Tyrosinase Biosensor for Phenol Monitoring in Water

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***Abstract*:** *Phenol and its derivatives are considered priority pollutants in water resources. They are introduced into the environment in various ways, but the concentrations of concern come with industrial wastes. Environmental legislation restricts phenolic content in surface water for drinking purposes to 1-10 μg/L; consequently, the detection of phenols at low concentrations in water samples is of major environmental concern. The chromatographic detection methods frequently used exhibit high detection limits necessitating laborious sample pre-concentration. Tyrosinase biosensors have been proposed for in situ monitoring of low phenol levels but enzyme leaching and electrode fouling limits their applicability. This paper reports on the development and validation of a phenol minisensor built on a solid supported bilayer lipid membrane platform incorporated with tyrosinase. The mechanism of response utilizes only the binding of phenol to the enzyme at the oxygenase phase, thereby reducing substantially electrode fouling, while physisorption is used to attach tyrosinase to the sensor with a simple, one-step, procedure that ensures an optimal environment for enzyme activity, prevents leaching and allows sensor reversibility for multi-assay purposes. The proposed sensor is prepared and calibrated within 25 min and can run 14 samples/h; exhibiting high tolerance to interfering ions, it can detect concentrations of 2.5 μg/L in surface water for drinking purposes and 6.1 μg/L in lake water.*

Keywords: *bilayer lipid membrane, biosensor, tyrosinase, phenol, water quality, environmental monitoring*

1. Introduction

Phenol and its derivatives are considered priority pollutants in water resources. They are introduced into the environment in various ways, but the concentrations of concern come with industrial wastes: paper manufacturing (as naturally-occurring wood components), agriculture (from pesticide degradation), petrochemical industry (during resin and plastics production), and coal processing (during coking). A highly toxic potential has been established for 165 phenols [1,2], while mutagenicity is suspected for some of the chlorophenols produced during chlorination processes [3]. Not surprisingly, environmental legislation restricts phenolic content in surface water for drinking purposes to 1-10 μg/L [4]. Consequently, the detection of phenols at low concentrations in water samples is of major environmental concern. The methods typically used for determining phenols are: gas chromatography (GC) combined with flame ionization (FID) [5] or mass spectrometric (GC–MS) [6] detection, and liquid chromatography (LC) combined with UV [7], electrochemical [8], or fluorescence [9] detection. Although chromatography offers supreme precision, accuracy and resolution, none of these methods can be packed into field instrumentation, while their high detection limits necessitate laborious sample pre-treatment, such as solid-phase extraction. Potential alternatives rely on capillary electrophoresis [10], immunoassays [11] and biosensors [12-14].

Biosensor technology mounts biochemical processes into sensitive and selective miniature detectors using less reagents and sample volumes [15]. The detection of the analyte is based on its interaction with a suitable biological species (enzyme); the biological information is translated into an electrical signal using a suitable transducer (e.g., amperometric). For water phenols, the tyrosinase-catalysed oxidation of the analyte seems to offer many possibilities for rapid electrochemical detection. Tyrosinase, a copper-containing unstable enzyme, catalyses two cascade reactions: the *ortho*-hydroxylation of phenols (monophenols) to catechols (diphenols) and

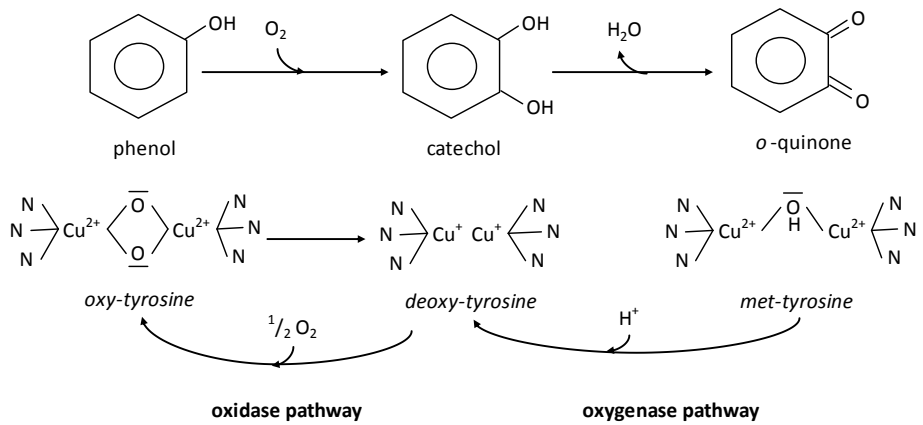

Fig. 1: Tyrosinase cascade degradation of phenols.

the dehydrogenation of catechols to *o*-quinones [16] (Fig. 1). The utilization of the enzyme in biosensor development has been limited to amperometry, investing on the redox character of the cascade reactions or the subsequent quinone reduction. A variety of problems reported refer mainly to (i) the immobilisation of the enzyme onto the sensor's surface [14], (ii) the leaching of enzyme to the solution [13], and (iii) the fouling of the electrode surface by the products of phenol oxidation [12].

