INSTITUTO SUPERIOR DE ENGENHARIA DO PORTO
MESTRADO EM ENGENHARIA QUÍMICA




DESENVOLVIMENTO DE UM BIOSSENSOR PARA DETERMINAÇÃO DE BISFENOL A

MARIANA SOUSA PRATA MONTEIRO DA SILVA
Julho de 2019

POLITÉCNICO
DO PORTO

DEVELOPMENT OF A BIOSENSOR TO DETERMINE BISPHENOL A

Dissertation subjected as a partial requirement to

obtain the master's degree in Chemical Engineering

Mariana Sousa Prata

Supervisor: Simone Morais (Instituto Superior de Engenharia do Instituto Politécnico do Porto)
Co-supervisor: Iria Bravo (Universidad Autónoma de Madrid)

Porto

July 2019

This study was financially supported from the European Union (FEDER funds through COMPETE) and National Funds (*Fundação para a Ciência e Tecnologia-FCT*) through PTDC/ASP-PES/29547/2017 (POCI-01-0145-FEDER-029547) by FCT/MEC with national funds and co-funded by FEDER.

Acknowledgments

To the Department of Chemical Engineering of *Instituto Superior de Engenharia do Porto* to make possible the opportunity to work at the REQUIMTE–LAQV/ISEP.

To Professor Simone Morais for giving me a chance of participating in this project and for all the support.

To Iria Bravo, for all the things she thought me and helped me to understand, for all the good and not so good working days and, all the support, I was very lucky to have you as my supervisor.

To all Electrochemistry Department researchers, specialty my fellow course colleagues.

To my family, for supporting my education and life choices.

And finally, to my friends, the best family I could ever choose, for hearing my complains, for their advice and for all the joyfully moments.

Abstract

The exposure to bisphenol A (BPA), a contaminant of emerging concern, is an environmental and public health problem; recently, it has been detected in a variety of daily products. Therefore, it is urgent to develop fast, simple, low cost, and portable determination techniques in order to assess the contamination by this chemical compound. Thus, the main objective of this study was the development of a biosensor based on laccase from *Trametes versicolor* to determine BPA. For that purpose, an electrochemical biosensor was developed using a screen-printed carbon electrode modified by the drop coating technique. The modification strategy consisted of two sequential phases: first, multi-walled carbon nanotubes were deposited onto the electrode surface, and once this film was dried, a composite composed by laccase (12.4 mg mL^{-1}), the ionic liquid 1-butyl-3-methyl imidazolium tetrafluoroborate and chitosan (5 mg mL^{-1}) in an optimum proportion of 5:2:3 (v/v/v) was drop-casted onto the previously modified surface. Cyclic and square-wave voltammetry were used for characterization of the biosensor construction and BPA electroanalysis. The developed enzymatic biosensor exhibited satisfactory electroanalytical performance with a sensitivity of (6.36 ± 0.09) × 10^{-2} µA/µM, linearity range of 0.5 – 8 µM, a limit of detection of 6.9 nM, relative standard deviation values of *ca.* 4.3% in the reproducibility assays and recoveries of 94.6 ± 5.0% in environmental waters. The proposed biosensor has the potential to be a useful device for BPA assessment.

Keywords: Bisphenol A; Laccase; Biosensor; Screen-printed carbon electrodes, Ionic liquid, Multi-Walled Carbon Nanotubes

Resumo

A exposição ao *bisfenol A* (BPA), um contaminante emergente, é uma preocupação ambiental e um problema de saúde pública; recentemente tem sido detetado numa variedade de produtos de uso diário. Assim sendo, é urgente desenvolver técnicas de determinação expeditas, portáteis, simples e de baixo custo, de modo a reduzir a contaminação por este composto químico. Deste modo, o principal objetivo deste trabalho foi o desenvolvimento de um biossensor, com base em *lacase* de *Trametes versicolor* para determinar o *bisfenol A*. Para tal, foi desenvolvido um biossensor eletroquímico utilizando elétrodos serigrafados de carbono modificados através da técnica de *gotejamento*. A estratégia de modificação consistiu em duas fases: primeiro, os nanotubos de carbono com paredes múltiplas foram depositados à superfície do elétrodo, e após secagem deste filme, um compósito constituído de *lacase* (12.4. mg mL^{-1}), líquido iónico (tetrafluoroborato de 1-butil-3-metilimidazolio) e quitosano (5 mg mL^{-1}), numa proporção ótima de 5:2:3 (v/v/v), foi imobilizado na superfície do elétrodo previamente modificado. As técnicas de voltametria cíclica e voltametria de onda quadrada foram aplicadas para a caraterização da construção do biossensor e para a eletroanálise do BPA. O biossensor enzimático desenvolvido apresenta um comportamento eletroanalítico satisfatório com uma sensibilidade de (6.36 ± 0.09) $\times 10^{-2}$ µA/µM, uma gama linear de 0.5 – 8 µM, um limite de deteção de 6.9 nM, um desvio padrão relativo de 4,3% nos ensaios de reprodutibilidade, e uma recuperação de 94,6 ± 5.0% em águas. O biossensor proposto poderá ser um dispositivo eficaz para a determinação de BPA.

Palavras chave: *Bisfenol A*; *Lacase*; Biossensor; Elétrodos de carbono serigrafados; Líquido iónico; Nanotubos de carbono com paredes múltiplas.

Table of contents

List of Tables

List of Figures

List of Abbreviations

A – amplitude

AD – arc discharge

AP – aminophenol

BPA – bisphenol A

BRB – Britton Robinson Buffer

CE – counter electrode

CEC – contaminants of emerging concern

CHI – chitosan

CNTs – carbon nanotubes

CV – cyclic voltammetry

CVD – chemical vapor deposition

EDCs - endocrine-disrupting chemicals

f - frequency

IL – ionic liquid

LA – laser ablation

LOD – limit of detection

LOQ - limit of quantification

MWCNTs – multi-walled carbon nanotubes

POPs - persistent organic pollutants

PPCPs - pharmaceutical and personal care products

RE – reference electrode

SPCE – screen-printed carbon electrode

SPE – screen printed electrode

SWCNTs – single-walled carbon nanotubes

WE – working electrode

1. Introduction

In this chapter, the general concepts related to biosensor development and the main aims of this research work are presented.

1.1. Work aims

The main aim of this work was to develop an electrochemical biosensor to determine bisphenol A. The following specific objectives were established:

- Bibliographic search on existing laccase-based biosensors to determine BPA;
- Selection of the metallic bare electrode and the modifying materials;
- Construction of the biosensor;
- Optimization of the chosen modifying materials quantities;
- Characterization of the electroanalytical features;
- Application of the developed biosensor.

1.2. Electrochemical (bio)sensors

Sensors are devices that can monitor physical, chemical or biological changes and convert them into a measurable signal. Sensors are composed by a recognition element, which enables the selective response to a specific analyte or a group of analytes, minimizing interferences from other sample components (Barsan et al., 2013), and a transducer, which is responsible for the signal conversion. When that recognition element is a biological system, the device is called a biosensor. The biological recognition element, i.e., a living organism or product derived from living systems, is in intimate contact or integrated with a transducer. Biosensors are designed with the purpose of detecting molecules of analytical significance, pathogens, and toxic compounds in order to provide rapid, accurate, and reliable information about the analyte examined. They have been used in many applications such as in medicine, agriculture, food safety,

homeland security, bioprocessing, environmental and industrial monitoring (Luong, Male, and Glennon 2008).

Taking the type of transducer into account (Table 1.1), (bio)sensors can be categorized into electrochemical, optical, and piezoelectric.

Table 1.1. Different types of (bio)sensors and their usual determination techniques (Zhu et al., 2017).

Type	Determination technique
Electrochemical	Impedimetric
	Potentiometric
	Amperometric
Optical	Colorimetric
	Interferometric
	Fluorescent
	Luminescent
Piezoelectric	Piezoelectric
	Acoustic wave

Electrochemical (bio)sensors are commonly used worldwide because of their easy management procedure compared to other analytical tools. They can provide information about an analyte in real time by using a selective recognition element that generates a signal, which is measured by a proper transducer, an electrode (Goda et al., 2019). The most important features that should be exhibited by (bio)sensors are the detection limit, response time, linearity, stability, sensitivity, and selectivity (The, 1999).

Screen-printed electrodes (SPEs) are low-cost and disposable devices designed to work with small amounts of sample volumes. Working with screen printed electrodes offers some advantages over traditional electrodes and the most important are the low cost, portability, solution volume, and operating temperature They are used in electrochemical analyses in environmental, clinical or agri-food areas and they can be based on carbon, gold, platinum, silver or carbon nanotubes inks. In Figure 1.1, an example of the type of screen-printed electrode used in this work is shown.

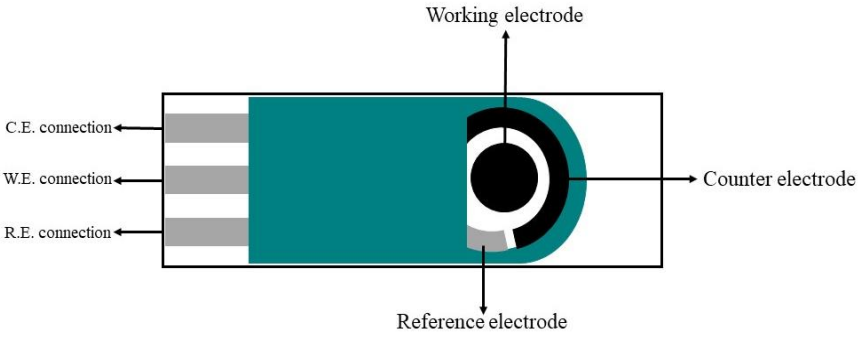

Figure 1.1. Scheme of a screen-printed carbon electrode.

Electrochemical biosensors are the most described ones in the literature, in fact, most enzymatic biosensors and immunosensors are based on electrochemical methods (The, 1999). They are part of a subclass of chemical sensors and combine the sensitivity, indicated by low detection limits, of electrochemical transducers with the high specificity of biological recognition processes. This biological recognition element can be of different types, such as enzymes, proteins, antibodies, nucleic acids, cell, tissues or receptors, which selectively react with the target. By means of the recognition event, an electrochemical signal is produced, related to the concentration of the analyte under study.

Based on the nature of the biological modification, electrochemical biosensors (Figure 1.2) can be classified as either biocatalytic or affinity sensors. Electrochemical biocatalytic or enzymatic biosensors are modified with biological elements (e.g.,

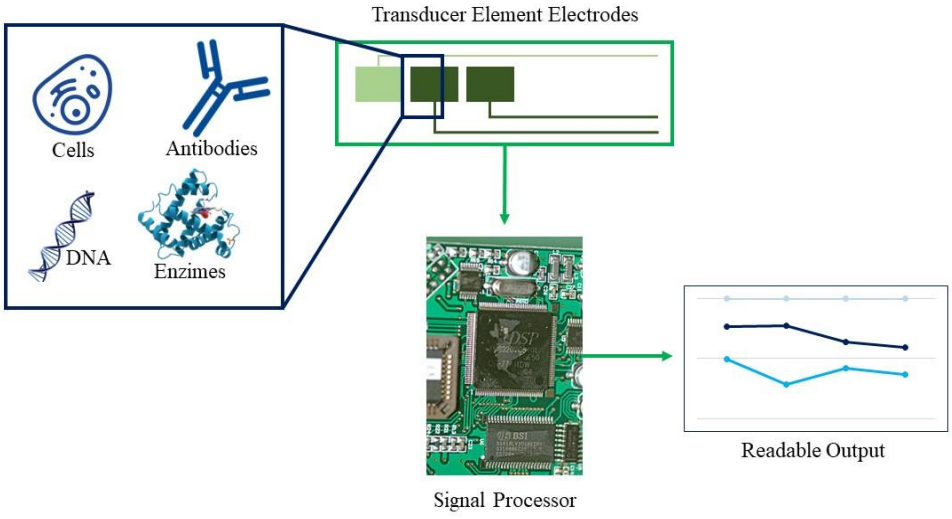

Figure 1.2. Scheme of an electrochemical biosensor.

enzymes) able to recognize a target and induce a catalytic response of an electroactive molecule. Meanwhile, electrochemical affinity sensors have a binding recognition element (e.g., antibodies) that releases a signal when it is coupled to the target.

