fact the former requires the preparation of a calibration curve for each sample, nullifying any prospect of routine use, whereas the latter, which is recognized as the most accurate and time saving option, cannot be applied in ion trap MS application because of the insufficient precision of this detector in measuring the relative proportion of isotopes in the labelled and natural TBT.

Materials and Methods

Reagents, solutions and reference materials

For TBT sediment extraction, hydrochloric acid and a methanolic solution of Tropolone (0.05% w/v) were used (HCl 34-37% superpure for trace analysis, Carlo Erba Reagents, Italy; methanol HPLC-Plus gradient, Carlo Erba Reagents, Italy; Tropolone purum ≥98.0% (GC) Fluka analytical, USA). A sodium acetate/acetic acid buffer solution (1.73 M, pH 4.74) was made up by mixing appropriate amounts of sodium acetate (CH_3COONa ACS-ISO, Carlo Erba Reagents, Italy) and acetic acid (RPE glacial for analysis ACS-ISO, Carlo Erba Reagents, Italy). A 2% (w/v) solution of sodium tetraethylborate ($NaBEt_4$, 98%; Aldrich, Italy) was prepared immediately before use in high purity water (18 MΩ·cm at 25°C) in a closed plastic glove-bag filled with nitrogen, in order to obtain an inert atmosphere. The deionized water used throughout the study was generated on-site by Milli-Q® Integral 5 Water Purification System (Merck Millipore, Vimodrone (MI), Italy). TBT standard (TBTCl, 98.5%) was purchased from Chiron AS (Trondheim, Norway), while deuterated internal standard (TBT-d27-Cl, 97%) was provided by C/D/N Isotopes Inc (Pointe-Claire, Canada). The stock solutions were prepared at a concentration of 1000 ng Sn μL^{-1} in methanol; from these, intermediate working standard solutions (1 ng Sn μL^{-1}) were prepared for calibration purposes.

100 μm polydimethylsiloxane (PDMS) fibres for SPME, purchased from Supelco (Sigma Aldrich, Italy), were conditioned according to Supelco's instructions before use. 20 mL headspace vials and associated screw caps with PTFE/silicone septum were purchased from Microcolumn srl (Lissone (MB), Italy).

For validation, three Certified Reference Materials, produced by the National Research Council of Canada, were used: SOPH-1, PACS-2 and PACS-3.

Blank sediment samples from Tyrrhenian Sea were used in the scope of method optimization and validation. Precisely, they were used as matrices for the spiking experiments (i.e. optimization of the solid/liquid extraction method, tests for evaluating the detection and quantification limits) and for preparing matrix-match standard solutions (i.e. linearity study, test for assessing the absence of matrix effects in calibration, SPME time profile, quantification). All samples were previously characterized for Total Organic Carbon content (TOC %) and grain-size composition.

Extraction from solid sample and derivatization

1.00 g of freeze-dried sediment (dry weight, d.w.) was extracted by sonication in ultrasonic bath (1 hour) with 4.875 mL of extraction solution (0.05% Tropolone in methanol) and 0.125 mL of HCl. The leaching was repeated twice, by the use of half of the reagent volumes. Once the extraction was completed, the supernatant was recovered by centrifugation (10 min at 2000 rpm). 1.5 mL of supernatant were transferred into a 20 mL headspace vial containing 8 mL of acetate buffer solution. Thus, a proper volume of deuterated internal standard was added to each vial. Afterwards, vials were crimped with caps provided with PTFE lined silicone septum. The analytes were ethylated at room temperature by adding 0.5 mL of a freshly prepared 2% water solution of $NaBEt_4$. Each vial was vortexed for two minutes to encourage the derivatization reaction before undergoing to SPME extraction.

(HS)SPME and GC-MS/MS analysis

The SPME was carried out in automated mode by using a TriPlus RSH liquid autosampler - SPME version (Thermo Fisher Scientific), interfaced to the GC-MS (Trace 1300 gas chromatograph coupled to ITQ 700 Ion Trap mass spectrometer, Thermo Fisher Scientific). (HS) SPME was performed after 20 minutes of incubation of the sample at 40°C, under constant agitation. The PDMS fibre was exposed in the headspace for 5 minutes at the same temperature and agitation conditions. Once the extraction step was completed, the fibre was desorbed for 3 minutes in the GC injection port (at 250°C) equipped with a 0.75 mm (I.D.) inlet liner (in splitless mode). Following desorption, the fibre was baked at 250°C for 5 minutes in the injector to prevent sample carry over (split valve open).