This paper presents a novel phenol minisensor, built on a solid supported bilayer lipid membrane (s-BLM) platform incorporated with tyrosinase. The mechanism of response utilizes only the binding of phenol to the enzyme at the oxygenase phase, thereby reducing substantially electrode fouling, while physisorption is used to attach tyrosinase to the sensor with a simple, one-step, procedure that ensures an optimal environment for enzyme activity, prevents leaching and allows sensor reversibility for multi-assay purposes. The sensor has been validated using spiked tap water and lake water samples.

2. Materials and Methods

2.1. Chemicals and Reagents

Egg phosphatidylcholine (egg PC Type XVI-E), HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanosulfonic acid), methemoglobin (bovine), teflon coated silver wires (diameters of 0.1, 0.25, 0.5 and 1.0 mm), and tyrosinase from mushroom (polyphenol oxidase, E.C.1.14.18.1, 5370 U/mg) were purchased from Sigma (Germany). The analyte was high purity solid phenol (Fluka >99.5%). All other chemicals were of analytical-reagent grade. Water was purified by passage through a Milli-Q cartridge filtering system (Milli-Q, Millipore, El Paso, TX, USA) and had a minimum resistivity of 18 MΩ cm. A 25-μL (graduated to 1 μL) Hamilton syringe (Hamilton Co., NV, USA) was used for administering the bioelement and the analyte in the electrochemical cell.

2.2. Apparatus

The bench-scale apparatus used in this study has been previously described [17]. The electrochemical cell had a capacity of 20 mL and a two-electrode configuration: a sensing electrode, i.e. the silver wire with an s-BLM, and an Ag/AgCl reference electrode. An external d.c. potential of 25 mV was applied between the electrodes and the ionic current through the BLM was measured with a digital electrometer (Model 6514, Keithley Instruments, Cleveland, OH, USA) having in-built current-to-voltage converter. LabVIEW (National Instruments Co., Austin, TX, USA) properly customized was used to store and process signal data. The sensing electrode was connected to the power supply source (Model 2400, Keithley Instruments, Cleveland, OH, USA) and the reference electrode was connected to the electrometer; the applied potential at the sensing electrode was positive relative to ground. The electrochemical cell and sensitive electronic equipment were placed in a grounded Faraday cage.