1.2.1. Amperometric biosensors

Amperometric biosensors are designated according to their transduction mechanism. During amperometric measurements, a potential is settled between the working electrode (WE) and the reference electrode (RE); the current is measured, and the generated signal is proportional to the concentration of the electroactive species. In the case of enzymatic biosensors, the detection principle is based on the electron transfer processes between the immobilized enzyme active center and the working electrode. In addition, alterations in analyte's oxidation state, as well as changes in other species involved in the reaction, may occur (Soares, 2011).

1.2.2. Electrochemical techniques

Electrochemical techniques are widely used to characterize the target analyte. They are expeditious methods that provide detailed information on the substrate and biological element interactions. Cyclic voltammetry and square wave voltammetry are the most important electrochemical techniques. (Soares, 2011).

1.2.2.1. Cyclic voltammetry

One of the most applied electrochemical techniques, cyclic voltammetry (CV) is the study of redox active macromolecules mechanisms and this information is obtained from cyclic voltammograms. CV allows the identification and determination of electrochemically active species as well as thermodynamic and kinetic information associated with an electrode-electrolyte system. The scan rate is fixed between mVs^{-1} to thousands of Vs^{-1}, and a cyclically ramped potential is applied to the working electrode. The resulting current response is recorded (Nakao et al., 2017).

The working electrode potential is anodically or catholically ramped from a starting potential to an ending potential value, during each cycle. This ending potential may or may not be similar to the starting potential. The result current response on these experiments is the result of a faradaic and non-faradaic process that occurs at the working electrode. The imposed electric conditions cause the reorganization of the ionic double layer at the solid-liquid interface, generating an electron transfer at the interface of the surface of the electrodes (Davis, 1985). This is the principle of faradaic and non-faradaic processes. The background current reflects the capacitive current. This current can be determined by recording blank measurements without redox active species present in solution (Izquierdo & Kranz, 2016).

1.2.2.2. Square-wave voltammetry

Square-wave voltammetry (SWV) is a very attractive method used in quantitative electroanalytical chemistry due to its superior sensitivity and applied for characterizing electron transfer processes of redox active species in catalysis, biology, and chem/biosensing applications. Current is measured at discrete points of time. SWV is carried out at a stationary electrode, being taken through a series of measurement cycles, without occurring renewal of the diffusion layer between cycles (Bard & Faulkner, 2001). The current is measured at the end of each potential step, diminishing the charging current contribution, thus improving the sensitivity and quality of the voltammetric data (Lovrić, 2010). Some advantages of SWV include the possibility to apply higher scan rates due to the minimization of the capacitive current (Izquierdo & Kranz, 2016).

The electrodes signal can be improved by modification with nanostructured materials. These materials are very attractive and have physical-chemical properties different from other macroscopic materials with the same composition (Wolf & Groves, 1994).

1.3. Modification strategies applied to biosensors

In order to make the electrode suitable for the application required, modification strategies are applied. This modification will influence the electrode electrochemical response. They are employed when: high sensitivity is required, in order to prevent the electrode poisoning, to pre-concentrate the analyte and, enhance its electrocatalytic properties. Chemically modified electrodes with nanostructured materials, compared to non-modified electrodes, reveal better analytical properties, as sensibility, selectivity and, stability (Seguro, 2017).

Nanostructured materials can be classified in terms of size in a range between 1 to 100 nanometers. Their particular properties are related not only with their composition, size, and shape but also with the functional groups present on their surface. These functional groups define their physical and chemical stability, reactivity, biocompatibility, selectivity, optical, catalytic, and electric properties and enable the enlargement of their range of application (Han et al., 2019).

Nanomaterials can enlarge the electrode surface area and improve the electron transfer rate when compared to normal materials. The main aim of using these materials as modifiers is the expansion of the electrode surface area, possibly resulting in an increase in the capacitative and surface faradaic currents. Carbon nanostructured materials, in particular, carbon nanotubes (CNTs) are extensively used because they have proved the increase of the electrochemical activity of the working surfaces. The application of nanotubes led to a better resolution of the peaks and lower detection limits in the analytical methods based on voltammetry (Donten & Stojek, 2012).

CNTs are one of the best examples of nanostructures among nanomaterials applied to modify electrodes surface. They have a simple chemical composition and atomic bonding configuration. CNTs can be classified into single walled or multi-walled. Single-walled carbon nanotubes (SWCNTs) are a rolled sheet of graphene, with cylindrical form, along a lattice vector in the graphene plane. Multi-walled carbon nanotubes (MWCNs) are no more than multi SWCNTs nested inside one another (Dai, 2002).

In this work, carboxylated MWCNTs were used as the first stage of electrode modification.

1.3.1. Multi-Walled Carbon Nanotubes

MWCNTs are composed of several concentric tubes of graphene fitted inside each other (Han et al. 2019). Although their low-cost production, they also have poor solubility and processability, caused by the attractive van der Waals interactions between the nanotubes. Due to that, MWCNTs exist aggregated and as bundles in their natural state. To overcome this issue, it is frequent the inclusion of various functional groups on their surface. These functional groups, introduced by chemical modification on the sidewall of the carboxylated CNTs, improve their dispersion in solution (Lee et al., 2008).

The physicochemical properties of MWCNTs depend on the synthesis and purification methods. The MWCNTs used in this study were produced by chemical vapor deposition. Nanotubes synthesis is made by three methods: arc-discharge, laser ablation, and chemical vapor deposition (Andrews et al., 1999).

Arc-discharge (AC) method occurs in an apparatus similar to a vaporization chamber, consisting of a vacuum chamber, containing two graphite electrodes and, two-rod tips distanced 1-2 mm (Dai 2002). Under a rarefied ambient and, low pressure, an electric arc is generated between the two graphite electrodes. Vaporization of carbon occurs on the anode due to the high temperature (>3000ºC). The vaporized carbon deposits on the cathode, producing the nanotubes. This method is applied either to single-walled carbon nanotubes (SWCNTs) and MWCNTs.

In the laser ablation (LA) method, a graphite electrode is vaporized by a laser. (Hutchison et al., 2001)The process occurs inside a furnace at 1200ºC on the presence of inert gas flow. The vaporization produces carbon particles. The inert gas drags these particles to the high-temperature zone, and they will set on a water cooled, made of copper, conical collector. This technique is also applied to the production of SWCNTs and MWCNTs.

In order to close the gaps in both previously mentioned methods, chemical vapor deposition (CVD) was developed. In CVD, nanotubes are formed by the decomposition of a gas. This phenomenon occurs in the presence of a catalyzer and proper pressure. This method is the cheapest of three and demands for low operating temperatures. Furthermore, it allows scaled output, ongoing operation and, the final product presents a

higher degree of purity, minimizing additional purification stages (K. A. Shah et al., 2018).

Synthesized nanotubes by AD and LA have better structural quality compared to the one produced by CVD. However, the required operational conditions restrict MWCNTs massive production. The nanotubes production demands for a certain purity degree. To ensure the best quality, they must be subjected to purification processes. These purification stages may cause unpleasant defects in nanotubes. Chemical methods, such as acid, basic and, oxidative treatments are the most common purification strategies. Physical methods are also applied in other to categorize nanotubes by size, standing out for being less destructive and, avoiding the damage of nanotubes. Ultrasounds, microfiltration and, magnetic purification, are also applied as purification techniques (Luis 2009).

Widely applied to modify electrodes, MWCNTs present themselves as a way to improve biosensors sensitivity and low detection limit (He et al., 2017). They held together with relatively weak van der Waals forces. The MWCNTs multi-layered structure further complicates their modeling properties (Thostenson et al., 2001).

1.3.2. Chitosan

Commercially produced from chitin by alkali deacetylation, chitosan, poly (1, 4) 2 amino – 2 – deoxy – D – glucose (Figure 1.3.) is an aminated polysaccharide abundant in nature. This hydrophilic polymer is industrially obtained through an alkaline treatment that hydrolyzes its amino acetyl groups, occurring deacetylation. After that, chitosan is transformed into flakes and milled to produce a fine mesh of chitosan (Riaz et al., 2016).

It can be found under many polymeric forms with unique structures, multifunctional properties, and various applications. Beyond all the benefits previously mentioned, chitosan is also used in diverse bio applications due to its NH_2 structural groups that give chitosan its compatibility and efficiency. It is insoluble at neutral and alkaline pH values, so in acid conditions the amine groups make chitosan become protonated and a soluble polysaccharide (Lizardi-mendoza & Valencia, 2016).

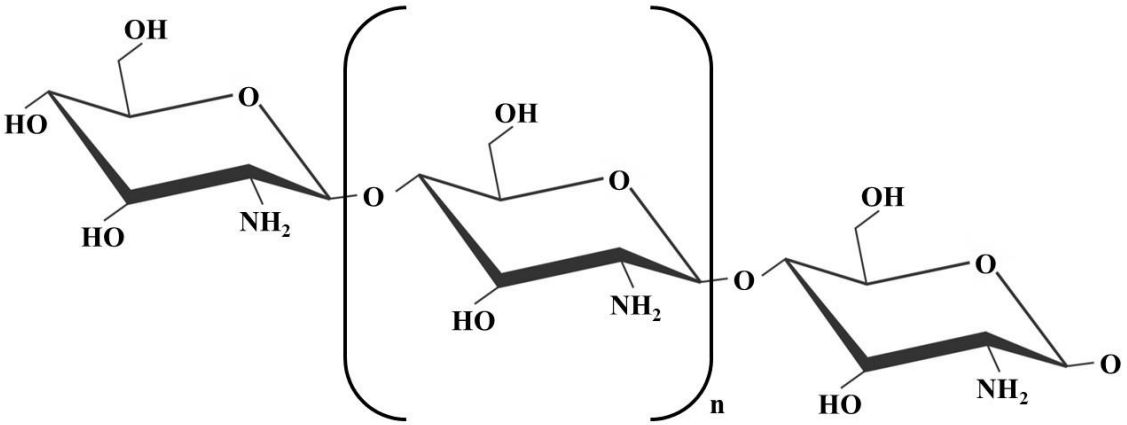

Figure 1.3. Chemical structure of Chitosan.

Chitosan was used as a modifying component in the biosensor in this work not only due to its biocompatibility with the chosen enzyme but also to help to stabilize the enzyme activity. It forms a semi-permeable gel layer with all the modifying components allowing them to remain on the electrode surface.

1.3.3. Ionic Liquids

Extensively used due to their unique properties, room temperature ionic liquids (IL) have been increasingly used as surface modifiers for biosensors (Jia et al., 2008). Ionic liquids offer a diversity of advantages over water and organic solvents, such as low volatility, excellent ionic conductivity, high thermal and electrochemical stability, vapor pressure near zero, wide electrochemical window and other adjustable properties like polarity, solvent miscibility, beyond other applications (Greaves & Drummond, 2008). Highly pursued in green chemistry applications, they are liquid state molten salts below 100ºC, composed of organic cations and (in)organic anions with a wide structural and physicochemical diversity according to cations and anions selection and modification (Liu & Yu, 2019).

Immobilization of IL on top of electrodes is a simple process that consists of physically mixing the liquid into the polymer matrix. Some biocompatible IL have been

used for biosensors for offering good stability and greater enzymatic activity, one example is 1-butyl-3-methyl imidazolium tetrafluoroborate *(*$BMIMBF_4$) (Figure 1.4.) (X. Lu et al., 2014).

Used as binders in electrochemistry, ILs have the ability to immobilize sensitive biomolecules onto the surface of the working electrode. Their viscosity deserves some attention once it influences the rate of mass transport in the solutions. Single used or combined in composites, they exhibit excellent biocompatibility, enhancing biomolecules activity and leading to more satisfactory and sensitive detection results (Kumar et al., 2017). They are two voltammetric sensors reported for bisphenol A detection (Chen et al., 2013; Yu et al., 2013) based on the electrode modification with ILs by a composite film (Wang & Hao, 2016).

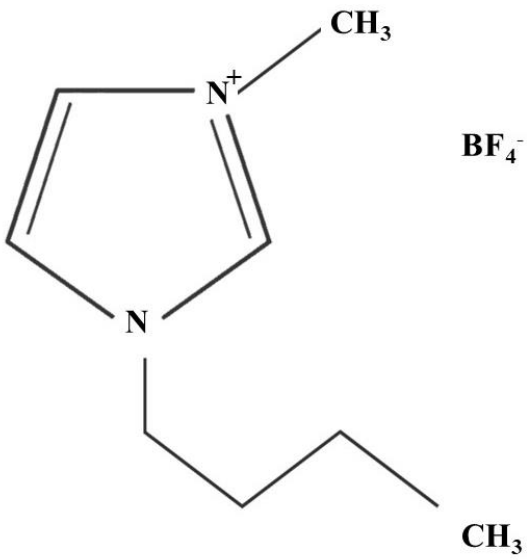

Figure 1.4. Chemical structure of 1-butyl-3-methylimidazolium tetrafluoroborate.