The analytical GC-MS/MS conditions are listed in Table 1. They were previously optimized as described elsewhere [24].

The peak area ratio between the analyte and the internal standard was computed for quantification purpose. In order to perform the external calibration, matrix-matched standard solutions were prepared by adding proper volumes of analyte and deuterated standard solutions into SPME vials containing 1.5 mL of TBT-free sediment extract and 8 mL of acetate buffer. As described in section 2.2., they underwent to the derivatization reaction by the addition of 0.5 mL of 2% solution of $NaBEt_4$.

Throughout this work, TBT concentrations are expressed as ng Sn g^{-1} of dry weight sediment (d.w.). Unless further indication, all tests were performed in triplicate (n=3).

Results and Discussion

Optimization

Solid/Liquid Extraction and Derivatization: In this study, the isolation of TBT from the sediment was carried out via solid/liquid extraction with a methanolic solution of tropolone (0.05%) in acidic environment [25]. Here we assessed the possible influence of the acidic condition in the extraction yield of TBT from sediments having different nature. To this purpose, we compared the analyte signal intensity obtained from the extraction of four spiked sediments (25 ng Sn g^{-1} of TBT) under two leaching conditions (HCl 1.2M vs. HCl 0.3M). The tested sediments differed for grain-size composition and total organic carbon amount (Table 2). Differences in the analyte signals were assessed through the ANOVA-nested design (p-level=0.05).

The extraction method applied in lower acidic condition (0.3M HCl) provided higher signal intensity for TBT, compared to the 1.2M HCl method (ANOVA nested design, $p<0.05$). As the same pattern was observed for the analogous deuterated compound, which was added after the acidic extraction, we can suppose that the major sensitivity was due to more favourable conditions for the equilibrium partition towards the fibre phase, and not to a different efficiency of the two solid/liquid extraction methods. According to Zuliani et al. [26] and references therein, it is likely that the weaker acidic condition allowed the co-extraction of lower amounts of sediment matrix constituents (i.e. fulvic acid) which can retain the analytes into the liquid phase via complexation. Although the pH in the two $NaBEt_4$ reactions media were statistically different (Table 2; t-test $p<0.05$), they were close and within the optimal pH range (pH 4-5), indicated by the literature for derivatization with $NaBEt_4$ [11, 19, 27]. In this respect, we excluded that the described behaviour might be due to different derivatization yields.

Besides providing major sensitivity for TBT, the extraction in presence of a lower concentration of HCl (0.3M) did not provided sample-to-sample differences in the analyte signal intensity (ANOVA, $p<0.05$). This result suggests that the method provides similar extraction yields for sediments having different nature (and

Table 1: GC-MS/MS operating conditions; quantification ions are in bold.

	GC	
Column	DB-5MS (length: 25m; ID: 0.2 mm; film thickness: 0.33 µm)	
Injector	SSL (split/splitless); T= 250°C; splitless time= 3 minutes	
Flow	Constant flow rate (1.0 mL min^{-1})	
Temperature program	50°C held for 3 min; 30°C min^{-1} to 140°C; 10°C min^{-1} to 160°C ; 5°C min^{-1} to 185°C; 30°C min^{-1} to 250°C, held for 4 minutes; 30°C min^{-1} to 300°C, held for 4 min	
MS transfer line	240°C	
	MS	
Ion source temperature	250°C	
Ionization	Electron Impact	
Mode	MS/MS	
	Scan Events	
Target molecules	**TBT**	**TBT-d27**
Retention time (minutes)	7.26	7.20
Excitation (V)	0.07	0.07
Precursor ion mass (m/z)	291	318
Product ion mass (m/z)	**179**	**189-190**
Product ion mass (m/z)	**234-235**	**253-254**
Product ion mass (m/z)	**265**	**318**

Table 2: Optimization of solid/liquid extraction method of TBT from sediment: characterization of the four sediment samples (Sed 1-4) used in the optimization experiment (percentage, grain-size composition and TOC content), and comparison of the pH values of the extracts and the $NaBEt_4$ reaction medium obtained through the use of tested extraction solution (1.2M HCl vs. 0.3M HCl methanolic solution of Tropolone).

							pH			
	Gravel %	Sand %	Silt %	Clay %	Silt+Clay %	TOC %	Extract		$NaBEt_4$ reaction medium	
							1.2M HCl	0.3 M HCl	1.2M HCl	0.3 M HCl
Sed 1	21.8	77.0	1.2	0.0	1.2	0.13	-0.77	0.28	4.47	4.96
Sed 2	22.8	55.1	11.1	11.0	22.1	0.27	2,15	5.90	4,85	4,98
Sed 3	17.5	55.3	10.8	16.4	27.3	0.35	1.16	3.67	4.85	4.99
Sed 4	12.4	85.1	2.5	0.0	2.5	0.20	-0.36	2.92	4.64	4.91

so different buffering capability) which highly influences the pH of extraction medium (see pH values of 0.3M HCl extracts; Table 2). Thus, for the final protocol, the addition of pure HCl leading to 0.3M concentration in the extraction medium was opted.