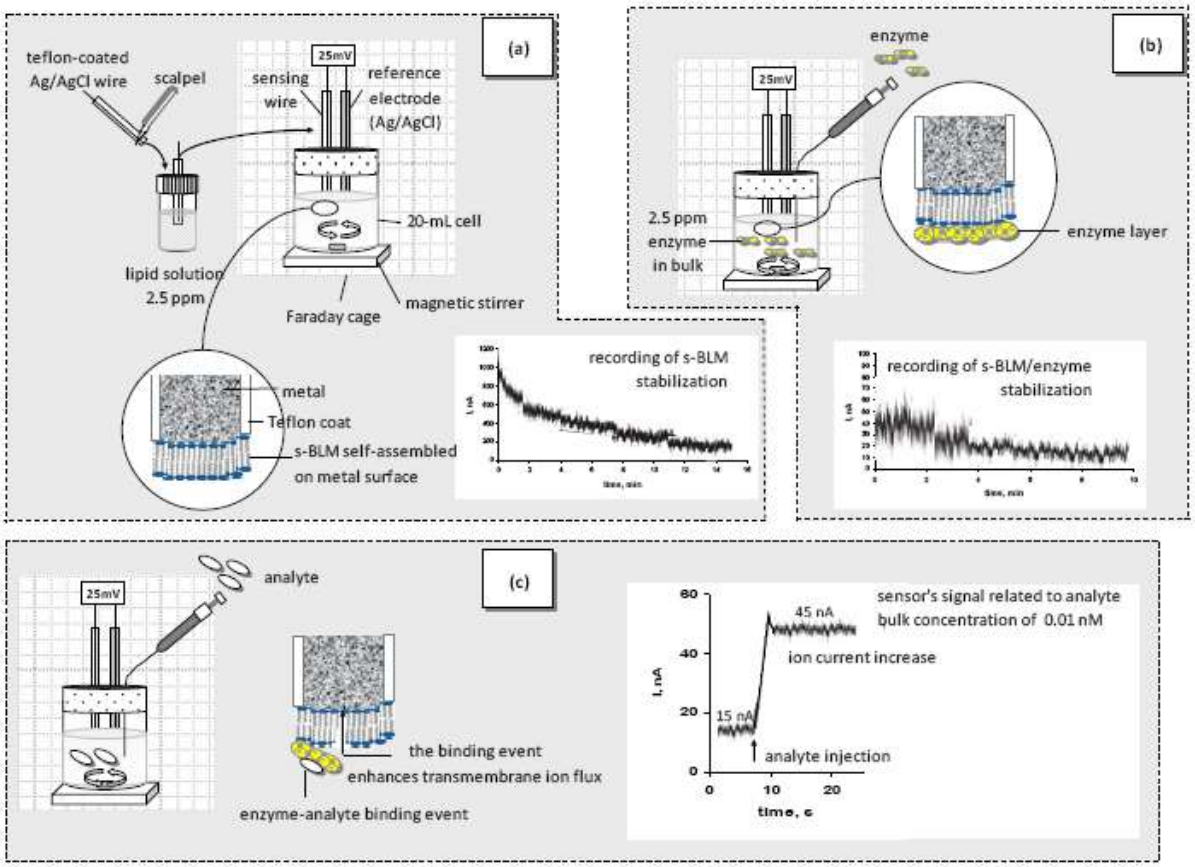

Fig. 2: Schematic illustration of the s-BLM tyrosinase biosensor: (a) preparation of s-BLM; (b) tyrosinase immobilization; (c) phenol determination.

2.3. Sensor Preparation, Calibration and Assay

s-BLMs were constructed from a stock lipid solution of 2.5 mg/mL (in 80% v/v n-hexane and 20% v/v absolute ethanol) according to established techniques [17]. For the optimization study, concentrations of 1-3 mg/mL were used. The tip of the silver wire was cut with a scalpel prior to its immersion into the lipid solution. After a few seconds, the wire was removed and placed into 0.1 M KCl aqueous solution buffered with HEPES; the electrochemical current was stabilized within 10-15 min. The membrane platforms remained stable and functional for >48h and exhibited limited air stability (ca. 10 min).

After membrane stabilization, 10 μL of a 5 mg/mL tyrosinase stock solution were injected into the electrochemical cell. The sensor stabilized within 6-8 min, giving a residual current of 15 nA (±2.5 nA, *n*=12). For the optimization study 5-40 μL injections of the stock enzyme solution were used. Sensor calibration was performed by stepwise additions of phenol solution, as 5 μL or 10 μL aliquots, freshly prepared from diluting a stock of 0.01 g/L in dimineralized water (Milli-Q, Millipore, El Paso, TX, USA). Sensor validation used phenol-spiked tap and lake water samples; the latter has been provided by Koumoundourou Lake (Attiki, Greece). Spiking solutions were freshly prepared from diluting the phenol stock solution; tap water 10-mL samples contained 9.4 μg/L phenol (close to the upper allowable level for drinking water) and lake water 5-mL samples were made to 18.8 μg/L. The spiked samples were measured immediately after preparation without any pre-dilution; the assay sample volume was 10 μL. All experiments were performed at 25 ± 1 ^{o}C under stirring. An overview of sensor preparation and assay methodology is presented in Fig. 2.

3. Results and Discussion

3.1. Analytical Development of the Sensor

The minisensor built on tyrosinase responded to stepwise additions of phenol by permanent ion current increases with magnitudes linearly correlated to the phenol concentration in the buffer solution. The time of response, i.e., the time to establish 99% of steady-state current (i.e., the signal) at 0.5 mm diameter sensing wire, was 10 s (± 0.75, *n*=31). A control experiment using pure membranes did not provide any measurable signal or any significant alteration of the electrical parameters of the sensor (background current and noise level).