For the present biosensor development, $BMIMBF_4$ was used due to its excellent ionic conductivity and high electrochemical stability in order to increase sensitivity. It is also biocompatible, which promotes the enzyme activity.

1.4. Laccase as a biological recognition element

Among the enzymes used to develop electrochemical biosensors, oxidoreductases are the most commonly employed (Ribeiro et al., 2014). Laccase (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) is a member of the blue multi-copper-oxidase family; it is a polyphenoloxidadase. These enzymes can be found mostly as extracellular enzymes in plenty of organisms, such as plants, fungi, insects, and bacteria. Laccases have demonstrated great potential in many study fields, such as agricultural, industrial, medical and environmental (Rodríguez-delgado et al., 2015). Laccase most common occurrence is associated with higher fungi and its producers are almost all wood-rotting fungi, *i.e*. *Coriolopsis polyzona*, *Trametes versicolor*, *Trametes ochracea*, *Trametes hirsute* among others. Laccase catalyzes the oxidation process of a variety of substrates, such as aminophenol, mono-, di-, and polyphenols, aromatic amines and ascorbate and, methoxyphenols, by a radical-catalyzed reaction mechanism and are able to reduce molecular oxygen into water (Claus, 2004; Fernández-fernández et al., 2012). The general reaction mechanism is represented in Figure 1.5.

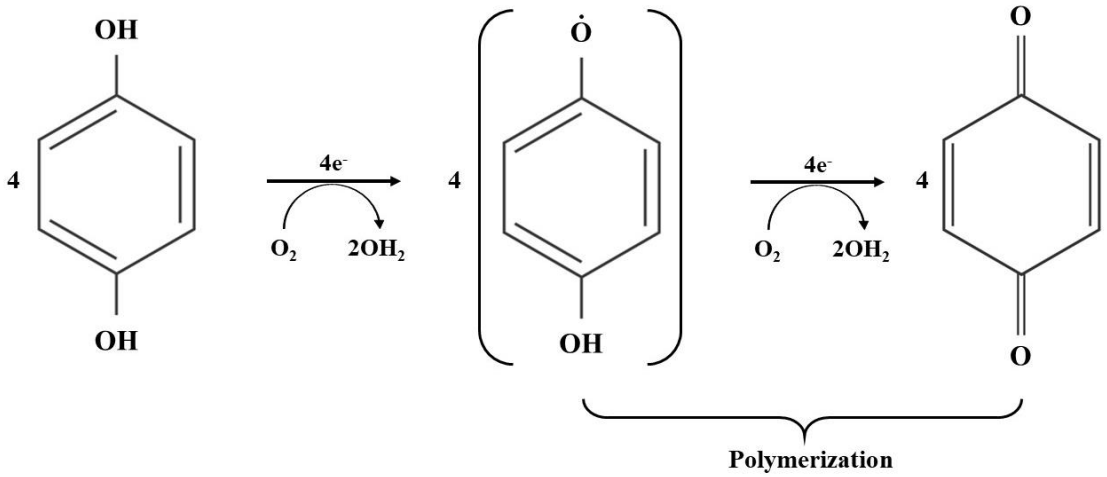

Figure 1.5. General reaction mechanism for phenol oxidation by laccase.

Among the biological agents, laccases represent an interesting enzyme group, offering great potential for biotechnological and bioremediation applications (Karigar & Rao, 2011). Distinguished for having an excellent biotechnological potential related to their ability to oxidize several substrates employed in industrial sectors, laccases are very

suitable for degradation of xenobiotics in the treatment of wastewaters or dye decolorization, beyond its application in biosensors construction (Duran et al., 2002). Development of biofuel cells, production of novel paper products, food industry applications, pulp paper industry, as bleaching product, or detection of phenols in wastewaters are only a few examples of laccase applications.

1.4.1. Laccase immobilization methods

Enzyme immobilization can be defined as the confinement of enzyme to a phase, either a matrix or support (i.e., inert polymers and inorganic materials), different from the one for substrates and products (Bickerstaff, 2003). Characteristics as stability, ability to maintain or increase enzyme activity, physical strength and regenerative capacity are the ones required in an ideal matrix (Datta et al., 2013). Different methodologies have been applied to immobilize laccase in biosensors surface. Table 1.2 summarized examples of its application to biosensors construction, categorized by the modification type.

Table 1.2. Methods for laccase immobilization applied to laccase-based biosensor construction (Fernández-fernández et al., 2012).

Laccase source	Support	Substrate	Reference
Entrapment			
Trametes villosa and Aspergillus niger	Polyaniline matrix		Timur et al., 2004
Trametes villosa	Polymers. Cetyl ethyl poly(ethyleneimine) and Nafion	Pyrocatechol	Yaropolov et al., 2005
Trametes versicolor	Sol-gel matrix of diglycerysilane	Phenols	Montereali et al., 2010
Adsorption			
Trametes versicolor	Graphite electrodes	Catechol	Portaccio et al., 2006
Trametes hirsuta	Graphite electrodes	Catechol	Shleev et al., 2006
Trametes versicolor	Glassy carbon electrode and colloidal suspension	ABTS and catechol	Mousty et al., 2007
Trametes versicolor	Sonogel-carbon electrodes	Phenols	ElKaoutit et al., 2007, 2008
Trametes versicolor	Mesoporous silica: MCM-41	Catechol	Xu et al., 2009
Covalent binding			
Myceliophthora thermophila (Denilite)	Glassy carbon electrodes and platinum electrode		Quan et al., 2004a, 2004b; Quan & Shin, 2004c, 2004b, 2004a; Xu et al., 2009
Rigidoporus lignosus	Self-assembled monolayer of 3-mercaptopropionic acid on a gold surface	ABTS and syringaldazine	Vianello et al., 2004
Rigidoporus lignosus	Mercury thin film electrode (MTFE)+gelatin	Catechol	Kirgöz et al., 2005
Coriolus hirsutus	Gold surface	Catechol	Solná & Skládal, 2005
Trametes versicolor	Graphite electrodes	Graphite electrodes	Portaccio et al., 2006
Trametes hirsuta	Aminated porous glass beads	Catechol	Shleev et al., 2006
Coriolus versicolor	Indium tin oxide electrode (TIO)		Tang et al., 2006
Rigidoporus lignosus	ECH-Sepharose (resin)	Phenols	Vianello et al., 2006
Fluka	Carbon electrode with magnetic core-shell		Zhang et al., 2007
Rhus vernicifera	Dendrymer on gold nanoparticles. Glassy carbon electrode	Catechin	Rahman et al., 2008

1.5. Contaminants of emerging concern

Nowadays, there is a significant preoccupation with the environment as a whole, that requires several actions to protect and restore its ecological integrity. It is necessary to characterize the compounds that are responsible for environmental pollution and create regulation to prevent a future imbalance of ecosystems and public health problems. Contaminants of emerging concern (CEC) can be classified as chemical substances for which no maximum levels have been laid down in EU legislation or substances which maximum levels have already been established but require further investigation (Beaman et al., 2008). CEC include a variety of synthetic chemicals used worldwide. These contaminants have been classified based on use, origin and/or effects. Potential sources include pesticides and herbicides, nanomaterials, phthalates, personal care products, additives to plastic, synthetic musk, brominated compounds, phytoestrogens, and pharmaceuticals (hormones, pain relievers, antibiotics, etc.) (Barrios-Estrada *et al.*, 2018; Hernandez-Vargas *et al.*, 2018).

Most of the times, CEC end at industrial wastewater and generate an enormous accumulation of pollutants. Also, there are many contaminants present in agriculture fields, urban parks, and residential yards, as a result of their application as fertilizers. Other compounds are discharged directly from industrial production processes into rivers or lakes and discharged chemicals move through the atmosphere and ocean currents. A series of ecotoxicological problems have been reported as the consequence of the increase in the levels of CEC. These compounds do not appear individually in the environment, which means that they can also lead to undesired synergistic effects (Beaman et al., 2008; Hernandez-Vargas et al., 2018).

Fish and shellfish have been identified as the food items typically showing the highest concentrations of several toxic elements; CECs have been also detected in seafood (Vandermeersch et al., 2015). CEC are not necessarily new pollutants since they include substances that have often been present in the environment, but whose presence and significance are only being evaluated now. CECs include persistent organic pollutants (POPs), pharmaceutical and personal care products (PPCPs), veterinary medicines, endocrine-disrupting chemicals (EDCs) and nanomaterials. Public health and

environmental concerns have made clear that it is urgent to develop criteria to assess and manage the potential risk of some CEC in the aquatic environment (Beaman et al., 2008).

1.5.1. Bisphenol A

BPA (2, 2-2 (4-hydroxyphenyl) propane or BPA) (Figure 1.6) is a CEC. It is a synthetic phenol, that was firstly reported by the Russian chemist Aleksandr Dianin in 1891 and synthesized via the condensation of acetone with two equivalents of phenol by Zincke in 1905 (Bisphenol A Global Industry Group, 2002).

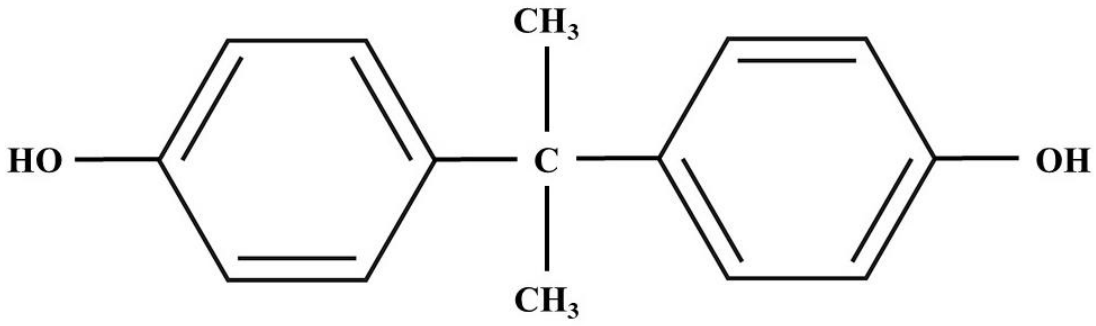

Figure 1.6. Molecular structure of bisphenol A.

BPA is a phenol derivative composed of a rigid aromatic ring and a flexible nonlinear aliphatic side chain. It is used worldwide in many applications (Jalal et al., 2018a) (Jalal et al. 2018), such as food and beverage containers, automobile components, electronic devices, sports safety equipment and medical instruments (Sheng et al., 2019). This compound is also used in the manufacture of phenolic resins, unsaturated polyester resins, thermal paper additive, flame retardants heat and carbonless paper coating. Connected by ester bonds, its polymers are easy to hydrolyze under heat, acidic or basic conditions, which leads to the potentially extensive exposure of the general population (Sheng et al., 2018).

Recently, there are some concerns about the effects that the continuous exposure to this compound may cause in public health (Li et al., 2016). Specifically, BPA acts as an endocrine disruptor, by competitive combination with estrogen receptors (Acconcia et al.,

2015). Endometrial hyperplasia, diabetes, heart disease, polycystic ovary syndrome, breast cancer, infertility, early childhood development, obesity, and other diseases are also associated with long exposure to this contaminant (Petrakis et al., 2017). There are also several disorders caused by BPA exposure at the brain level. Some studies in mice have shown that this exposure affects and alters the differentiation and connectivity of neurons and synaptic plasticity. Therefore, regarding BPA production and applications, multinational governments are creating and implementing rules and regulations to restrict its application amount or ban its use in infant products (Pang et al., 2019)

Quantification of BPA in environmental (surface waters, seawaters, sludge, sediments, groundwater, wastewaters, etc.) and food samples is usually performed by one or a combination of various chromatographic techniques (Álvarez-Muñoz et al. 2018, Santos et al. 2010).