The optimal conditions for derivatization reaction with $NaBEt_4$ have been extensively addressed in the literature. Thus, in this study the derivatization reaction was carried out at room temperature, consistently with the methods published by Carvalho et al. (2007), Delgado et al. (2007), and Carpinteiro et al. (2004) [13-15]. After the addition of $NaBEt_4$ (0.5 mL), each vial was vortexed for two minutes [11,16], as the agitation improves the reaction kinetics [13]. Then, each sample underwent SPME.

SPME time: The SPME optimization study mainly focused on the choice of the fibre extraction time, which is highly influenced by the nature of the sample matrix. The SPME time profile was obtained by repeated measures (n=3) of matrix-matched standard solutions at increasing sorption time (up to 90 min). The results plotted in Figure 1 pointed out a change in the sorption dynamic after 40 minutes of fibre extraction, as evidenced by the decrease of the curve slope. Also other methodological studies on SPME extraction of butyltins from abiotic environmental samples (i.e. sediment, soil and sludge), found that the equilibrium was reached after 30-40 minutes of fiber extraction time [12-14, 19-21, 26]. Differently from these methods, we selected a pre-equilibrium SPME extraction time of 5 minutes by observing that the analyte areas, corresponding to the sediment target concentration (2 ng Sn g^{-1} d.w.), were sufficiently high after 5 minutes and increase rapidly within the working range (Figure 2). In fact, because SPME is an equilibrium-extraction method, the fibre sorption time determines the amount of analyte that is extracted, and thus it controls the sensitivity of the method. Nevertheless, if the achieved analytical sensitivity is sufficient for the quantitative analysis, it is not necessary to reach the equilibrium [11, 20, 22]. This choice was supported by the combined use of a SPME autosampler, which keeps constant SPME conditions, and isotope labelled internal standard, which normalizes minor variation [20]. The three-phase extraction equilibrium (extract/headspace/fiber) highly depends on temperature, which influences analytes' solubility and their distribution coefficient between headspace and fiber coating. Higher temperatures can favor the partition of TBT into headspace, but also induce the desorption of MBT from the fiber. Thus the fiber extraction temperature of 40 °C was chosen as compromise between the optimum partition coefficient of TBT and MBT into the 100 µm PDMS fiber [11, 13, 16].

Validation

The method performances evaluated during the validation procedure were: linearity, matrix effect calibration, sensitivity (limit of detection and limit of quantitation), trueness and measurement uncertainty. Selectivity and blank interferences/contamination were also assessed at the begin of the validation process.

Selectivity and blank interferences/contamination: Repeated analyses of sediment matrix blanks, proceeded according to an inter-day experimental design, pointed out the high confidence in the identification of the target analyte. In fact, no background interference was found at the analyte retention time (data not shown). This is mainly due to the combined use of SPME in headspace mode, which

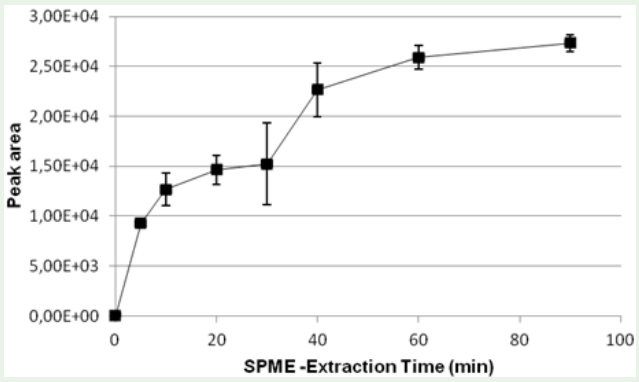

Figure 1: SPME extraction time profile; mean values and standard deviation of TBT peak area (n=3); SPME vial content: 10 ng Sn (matrix match standard solution).

