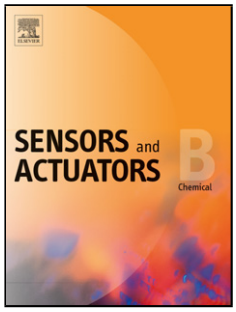

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DETERMINATION OF PARALYTIC SHELLFISH TOXINS USING POTENTIOMETRIC ELECTRONIC TONGUE

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Highlights

- Potentiometric electronic tongue for detection of paralytic shellfish toxins
- Electronic tongue quantifies three toxins in clean acidic mussel extracts
- Individual sensor quantifies dcSTX toxin in acidic mussel extracts
- Joint Y-PLS effective for calibration transfer from buffers to bivalve extract

Abstract

Paralytic shellfish toxins (PSTs) are monitored in commercial bivalves in several countries in the world due to their toxicity to human consumers. The present work examines the application of an electronic tongue based on potentiometric chemical sensors to the quantification of PSTs in mussel extracts. The electronic tongue comprised six miniaturized sensors with solid inner contact and plasticized polyvinylchloride membranes. Calibration models were calculated by PLS regression using measurements in sixteen model mixed solutions containing four PSTs commonly found in bivalves from the Portuguese coast. Transfer of the calibration models to sample matrix was done by joint-PLS regression using measurements in five mussel extracts spiked with PST standards. Quantification of PSTs in extracts of naturally contaminated mussels, using the electronic tongue and updated calibration model, was in agreement with values of the chromatographic reference method. Those sensors alone or combined in an electronic tongue are useful tools for rapid screening of PST in bivalves.

Keywords: electronic tongue, potentiometric chemical sensors, paralytic shellfish toxins, bivalves, calibration transfer

1. Introduction

Paralytic shellfish toxins (PSTs) are a broad group of neurotoxins produced by species of marine dinoflagellates belonging to the genera Alexandrium, Pyrodinium and Gymnodinium [1]. PSTs are causative agents of paralytic shellfish poisoning (PSP) in humans, which is manifested by neurological and gastrointestinal symptoms and may lead to death in severe cases [2-3]. During toxic algal blooms filter-feeding bivalves accumulate these phytoplankton species and, consequently, PSTs. More than 50 paralytic shellfish toxins (PSTs) have been reported, comprising saxitoxin (STX) and its analogues. PSTs are generally divided into various sub-groups based on substituent side chains such as carbamate, sulfate, hydroxyl, hydroxybenzoate, or acetate [1]. The better-known toxins are included in the following groups: the carbamate toxins, to which STX and neosaxitoxin (NEO) belong, the N-sulfocarbamoyl group, which includes GTX5; and the decarbamoyl compounds, which includes dcSTX (Fig. 1). N-sulfocarbamoyl and decarbamoyl are the dominant toxins for the species *Gymnodinium catenatum* [4-7]. The association of *G. catenatum* blooms with PSP episodes has been reported in NW Spain, Pacific coast of Mexico, Australia, Japan [8] and Portugal [9]. Due to the potential severity of the symptoms, several countries run national monitoring programmes of PSTs in commercial bivalves to alert the consumers about bivalves' toxicity episodes [10], i.e. PST concentrations above regulatory limits [11].

Currently, the official reference method for the detection of PSTs in European Union is the liquid chromatography (LC) with fluorimetric detection (FLD) [12-13]. As LC-FLD is a laboratorial technique involving the use of expensive apparatus that must be operated by highly skilled personnel, the development of a less costly and less complex assays and probes for marine toxins detection is of practical interest. Several biosensors and immunoassays have been proposed for individual PSTs' detection, along with nerve cell and sodium channel based assays [14-18]. Antibody-based assays and biosensors can achieve very low limits of detection, but usually only for a small proportion of known PSTs, mainly STX and NEO excluding N-sulfocarbamoyl and decarbamoyl toxins, which are dominant in contaminated bivalves from the Portuguese coast [16-17]. Additionally, antibodies require an animal host for their production. Nerve cell and sodium channel based methods produce toxicity estimation, which is well correlated with the mouse bioassay but involve laborious preparation procedures and have long response times and, most importantly, lack stability, which leads to low reliability and reproducibility of measurements [15].