However, the routine and extensive application of these methods are not free of disadvantages considering the common usage of organic solvents (being most of them not green) and the typical complex extraction and purification steps needed before analysis. Therefore, (bio)sensors have progressively paved their way as cost-effective routine screening tools, serving as expeditious and complementary methods to the traditional ones.

Nowadays, BPA has been detected in a variety of dairy products and for that reason, it is urgent to develop fast determination techniques in order to decrease the contamination with this chemical compound. As mentioned, gas chromatography-mass spectrometry, high-performance liquid chromatography, fluorometry, and enzyme-linked immunosorbent assays have been applied to identify BPA but investigators, with the purpose of enhancing determination and measurements methods, are making efforts to create even better devices (Li et al., 2016).

Into the aquatic environment, BPA can occur not only through effluent from wastewater treatment plants and landfill sites but also from the migration of BPA-based products into rivers and marine water. This contaminant is toxic to aquatic organisms in the range of 4.4 – 44 μM. Studies conducted in China, Japan, Germany, the Netherlands, Spain, and the United States revealed levels of BPA, in river water, between 2.2×10^{-6} μM and 3.5×10^{-2} μM, except in one sample (9.2×10^{-2} μM) (Kang et al., 2007).

BPA determination through conventional electrochemical sensors is difficult because of the weak response and adsorption of BPA on the sensing interface that contaminates the electrode surface, decreasing the sensitivity. To reduce this problem, electrochemical enzymatic based biosensors, modified with various types of advanced materials in order to increase electrodes surface area have been studied. BPA biosensors based on laccase found in the literature are listed in Table 1.3. The available studies are scarce (only 4). The reported limits of detection ranged from 6.0×10^{-3} to 35 μM with applications in tomato juice samples, spiked plastic bottles, and commercial plastic products. This limited information clearly indicates that there is a gap concerning this research topic.

Table 1.3. Previously published laccase-based biosensors for BPA detection.

Transducer	Immobilization	Detection technique	Linear range (µM)	Limit of detection (µM)	Matrix	Reference
Graphite SPEs	Adsorption on thionine–carbon black electrode	amperometry	0.5 - 50	0.2	tomato juice samples	Cammarota et al., 2013
Graphite SPEs	Nafion	amperometry	120 - 500	35	-	Scognamiglio et al., 2012
SPCE	Ag-ZnONPs functionalized MWCNTs	CV	0.5 - 2.99	6.0×10^{-3}	spiked plastic bottles	Kunene et al., 2018
SPCE	Graphene-decorated AuNPs and chitosan	CV and DPV	-	0.023	commercial plastic products	Fartas et al., 2019

SPEs – screen printed electrodes; SPCE – screen printed carbon electrode; MWCNTs – multi walled carbon nanotubes; AuNPs - gold nanoparticles; ZnONPs - zinc oxide nanoparticles; CV – cyclic voltammetry; DPV – differential pulse voltammetry.

2. Materials and Methods

In this chapter, all the strategies applied to the construction and characterization of the biosensor are described, as well as the used detection methods, experimental conditions.

2.1. Reagents

The polyphenol oxidase laccase enzyme from T. *versicolor* (EC 1.10.3.2; 0.53 U/mg), Chitosan ≥ 75% from shrimp shells (deacetylated) (CAS: 9012 – 76 – 4), 1-butyl-3-methylimidazolium tetrafluoroborate (BMIMBF4) ≥ 97.0% (HPLC), 4-aminophenol ($H_2NC_6H_4OH$) ≥ 98% and bisphenol A ($(CH_3)_2C(C_6H_4OH)_2$) ≥ 99% were purchased from Sigma-Aldrich (Germany).

Multiwalled carboxylic functionalized carbon nanotubes (>8% carboxylic acid functionalized), with an average 9.5 nm diameter and 1.5 μm length, were also obtained from Sigma-Aldrich (Germany).

Orthophosphoric acid (H_3PO_4, 85 wt. % in H_2O), boric acid (H_3BO_4 ≥ 99.5%) and, glacial acetic acid (CH_3COOH ≥ 99%) were used to prepare phosphate buffer (0.04 M, pH 5.0); they were bought from Riedel-de-Haën (Germany) as well as potassium hydroxide (KOH ≥ 85%).

Ethanol (EtOH; 99.8%) and sodium hydroxide (NaOH; 98%) were obtained from Panreac (Barcelona, Spain).

All solutions were prepared with ultrapure water (ρ=18.2 MΩ cm^{-1}) obtained by a Milli – Q Simplicity 185 system (Millipore, Molsheim, France).

2.1.1. Solutions

A 0.04 M Briton-Robinson Buffer (BRB) was used as the electrolyte. For its preparation, H_3PO_4 0.04 M, H_3BO_4 0.04 M and CH_3COOH 0.04 M stock solutions were

prepared. Then, aliquots of the stock solution were taken, pH was adjusted with a 2.5% NaOH solution and volume was adjusted with ultrapure water.

The enzyme was daily dissolved in ultrapure water at 12.4 mg mL^{-1}.

A MWCNTs suspension was prepared at 1 mg mL^{-1} by dissolving the appropriate amount in absolute ethanol (99.8%) and keeping the solution in the ultrasonic bath (Bandelin, Sonorex Digital 10P) for approximately 20 minutes before each use.

Bisphenol A was dissolved in ethanol to obtain a 100 mM concentration solution. It was diluted 10 times in water and then the working solution was prepared in BRB pH=5.0. This solution was daily prepared.

Chitosan is insoluble at neutral and alkaline pH values (S. Lu et al., 2004), so it was prepared a 3% CH_3COOH to dissolve it with the concentration of 5 mg mL^{-1}. After its dissolution, it stood in the ultrasound bath for approximately 30 minutes. This solution was daily prepared.

2.2. Instrumentation

Electrochemical measurements were performed with a potentiostat-galvanostat, AUTOLB model PGSTAT12 (Metrohm, The Netherlands) controlled by a computer through the Model Nova version 11.2 software.

Screen Printed Carbon Electrodes (SPCEs) from DropSens Metrohm (Spain) were used. Each SPCE contains a carbon-based working electrode and counter electrode, and one pseudo-reference electrode made of silver. Through an SPE connector, the electrodes were connected, and the measurements were made with the support of the previously mentioned software.

CV and SWV were applied to evaluate the construction of the enzymatic biosensor.

2.3. Biosensor preparation

For the biosensor development, 5 µL of laccase solution was mixed with 2 µL of $BMIMBF_4$ and 5 µL of chitosan solution in order to form a homogeneous composite.

SPCEs were modified by drop coating 5 µL of a 1 mg mL^{-1} MWCNTs alcoholic suspension. After ethanol evaporation, at room temperature, the previously described composite was drop coated onto the nanostructured working electrode (Figure 2.1). Then, the prepared biosensors were kept at 4ºC overnight.

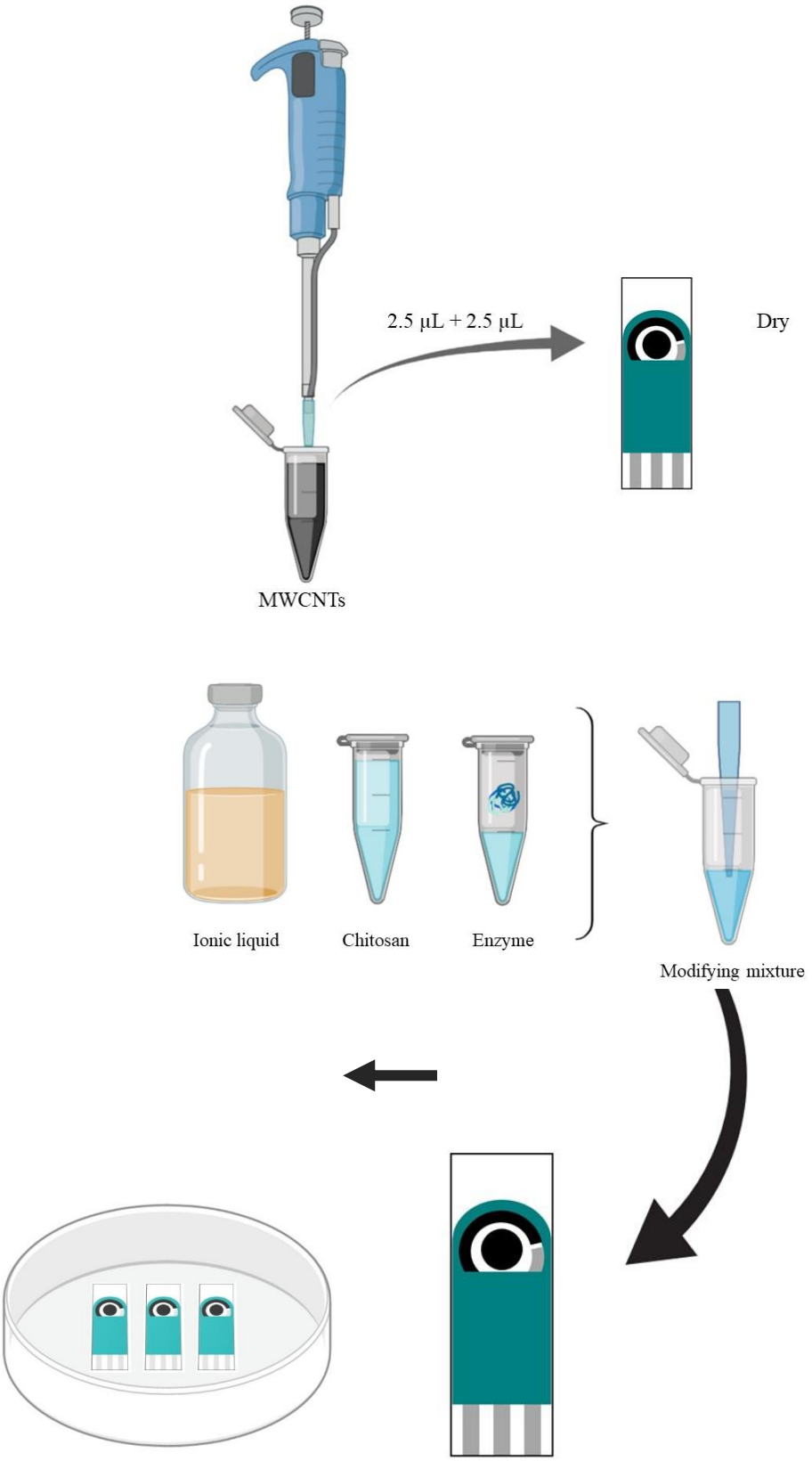

Figure 2.1. Representative scheme of the biosensor construction.

3. Results and discussion

The main objective of this work was to develop an electrochemical biosensor to determine Bisphenol A based on the enzyme laccase. With the aim of obtaining the best response, beyond the enzyme, different materials were employed as electrode modifiers, such as carbon nanotubes, the ionic liquid $BMIMBF_4$, and chitosan. The different modification steps were optimized and characterized. All experiments were performed at room temperature.

3.1. Construction stages and characterization of the electrochemical behavior of the biosensor in the presence of BPA

In order to develop a biosensor based on laccase enzyme, the first step was to observe the effect of the enzyme on the electrode. For that purpose, an SPCE was modified with 5 µL of laccase and its voltammetric response in the presence of BPA was compared with that of a non-modified electrode (Figure 3.1)

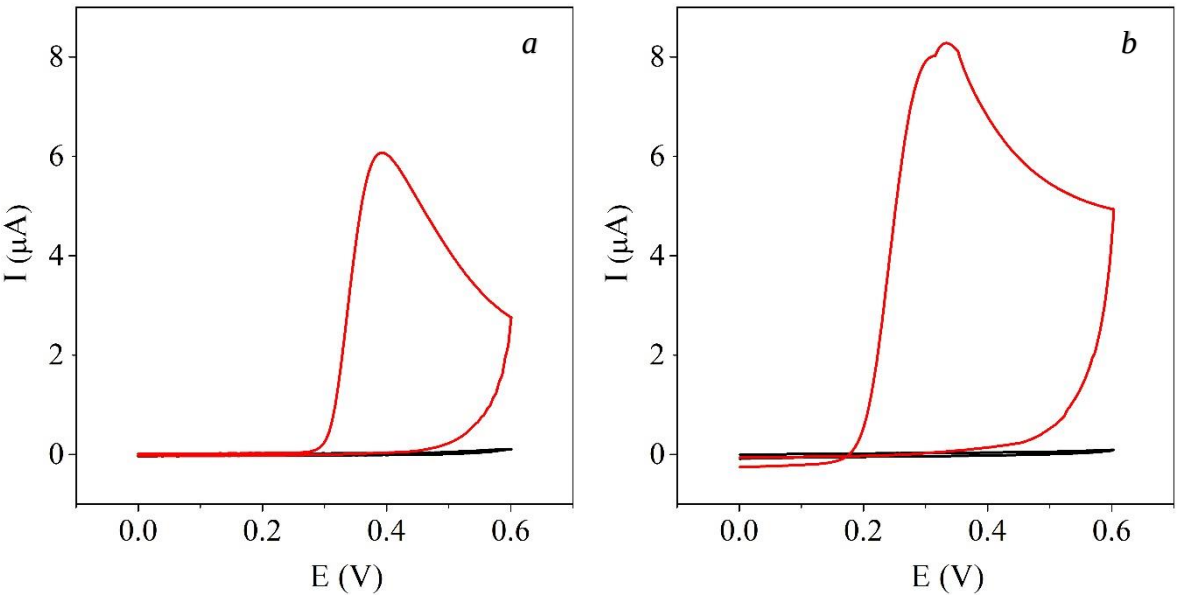

Figure 3.1. Cyclic voltammogram of **a)** a non-modified SPCE and **b)** a SPCE modified with laccase in the absence (—) and in the presence (—) of 0.5 mM BPA in 0.04 M BRB pH=5.0. Scan rate: 10 mV s^{-1}.