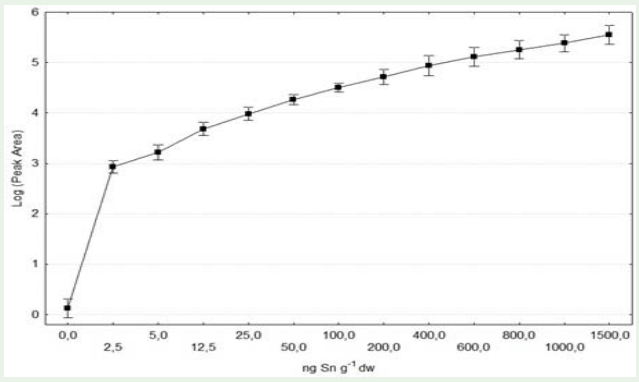

Figure 2: Mean and whiskers plot of TBT peak area (log- transformed) versus TBT sediment concentration. SPME time= 5 min; mean values and 95% confidence interval (n = 15).

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Table 3: Calibration models: PG value ($F_{1-0.05}$ = 4,9646 on the hypothesis of polynomial regression) and fitted regression (model order, correlation coefficients, r^2; regression coefficients a, b, b^2).

	PG value	Order	r^2	a	b	b^2
Regression curve 1	0.09	1	0.688889	0.110417	0.044	
Regression curve 2	*10.01*	*2*	*0.690972*	*0.261806*	*0.035*	*0.000007*
Regression curve 3	0.09	1	0.690972	0.428472	0.036	
Regression curve 4	2.07	1	0.6875	0.282639	0.038	
Regression curve 5	1.00	1	0.679167	1.002	0.032	

minimizes the co-sorption of matrix components, and tandem mass spectrometry (GC–MS/MS), which focuses on selective precursor-product ions relations. Despite this, it is always advisable to carry out a preventing control of reagent contamination, by performing reagent blanks regularly.

Linearity: The linearity study was carried out by modelling the TBT concentrations through both linear and quadratic least-square parametric regression models. To this purpose, 13 matrix match standards were prepared within the concentration range of 0–1500 ng Sn g^{-1} (the point 0 ng Sn g^{-1} was the blank sample). Each concentration was analysed in triplicate. The ISO 8466 approach [28] was used to compare the fitting capacity of the linear and the quadratic regression models for calibration purpose. Basically, this approach consists in comparing the residual error associated to the linear regression with the residual error caused by a second order polynomial regression applied to the same data, according to the following Equation (1):

$$PG = \frac{(N-2)S_{res}^2 - (N-3)S'^2_{res}}{S'^2_{res}} \quad (1)$$

In Equation (1) S_{res} indicates the residual standard deviation for the linear model, S'_{res} is residual standard deviation for the polynomial model, and N is the regression point number. The value PG is compared with the Fischer critical value ($F_{1-\alpha}$; degrees of freedom 1 and N-3). When $PG \leq F_{1-\alpha}$, it is inferred that the polynomial calibration function does not provide an improved adjustment, and so the calibration function is linear.

Table 3 reports the results of the linearity studies. According to the ISO 8466 approach [28], TBT responses resulted linear over the studied range, except for curve 2. However, by considering the positive value of the second order coefficient (usually negative because of the kinetics of the three-phases equilibrium), the quadratic fitting of curve 2 is likely due to noisy data interpolation. Thus, we accepted the linear regression also for curve 2 (r^2=0.990; a=-0.483; b=0.044).

Matrix effects in calibration: Among the methods suitable to quantify data obtained from the combined use of SPME and ion trap tandem mass spectrometry (standard addition; matrix-matched signal ratio external calibration), the matrix-matched signal ratio external calibration is the most feasible for routine analysis. As previously mentioned, the most sensitive and accurate isotope dilution approach, cannot be used in ion trap mass spectrometry applications because this detector does not allow the precise measurement of the small mass difference between the labelled and natural TBT.

When external matrix-matched calibration curves are used for the quantification of analyte concentrations in sediments, it is important to be aware that the specific sample nature can highly influence the equilibrium partition and thus the signal intensity. By considering the extreme heterogeneity and unpredictability of sediments as analytical matrix, it is highly recommended to use the internal standard for signal normalization and it is mandatory to test the lack of significant matrix effects in the area ratio measurements.

