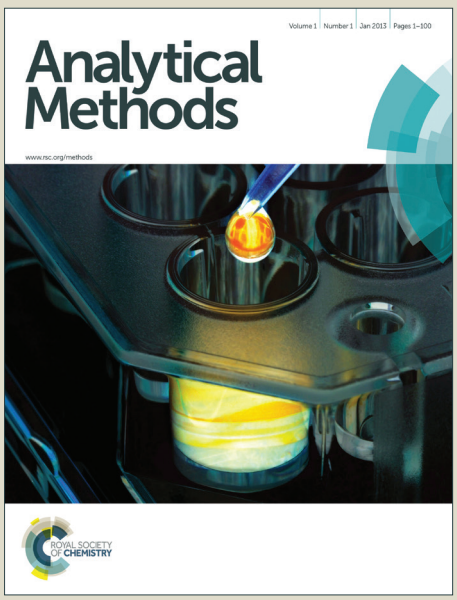
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

A vortex-assisted MSPD method for triclosan extraction from fish tissues with determination by LC-MS/MS

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

In this study, a simple, rapid and sensitive analytical method for the detection of TCS from *Poecilia vivipara* tissues (muscle, gills, brain, liver, gonads and whole fish) was developed. A matrix solid phase dispersion (MSPD) extraction method followed by analysis with a liquid chromatography tandem mass spectrometry (LC-MS/MS) system was developed and the application of the multivariate statistical approach (experimental design) was used to optimize the extraction conditions. The results showed that the method is accurate as robust and highly reproducible, since high recoveries were achieved. The analytical method showed high extraction yields for the determination of this compound in a complex matrix such as tissue. Moreover, the extraction procedure is very fast and it is possible to perform on a small sample aliquot. Besides, the extraction and clean up are performed in a single step. The LOQ value in fish tissue was 0.083 μg g^{-1} and LOD was 0.016 μg g^{-1} . The RSDs for repeatability and intermediate precision studies were in the range of 1.1 to 8.9% and from 0.2% to 8.9%, respectively. Adequate linearity with correlation coefficients (r) higher than 0.99 was obtained for the range of 0.005 to 0.25 μg g^{-1}. Quantitative recoveries (≥80%) and satisfactory precision (average 9%) were obtained. The application of the vortex-assisted MSPD method to the analysis of real samples shows TCS in some liver and gills fish samples at trace levels.

Introduction

Labeled as emerging organic contaminants, pharmaceuticals and personal care products (PPCPs) represent a class of environmental organic pollutants present in human and veterinary medicine and have caused concern due to their extensive use.[1] After their release for the aquatic systems, they might interact with different organisms, leading to deleterious effects through modes of action yet to be understood.[2, 3]

Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether) (TCS) is a broad-spectrum antibacterial and antifungal agent present in many personal care products such as soaps, deodorants, skin creams, oral healthcare products and household cleaning products. It is also frequently infused in an increasing number of products, such as kitchen utensils, toys, food packing materials, trash bags, shoes and textiles, because of its capacity to inhibit microbial growth.[4-6]

After these products are used and rinsed down the drain, TCS can enter the waste stream and be transported to wastewater treatment plants (WWTPs), where is not completely removed.[7] Typically, 70 to 98% is removed through sorption to the solid phase or biodegradation, but the complete removal of this chemical by conventional methodologies for both wastewater treatment and drinking water production is improbable.[8, 9] Therefore, the remaining TCS in WWTP effluent reaches the aquatic environment, and it has been found in sewage treatment plant (STP) influents and effluents, natural water bodies like rivers and lakes, and even into organisms.[5, 6, 10-13] According to Montagner *et al.*[10] this reality is even more concerning in developing countries where the effluent of WWTPs is the main anthropogenic source of contamination into the river, and either the sanitation system is often ineffective, or the sewage is disposed of into the environment without any treatment.