Chemical sensors represent an interesting alternative to the methods described above, mainly due to their robustness and low cost. However, there are only few reports on chemical

sensors for the detection of STX [19-24]. In our previous work, a range of potentiometric chemical sensors with sensitivity to four PSTs commonly found in Portuguese waters, namely GTX5, C1&2, dcSTX and STX have been developed [25]. While sensors responded to all four toxins, they had low selectivity to them, which complicates their use as selective sensors when several toxins are simultaneously present, which is a typical scenario. However, sensitivity and low selectivity make these sensors interesting candidates for the development of an electronic tongue sensor system, which by combining an array of partially selective sensors and chemometric tools for data processing allows performing quantitative analysis and classification of multicomponent media [26]. While electronic tongues have been applied to a wide range of analytical tasks [27-28], only few works addressed their use for detection of toxins produced by microorganisms. Examples are the quantification of microcystin MC-LR, a toxin produced by some species of cyanobacteria, using a potentiometric electronic tongue [29-30] and an array of impedance sensors modified with antimicrobial and endotoxin neutralizing proteins applied for the detection of endotoxins produced by gram-negative bacteria [31]. No reports of electronic tongues application to the detection of marine toxins have been found.

To the best of our knowledge, the present work describes the first electronic tongue, based on potentiometric chemical sensors, for the simultaneous detection of PSTs in bivalve extracts. Quantification of PSTs in extracts of naturally contaminated mussels, using an electronic tongue and updated calibration model, was compared with measurements by the chromatographic reference method. These results have been partly presented at the ISOEN conference [32].

2. Materials and methods

2.1. Reagents and materials

Sodium hydrogen phosphate and dihydrogen phosphate, aniline, tris(hydroxymethyl) aminomethane (BioPerformance Certified), acetonitrile (LC grade), acetic acid, methanol and ammonium formate were from Sigma Aldrich; ethanol, sodium hydroxide, hydrochloric acid, sulfuric acid, sodium nitrate, potassium nitrate and calcium nitrate were from Panreac; tetrahydrofuran (Chromasolv) was from Fisher and hydrogen peroxide and hydrochloric acid were from Merck. All reagents were p.a. (for analysis) unless stated otherwise. Standard solutions of PSTs (STX, dcSTX, GTX5 and C1&2) were certified reference material from the Institute for Marine Biosciences, National Research Council, Halifax, Canada. When working with PSTs, long sleeved lab coat and non-permeable nitrile or latex gloves should be used.

Toxin containing waste should be decontaminated using a 10% solution of sodium hypochlorite during 30 minutes and disposed of down the drain with plenty of water.

High molecular weight polyvinyl chloride (PVC), dibutyl phthalate (DBP), potassium tetrakis(4-chlorophenyl)borate (K-TCPB) and ionophores were from Fluka. Six ionophores were used: calix[6]arene (1), calix[4]arene-25,26,27,28-tetrol (2), 1,4,7,10,13-pentaoxa-16-azacyclooctadecane (3), 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane (4), octadecyl 4-formylbenzoate (5) and 4,6,11,12-tetrahydro-3-methyl-1-phenyl-1H-pyrazolo[3′,4′:4,5]pyrimido[1,2-b]quinazolin-5-ium tetrafluoroborate (6). Screen-printed electrodes (SPEs) with gold working and auxiliary electrodes and silver reference electrode were from DropSens (Spain). Octadecyl bonded phase silica C18 solid phase extraction cartridges (500 mg/3 mL) were from Supelclean, Supelco, USA. Ultrapure water produced by Merck Millipore Water System (18 MΩcm-1) was used for solution preparation and sensors' washing.

2.2. Preparation of bivalve extracts

Extracts of four composite samples of mussel whole soft tissues were prepared; two have been naturally contaminated with PSTs, and two were free of PSTs (controls). The contaminated specimens were collected during PST outbreaks at Sagres and Ria de Aveiro, Portugal, in 2012 and 2015, respectively. Mussels free of PSTs were collected in 2016 at Ria de Aveiro during absence periods of *G. catenatum* blooms. Specimens were sacrificed, dissected and composite samples (n=20) of whole soft tissues were prepared and stored at -25 oC until further analysis.

Extract preparation was carried out according to the official AOAC method, which consists in two steps, an acid extraction and a clean-up [13]. Briefly, for the acid extraction, a mixture of 3 mL of 1% acetic acid and 5 g of bivalve flesh was heated for 5 min in water bath. The tubes were cooled in ice water and centrifuged at 3600 x g for 10 min at room temperature. The supernatant was saved, and the pellet was extracted again with 3 mL of 1% acetic acid. The two supernatants were combined, and the final volume brought up to 10 mL with ultrapure water constituting acidic extract. For the clean-up, a C18 solid phase extraction cartridge was preconditioned with 6 mL of methanol followed by 6 mL of ultrapure water. Then 1 mL of the acidic extract (0.5 g bivalve equivalent) was loaded into the cartridge and the eluent was collected. The cartridge was washed with 2 mL of ultrapure water and the washing was combined with the eluent producing clean extract.