The absence of significant matrix effects was assessed by the t-test on the null hypotheses of parallelism of different matrix-matched calibration curves. To this scope, we compared four matrix-matched external standard curves (concentration range: 0-400 ng Sn g^{-1}) prepared by using four sediment blank samples, differing for the percentage of TOC and grain-size composition. The results of the t-test on the null hypotheses of parallelism are summarized in Table 4. They confirm the ability of the internal standard to normalize sample dependant- differences in the three phases equilibrium partition of TBT, as well as fluctuations typical of MS detection.

Limit of detection and limit of quantitation: In (HS)SPME-GC-MS/MS methods, the design of the experiments for establishing limits of detection and quantification has to consider: 1) the high selectivity achievable by coupling SPME with tandem mass spectrometry, and 2) the implication arising from the equilibrium extraction approach. In fact, the second MS analysis makes almost null the baseline noise, whereas the matrix, making part of the equilibrium liquid phase, controls the analyte transport to the fibre coating and, thus, the method sensitivity. In the light of this feature, we considered the EPA method for Method Detection Limit (MDL) and Minimum Level of

Table 4: T-test for parallelism hypothesis with p-value *0.01*: for each sample matrix (M1-M4) a linear regression was calculated on three repeated measurements of nine concentrations (0-400 ng Sn g^{-1} dw); mean values and Total Sum of Squares (TSS) of x and y (TBT concentrations on sediments and area ratio between the analyte and the isotope-labelled internal standard, respectively), intercept (a) and angular coefficient (b) of the linear regression, t-student values and critical value (in the brackets); the characterization of the sediment blanks in terms of TOC% and grain-size composition is presented in the upper part of the table.

					M1	M2	M3	M4
TOC%					0.06	3.61	1.60	1.47
Gravel%					0.0	11.0	0.0	17.0
Sand%					97.5	52.7	83.1	76.0
Silt+clay%					2.5	36.3	16.9	7.0
		Mean	**TSS**	**Regression coefficients**	**t-value (critical value, N-4; p=0.01)**			
					M1	**M2**	**M3**	**M4**
M1	**x**	88.33	429263	a= 0.325				
	y	3.67	618	b= 0.038				
M2	**x**	77.50	304650	a= 0.067	0.74 (2.69)			
	y	3.52	608	b= 0.045	-1.90 (2.69)			
M3	**x**	91.63	421612	a= 0.425	-0.30 (2.68)	-1.02 (2.69)		
	y	3.91	621	b= 0.038	-0.07 (2.68)	1.80 (2.69)		
M4	**x**	87.88	436845	a= 0.239	0.26 (2.68)	-0.49 (2.69)	1.31 (2.68)	
	y	4.01	874	b= 0.043	-1.57 (2.68)	0.41 (2.69)	-1.79 (2.68)	

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Quantitation (ML) [29] as the most proper to provide robust insights of method sensitivity. MDL is defined as "the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero, and is determined from analysis of a sample in a given matrix containing the analyte", while ML as "the lowest level at which the entire analytical system must give a recognizable signal and acceptable calibration point for the analyte" [29].

Thus, the values of MDL and ML were calculated according to EPA recommendation. Replicated measurements (m=10) of sediment samples, spiked at TBT concentration of 0.50 ng Sn g^{-1} dw, were carried out. Thus the MDL was computed as the product between the measurements standard deviation and the t-student value (df=n-1), and the ML as 3.18 times the corresponding MDL value.

As further confirmation, DL (Detection Limit) and QL (Quantitation Limit) were also determined by applying the ICH approach [30], which is based on the uncertainty of calibration curves in the range of QL.

To this purpose, a calibration curve in the range of the expected QL, was used in order to calculate DL and QL. Calculations were made according to Equations (2) and (3):

$$DL = \frac{3.3 * \sigma}{S} \quad (2)$$

$$QL = \frac{10 * \sigma}{S} \quad (3)$$

where σ is the residual standard deviation of the regression line, and S is the slope of the calibration curve.

The limits obtained with two approaches were comparable and they pointed out a good sensitivity of the method. The detection limit, expressed as both MDL and DL, was 0.2 ng Sn g^{-1}. The quantitation limit was 0.5 ng Sn g^{-1} when computed as ML, and 0.6 ng Sn g^{-1} when calculated as QL. Furthermore, the ML (EPA approach) and QL (ICH approach) values obtained match the minimum performance criteria required by the Directive 2009/90/EC [4] for methods to be used within the scope of WFD monitoring. Indeed, they are below, or equal, to 30% of the value of the target EQS (i.e. 1.5 ng TBT^{+} g^{-1}, equivalent to 0.6 ng Sn g^{-1}).