Once released into the environment, TCS is susceptible to biodegradation, particularly under aerobic conditions, and the ionized form is subject to photodegradation, what may be a possible elimination process for TCS in surface water. Methylation of triclosan also occurs in the environment and entails transformation of the compound from a phenol to an ether. This compound is more persistent, lipophilic, bio-accumulative and less sensitive towards photo-degradation in the environment. On the other hand, triclosan is quite stable against hydrolysis. [2,14]

Therefore, due to the widespread use of triclosan, this compound has been detected in wastewater, sediments and receiving waters.[4, 8, 14, 15] Besides, TCS is a ubiquitous pollutant, detected in all environmental compartments, being reported in surface waters around the world, and ranking among the ten most commonly detected PPCPs in frequency and concentration.[5, 8 16, 17]

There are some reports describing the potential effects of TCS

on the biota, but the environmental impacts caused by this pollutant are only beginning to be understood. Toxicity tests showed that TCS is toxic to animals so it may impose a potential risk.[17-20] Since TCS is a relatively stable lipophilic compound, it is readily bioavailable to aquatic organisms and bioaccumulation into aquatic organisms has been reported.[8, 19, 21-25]

To date, only few studies have monitored TCS levels in freshwater fish. Valters *et al.*[26] detected TCS in the plasma samples of 13 species of fish collected from the Detroit River, in the range of 750 to >10 000 pg g^{-1} of wet weight.

A large monitoring program on TCS (period 1994–2003 and 2008) and its metabolite methyl-triclosan (MTCS; period 1994–2008) was conducted by Boehmer *et al.*[27] and Rudel *et al.,*[28] who extracted muscle tissue's samples from breams (*Abramis brama*) by pressurized liquid extraction. TCS concentrations ranged from below the limit of quantification (LOQ) up to 3.4 ng g^{-1}.[28, 29] Mottaleb *et al.*[30] developed two screening methods for simultaneous determination of ten extensively used personal care products (PCPs) and two allylphenol surfactants in fish. TCS was detected in 11 out of 11 environmental samples at concentrations ranging from 17 to 31 ng g^{-1}.

As mentioned above, most methods reported to date deal with the determination of TCS in environmental matrices like water and sediments.[8, 31] Since the occurrence of TCS in different matrices in the aquatic environment can lead to effects and consequently potential impacts, it is needful the determination of this compound in organisms. In this context, a few papers have been published, because is a challenge to deal with the small amount of tissue or tiny organisms, the low concentration of the analytes and complex sample matrix.[32] Therefore, there is an urgent need for the development of methods with enough sensitivity and accuracy to detect TCS in different tissues to allow the advance in the understanding of its mechanism of action, the toxicokinetic parameters and possible sublethal effects in biota. Thus, the combination of analysis in the environment, distribution in the tissues, and effect biomarkers would help to clarify the link between the presence of TCS in specific tissues and the associated early biological effects.

From an analytical perspective, due to the low concentrations of these chemicals in complex matrices such as biota, the analytes need to be extracted and sometimes pre-concentrated before analysis. In this sense, the common approaches developed for tissue samples analysis usually require large volumes of organic solvents and are time consuming. Besides, the major applications have been focused on liquid samples with less attention to the solid ones. In recent years, other extraction strategies have been applied as an alternative to Soxhlet extraction of PPCPs, such as the matrix solid-phase dispersion (MSPD), ultrasound-assisted extraction (UAE), and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe).[4, 14, 33-35]

MSPD allows the simultaneous extraction and clean-up of the analytes from solid, semisolid and highly viscous samples, and the main advantages are feasibility, low costs, rapidity, flexibility and versatility due to the variety of combinations of sorbents and elution solvents. Besides, this technique allows a reduction in sample to the minimum amount that will provide reliable results, being especially important when the biological sample is scarce, beyond reducing the analysis time and number of steps.[36, 37]

Regarding the quantification method, triclosan has been successfully detected by gas chromatography (GC–MS)[33, 38] or liquid chromatography– mass spectrometry (LC–MS),[15] especially if coupled to tandem mass spectrometry (LC-MS/MS).[39]

In the light of the above, the goal of this study was to develop and validate a simple, rapid and sensitive analytical method for the detection of TCS in fish tissues (muscle, gills, brain, liver, gonads and whole fish). For that, an easy MSPD extraction method followed by analysis with LC-MS/MS was developed and the procedure is described. An advance in the MSPD development was the use of a vortex instead of a vacuum manifold for the elution step, which prevented the analyst to be much exposed to the solvent and sample handling. As well the application of the multivariate statistical approach (experimental design) was also used to optimize the extraction conditions.