Prior to LC analysis, the pH of the extracts were adjusted to 6.5 with 0.2 molL^{-1} NaOH. Prior to measurements using sensors, the extracts pH were adjusted to 7 by the addition of 1 mol L^{-1} Tris-base solution.

2.3. Quantification of PSTs in bivalve extracts by LC-FLD

PST quantification by LC-FLD was carried out according to the official AOAC method [13]. The procedure used in the oxidation of PSTs was based on [13] with a procedural modification due to dominance of N-sulfocarbamoyl and decarbamoyl compounds in the *G. catenatum* toxic profile [33]. Toxin oxidation procedure, chromatographic conditions and details in PSTs quantification are described in [34].

Instrumental quantification limits (nmol L^{-1}) were 6.0 (C1&2), 7.0 (GTX5), 20 (dcGTX2&3), 35 (dcSTX) and 67 (dcNEO). Recovery experiments of the analytical procedure were carried out using PST-free clam tissues spiked at two concentration levels as described in [34]. Intervals of the mean recoveries for the quantified PSTs were: 71-74% (C1&2), 97-98% (GTX5), 85-107% (dcGTX2&3), 77-114% (dcSTX) and 55-56% (dcNEO) [34]. Repeatability values in terms of relative standard deviation were from 1 to 11%.

Total toxicity values in bivalve samples were estimated in terms of µg STX di-HCl equivalents per kg of tissue, multiplying the toxin concentration by the toxicity equivalence factor (TEF) of each individual compound [35]. In the case of isomeric pairs as dcGTX2&3 and C1&2, the highest TEF was used for each pair. The regulatory limit (RL) for PSTs is 800 µg STX di-HCl equivalents per kg of bivalve tissue [11].

2.4. Sensor fabrication and potentiometric measurements

Potentiometric sensors with solid inner contact were fabricated using screen-printed electrodes (SPE). Firstly, the SPE working electrode surface was rinsed with ethanol and ultrapure water and cleaned by cycling potential for 5 cycles between -0.2 and +1.2 V at 50mV/s in 50 mmol L^{-1} sulfuric acid. Solid contact was prepared by electropolymerization of aniline in deaerated aqueous solution of 50 mmol L^{-1} aniline in 1 mol L^{-1} hydrochloric acid by cycling potential for 100 cycles between -0.23 and +0.85 V at 50 mV/s. Sensors were washed with deionized water, conditioned for 2 h in 1 mmol L^{-1} hydrochloric acid and dried. All controlled-potential experiments were performed with an EZstat-Pro EIS (NuVant Systems Inc., Indiana, USA). Platinum wire served as the counter electrode and Ag/AgCl (KCl 3 mol L^{-1}) served as a reference electrode.

Membranes were prepared by dissolving PVC (33 % w/w), dibutyl phthalate (66 % w/w), ionophore (1 % w/w) and lipophilic salt (0.5 % w/w) in tetrahydrofuran. The correspondence between membranes and ionophore compounds were the following: calix[6]arene (1), calix[4]arene-25,26,27,28-tetrol (2), 1,4,7,10,13-pentaoxa-16-azacyclooctadecane (3), 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane (4), octadecyl 4-formylbenzoate (5) and 4,6,11,12-tetrahydro-3-methyl-1-phenyl-1H-pyrazolo[3′,4′:4,5]pyrimido[1,2-b]quinazolin-5-ium tetrafluoroborate (6). Each membrane was drop casted on the solid contact of the SPE and left to dry at room temperature. Prior to use, the sensors were conditioned in ultrapure water for 2 h.

Potentiometric measurements were carried out using custom-made high input impedance digital voltmeter (Sensor Systems LLC., St. Petersburg, Russia) connected to a PC for the data acquisition. Sensor potentials were measured vs. SPE own pseudo-reference electrode. Measuring time was 5 minutes and an average value of the last three measurements was used. Between measurements, sensors were washed with ultrapure water until they reached a stable potential.

2.5. Solution preparation for measurements with sensors

Individual calibrating solutions of STX, dcSTX, GTX5 and C1&2 were prepared in mussel extracts free of PSTs after the acid extraction and clean-up processes. Calibration solutions were prepared by the addition of toxin standards to the extracts. Concentration range of the individual solutions was from 0.05 to 5.5 μmol L^{-1}.