Table 5: Trueness and precision study: AOAC Expected Recovery range reported for demonstration of fitness of recoveries obtained for certified reference materials; Target RSD % by Horwitz describes predicted relative standard deviation at appropriate concentration levels.

		SOPH-1	PACS-2	PACS-3
Measured concentration (ng Sn g^{-1} dw)	**Mean**	113	926	451
	St.dev	14	53	44
Certified Reference value (ng Sn g^{-1} dw)	**Assigned Value**	125	832	410
	Error	7	95	40
Trueness	**% Recovery**	90%	111%	110%
	AOAC Expected Recovery	*80-110%*	*80-110%*	*80-110%*
Intermediate Precision (R)	**RSD%**	12%	6%	10%
	Target RSD%-Horwitz	*22%*	*16%*	*18%*
	n	3	3	3
Repeatability (r)	**RSD%**		4%	
	n		3	

Trueness, precision and measurement uncertainty: The trueness study addressed all possible aspects of intra-laboratory bias (e.g. solid sample extraction, calibration). It was carried out by analysing Certified Reference Materials and it was evaluated in terms of percentage recovery compared to the certified concentration.

The results, reported in Table 5, showed that the recoveries obtained are comparable with the acceptable percentage recovery range established by the Association of Official Analytical Chemistry (AOAC; [31]) for analyses of a similar concentration range (80–110%).

The method precision was evaluated in terms of within laboratory repeatability (intra-batch; r) and intermediate precision (inter-batch; R). The former mainly indicates the repeatability of the solid/liquid extraction method, whereas the latter represents the general consistency of data produced by a single laboratory. In fact R is affected by several batch-to-batch variation sources, like the SPME fibre, reagent commercial lot, the blank sediment sample used for preparing the matrix-matched calibration curves, the instrument tune and the analyst. Both r and R were expressed in terms of RSD%, being the standard deviation proportional to the concentration.

The within laboratory repeatability (intra-batch; r), which was estimated on the results of three replicated extraction of one of the available certified materials at the same time (i.e. PACS-2, intra-day analysis, single batch), resulted 4% (Table 5). It is likely that the simplicity of the protocol for the solid/liquid extraction and sample preparation for the SPME analysis positively contributed to this result. The minimal sample manipulation lessened variation due to possible losses of the analyte, which is a common weakness of extraction techniques involving separation, clean-up, solvent changes and concentration steps.

The intermediate precision (inter-batch; R) was evaluated at three representative levels of sediment contamination by analyzing the Certified Reference Materials during three different days (m=3; main changing variables: SPME fibre, reagent commercial lots, sediment blank samples used for preparing the matrix-matched calibration curves). The results, reported in Table 5, were compared with Horwitz values which were computed by applying the Horwitz function to the estimated concentrations [32]. By considering Horwitz values as independent fitness-for-purpose criteria for method precision, we observe that TBT concentration measurements were more precise than the predicted ones.

Measurement uncertainty: The expanded uncertainty was calculated in order to verify the method compliance to the minimum performance criteria required by the Commission Directive 2009/90/EC (i.e. expanded uncertainty of measurement of 50% or below estimated at the level of EQS; k=2). The EURACHEM/CITAC guide [33], which interprets the ISO/IEC Guide 98-3:2008 to the Expression of Uncertainty in Measurement [34] in the field of analytical chemistry, was followed to this purpose.

The uncertainty study covered the full scope of the method (i.e. heterogeneity of sediment composition, working concentration range

of the analyte) and it was representative of the normal operation required by the protocol. Basically, after the evaluation of all the potential sources of uncertainty associated to measurements, three individual uncertainty components were computed: 1) the inter-batch method precision uncertainty (u(P)/P); 2) the method recovery uncertainty (u(Rm)); and 3) the run-to-run (HS)SPME-GC-MS/MS precision uncertainty (alias analytical response uncertainty; $u(P_{SPME-GC-MS/MS})/P_{SPME-GC-MS/MS}$). They covered the following potential sources of uncertainty:

- **inter-batch precision experiment:** uncertainty associated to the calibration curve (i.e. interpolation, matrix differences between standards and unknowns, concentration of working standards solution), reagent purity, GC-MS tune conditions, repeatability among SPME fibres;
- **trueness experiment:** bias and uncertainty of solid/liquid extraction efficiency, sediment samples weight, standard purity, concentration of working standards solution. Furthermore, the recovery uncertainty estimation included the influence of the matrix effects in the calibration (matrix differences between standards and reference material), which has been already considered in the intra-batch precision experiment, but that was not possible to exclude;
- **(HS)SPME-GC-MS/MS precision experiment:** run-to-run variation on all the conditions affecting the efficiency of fibre extraction and instrumental detection (i.e. fibre state and aging, all variables influencing the three-phases equilibrium, MS/MS detection stability, peak areas integration), volumes of reagents, sample extracts and internal standard solution.