Experimental

Chemicals and reagents

Triclosan analytical standard (purity >99%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Methanol and acetonitrile of chromatographic grade were supplied by J.T. Baker (Edo. de Mex., México), ammonium acetate >98% by Sigma Aldrich (São Paulo, Brazil) and all the other reagents were of analytical grade. The stock standard solution was prepared in methanol at the concentration of 1000 mg L^{-1}. The working standard solution was prepared at 10 mg L^{-1} by mixing the appropriate amount of the stock standard solution and diluting with methanol, and it was used for sample spiking and for preparing the calibration curves. Working standard solutions were prepared monthly, while the dilutions were prepared daily. Ultrapure water was obtained by Direct Q UV3® water purification system (Millipore, Bedford, MA, USA).

Apparatus and chromatographic conditions

Analyses were performed on a Waters Alliance 2695 Separations Module HPLC, equipped with a quaternary pump, an automatic injector and a thermostatted column compartment (Waters, Milford, MA, USA). The chromatographic separation was performed with a Kinetex C18 (3.0 mm × 50 mm i.d., 2.6 µm film thickness) column Phenomenex (Torrance, CA, USA). The mobile phase components were (A) ultra-pure water with 10 mM ammonium acetate and (B) methanol (80:20, v/v). A Quattro micro API (triple quadrupole) mass spectrometer, equipped with a Z-spray electrospray (ESI) ionization source, from Micromass (Waters, Milford, MA, USA) was used. Drying gas, as well as nebulizing gas, was nitrogen generated from pressurized air in an NG-7 nitrogen generator (Aquilo, Etten-Leur, NL). The nebuliser gas flow was set to 50 L h^{-1} and the gas flow desolvation to 450 L h^{-1}. For the operation in the MS-MS mode, collision gas was Argon 5.0 (White Martins, Rio Grande do Sul, Brazil) with a pressure of 3.5×10^{-3} mbar in the collision cell.

Optimization of the MS-MS conditions, choice of the ionization mode, identification of the parent and product ions, and selection of the most favorable cone and collision voltages for the analysis of the target analyte were performed with direct infusion of standard solution. Analytical instrument control, data acquisition and treatment were performed using the software

MassLynx, version 4.1 (Micromass, Manchester, UK).

Fish samples

The fish *Poecilia vivipara* Bloch & Schneider, 1801 (CYPRINODONTIFORMES, POECILIIDAE) is found in both estuarine and river waters, showing its high tolerance to a wide range of salinity. It is a small fish (2-5 cm long) that distributes along all the Atlantic coast of South America, being one of the most common fish species in small ponds, rivers, and coastal lagoon ecosystems in Brazil. Many species of this family are known for their tolerance to organic contamination, dwelling in both clean waters and waste streams containing domestic sewage. Due to these facts and because of its short life span and ease of handling and breeding, the guppy *P. vivipara* is a good candidate for fish model to be used in ecotoxicological research in Brazil and other tropical countries.[40-44]

Animals were collected in streams in Rio Grande (RS, Brazil) and Florianópolis (SC, Brazil) and transferred to the laboratory, where tissues of interest were extracted (muscle, gills, brain, liver and gonads), put in calcinated-aluminum foil and frozen (-80ºC) until analyses. Fish collection and transportation activities were authorized by ICMBio (Chico Mendes Institute of the Ministry of the Environment), license number 35454. Procedures involving animal handling and experiment were approved by the Ethics Committee for Animal Use-FURG (P029\2012).