In the case of the bare electrode (fig. 3.1 a), in the absence of BPA solution, no redox peaks are observed, whereas, in the presence of BPA an anodic peak at 0.39 V with a current of 6 µA is observable. When the biological recognition element, laccase, is present (fig. 3.1 b) on the electrode surface, as well as in the case of the bare electrode, no redox peaks are observable. However, in the presence of BPA, an anodic peak at 0.33 V with 8.3 µA can be seen. The presence of laccase as a modifying element decreases the peak potential and enlarges the intensity of the signal, thus enhancing the sensitivity.

Since enzyme stability is one of the most important issues in the biosensor development, an entrapment polymer, chitosan, was employed, due to its advantages compared to other natural polymers, namely, non-toxicity and biocompatibility (Peniche et al., 2003). This polymer forms a film on the surface of the electrode and facilitates the modification and prevent leaching problems (Naghdi et al., 2019). To test the utility of chitosan in this biosensor development, a composite of 5 µL of laccase and 5 µL of chitosan was prepared and drop coated onto the working electrode. The voltammetric response in the presence of BPA is represented in Figure 3.2.

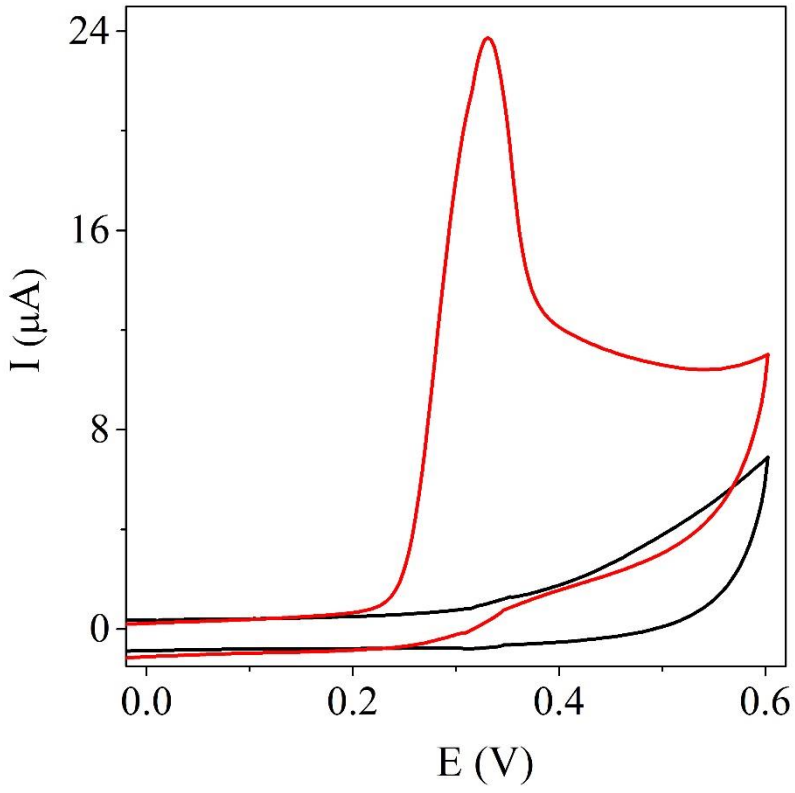

Figure 3.2. Cyclic voltammogram of an SPCE modified with a composite of laccase and chitosan in the absence (—) and in the presence (—) of 0.5 mM BPA in 0.04 M BRB pH=5.0. Scan rate: 10 mV s^{-1}.

As it can be observed, in the absence of BPA, chitosan causes an increase in the capacitive current (black curve) when compared to the previous experiments. In the presence of BPA, an anodic peak at 0.33 V with 23.7 µA can be observed. The signal intensity is increased, compared with the electrode modified only with laccase, mainly caused by the enhancement of the stability of the signal since the enzyme is retained in the electrode surface. Still, in order to improve the ionic conductivity and electrochemical stability and, therefore, enhance the signal, an ionic liquid was also added to the composite. The selected ionic liquid was $BMIMBF_4$, and 2 µL of it were added to the previous composite that already contained the enzyme and natural polymer. The results of this experiment are presented in Figure 3.3.

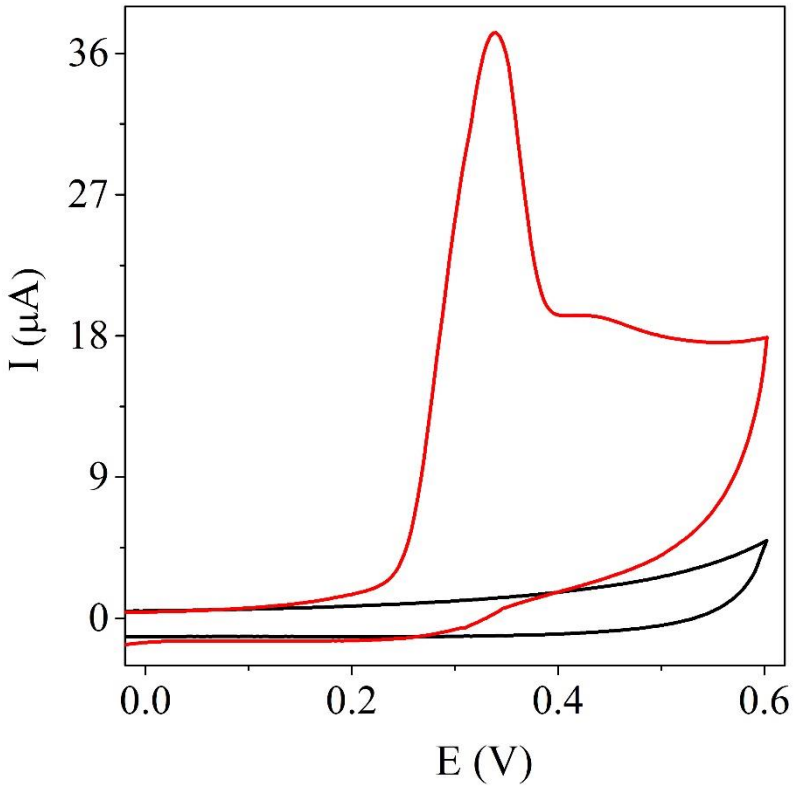

Figure 3.3. Cyclic voltammogram of an SPCE modified with a composite of laccase, chitosan $BMIMBF_4$ in the absence (—) and in the presence (—) of 0.5 mM BPA in 0.04 M BRB pH=5.0. Scan rate: 10 mV s^{-1}.

Even in the presence of the ionic liquid, no electrochemical response is observed in the absence of BPA (Figure 3.3). In the presence of BPA, the anodic peak at 0.33 V is clearly observed, in this case, with an increase of approximately 60% in its intensity, when compared to the electrode modified only with the composite of laccase and chitosan. This indicates that $BMIMBF_4$ is behaving as predicted.

Finally, with the aim of increasing, even more, the sensitivity of the biosensor in the study, MWCNTs were employed as electrode surface modifiers. As described in the literature (Jin et al., 2017), the use of MWCNTs as modifying elements enlarges the electrode surface area and improves the electron transfer rate. With the objective of obtaining the best possible response, two different strategies were studied. In the first one, the working electrode was modified with a composite of MWCNTs, laccase, chitosan, and $BMIMBF_4$. The second strategy consisted of the modification of the electrode with 5 µL of MWCNTs and, once dried, the previously mentioned laccase, chitosan, and the ionic liquid composite was deposited onto it. When comparing the obtained results, the second option was the best alternative to modify the electrode, because it was the one that offered the best signal. Therefore, the final biosensor was a SPCE containing the first layer with 5 µL of MWCNTs, and then a composite layer consisting of 5 µL of laccase, 5 µL of chitosan and 2 µL of $BMIMBF_4$. The voltammetric response of the developed biosensor in the presence of BPA is presented in Figure 3.4.

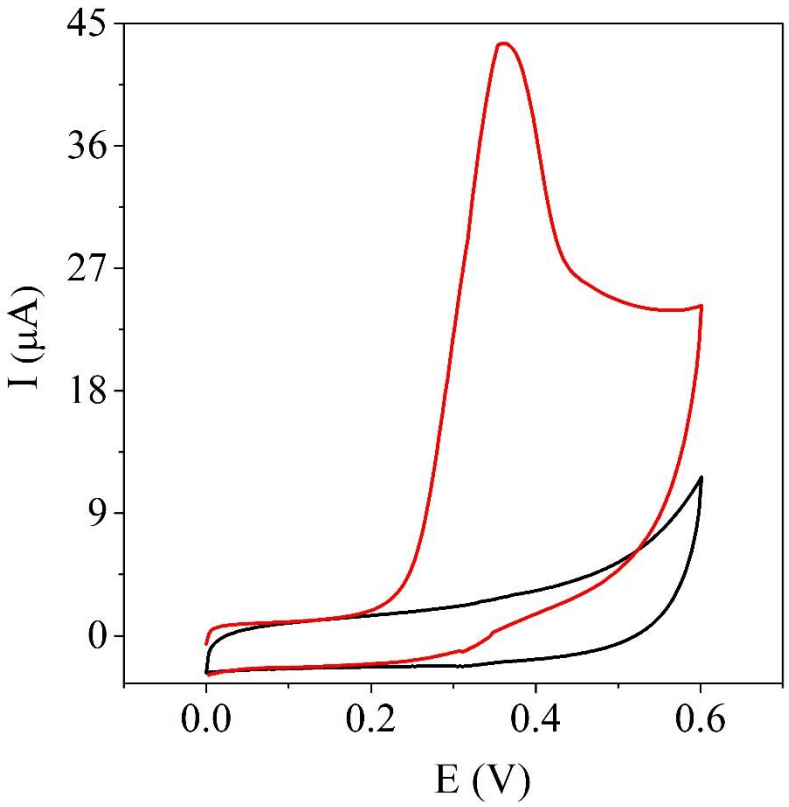

Figure 3.4. Cyclic voltammogram of an SPCE modified with MWCNTs and a composite of laccase, chitosan and $BMIMBF_4$ in the absence (—) and in the presence (—) of 0.5 mM BPA in 0.04 M BRB pH=5.0. Scan rate: 10 mV s^{-1}.

In the presence of BPA, an anodic peak at 0.36 V and 43.5 µA is detected. An increase of approximately 16% in intensity is obtained when compared to the electrode modified with the same composite but without MWCNTs.

Considering the results obtained, it can be concluded that the best response is obtained with the SPCE modified with MWCNTs and a composite of laccase, chitosan, and $BMIMBF_4$, and therefore, this was the platform employed for the biosensor development. Thereafter, the influence of the pH and quantities of all modification components were studied and optimized.