The batch-to-batch precision study was carried out by replicated analyses of certified materials and reference samples which cover the TBT concentration working range and different sediment composition. Each sample was analysed a total of three times in separate extraction batches. For each batch, matrix-matched calibration standards were prepared by using different sediment blank samples; furthermore fresh reagents and new SPME fibres were used. As the standard deviation was dependant on the concentration, the precision was estimated in terms of RSD. Thus, the u(P)/P was computed according to the Equation (4) of the pooled RSD:

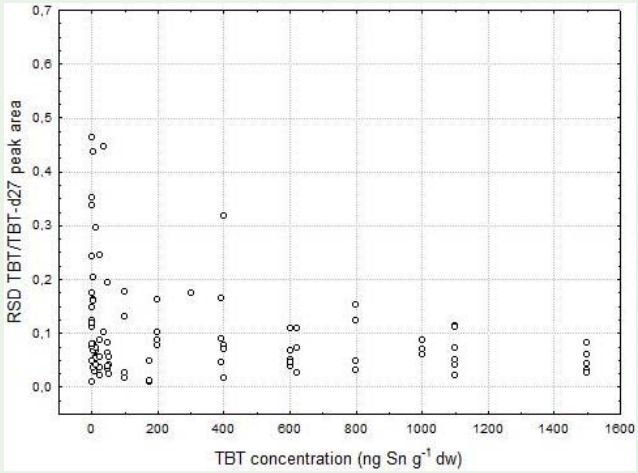

Figure 3: RSD of TBT/TBT-d27 Area Ratio measurements (n=3) versus sediment sample concentrations.

$$RSD_{pool} = \sqrt{\frac{(n_1 - 1) * RSD_1^2 + (n_2 - 1) * RSD_2^2 + ...}{(n_1 - 1) + (n_2 - 1) + ...}} \quad (4)$$

where RSD_i is the relative standard deviation calculated for the i- reference sample and ni is the respective number of replicated analyses.

The uncertainty of the method recovery, $u(R_m)$, was estimated through the analyses of three aliquots of a certified material (PACS-2; intra-day analyses, single batch). Then the following Equation (5) was applied to the obtained results:

$$u(\overline{R}_m) = \overline{R}_m * \sqrt{\left(\frac{s_{obs}^2}{n * \overline{C}_{obs}^2}\right) + \left(\frac{u(C_{CRM})}{C_{CRM}}\right)^2} \quad (5)$$

where R_m is the mean recovery, sobs is the standard deviation of the results from the replicate analyses of the PACS-2, n is the number of replicates, Cobs is the mean concentration observed, CCRM is the certified concentration and u(CCRM) is the standard uncertainty of the certified value (i.e. half of the expanded uncertainty reported in documentation supplied with PACS-2; coverage factor k=2).

The SPME-GC-MS/MS precision uncertainty, $u(P_{SPME-GC-MS/MS}/P_{SPME-GC-MS/MS})$, was computed by pooling the RSD obtained by triplicate SPME-GC-MS/MS measurements (n=3) according to Equation [4]. The estimation was based on 90 RSD data (m=90), referring to samples spanning the whole concentration working range and showing heterogeneity in matrix composition.

Table 6: Measurement uncertainty study: single components uncertainty (Inter-batch precision, recovery, run-to-run SPME-GC-MS/MS precision); Standard and Expanded Uncertainty; estimated Standard and Expanded Uncertainty at TBT EQS level.