Sixteen mixed model solutions containing PSTs were prepared on the background of 0.1 mmol L^{-1} Tris-HCl buffer with pH 7. These model solutions contained GTX5, C1&2 and dcSTX in proportions reflecting the relative toxin concentrations detected in bivalves from Portugal [16] (Table 1). Because STX is most commonly found worldwide, although usually absent or present at low concentrations in Portugal, it was included in the mixed solutions as well. The toxin concentrations were chosen based on estimated total toxicities in bivalve tissues (μg STX di-HCl equivalents per kg) above the regulatory limit (RL) for PSTs. The corresponding estimated toxicities ranged from 1.7 to 14 times the RL in the mixed solutions.

Compositions of the mixed solutions were defined using 4**(5-3) fractional factorial design. Compositions of five solutions for the calibration transfer were selected by Kennard-Stone algorithm. Kennard-Stone algorithm is commonly used for selection of samples uniformly distributed over the object space [36]. This sequential procedure consists in choosing the next sample as the one that is most distant from those already selected. Two samples that

are the most distant from each other serve as a starting point. The distance is usually the Euclidean distance. Transfer solutions were prepared in mussel extract free of PSTs after clean-up. Mussel extract preparation is described in the section 2.2.

2.6. Data processing

Sample recognition was done using Principal Component Analysis (PCA). Calibration models were calculated with respect to PST concentrations by PLS regression using measurements in 16 model solutions. Models were calculated for each toxin individually and validated by leave-one-out validation [37].

Table 1. Concentrations (µmol L^{-1}) of STX, dcSTX, GTX5 and C1&2 in sixteen mixed model solutions at four concentration levels. Mixed model solutions selected for calibration transfer are shown in italic/bold.

Solutions	Concentration, µmolL^{-1}			
	STX	dcSTX	GTX5	C1&2
1	***0.19***	***0.19***	***0.24***	***0.63***
2	***0.38***	***0.37***	***0.24***	***1.7***
3	0.76	0.84	0.24	3.6
4	1.5	1.5	0.24	7.2
5	0.76	0.37	0.56	0.63
6	1.5	0.19	0.56	1.7
7	0.19	1.5	0.56	3.6
8	***0.38***	***0.84***	***0.56***	***7.2***
9	1.5	0.84	1.3	0.63
10	***0.76***	***1.5***	***1.3***	***1.7***
11	0.38	0.19	1.3	3.6
12	0.19	0.37	1.3	7.2
13	***0.38***	***1.5***	***2.5***	***0.63***
14	0.19	0.84	2.5	1.7
15	1.5	0.37	2.5	3.6
16	0.76	0.19	2.5	7.2
Range	0.19-1.5	0.19-1.5	0.24-2.5	0.63-7.2

Calibration transfer was done by Joint-Y Partial Least square regression (JY-PLS). The Joint-Y PLS regression was first developed in the frame of Process Analytical Technology to tackle transfer of the operating conditions from one production site to the other in order to maintain the same quality of the final product [38-39]. Such transfer can be done by modelling a common latent variable space of the operating conditions (X) and the product parameters (Y) measured at production sites a and b, Xa and Ya and Xb and Yb, respectively. JYPLS consist in modelling joint Y matrix combining Ya and Yb using matrices Xa and Xb. It was demonstrated that JYPLS can be also used for the calibration update, or transfer considering initial calibration data and update calibration samples as matrices Xa and Xb, respectively, and concentrations in the initial data set and update samples' set as Ya and Yb, respectively [40]. In this study, calibration transfer was done by using update data set of five model solutions prepared in bivalve extract free of PSTs. Selection of the number of latent variables to use in the updated calibration model was done by using a plot of the regression coefficients' standard errors vs. Root Means Square Error of the transfer data set. Prior to calculations, sensor potentials were standardized using means and standard deviations of the update samples. All algorithms were implemented in MATLAB, v. 7.12 (release 2011a).

3. Results and discussion

3.1. PST quantification in model mixed solutions

On the basis of the results obtained in a previous study [25], six sensors were selected to be used in the electronic tongue for PST determination. All six sensors responded to STX and dcSTX, two sensors to GTX5 and three to C1&2. Detection limits were in the ranges of 0.2-0.5 μmol L^{-1} for STX and dcSTX, and 0.08-1.8 μmol L^{-1} for GTX5 and C1&2. These limits allow the detection of toxins in bivalve matrix at concentrations close to the regulatory limits. Selectivity of all sensors to toxins was low, which makes individual sensors inapplicable to the simultaneous detection of various PSTs. For example, logarithm of selectivity coefficient to STX in the presence of dcSTX varied from 0.3 to -1 for studied sensors [25]. However, as the sensors displayed cross-sensitivity to four PSTs, these can be detected when sensors are combined in the multisensor system. The electronic tongue capability to quantify PSTs was first evaluated in mixed solutions prepared in buffer.