3.2. Operational parameters

3.2.1. pH influence in the biosensor response

pH is an important parameter for biosensing, and the catalytic activity of the enzyme is strongly dependent on this parameter, so its optimization is important and a crucial stage in this study. Laccase has an optimal range where the maximum activity occurs and it is between 2.0 and 6.0 (Kolomytseva et al., 2017). The intensity of the current is related to the optimal pH value for which the enzyme presents its best activity. In other words, a higher conversion of BPA into a product by the enzyme results in a higher intensity of the signal, so the sensitivity will increase too. On the other hand, the peak potential (where the oxidation of the product occurs) may also depend on the pH, depending if there are protons involved in the reaction.

To optimize the pH value, a range of values between 4.0 and 6.0 were studied. Distinct BRB solutions were prepared, and CV was applied in other to identify the higher intensity of the signal. The experimental results are represented in Figure 3.5 and Figure 3.6.

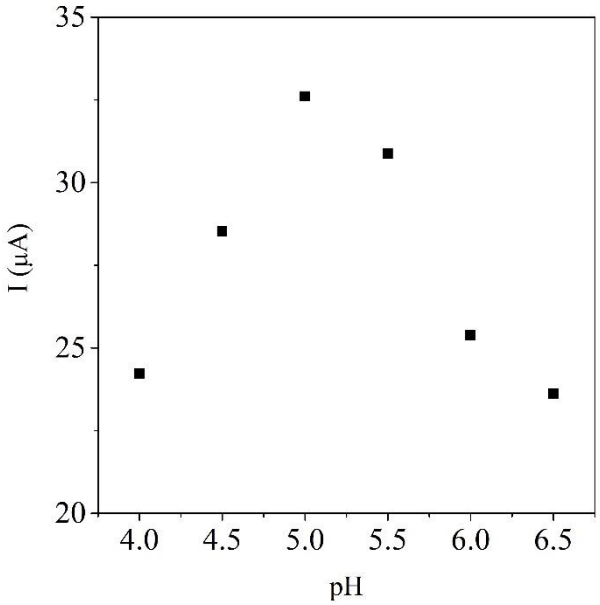

Figure 3.5. Influence of pH on peak current of 0.5 mM BPA in 0.04 M BRB pH=5.0. using the developed enzymatic biosensor. Scan rate: 10 mV s^{-1}.

The maximum peak current is evidenced at pH 5.0 (Figure 3.5) which was selected as the optimum value. This is in accordance with previous studies (Ribeiro et al., 2014) and proves that the immobilization procedure does not affect negatively the enzyme activity. It is also observable that for higher pH values the peak potential displaces to lower values (Figure 3.6).

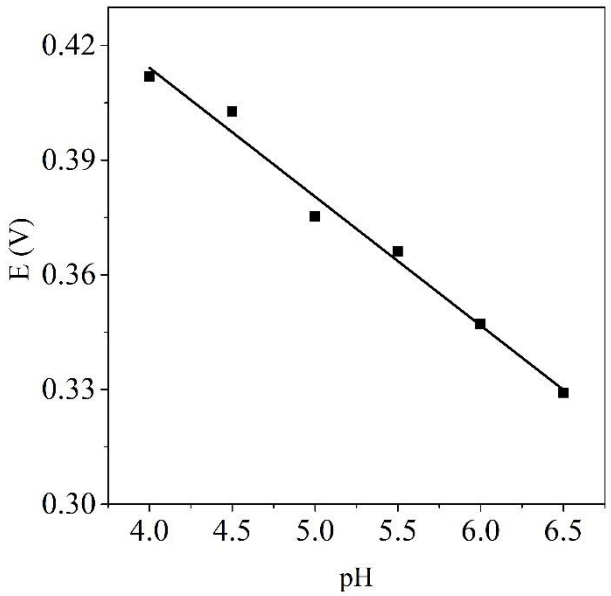

Figure 3.6. Influence of pH on peak potential of 0.5 mM BPA in 0.04 M BRB pH=5.0. using the developed enzymatic biosensor. Regression equation: Ep=0.5489±0.0105 – 0.0337±0.0020 pH. Scan rate: 10 mV s^{-1}.

Using the experimental data (Ep *vs* pH), a slope of 33.7 mV/pH was obtained. The theoretical value predicted by the Nernst equation is 59 mV/pH, which indicates that the electron transfer process may not be a simple one-electron one proton process (A. H. Shah et al., 2015). Since the slope is approximately half of the theoretical value, this means that in this reaction an exchange of the double of electrons compared to the number of involved protons occurs.

3.2.2. Optimization of the other experimental parameters

Once the biosensor design was selected, the optimization of each modification step was carried out in the presence of 0.5 mM BPA in order to maximize the sensitivity and repeatability. For this study, the amount of the modifying components was kept constant, varying only one each at time.

To optimize the amount of enzyme (Figure 3.7), 4 electrodes were modified with different amounts of laccase (4, 5, 6 and, 8 µL). The highest intensity was reached with the use of 5 µL of the enzyme, so this amount was chosen as the optimal.

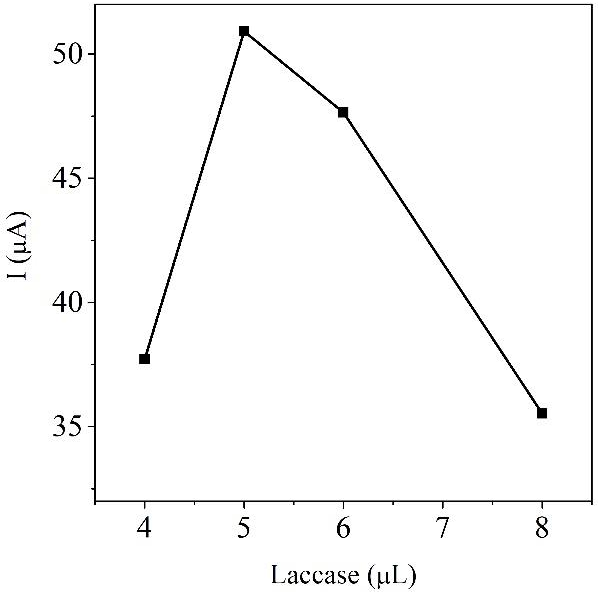

Figure 3.7. Biosensor response to different amounts of Laccase.

To determine the optimal quantity of chitosan (Figure 3.8), 2, 4, 5 and, 6 µL of a 5 mg mL^{-1} chitosan solution were employed. As observed, the quantity that provides the highest intensity is 5 µL, so this was selected as the optimal amount.

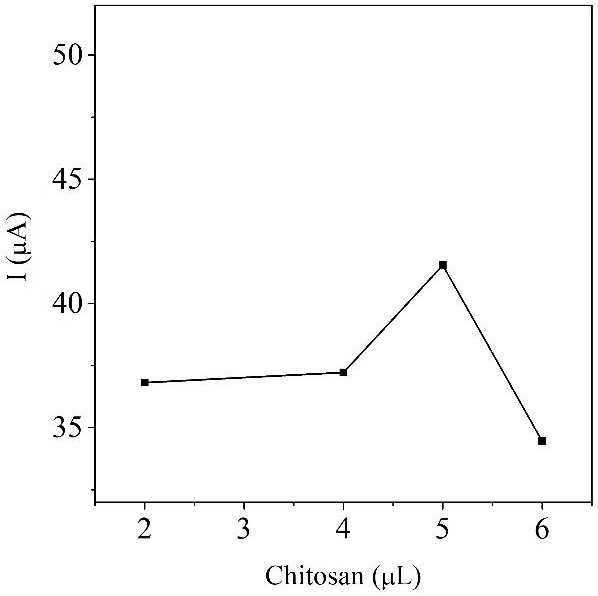

Figure 3.8. Biosensor response to different amounts of Chitosan.

To optimize the quantity of $BMIMBF_4$ (Figure 3.9), 5 electrodes were modified with ionic liquid volumes of 0.5, 1, 2, 3, and 4 µL. The results showed that the highest intensity was reached with 2 µL, so it was the chosen quantity.

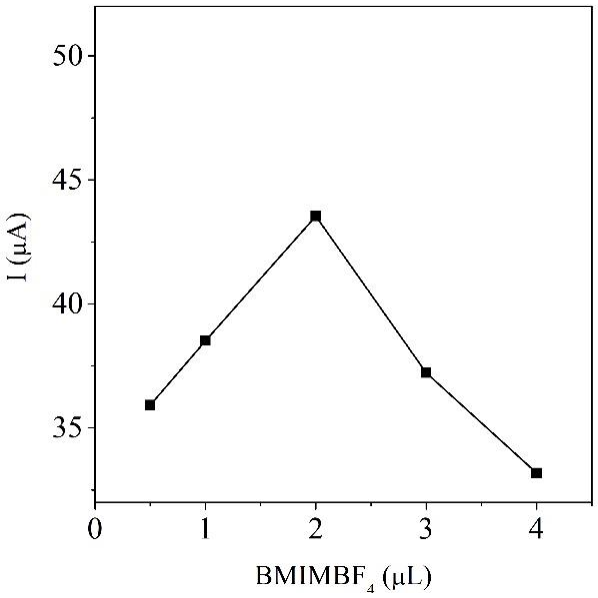

Figure 3.9. Biosensor response to different amounts of $BMIMBF_4$.

In the case of MWCNTs (Figure 3.10), 4 electrodes were modified using 2, 4, 5, and 6 µL of MWCNTs suspension. It can be observed that the signal intensity increases as the amount of MWCNTs increases and the optimal value was selected as being 5 µL.

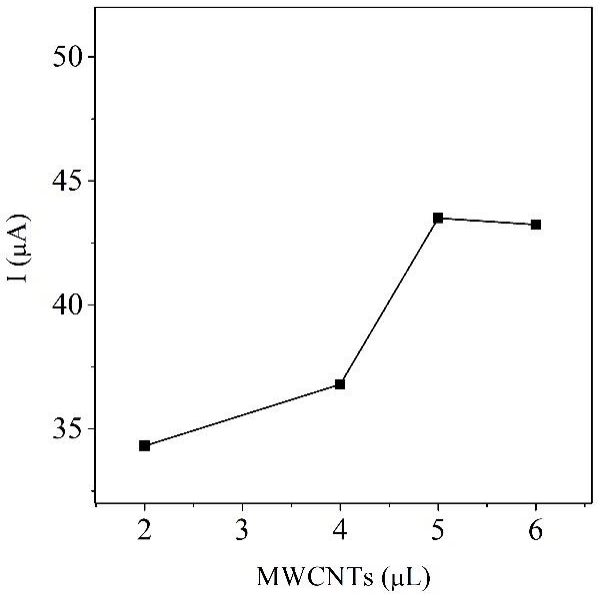

Figure 3.10. Biosensor response to different amounts of MWCNTs.

3.3. Characterization of the biosensor

3.3.1. Electrochemical behavior

The modified electrode performance in the presence of 50 µM 4-aminophenol (4-AP) was studied. This compound was chosen as a redox model and electroactive indicator since the electrochemical behavior of 4-aminophenol laccase enzymatic product is an extensively studied process (Oliveira et al., 2014; Rodríguez-Delgado et al., 2015).

In Figure 3.11, the different responses for a non-modified SPCE, an SPCE modified with MWCNTs, an SPCE modified with MWCNTs and a composite of $BMIMBF_4$ and chitosan, and an SPCE modified with MWCNTs and a composite of $BMIMBF_4$, laccase, and chitosan in the presence of 4-AP are presented. The potential values for anodic and cathodic peaks for the different modifications are shown in Table 3.1.

In the case of the non-modified SPCE, a quasi-reversible redox pair appears, which corresponds to the formation of a quinone-imine species (Figure 3.12) (Giles et al., 2003).