MSPD procedure

MSPD procedure was performed in muscle, gills, brain, liver, gonads and whole fish (*Poecilia vivipara*). For the optimization of the MSPD procedure, muscle fish samples were used. In this study, different combinations of dispersants, solvent, time of mixture and amount of sample were tested.

Spiked tissue samples for the method optimization were prepared by adding 50 µL of a 5 mg L^{-1} standard of triclosan in methanol. This volume enabled the solvent to thoroughly cover the sample tissue. The solvent was slowly evaporated at room temperature for at least 1 h before the extraction.

Aliquots of 0.3 g of sample were pooled and spiked with a standard solution, and gently homogenized after solvent evaporation (approximately one hour). Because of the tissues collected from fish were not abundant, the rate of 0.3 g is a pool of tissue, for all tissues tested. The samples were blended and dispersed with 0.5 g C18 and 0.5 g of sodium sulfate for 5 min to obtain a homogeneous mixture, which was carefully transferred into a 15 mL centrifuge polypropylene tube; 5 mL acetonitrile was added, and the content was thoroughly vortexed for 1 min. Then, the tubes were placed into a centrifuge at 8000 rpm for 15 min. The extract was collected and 10 µL was injected into the LC-MS/MS.

Method Validation

Limits of detection (LODs) and quantification (LOQs) were calculated for a relation S/N = 3 or 10, respectively, and from blank samples spiked with TCS in the corresponding matrix matched sample. LOD and LOQ were determined by the injection of different concentrations of analytes diluted with the muscle extracts and were confirmed experimentally. The LOQ is defined as the lowest validated spiking level meeting the method performance acceptability criteria (mean recoveries were in the range 70-120%, with an RSDr ≤ 20%).

The linearity of the method was evaluated through matrix matched calibration in concentrations ranging from the LOQ of each compound to a concentration equivalent to 50-fold LOQ value. Three replicates of at least five concentration of calibration standard were injected. Dilutions of the standard solution of TCS with the blank extract from the matrix extracted by MSPD were performed. An external calibration, at the same concentrations, was also performed by the dilution of the standard solution of TCS in methanol.

The accuracy of the method was evaluated through the recovery assays, in compliance with INMETRO and SANCO.[45, 46] Blank tissue samples were fortified by adding a known volume of standard solution in 0.3 g (fish muscle) samples at the beginning of the process. The levels of fortification were at concentrations equivalent to the LOQ, 2-fold LOQ and 10-fold LOQ. Each fortification level was extracted in triplicate and injected three times (n=9). For the other tissues tested, the fortification was done only at the highest concentration (10-fold LOQ), because of the small amount of fish tissues.

The precision of the method was evaluated considering the repeatability and the intermediate precision. Repeatability was studied with nine determinations. The samples were extracted by MSPD in three different fortification levels, in triplicate.

The study of the matrix effect (ME) was performed according to equation 1,[47] by comparing the slopes in matrix matched calibration solutions prepared in blank tissue extract to those standard solutions prepared in solvent. The extent of the effects due to the matrix components was rated according to the % signal enhancement (+) or suppression (-).

$$ME\% = 100 \times \left(1 - \frac{Sm}{Ss}\right) \quad (1)$$

where Ss is the slope in solvent, Sm is the slope in matrix. No matrix effect is observed when ME (%) is equal to 100%. Values above 100% indicate ionization enhancement, and values below 100% show ionization suppression.

Quality control

Internal quality controls were used, such as the use of a blank

Table 1. 2^4 Full Factorial Design matrix with the observed and predicted TCS recoveries and the relative deviations

Treatment	Sample mass (g)	Solid support mass (g)	Solvent	Dispersion time (min)	Observed TCS recovery (%)	Predicted TCS recovery (%)	Relative deviation (%)
1	-1 (0.2)	-1 (0.5)	-1 (acetonitrile)	-1 (0.5)	72	77	8
2	1 (0.5)	-1 (0.5)	-1 (acetonitrile)	-1 (0.5)	57	57	0
3	-1 (0.2)	1 (1.5)	-1 (acetonitrile)	-1 (0.5)	78	77	1
4	1 (0.5)	1 (1.5)	-1 (acetonitrile)	-1 (0.5)	55	57	3
5	-1 (0.2)	-1 (0.5)	1 (methanol)	-1 (0.5)	65	58	11