Table 2 gives the parameters of the predicted and measured curves obtained for the calibration and validation data for the PLS calibration models. Lower R^2_{adj} obtained for STX and dcSTX prediction are most likely due to narrower concentration ranges and toxin

concentrations in the mixed solutions being close to the detection limits (see Table 1). Errors (RMSECV) for STX and dcSTX were 0.3 and 0.2 µmol L^{-1}, respectively, while higher values were observed for GTX5 (0.5 µmol L^{-1}) and C1&2 (0.7 µmol L^{-1}). The latter was due to higher prediction errors for the solutions, in which high concentrations of either STX or dcSTX were present, pointing to lower selectivity of the electronic tongue sensors to GTX5 and C1&2. Obtained RMSE values indicate that the electronic tongue is capable of quantifying all four toxins.

Table 2. Root Mean Square Errors (RMSE) and parameters of predicted vs. measured curves (intercept, slope and adjusted R2) for concentrations of STX, dcSTX, GTX5 and C1&2 in the model mixed solutions predicted by the electronic tongue and PLS regression calibration model.

Toxin		LV	Intercept	Slope	R^2_{adj}	RMSE, µmolL^{-1}
STX	Calibration	2	0.08	0.93	0.68	0.26
	Validation		0.23	0.72	0.53	0.33
dcSTX	Calibration	2	0.03	0.98	0.81	0.19
	Validation		0.02	1.02	0.71	0.23
GTX5	Calibration	2	0.14	0.90	0.87	0.30
	Validation		0.31	0.78	0.76	0.45
C1&2	Calibration	3	-0.14	1.09	0.97	0.47
	Validation		-0.09	1.12	0.92	0.73

3.2. Study of the matrix effect of bivalve extracts

Prior to application of the electronic tongue to PST quantification in bivalve extracts, matrix effects of the extracts on the sensor response were assessed, through calibration measurements in mussel acidic extracts free of PSTs spiked with individual solutions of toxins, with and without clean-up. Only sensor based on the ionophore 5 (further on sensor 5), responded to STX and dcSTX in acidic extracts (without clean-up), but did not respond to GTX5 or C1&2. Otherwise, sensor 5 displayed sensitivity to the four toxins in the buffer solutions [25]. Slopes, which were superNernstian in buffer, decreased to the values closer to the response to double charged cations in mussel extract: 26 instead of 69 mV/pX for STX and 32 instead of 62 mV/pX for dcSTX, as shown in the Fig. 2a and b. Reproducibility of the sensor response was not affected by the matrix since standard deviation of slopes remained at the level of about 1

mV/pX. Detection limit in the extract was estimated to be slightly lower for both toxins, i.e. 0.2 instead of 0.6 µmol L^{-1} due to the slope decrease [42].

Calibration measurements in toxin solutions prepared in uncontaminated mussel extract after acidic digestion followed by clean-up have shown that all sensors responded to the four toxins studied. While clean-up procedure removed compounds interfering with the sensor response in bivalve extracts, sensor characteristics in extracts were different from buffer similarly to the sensor 5 response in acidic mussel extract. Slopes of the electrode functions decreased 2 to 3 times becoming close to the values for the double and triple charged ions, while reproducibility and detection limits did not change. Differences in the sensor responses in buffer solutions and mussel extracts are illustrated by the PCA score plot of the measurements with six sensors in two sets of mixed toxin solutions with the same concentrations (prepared in the Tris buffer and in mussel extracts after clean-up) (Fig. 1S).

3.3. PST profiles in mussel samples

Table 3 shows the concentration of dcSTX, GTX5 and C1&2 measured by the reference method [13] in the four composite mussel samples. Samples 1 and 2 exhibited toxins below the LOQs of all toxins determined, most likely reflecting the absence or low density of toxic cells of Gymnodinium catenum in the environment. Samples 3 and 4 showed relatively high concentrations of dcSTX, GTX5 and C1&2, which are indicative of mussels exposed to a toxic algal bloom [16].

Table 3. Concentrations (µmol L^{-1}) of dcSTX, GTX5 and C1&2 in mussel cleaned extracts determined by LC-FLD; mean values with standard deviation in the parenthesis are shown (n=3).