The rapid response of the tyrosinase-containing sensor to phenol (10 s), suggests a rapid alteration of ion transport through the bilayer. The increases of the transmembrane ion flux could be attributed to changes of the packing and fluidity of the membrane lipids, induced by the interaction between tyrosinase and phenol. Several enzyme studies suggest that the monophenolase reaction mechanism could involve the nucleophilic attack of the oxygen atom belonging to the phenol's hydroxyl group on the copper atoms of the enzyme's active site; this would bring about finite changes at the secondary and tertiary structure of the membrane-bound enzyme, opening conduction pathways through the bilayer; similar enzyme-induced bilayer modifications has been previously reported for a variety of sensors.

The optimization of the analytical signal followed the protocol established previously for similar biosensor platforms [15]. An overview of the investigation and the optimal operational conditions are presented in Table I. The 2.5 mg/mL lipid solution had a 100% success rate in constructing an s-BLM; the use of more dilute lipid solutions showed a success rate of 60%, whereas higher concentrations resulted in prolonged stabilization times.

TABLE I: Sensor Optimization Parameters

Variables	Studied Range	Optimal Value
pH	5.0-8.5	8.0
tyrosinase (at bulk electrolyte)	1.25-10 μg/mL (ppm)	2.5 μg/mL (ppm)
Sensing wire diameter	0.1-1.0	0.5
Membrane composition	100% PC	100% PC
Lipid solution	1-3 mg/mL	2.5 mg/mL
Regeneration time (at pH 8.5)	2-15 min	10 min

The optimal bulk concentration of tyrosinase was found to be 2.5 ppm; lower levels did not provide adequate sensitivity for detection (using a phenol bulk concentration of 0.05 nM), while increasing tyrosinase loading caused permanent membrane destabilization. Sensing wires with diameters smaller to 0.5 mm exhibited high noise levels that increased detection limit (estimated for S/N=3, i.e. the concentration of phenol providing a difference of 45 nA with respect to 15 nA residual current), whereas larger diameters increased the residual current to 60 nA limiting the analytical range (only currents up to 650 nA are reliable for sensing, as larger currents may indicate membrane destabilization and not analytical signals).

The effect of pH was a critical issue in the experimental design. Various studies report on optimum pH values between 6 and 7, focusing mainly on the oxidase activity of tyrosinase. The enzyme is rather unstable; it can be inactivated by its substrate, and, further, the sensor performance can be altered by the formation of radicals during electrochemical reduction of the enzymatically generated quinones, causing electrode fouling [12]. These disadvantages have been extensively discussed in literature, whereas various solutions have been proposed with bi-enzyme systems or complex multilayer designs [12-14]. Since the goal of the present work was to construct a simple and reliable sensor based only on phenol hydroxylation and avoiding completely the catechol oxidase activity of the enzyme, the authors studied the response of the tyrosinase-containing membrane within the pH-range 5.0-8.5 with a view to highlighting a suitable pH value. At pH values < 5.5, the noise level was prohibitively high, with transients up to 100 nA, possibly to due to enzyme inactivation and/or desorption from the membrane (at this pH value, the produced catechols may bind tyrosinase in the oxygenase mode, i.e. as

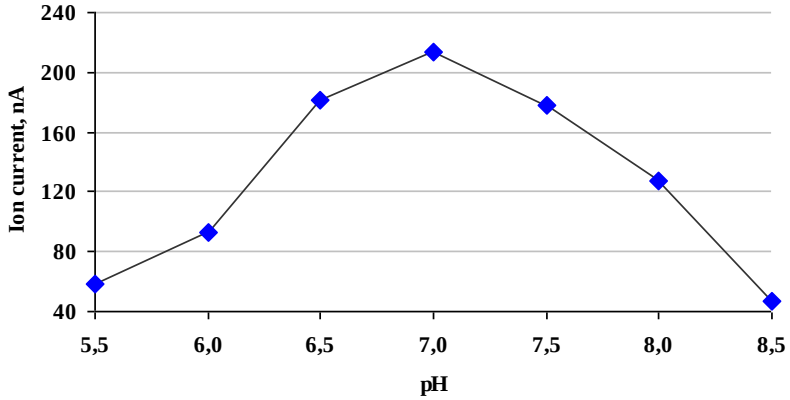

Fig. 3: Effect of pH on sensor's response to 0.05 nM phenol .

if they were phenols [16]). At pH values > 8.5, sensitivity of the sensor towards 0.05 nM of phenol diminished (Fig.3). The highest signal was achieved at pH 7.0, but membrane destabilisation became evident after 2 consecutive injections, with an increase of background current to 420 nA and the appearance of transient signals of high magnitude, prohibiting any further use of the sensor. At pH 8.0, sensor's response dropped to 60%, but no membrane instability was observed after 5 consecutive injections (limited by the high residual ion current reached). The attempts made to increase enzyme loading in order to compensate for the loss of sensitivity led either to membrane instability or noise increase.