6	1 (0.5)	-1 (0.5)	1 (methanol)	-1 (0.5)	55	50	10
7	-1 (0.2)	1 (1.5)	1 (methanol)	-1 (0.5)	56	58	4
8	1 (0.5)	1 (1.5)	1 (methanol)	-1 (0.5)	46	50	9
9	-1 (0.2)	-1 (0.5)	-1 (acetonitrile)	1 (1.5)	61	62	1
10	1 (0.5)	-1 (0.5)	-1 (acetonitrile)	1 (1.5)	56	57	2
11	-1 (0.2)	1 (1.5)	-1 (acetonitrile)	1 (1.5)	60	62	3
12	1 (0.5)	1 (1.5)	-1 (acetonitrile)	1 (1.5)	53	57	8
13	-1 (0.2)	-1 (0.5)	1 (methanol)	1 (1.5)	64	69	8
14	1 (0.5)	-1 (0.5)	1 (methanol)	1 (1.5)	78	77	2
15	-1 (0.2)	1 (1.5)	1 (methanol)	1 (1.5)	76	69	10
16	1 (0.5)	1 (1.5)	1 (methanol)	1 (1.5)	81	77	5
17	0 (0.35)	0 (1.0)	0 (acetonitrile)	0 (1.0)	59	74	25
18	0 (0.35)	0 (1.0)	0 (acetonitrile)	0 (1.0)	72	74	3
19	0 (0.35)	0 (1.0)	0 (acetonitrile)	0 (1.0)	75	74	1
20	0 (0.35)	0 (1.0)	0 (methanol)	0 (1.0)	80	74	7
21	0 (0.35)	0 (1.0)	0 (methanol)	0 (1.0)	82	74	10
22	0 (0.35)	0 (1.0)	0 (methanol)	0 (1.0)	77	74	4

matrix extract to eliminate false positives due to a possible contamination during the extraction procedure, either in the instrument or in the chemicals. The extraction of a spiked blank sample at 10-fold LOQ concentration to check the extraction efficiency, as wells as an analytical curve to evaluate the sensitivity and the linearity in the working range of concentrations, were carried out. A reagent blank (acetonitrile) was also injected after every six sample injections to check for carryover and to perform simple cleaning of the chromatographic system. No carry over phenomena was noticed.

Statistical analyses

All statistical analyses, including one-way analysis of variance (ANOVA), were performed using the GraphPad InStat software (Version 3.00, 1997) and Statistic 8.0 software (Copyright 1984-2007, Statsoft) A 95% significance level was adopted for all comparisons.

Results and Discussion

LC-MS/MS analysis

After the optimization of the collision cell energy of the triple quadrupole, two different parent ion-product ion transitions were selected, one for quantification and one for qualification, and these ions were monitored under time-scheduled multiple reaction monitoring (MRM) conditions. The compound was identified at two transitions plus the retention time to ensure unequivocal identification. TCS showed more efficient ionization in the negative mode. The detection of TCS was performed by monitoring the MRM transitions from *m/z* 286.7 (parent ion) to the *m/z* 34.8 (product ion), and from *m/z* 288.7 (parent ion) to the *m/z* 34.8 (product ion). *The most intense one (286.7>34.8) was used for the quantification. The ratio between the signals of two transitions was used for the confirmation of the compound, as suggest by SANCO guidelines for method validation.* The cone voltage was 20 V and the collision energy was 8 eV. The total run time was 5 min.