Mussel samples	Toxins in mussel extract (µmol L^{-1})		
	dcSTX	GTX5	C1&2
1	<0.035	<0.007	<0.006
2	<0.035	<0.007	<0.006
3	0.33 (0.13)	0.85 (0.025)	0.99 (0.12)
4	0.072 (0.003)	0.30 (0.009)	0.65 (0.078)

STX concentration was below quantification limits in all four samples, which is in line with previous works documenting the PST profiles in bivalves from Portugal [6,16]. Total toxicity was estimated accounting with the concentrations of all toxins (dcSTX, GTX5, C1&2, dcGTX2&3 and dcNEO) currently measured in the laboratory [16]. Toxicity of sample 3 (1948 µg STX di-HCl equiv. kg^{-1}) exceeded the regulatory limit (800 µg STX di-HCl equiv. kg^{-1}) while toxicity of sample 4 (629 µg STX di-HCl equiv. kg^{-1}) was slightly below. Toxins dcSTX, GTX5 and C1&2 accounted for 70% of total toxicity of sample 3 and 80% of sample 4.

3.4. Quantification of dcSTX in bivalve extracts using sensor 5

Response of the sensor 5 to STX and dcSTX in acidic mussel extracts emphasizes its suitability for quantification of these toxins. As STX is rarely present in PST profile in bivalves from Portuguese coast, this sensor was employed for selective quantification of dcSTX in two acidic extracts (samples 3 and 4, Table 3) of mussels contaminated by PSTs. Quantification of dcSTX was done by double standard addition to account for the small fluctuation of the standard potential and slope and improve accuracy of the analysis [41]. Table 4 shows dcSTX concentrations measured using sensor 5 and concentrations estimated in acidic extracts based on values obtained by LC-FLD in cleaned extracts. Values obtained by the two methods were not significant different according to the t-test: p values were 0.58 and 0.44 for the samples 3 and 4, respectively.

Table 4. Concentration of dcSTX in two contaminated mussel samples estimated by LC-FLD and measured by sensor 5. Cleaned bivalve extracts were analysed by LC-FLD and concentrations in acidic extracts were calculated taking into account sample dilution in the process of clean-up (ca. three times). Averages of three measurements with standard deviations in the parenthesis are shown.

Mussel samples	Concentration of dcSTX, $\mu molL^{-1}$	
	LC-FLD	Sensor
3	0.99 (0.04)	1.04 (0.04)
4	0.29 (0.01)	0.32 (0.06)

3.5. PST quantification in bivalve samples using the electronic tongue

Since compounds present in the bivalve extract affected both standard potentials and slopes of the electrode function of the sensors as shown in the Figs. 2 and 1S, calibration models based

on measurements in the buffer solutions should not be applicable for quantification of PSTs in bivalve extracts. Indeed, concentrations of dcSTX, GTX5 and C1&2 predicted in contaminated mussel extract using these models deviated significantly from the reference method as shown in the Table 5. These results emphasize the inadequacy of the calibration models made in buffer solutions and the necessity to recalibrate sensor array in solutions prepared using bivalve extracts.

Table 5. Concentrations of STX, dcSTX, GTX5 and C1&2 in contaminated mussel sample (3), measured by the LC-FLD and electronic tongue without and after calibration transfer. Averages of three measurements with standard deviations in the parenthesis are shown.

Concentration, $\mu molL^{-1}$	dcSTX	GTX5	C1&2
LC-FLD	0.33 (0.13)	0.85 (0.025)	0.99 (0.12)
Electronic tongue (without calibration transfer)	2.0 (0.1)	0.37 (0.01)	0.061 (0.005)
Electronic tongue (after calibration transfer)	0.27 (0.07)	0.9 (0.2)	0.8 (0.1)

The procedure of the bivalve extract preparation is quite cumbersome and large volumes of the extract cannot be easily obtained. Thus, instead of recalibrating the electronic tongue in the full set of the calibration solutions prepared on the background of the cleaned bivalve extract, calibration transfer was performed. Calibration transfer consist in adapting an existing calibration model to the new experimental conditions, most often to account for the alteration or temporary drift of sensor characteristics. In this work, calibration transfer was used to account for the matrix effect of the samples. The advantage of the calibration transfer compared to re-calibration is that it requires restricted number of new solutions to be measured. Calibration transfer was carried out by JY-PLS regression for each toxin individually [40]. Five mixed solutions prepared in PST free mussel extracts were measured using sensor array and used as data set corresponding to new experimental conditions in JY-PLS algorithm. In this case cross-validation was not feasible for the selection of a number of latent variable to use in the updated calibration model due to restricted number of samples in the transfer data set. Thus, selection of number of latent variables was done using a plot of the regression coefficients' standard errors vs. root mean square errors of update data set as proposed in [43]. This plot allows selection of the most parsimonious model, which represents the best compromise between bias (RMSE) and variance of predicted y values (b-coefficients standard errors) for the

updated calibration model. Number of latent variables minimizing bias without increase of the variance was one for models for GTX5 and C1&2 and two for dcSTX prediction (Fig. 2S).