Fig. 4 shows the calibration plot for phenol at the optimized conditions. The ion current values (I) are linearly related to phenol concentration (C_{phenol}) in solution: I (nA) = 3124.3 C_{phenol} (nM) + 3.99. The coefficient of determination r^2 was found to be 0.9997 (n=31) and the reproducibility of response was estimated to ± 8-12% for within-day analyses (as relative standard error, n=31, 5.95% confidence limit) and 15.85% for between-days and analysts analyses (n=24, 5.95% confidence limit). The detection limit was estimated to 0.013 nM (1.24 ng/L), providing an analytically useful concentration range between 0.013 - 0.16 nM (1.24-15 ng/L) phenol (as bulk electrolyte concentrations). Some drift of the ion current with time was noticed, especially at high phenol concentrations; however, the maximum value observed was 1 nA/min.

Analytical validation included, also, sensor reversibility studies. It has been previously demonstrated that this s-BLM platform responds to both, increases and decreases of analyte concentration, although it is impossible to totally extract the bioelement from the membrane without destroying the sensor [17]. In this report, sensor reversibility could be achieved, to some extent, by gradually removing electrolyte volume from the electrochemical cell and replacing it with analyte-free electrolyte until an acceptable background current was reached. Yet, memory and carryover effects could not be ruled out [17]; thus the authors suggested the use of newly constructed sensors for reliable detections. This, actually, has proved to be time-consuming, laborious, and impractical with large sample rates [15]. Dipping the sensing electrode into strongly acidic or alkaline buffer could be a viable alternative; as the membrane cannot tolerate pH values < 4.5 (due to lipid oxidation), only alkaline treatment has been considered herein. Using the largest phenol concentration tested in the present study (0.16 nM), the sensing wire was removed from the electrochemical cell and placed in pH 8.5 buffer (0.1M KCl with HEPES); the results demonstrated that 10 min at pH 8.5 were adequate to regenerate the sensor, as indicated by a reduction of ion current to 28 nA (± 1.5 nA, n=12). When transferred to a phenol-free electrochemical cell, no decline of phenol sensitivity was detected; this clearly indicates insignificant or none desorption of the bioelement from the lipid membrane. The number of repetitive assay cycles of high phenol level samples, including measuring, regeneration, re-stabilisation and re-measuring, was 8 without observing any statistically significant reduction in signal magnitudes; the number of samples that could be assayed in a single format increased as phenol levels reduced in the assayed samples, reaching 15 for 0.02 nM phenol.