MSPD optimization

The MSPD was carried out using the modified MSPD (vortex-assisted MSPD). In this procedure, the elution step was changed by transference of the sample plus solid support, after blending, to a 15 mL polypropylene vessel. Afterward, the elution solvent was spiked into the vessel and stirred with aid of a vortex. It is important to point out that this method was based on previous studies of our research group, and it shows advantages in comparison to the original MSPD procedure, such as quickness and easiness, and still avoids the formation of preferential ways into the column.[36, 48]

Initially, the influence of the type of solid support was evaluated in the TCS recovery, where C18, diatomaceous earth and silica were compared. For the initial experiments, 0.3 g of sample spiked at 50 μg L^{-1}, 0.5 g of solid support, acetonitrile as the elution solvent and dispersion time of 5 min were used. C18 showed the highest recovery (94%) and the lowest RSD (8 %). The better results employing C18 can be attributed to the lipophilic characteristic of fish tissues, whereas the C18 phase can act as a solvent assisting the rupture of the cell membranes from biological and food samples. In addition, the use of C18 can act simultaneously as a clean-up, allowing the disruption of the sample architecture, and there is the possibility of dispersion of the lipophilic matrix on the C18 surface.[49] Previous studies employing the MSPD have also used C18 as a solid support for the extraction of 7 pesticides from fish liver and crab hepatopancreas samples. The recoveries were from 61 to 122% for crab hepatopancreas and from 57 to 107% for fish liver, with RSDs lower than 21 and 26%, respectively.[36]

After carried out the choice of C18 as a solid support, the influence of other important MSPD parameters in the TCS recovery such as sample mass (0.2-0.5 g), solid support mass (0.5-1.5 g), type of elution solvent (acetonitrile or methanol), and time of dispersion (0.5-1.5 min) were evaluated in 2 levels using a 2^4 full factorial design with 22 treatments (6 central points with triplicate for each solvent), after spike of 50 μg L^{-1}. The results are expressed in terms of recovery (Table 1).

The influence of the variables was evaluated through the analysis of effects (with 95% confidence level). Among the variables evaluated in this study, only the sample mass and the dispersion time showed a significant effect ($p<0.05$) on the TCS recovery. The sample mass showed the most pronounced negative effect (22%). It means that the lower the sample amount, the higher the signal intensities, which in turn allows the use of little sample mass. This fact is advantageous considering the small size of fish used in this study.

The dispersion time is the necessary time to promote a homogeneous mixture during the blending of the sample with the solid support, which consequently affect the distribution of target analytes of the samples.[49, 50] The dispersion time showed a

positive effect (6%) indicating that the recoveries can increase using dispersion times higher than 1.5 min. Most of the studies that employed the MSPD had used dispersion times higher than 1.5 min.[36, 37, 48] For the solid support mass, no significant effect was observed, showing that 0.5 g (the lowest value) can be used for the further experiments, to decrease its consumption.

The type of solvent and the solid support bonded-phase are important MSPD parameters, since their relative polarity plays an important role in what will remain on the blended phase and what will be extracted.[51] The type of solvent also showed no significant effect. For the solvents (acetonitrile and methanol), recoveries were from 46 to 82%. Acetonitrile was chosen for the further experiments due to some advantages reported in the literature, such as efficiency to extract TCS from solid matrices and low affinity for lipids.[33] In addition, the RSD (12%) obtained for acetonitrile in the central point was suitable considering the variation associated to MSPD.

Table 2 ANOVA parameters

Variation source	SS [a]	DF [b]	MS [c]	F_{com} [d]	F_{tab} [e]
Regression	1954.1	6	325.7	8.33	2.8
Residual	586.3	15	39.1		
Total	2540.4	21			

a SS: Sum of squares; b DF: Degree of freedom; c MS: Mean squares; dFcom: Computed F value; eFtab: Tabulated F value.

Using the data obtained from factorial design, a quadratic regression model was evaluated by ANOVA with 95% of confidence level, employing the usual Fisher *F*-tests, according to Table 2. It is important to elucidate that the regression model was simplified by removing terms that were not statistically significant (p>0.05), according to Equation 2.

$$R_{TCS} = 74.2 - 3.2m_S - 10.9m_S^2 + 2.8t_D + 3.1m_S.S_E + 4.0m_S.t_D + 6.8S_E.t_D \quad (2)$$

where, R_{TCS} is the TCS recovery; m_S is the sample mass; t_D is the dispersion time; S_E is the type of elution solvent.