Results of prediction of concentrations of PSTs in contaminated mussel extract using electronic tongue and updated calibration models were found to be in agreement with the reference method. Values obtained by the two methods were not significant different according to the t-test: p values were 0.52, 0.67 and 0.10 dcSTX, GTX5 and C1&2, respectively (Table 5). Results of this study suggest the use of calibration transfer to account for the matrix effect of samples.

3.6. Advantages and limitation of electronic tongue for screening of PSTs in bivalves

Present study points to the adequacy of electronic tongue and the sensor 5 alone as screening tools for PST detection in bivalves. Sensor 5 can be employed for selective detection of dcSTX in bivalves exposed to *G. catenatum* blooms as marker of PST level. Though dcSTX is one of the toxins present in this typical toxin profile it remains during the post-bloom conditions due to biotransformation of N-sulfocarbamoyl toxins. .In addition, bivalve species as *Spisula solida* presents a toxin profile dominated by decarbamoyl compounds as dcSTX reflecting intense biotransformation during exposure to blooms [16].

In bivalve samples with other PST profiles, i.e. dominated by STX, this sensor can be used for quantification of STX or the sum of STX and dcSTX. Furthermore, this sensor can be used in acidic extract without clean-up, which is easier and faster to prepare and is more readily available. Use of double standard addition procedure makes unnecessary frequent sensor calibration ensuring high accuracy of the measurements.

To the best of knowledge, there are no previous reports of the chemical sensors for the detection of PSTs others than STX. Fluorescence optical sensors with detection limit to STX of about 50 µmol L^{-1} (149 mg STX di-HCl equiv. kg^{-1}) have been reported in [21]. Much lower detection limit of about 1 nmol L^{-1} (3 µg STX di-HCl equiv. kg^{-1}) was reported for the surface plasmon resonance sensor [19]. However, those studies were of the exploratory nature and none of the reported sensors was tested in the bivalve extracts either contaminated or spiked.

Though the electronic tongue proposed in this study requires cleaned bivalve extracts for its proper functioning, it allows performing more detailed characterization of toxin profile in bivalve extracts compared to the individual sensor 5. The electronic tongue is capable to quantify three toxins responsible for 70 to 80% of the total bivalve toxicity related to PST outbreaks. Simultaneous detection of three toxins also means that calibration model calculated using a set of solutions with known toxins concentrations at varying levels is necessary.

However, such calibration needs to be done only once and can be maintained by regular re-calibration in a small number of standards by employing calibration transfer by JY-PLS.

As a consequence of the sparsity of the research in the field of chemical sensors for marine toxins, there are no reports of the electronic tongue sensor systems applications for toxin detection. Furthermore, only few works addressed application of the multisensory systems to the detection of toxins produced by microorganisms. Comparison of these works with the results of the current study is difficult as they targeted toxins, i.e. PSTs, endotoxins and microcystin LR, that are chemically different, and employed different experimental protocols. Endotoxins detection aimed at the discrimination of the samples containing them without quantification of the toxins [31]. Quantification of microcystin LR reported in [29-30], was done using measurements with sensor array in tap water spiked with varying amounts of cyanobacterial culture and toxin concentration determined by the reference method to calibration. Microcystin quantification was done in the concentration range from 2 to 300 nmol L-1 with RMSECV values of 0.42 in concentration log units. In this study higher concentrations has been used, from 0.19 to 7.2 µmol L^{-1}, while RMSECV values were lower – 0.12 – 0.21 in concentration log units.

4. Conclusions

The electronic tongue based on six potentiometric chemical sensors was applied to the quantification of PSTs in model solutions and bivalve extracts prepared by acidic extraction and clean-up using solid phase extration C18 cartridges. Since compounds in bivalve extracts affected responses of the other sensors to toxins, transfer of the calibration models calculated using measurements in buffer solutions was necessary. Toxin concentrations in naturally contaminated mussel extract predicted by the calibration models updated using JY-PLS regression and five PST solutions prepared using bivalve extract were) indicated values close to expected ones that were measured by the reference method, LC-FLD. Furthermore, one of the sensors of the array, sensor 5, was successfully employed for selective detection of dcSTX in the acidic bivalve extracts.

Both electronic tongue and sensor 5 can be useful tools for rapid screening of PST in bivalve extracts.

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Figure legend

Fig. 1. Chemical structures of carbamoyl, sulfocarbamoyl and decarbamoyl groups of paralytic shellfish toxins.