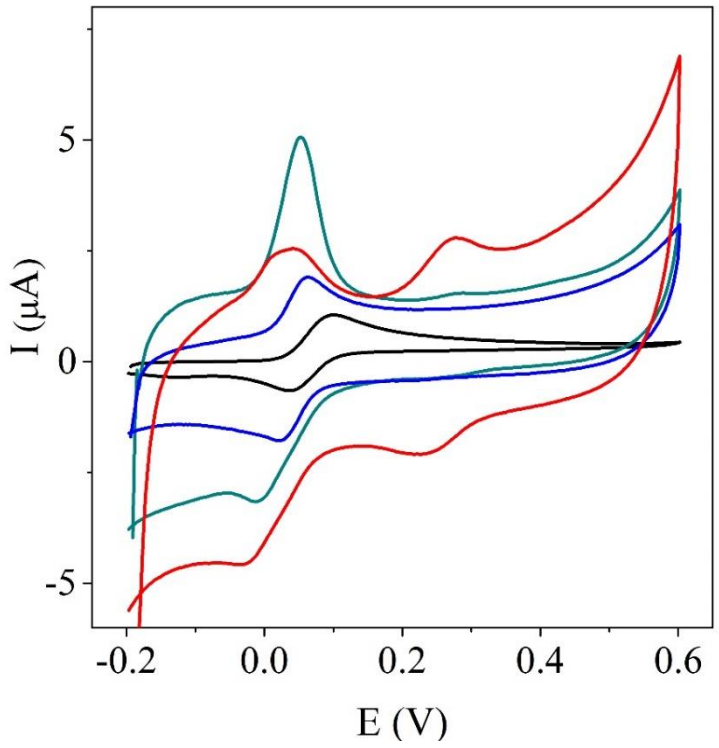

Figure 3.11. Cyclic voltammograms of bare (—) and modified with CNTs (—), CNTs and a composite of $BMIMBF_4$ and chitosan (—) or CNTs and a composite of ionic liquid, laccase and chitosan (—) CSPEs in the presence of 50 µM 4-aminophenol in BRB 0.04 pH = 5.0. Scan rate: 10 mV s^{-1}.

Table 3.1. Anodic and cathodic potential peaks of -aminophenol for the different modification strategies.

SPCE modification	E_a (V)	E_c (V)	ΔE (V)
Non	0.10	0.03	0.07
MWCNTs	0.05	-0.10	0.15
MWCNTs and a composite of $BMIMBF_4$ and chitosan	0.06	0.02	0.04
MWCNTs and a composite $BMIMBF_4$, laccase, and chitosan	0.03	-0.02	0.04
	0.28	0.24	0.04

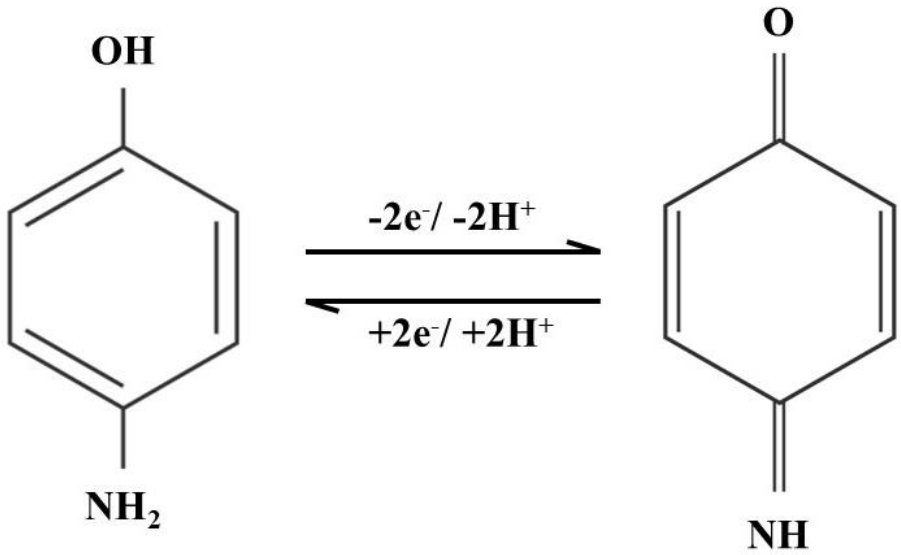

Figure 3.12. Formation mechanism of a quinone-imine.

When the electrode is modified with MWCNTs, the redox pair is shifted to more negative potentials. Moreover, both anodic and cathodic peaks show a big current increase due to the greater surface area. In the case of the SPCE modified with MWCNTs and a composite of $BMIMBF_4$ and chitosan, a decrease in the current is observed, as well as a more reversible behavior. When looking at the SPCE modified with MWCNTs and a composite of $BMIMBF_4$, laccase, and chitosan voltammogram, one can see the mentioned redox pair, with a higher cathodic current, and a new one at higher potential, corresponding to the oxidation and reduction of the enzymatic product. Accordingly, for this enzymatic biosensor, the shape of cyclic voltammograms obtained suggests that the reaction involved is predominantly controlled by a diffusion process (Oliveira et al., 2014).

In order to confirm that the reaction is controlled by diffusion, the scan rate dependence of the 4-AP-peak current was studied. For diffusion controlled processes, the Randles-Sevcik equation (at 25 ºC) applies (Bard & Faulkner, 2001):

$$i_p = 268{,}6 \times \sqrt{n^3} \times A \times \sqrt{D} \times C \times \sqrt{v} \qquad (1)$$

where i_p is the current in amps, n is the number of electrons transferred in the redox event, A the electrode area in cm^2, D the diffusion coefficient in cm^2/s, C the concentration in mol/cm^3 and, v the scan rate in V/s.

According to the previous equation, the current is proportional to the concentration and increases with the square root of the scan rate, in other to maintain the equilibrium ratio between the reduced and the oxidized forms of the redox couple predicted by the Nernst equation. Therefore, a plot of i_p *vs* $v^{1/2}$ was used to study this reaction. In Figure 3.13, a linear relation between i_p vs $v^{1/2}$ is observable, which proves that the enzymatic process is controlled by diffusion.

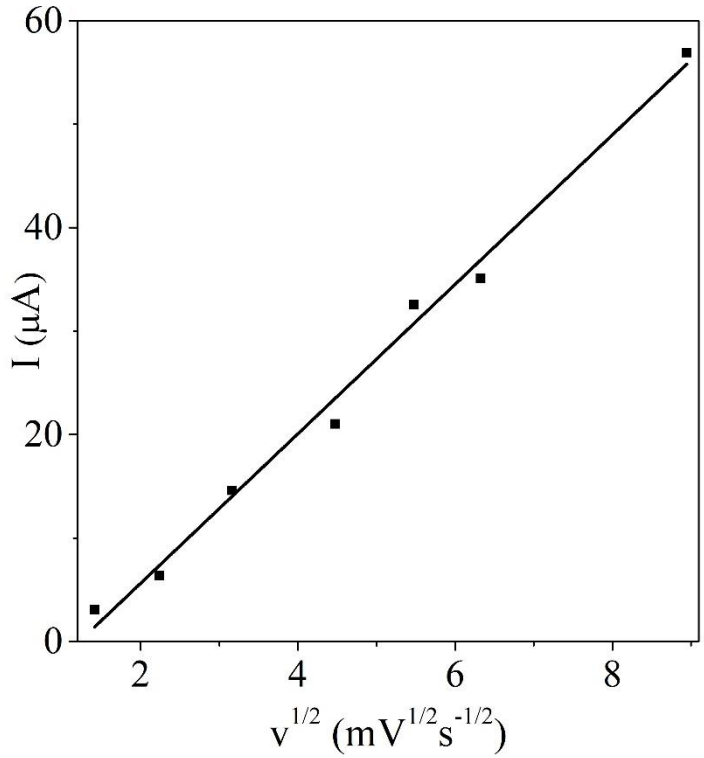

Figure 3.13. Plot of ip vs $v^{1/2}$.

3.3.2. Electroanalytical performance

The main objective of this work was the development of a BPA biosensor. BPA biosensing requires characterization of numerous electroanalytical parameters such as the linear range, limit of detection and quantification, and the sensitivity. According to the real BPA levels in the environment (Huang et al., 2012; Kang et al., 2007), high accuracy

and sensitivity coupled with low linear range and detection limit are desirable. In order to confirm the suitability of the proposed biosensor, its electroanalytical characteristics were evaluated.

Square-wave voltammetry was chosen as the determination technique since it offers higher sensitivity than the other voltammetric techniques. SWV parameters were optimized in order to obtain well-defined peaks, with the highest intensities. The amplitude in SWV is important information in kinetic studies, once it strongly affects a wide range of electrode mechanisms. In kinetically controlled electrode processes, the amplitude determines the actual electrode potential at which the current is sampled. Square wave frequency ($f = 1/\tau$, where τ represents the duration of the potential cycle) represents the critical time window of the voltammetry experiment. In SWV, the scan rate is defined as the product of the frequency and the potential step (ΔE) of the staircase potential ramp, $v = f\Delta E$. The frequency is the main tool in both mechanistic and kinetic studies of electrode processes (Mirceski et al., 2013). The best results were attained using the amplitude (A) = 40 mV, frequency (f) = 1 Hz, and step = 5 mV.

The optimum SWV parameters were used to obtain the electroanalytical features (Table 3.2 and Figure 3.14). The results were obtained from three experiments with three biosensors equally prepared. Figure 3.14 displays the obtained SWV voltammograms and the respective calibration curve obtained in the range of 0.5 and 8 µM BPA. The curve $\Delta I = f([BPA])$ in Figure 3.14 b) shows that the intensity of current increases linearly with the BPA concentration (I (µA) = 0.0636 [BPA, µM]– 0.02158; $R^2 = 0.998$) in a studied concentration range. Sensitivity was calculated by the slope of the calibration curve and the value was $(6.36 \pm 0.09) \times 10^{-2}$ µAµM^{-1}, higher than the one obtained from (Fuzi Mohamed Fartas, Jaafar Abdullah, Nor Azah Yusof, Yusran Sulaiman, 2019).

The limit of detection (LOD) and limit of quantification (LOQ) were calculated through the statistical parameters of the calibration curve and with the following expressions (Miller & Miller, 2004):

$$LOD = \frac{3 \times S_{y/x}}{slop} \quad (2) \qquad LOQ = \frac{10 \times S_{y/x}}{slop} \quad (3)$$

where $S_{y/x}$ is the standard deviation of the linear regression. This standard deviation can be calculated by the next expression:

$$S_{y/x} = \sqrt{\frac{\sum_i (y_i - \bar{y})^2}{n_i - 2}} \qquad (4)$$

where y_i is the response for a certain level of concentration, $\bar{y}$ the response predicted by the calibration curve and, n_i the number of points used the construct the calibration curve.

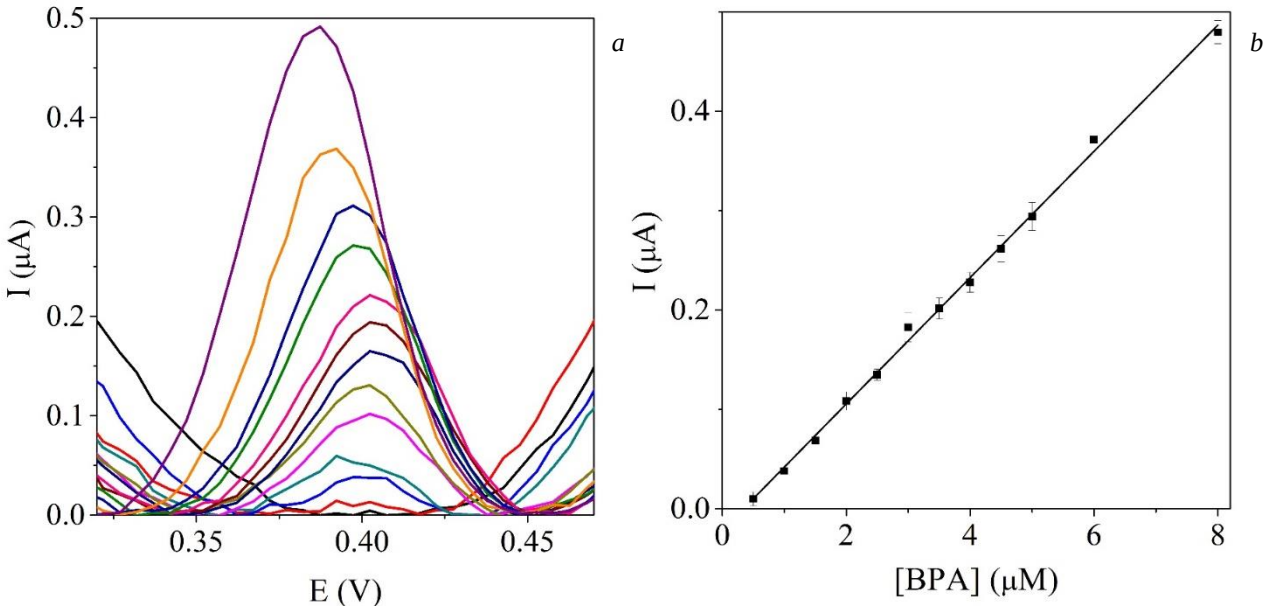

Figure 3.14. a) Square wave voltammograms a (f = 1 Hz- E_{sw} = 40 mV - E_{step} = 5 mV) in the presence of [0.5 – 8] µmol/L of BPA in 0.04 BRB pH=5.0; b) respective calibration curve.