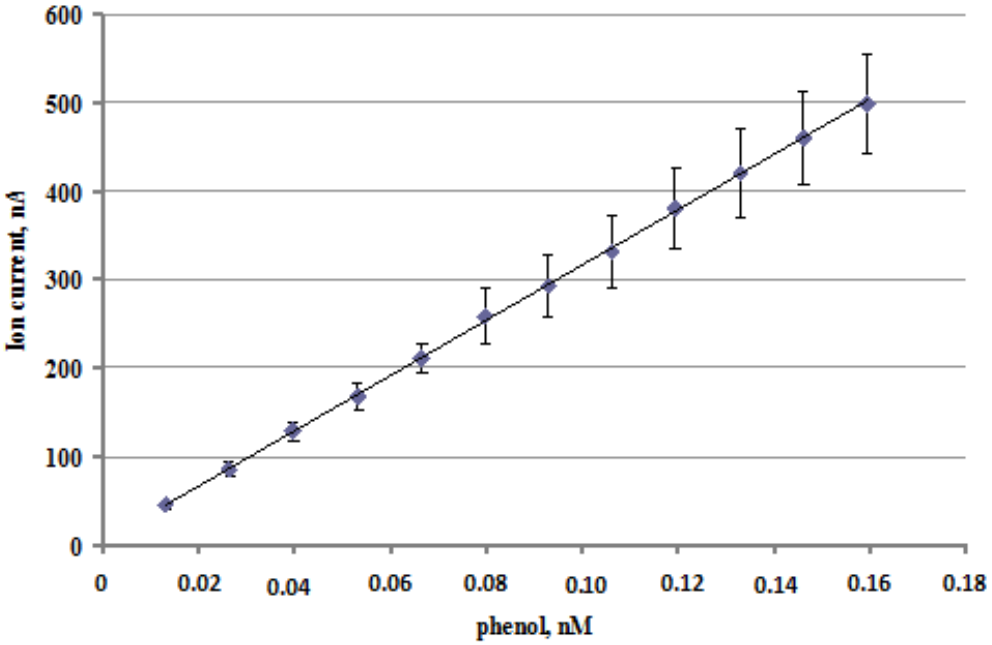

Fig. 4: Calibration curve for phenol detection. Experimental conditions: pH 8.0; s-BLM composed of PC; 0.5 mm Teflon coated Ag wire; 0.1 KCl solution with HEPES; 2.5 ppm tyrosinase. Error bars denote standard deviation (*n*=31).

Interference studies included a number of anions (nitrates, sulfates, sulfides, carbonates, and phosphates) and cations (calcium, magnesium, chloride and ammonium ions), expected to be present in water samples. The results indicated a high sensor tolerance as the interfering ions showed a determinant error of less than 5% for bulk electrolyte concentrations up to the mM range.

Size reduction of the sensor was not considered in the present study, although preliminary results indicate that the cell capacity can be reduced to 10 mL and analysis volumes to 1 μL. In electrochemical configurations there are always limitations posed by the minimum necessary distance between the electrodes. An investigation on reducing the noise effect from smaller diameter wires is currently underway.

3.2. Sensor Validation

The injection of a lake water control (phenol-free) sample raised slightly noise levels and the detection limit was set to 0.03 nM; however, no discernible activity from the electrode was detected. Tap water did not produce any effect. All samples were analyzed on the same sensor, using the regeneration protocol described above; the mean analysis rate per sensor was 14 samples/h. Phenol concentrations in the samples were estimated by the formula: C (μg/L) = 0.06 I (nA) - 0.24.

The results from the analyses of the spiked tap water samples are presented in Table II; recovery ranged between 93-105%. No positive or negative trends, indicative of standard errors, were observed. These results verify the analytical development data, thereby proving the suitability of the minisensor for drinking water monitoring. The lowest phenol level that can be reliably detected in surface water for drinking purposes is estimated to 2.5 μg/L.

Table III shows the results from the lake water samples. The minisensor gave consistently higher values, possibly due to matrix effects; yet, the maximum deviation observed was only 6.6%, rendering the minisensor quite capable for measuring contaminated and complex field samples. Nonetheless, the effect of organic matter, needs to be further investigated and the results obtained in the present study should be further verified with large-scale water sampling. The lowest phenol level that can be reliably detected in lake water is estimated to 6.1 μg/L.

TABLE II: Determination of Phenol in Tap Water Samples

Sample Nr	Phenol in sample (μg/L)	Phenol detected (μg/L)	% relative error
1	9.4	9.06	+3.617
2	9.4	9.36	+0.426
3	9.4	9.84	-4.681
4	9.4	9.48	-0.851
5	9.4	9.66	-2.766
6	9.4	9.18	+2.340
7	9.4	9.90	-5.319
8	9.4	9.84	-4.681
9	9.4	9.12	+2.979
10	9.4	8.76	+6.809

TABLE III: Determination of Phenol in Lake Water Samples

Sample Nr	Phenol in sample (μg/L)	Phenol detected (μg/L)	% relative error
1	18.8	19.26	-2.447
2	18.8	18.96	-0.851
3	18.8	20.04	-6.596
4	18.8	19.56	-4.043
5	18.8	19.86	-5.638
6	18.8	19.80	-5.319

4. Conclusions

A phenol minisensor has been presented herein, based on a solid supported bilayer lipid membrane platform incorporated with tyrosinase. The minisensor developed has been successfully validated for tap and lake water samples. The detection limit achieved with the sensor (at S/N=3) was 1.24 ng/L, significantly lower that the detection limits reported for chromatographic [5-9] and biosensor [12-14] approaches. The proposed sensor exhibits high tolerance to interfering ions and can detect concentrations of 2.5 μg/L in surface water for drinking purposes and 6.1 μg/L in lake water. Future research is focusing on eliminating the matrix effects observed in the lake samples and, also, in the simultaneous determination of diphenols and phenol derivatives.

5. Acknowledgements

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