The Fisher *F*-test showed significance for the regression model, since the computed *F* value was 3 times higher than the calculated *F* value. The relative deviations between the predict and the observed values ranged from 0 to 25%, showing that the model can be considered significant and predictive. The response profile that represents the TCS recovery as a function of significant variable sample mass and dispersion time are shown in Fig. 1.

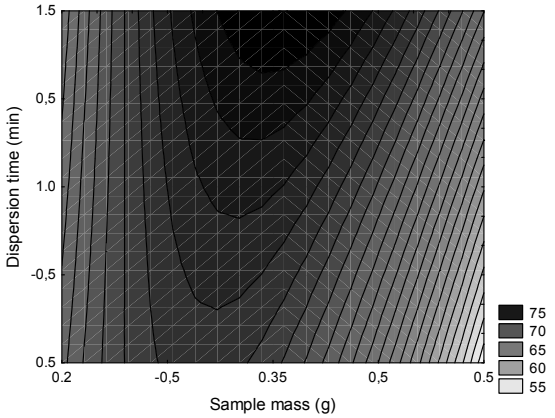

Fig.1 Response profiles representing the TCS recovery as a function of variable sample mass and dispersion time

According to the response profile (Fig. 1), the area with the highest TCS recovery (about 75%) included 0.35 g of sample and 1.5 min of dispersion. It means that is possible to increase the recoveries using sample mass values around 0.3 g and dispersion times higher than 1.5 min. Thus, based on the results of full factorial design, a new experiment was carried out. Due to the low quantity of available sample, an experiment was performed (n=3) with 0.3 g of sample and dispersion time of 5 min to evaluate the TCS extraction efficiency. The other variables were kept constant (0.5 g of C18 and acetonitrile as the elution solvent). Using as comparison the TCS area obtained after spike of 50 μg L^{-1} on the MSPD extract, the recovery significantly increased (99.35 % with RSD of 12%). Therefore, 0.3 g of sample mass and 5 min of dispersion time were chosen as optimum conditions.

Method validation

The LOQ of the method, in other words, the lowest validated spiking level meeting the method performance acceptability criteria, was 0.005 μg L^{-1} (0.083 μg g^{-1}), presenting the same magnitude order as those previously obtained for fish tissue samples.[4, 52, 53]

Table 3 Recovery values (n=9) and RSD obtained with the MPSD method at three concentration levels in fish muscle matrices

1 LOQ (R% ± RSD)	2 LOQ (R% ± RSD)	10 LOQ (R% ± RSD)
87 ± 5	90 ± 3	90 ± 2

Linearity was studied by injecting 10 μL spiked blank matrix extracts into concentrations ranging from the LOQ to 50-fold LOQ. The first calibration level was always equivalent to the LOQ of the compound. The concentration levels of the analytical curve were 0.005; 0.01; 0.025; 0.05; 0.1 and 0.25 μg L^{-1}. Linear calibration curves were plotted by concentration's least-squares regression versus the peak area of the calibration standards. Adequate linearity with correlation coefficients (r) higher than 0.99 was obtained.

Table 4 Recovery values (n=9), RSD obtained with the MPSD method in fish gills, liver, gonads, brains and whole fish matrices

Sample	Spiked (μg g^{-1})	R (%) ± RSD
Gill	0.83	85 ± 11
Liver	0.83	108 ± 9.9
Gonads	0.83	97. ± 13
Brain	0.83	86 ± 13
Whole fish	0.83	79 ± 10

Recovery data were calculated and compared with the appropriate working standard solutions prepared with the muscle extracts. The TCS-free tissues were fortified at three different concentrations (1, 2 and 10-fold LOQ) and residues were quantified by using the matrix-matched standard. Average recoveries ranged from 80 to 104%, with RSD from 2 to 5%. For the other tissues (gills, liver, gonads, brain) and whole fish, the recoveries were calculated by matrix matched calibration with the respective extract fortified at one concentration (10-fold LOQ) and the results are in compliance with SANCO),[45] between 60 and 140% (Tables 3 and 4).