	R1	R2	R3
STX	$OCONH_2$	H	H
dcSTX	OH	H	H
GTX5	$OCONHSO_3$	H	H
C1	$OCONHSO_3$	OSO_3	H
C2	$OCONHSO_3$	H	OSO_3

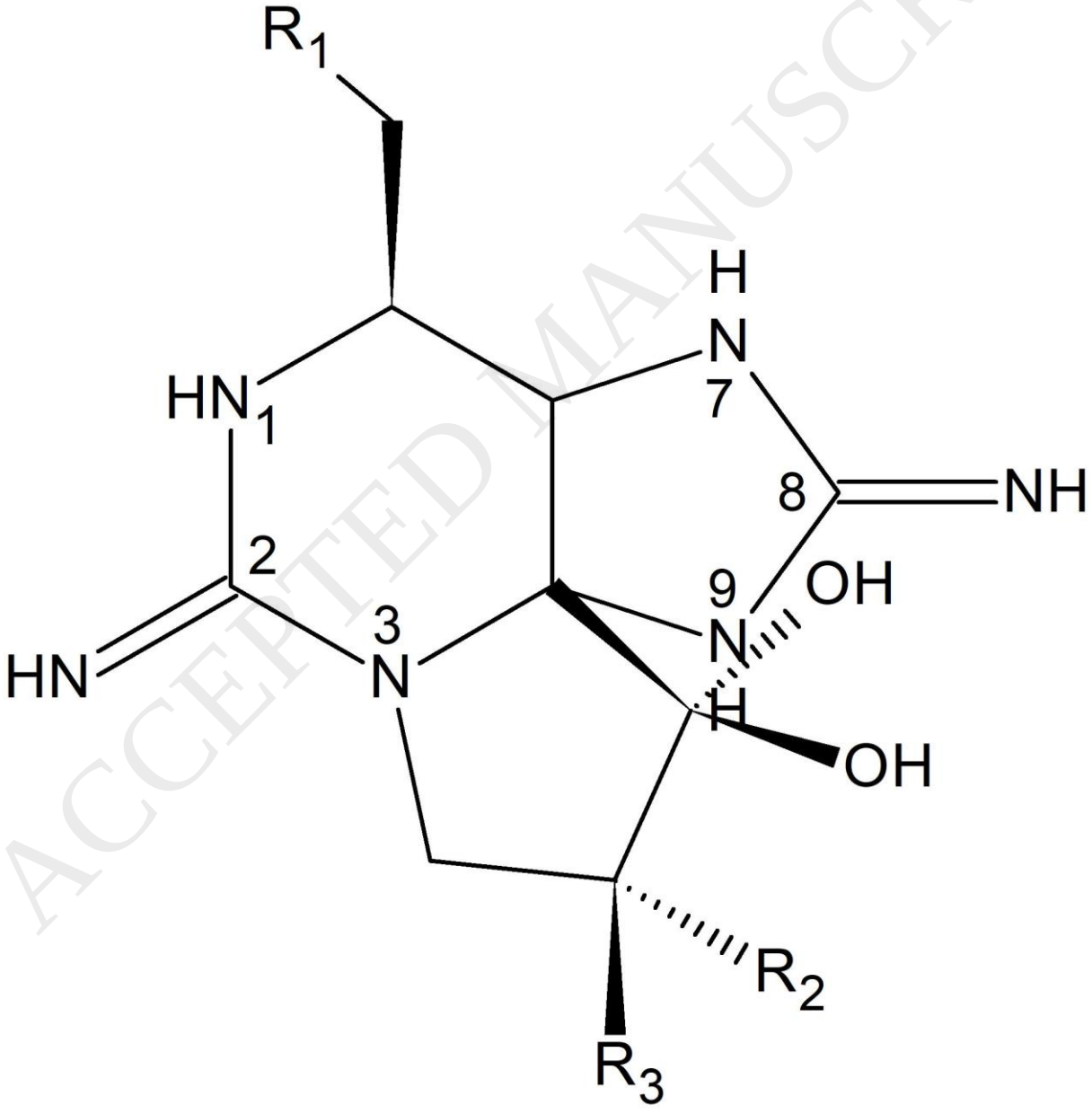

Fig. 2. Responses of the sensor 5 in the solutions of STX (a) and dcSTX (b) prepared in Tris buffer (▲) and mussel extract after cleanup (○). Sensor parameters calculated in the concentration range from 1.3 to 6.5 μmolL^{-1} are shown in the insets: S – slope of the electrode function and E^0 – standard deviation.