		TBT
INTER-BATCH PRECISION UNCERTAINTY STUDY		
SOPH-1	RSD (m=3)	0.12
PACS-3	RSD (m=3)	0.10
PACS-2	RSD (m=3)	0.06
	u(P)/P	**0.10**
RECOVERY UNCERTAINTY STUDY ON PACS-2		
Observed concentration	Mean and standard deviation (ng Sn g^{-1}; m=3)	927±42
Certified value	Assigned value and expanded uncertainty (k=2; ng Sn g^{-1})	832±95
Recovery	Mean (R_m)	1.11
	u(R_m)	**0.07**
	t-test value (H_0: R_m=1)	-1.63
RUN-TO-RUN SPME-GC-MS/MS PRECISION UNCERTAINTY STUDY (m=90; n=3)		
	$u(P_{SPME-GC-MS/MS})/P_{SPME-GC-MS/MS}$	**0.14**
	Standard Uncertainty	0.19
	Expanded Uncertainty (k=2)	0.37
	Standard Uncertainty at EQS (ng Sn g^{-1})	0.38
	Expanded Uncertainty at EQS (k=2; ng Sn g^{-1})	0.76

Citation: Noventa S, Formalewicz M, Barbaro J, Gion C, Rampazzo F, Gabellini M, et al. Quantitative Headspace Solid-Phase Microextraction Gas Chromatography Mass Spectrometry (HS-SPME-GC-MS/MS) Method for the Determination of Tributyltin in Sediment: Validation according to EU Directive Requirement. SM Anal Bioanal Technique. 2017; 2(1): 1007.

Table 7: (HS)SPME-GC-MS/MS precision study: RSD (m=90; n=3); basic statistics of RSD of replicated SPME measurements (n=3); descriptive statistics.

						Percentile			
	Mean	Std.Dev.	Median	Min	Max	5th	25th	75th	95th
TBT/TBT-d27	0.106	0.097	0.074	0.01	0.463	0.016	0.045	0.131	0.338
TBT-area	0.229	0.201	0.17	0.012	0.871	0.016	0.075	0.319	0.618

Finally, the individual uncertainty components were combined following the appropriate rules to give the combined standard uncertainty for the method as a whole. Then, the standard uncertainty was multiplied for the coverage factor (k=2) to obtain the expanded uncertainty.

Considering the expanded uncertainty value obtained at the reference EQS, the method complies the minimum performance criteria required for measurements within the scope of WFD monitoring. In fact, the value obtained (0.76 ng Sn g^{-1}) is below the 50% of the EQS, as required by the Commission Directive 2009/90/EC [4].

By analysing the single contribution provided by each component to TBT standard uncertainty, it is possible to notice that the precision of the (HS)SPME-GC-MS/MS analysis ($u(P_{SPME\text{-}GC\text{-}MS/MS})/P_{SPME\text{-}GC\text{-}MS/MS}$) provided the highest contribution. From Table 7 it is possible to see that TBT area ratios showed repeatability median values of 7.4%, and 75% percentiles value of 13.1%. However, the distribution of TBT precision data has rather extended tails, with 20% of replicated measurements having RSD% between 13.1% and 33.8%. Figure 3, showing the distribution of RSD versus the sample concentration, illustrates that this deviation from the average trend is mainly due to low concentration samples. This is consistent with the general precision pattern of repeated measurements, showing greater variation at low concentration.

The comparison of the analytical precision with SPME based methods reported in the literature is almost inconclusive. In fact, in most studies focusing on the butyltins analysis in sediments, these data are omitted (they only provide the overall method precision) or are almost incomparable, being derived from small datasets or repeated analyses of standard solutions without sediment extract [11-15, 20]. By considering that the same GC-MS/MS method, applied to liquid injections of standard solution of TBT and the deuterated standard, provided RSD% of 2.0% for TBT/TBT-d27 area (n=5), we supposed that the major contribution to the analytical precision is provided by the fibre extraction, rather than the instrumental ion trap tandem mass spectrometry. Further investigation is needed in order to improve this method weakness.

Finally, as regards the recovery uncertainty, the t–test on the hypothesis of no bias in TBT recoveries (R_m=1) provided t-value lower that the coverage factor k=2 (Table 6). This stated that Rm were not significantly different from 1 and thus there was no need of recovery correction.

Conclusion

This study validated a (HS)SPME–GC–MS/MS method for the analysis of TBT concentration in sediment samples. SPME offers important benefits in terms of speed and almost solventless use, resulting in low-cost analytical methods free of health hazards. Furthermore the technique is simple and easy to automate making it attractive for routine application. The figures of merit indicated that the method is valuable for routine measurements of the existing TBT levels in sediments, according to the requirements of the WFD and related legislation. This result is particularly worthy in view of the possible adoption, by other EU Member States, of analogous EQS of TBT in sediment, alternative to those established for surface water. The validation process pointed out that this ion trap MS based method fulfils the legislative requirements and so it can be adopted by laboratories performing SPME analyses without instrumentation allowing the isotopic dilution quantification.

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