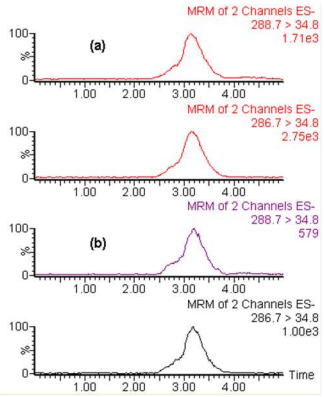

Fig.2 LC-MS/MS chromatogram obtained by fish liver extract spiked at the level of 0.01 mg L^{-1} (a) and fish liver extract sample (b)

Precision was also in accordance with the validation criteria. The RSDs for repeatability and intermediate precision studies were in the range of 1.1 to 8.9% and from 0.2% to 8.9%, respectively. Fig. 2 shows the chromatogram of a spiked fish liver and a sample of fish liver.

The slopes of the standard curves prepared in methanol and the extracts may serve as an indicator of the matrix effect (ME (%)). When the slope of the analytical curve prepared by spiking blank matrices extract with known amounts of TCS was compared to the slope of the analytical curves prepared in methanol, no significant matrix effect (<15%) was found. Nevertheless, a matrix matched calibration was used to improve the accuracy of the quantification in this study.

Method Application

To assess the applicability of the method, 30 specimens of *Poecilia vivipara* were collected in two different estuaries impacted by sewage disposal (Rio Grande-RS and Florianópolis-SC, Brazil), and samples of whole fish, gill, liver, gonads, muscle and brain were extracted and analyzed. In order to assure the quality of the results and to eliminate false positives when the proposed methods were applied, blank samples and matrix matched calibration curves were prepared and analyzed daily.

In fish samples collected in Rio Grande, TCS was not detected in any tissue or in the whole fish. On the other hand, when fish were collected in Florianópolis, TCS was found in gill and liver samples, at concentrations higher than the LOD (16.6 ng g^{-1}), but lower than LOQ (83.3 ng g^{-1}). TCS was not detected in muscle, brain, gonads and the whole fish samples. Currently, there are a few reports on the presence of TCS in fish muscle tissue samples, where the compound was found in the range of 0.3 - 31 ng g^{-1}.[27, 30, 52] To date, this research is amongst the first to study the determination of TCS in different tissue of fish, and can be useful to trigger research on deleterious effects of TCS and other PPCPs, especially in freshwater and brackish fish.

Conclusion

The sample preparation approach developed in this study constitutes the first application of the MSPD technique for the extraction of TCS in sample tissues of *P. vivipara*, which is a promising fish model for ecotoxicological studies in tropical and subtropical environments. The method enables extraction with low solvent consumption and short analysis time in fish tissue, which can be an important tool for biomonitoring studies in impacted ecosystems.

The application of the vortex-assisted MSPD method for the analysis of field samples shows TCS in some liver and gills fish samples at trace levels. The data shows that the compound is present in the environment and bioavailable to *P. vivipara*, being detected at a range of ng g^{-1}. This result supports the importance of carry out studies on the TCS determination in biota as well the use of these methodologies in further research focused on the distribution and persistence of TCS in various fish tissues. The combination of the toxicokinetics parameters and early biological effects can provide useful information on potential impacts to the aquatic life.

Notes and references

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Acknowledgments

The authors acknowledge the financial support and fellowships granted by the Brazilian agencies CAPES, FINEP and FURG. Part of this study was supported by a grant from the Brazilian Agency FAPERGS/CNPq (process number 010/0022-0), CNPq/CAPES (process number 552318/2011-6), CNPq (process number 477083/2011-00), Instituto Nacional de Ciência e Tecnologia em Toxicologia Aquática (INCT-TA/CNPq), FAPERGS (process number 11/0816-3), and FAPERGS/ PROCOREDES (process number 0905342). E.G. Primel got a productivity research fellowship from the Brazilian Agency CNPq (DT 311605/2009-5). A.L.V. Escarrone got a doctoral fellowship from the CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

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