The attained LOD and LOQ were 6.9 and 21.9 nM, respectively. In terms of LOD, the biosensor developed shows a better limit of detection compared to the other BPA biosensors based on laccase found in the literature (Cammarota et al., 2013) and (Scognamiglio et al., 2012). All the optimal analytical parameters are summarized in Table 3.2.

Table 3.2. Electroanalytical parameters of the developed laccase-based biosensor.

LOD (nM)	LOQ (nM)	Linear range (µM)	Sensitivity (µA/µM)	R.S.D. (%) of reproducibility assays
6.9	29.1	0.5 - 8	$(6.36 \pm 0.09) \times 10^{-2}$	4.3

The reproducibility was calculated from the standard deviation values, which reached 4.3%. Thus, it can be considered that the developed biosensor displays satisfactory reproducibility.

The developed biosensor was applied for the analysis of BPA in river water samples. The recovery assay was performed at a 1 µM spiking level by the standard addition method. The obtained recovery was 94.6%, with an RSD value of 5%. This result confirms that the developed biosensor can be used for the determination of BPA in river water samples.

Overall, the attained parameters indicated that the proposed biosensor presents adequate electroanalytical performance, i.e., wide linear range, low LOD and LOQ, good reproducibility and accuracy, to be applied for BPA detection.

4. Conclusions

The contamination by BPA is an important environmental and health issue and for that reason, it is important to develop suitable portable, quick, low cost and simple tools for its detection and quantification. In this work, a laccase-based biosensor was successfully developed to determine BPA. This biosensor was based on the modification of a screen printed carbon electrode with a layer of MWCNTs, followed by the subsequent drop-cast of a homogeneous composite constituted by laccase (12.4 mg mL^{-1}), the ionic liquid 1-butyl-3-methyl imidazolium tetrafluoroborate and chitosan (5 mg mL^{-1}) in an optimum proportion of 5:2:3 (v/v/v). All the modification stages, as well as the optimization of the quantities of all modification elements were evaluated using cyclic voltammetry and square-wave voltammetry. The effect of pH and scan rate were also studied and indicated that an exchange of the double of electrons compared to the number of involved protons occurs in the BPA reaction mechanism. In addition, the prepared biosensor was electrochemically characterized using a redox probe, 4-aminophenol, suggesting that the process is controlled by diffusion. The developed enzymatic biosensor displayed appropriate electroanalytical performance with a sensitivity of $(6.36 \pm 0.09) \times 10^{-2}$ µA/µM, linearity range of 0.5 – 8 µM, a limit of detection of 6.9 nM, relative standard deviation values of 4.3% in the reproducibility assays and recoveries of 94.6 ± 5.0% in environmental waters. The excellent electric conductivity, together with the enlargement of electrodes surface area promoted by the MWCNTs, combined with the suitable biocompatibility, viscosity and ionic conductivity of the selected IL and chitosan provided the appropriate conditions for the immobilization of laccase and for enhancing its activity. Still, further characterization, mainly based on scanning electron microscopy, Fourier transform infrared spectroscopy, electrochemical impedance spectroscopy, and accuracy and repeatability assays are needed to obtain a comprehensive assessment of the overall performance of the biosensor.

References

Acconcia, F., Pallottini, V., & Marino, M. (2015). Molecular mechanisms of action of BPA. *Dose-Response*, *13*(4), 1–9. https://doi.org/10.1177/1559325815610582

Álvarez-Muñoz, D., Rodríguez-Mozaz, S., Jacobs, S., Serra-Compte, A., Cáceres, N., Sioen, I., … Barceló, D. (2018). Pharmaceuticals and endocrine disruptors in raw and cooked seafood from European market: Concentrations and human exposure levels. *Environment International*, *119*(July), 570–581. https://doi.org/10.1016/j.envint.2018.07.006

Andrews, R., Jacques, D., Rao, A. M., Derbyshire, F., Qian, D., Fan, X., … Chen, J. (1999). Continuous production of aligned carbon nanotubes: A step closer to commercial realization. *Chemical Physics Letters*, *303*(5–6), 467–474. https://doi.org/10.1016/S0009-2614(99)00282-1

Bard, A. J., & Faulkner, L. R. (2001). Electrochemical methods, fundamentals and applications. In *John Wiley & Sons, Inc* (Vol. 60). https://doi.org/10.1021/ed060pa25.1

Barrios-Estrada, C., de Jesús Rostro-Alanis, M., Muñoz-Gutiérrez, B. D., Iqbal, H. M. N., Kannan, S., & Parra-Saldívar, R. (2018). Emergent contaminants: Endocrine disruptors and their laccase-assisted degradation – A review. *Science of the Total Environment*, *612*, 1516–1531. https://doi.org/10.1016/j.scitotenv.2017.09.013

Barsan, M. M., Emilia Ghica, M., & Brett, C. M. A. (2013). Electrochemical biosensors. *Portable Biosensing of Food Toxicants and Environmental Pollutants*, 33–69. https://doi.org/10.1201/b15589

Beaman, J., Co-chair, W., Eignor, D., & Huff, L. (2008). *AQUATIC LIFE CRITERIA FOR CONTAMINANTS OF EMERGING CONCERN Prepared by the*.

Bickerstaff, G. (2003). Immobilization of Enzymes: A Literature Survey. *Immobilization of Enzymes and Cells*, *1051*. https://doi.org/10.1385/0896033864

Bisphenol A Global Industry Group. (2002). *BISPHENOL A : INFORMATION SHEET (Discovery and Use)*. *343*(October), 2002.

Cammarota, M., Lepore, M., Portaccio, M., Di Tuoro, D., Arduini, F., Moscone, D., & Mita, D. G. (2013). Laccase biosensor based on screen-printed electrode modified with thionine-carbon black nanocomposite, for Bisphenol A detection. *Electrochimica Acta*, *109*, 340–347. https://doi.org/10.1016/j.electacta.2013.07.129

Chen, X., Ren, T., Ma, M., Wang, Z., Zhan, G., & Li, C. (2013). Voltammetric sensing of bisphenol A based on a single-walled carbon nanotubes/poly{3-butyl-1-[3-(N-pyrrolyl)propyl] imidazolium ionic liquid} composite film modified electrode. *Electrochimica Acta*, *111*, 49–56. https://doi.org/10.1016/j.electacta.2013.07.211

Claus, H. (2004). Laccases: Structure, reactions, distribution. *Micron*, *35*(1–2), 93–96. https://doi.org/10.1016/j.micron.2003.10.029

Dai, H. (2002). Carbon nanotubes: Synthesis, integration, and properties. *Accounts of Chemical Research*, *35*(12), 1035–1044. https://doi.org/10.1021/ar0101640

Datta, S., Christena, L. R., & Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. *3 Biotech*, *3*(1), 1–9. https://doi.org/10.1007/s13205-012-0071-7

Davis, G. (1985). Electrochemical techniques for the development of amperometric biosensors. *Biosensors*, *1*(2), 161–178. https://doi.org/10.1016/0265-928X(85)80002-X

Donten, M., & Stojek, Z. (2012). Nanoparticles and Nanostructured Materials. *Functional Nanoparticles for Bioanalysis, Nanomedicine, and Bioelectronic Devices Volume 1*, *1*, 313–325. https://doi.org/10.1021/bk-2012-1112.ch012

Duran, N., Durán, N., Rosa, M. A., & D'Annibale, A. (2002). Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. *Enzyme and Microbial Technology*, *31*(7), 907–931. https://doi.org/10.1016/S0141-0229(02)00214-4

ElKaoutit, M., Naranjo-Rodriguez, I., Temsamani, K. R., De La Vega, M. D., & De Cisneros, J. L. H. H. (2007). Dual laccase - Tyrosinase based sonogel-carbon biosensor for monitoring polyphenols in beers. *Journal of Agricultural and Food Chemistry*, *55*(20), 8011–8018. https://doi.org/10.1021/jf0711136

ElKaoutit, M., Naranjo-Rodriguez, I., Temsamani, K. R., Hernández-Artiga, M. P.,

Bellido-Milla, D., & Cisneros, J. L. H. H. de. (2008). A comparison of three amperometric phenoloxidase-Sonogel-Carbon based biosensors for determination of polyphenols in beers. *Food Chemistry*, *110*(4), 1019–1024. https://doi.org/10.1016/j.foodchem.2008.03.006

Fartas, F. M., Abdullah, J., Yusof, N. A., Sulaiman, Y., Saiman, M. I., & Zaid, M. H. M. (2019). Laccase Electrochemical Biosensor Based on Graphene-Gold/ Chitosan Nanocomposite Film for Bisphenol A Detection. *Current Analytical Chemistry*, *15*, 1–2. https://doi.org/10.2174/1573411015666190117114804

Fernández-fernández, M., Sanromán, M. Á., & Moldes, D. (2012). Recent developments and applications of immobilized laccase. *Biotechnology Advances*. https://doi.org/10.1016/j.biotechadv.2012.02.013

Giles, G. I., Collins, C. A., Stone, T. W., & Jacob, C. (2003). Electrochemical and in vitro evaluation of the redox-properties of kynurenine species. *Biochemical and Biophysical Research Communications*, *300*(3), 719–724. https://doi.org/10.1016/S0006-291X(02)02917-0

Goda, E. S., Singu, B. S., & Yoon, K. R. (2019). PT. *Microchemical Journal*. https://doi.org/10.1016/j.microc.2019.04.011

Greaves, T. L., & Drummond, C. J. (2008). Protic Ionic Liquids: Properties and Applications. *ChemInform*, *39*(18), 206–237. https://doi.org/10.1002/chin.200818249

Han, T., Nag, A., Mukhopadhyay, S. C., & Xu, Y. (2019). SC. *Sensors & Actuators: A. Physical*. https://doi.org/10.1016/j.sna.2019.03.053

He, Y., Chen, X., & Lyu, W. (2017). Author ' s Accepted Manuscript. *Biosensors and Bioelectronic*. https://doi.org/10.1016/j.bios.2017.07.018

Hernandez-Vargas, G., Sosa-Hernández, J. E., Saldarriaga-Hernandez, S., Villalba-Rodríguez, A. M., Parra-Saldivar, R., & Iqbal, H. M. N. (2018). Electrochemical biosensors: A solution to pollution detection with reference to environmental contaminants. *Biosensors*, *8*(2), 1–21. https://doi.org/10.3390/bios8020029

Huang, Y. Q., Wong, C. K. C., Zheng, J. S., Bouwman, H., Barra, R., Wahlström, B., … Wong, M. H. (2012). Bisphenol A (BPA) in China: A review of sources,

environmental levels, and potential human health impacts. *Environment International*, *42*(1), 91–99. https://doi.org/10.1016/j.envint.2011.04.010

Hutchison, J. L., Kiselev, N. A., Krinichnaya, E. P., Krestinin, A. V., Loutfy, R. O., Morawsky, A. P., … Zakharov, D. N. (2001). Double-walled carbon nanotubes fabricated by a hydrogen arc discharge method. *Carbon*, *39*(5), 761–770. https://doi.org/10.1016/S0008-6223(00)00187-1

Izquierdo, J., & Kranz, C. (2016). Electrochemical techniques for investigating redox active macromolecules. *European Polymer Journal*, *83*, 428–449. https://doi.org/10.1016/j.eurpolymj.2016.05.016

Jalal, N., Surendranath, A. R., Pathak, J. L., Yu, S., & Chung, C. Y. (2018a). Bisphenol A (BPA) the mighty and the mutagenic. *Toxicology Reports*, *5*(June 2017), 76–84. https://doi.org/10.1016/j.toxrep.2017.12.013

Jalal, N., Surendranath, A. R., Pathak, J. L., Yu, S., & Chung, C. Y. (2018b). Bisphenol A (BPA) the mighty and the mutagenic. *Toxicology Reports*, *5*(December 2017), 76–84. https://doi.org/10.1016/j.toxrep.2017.12.013

Jia, F., Shan, C., Li, F., & Niu, L. (2008). Carbon nanotube/gold nanoparticles/polyethylenimine-functionalized ionic liquid thin film composites for glucose biosensing. *Biosensors and Bioelectronics*, *24*(4), 945–950. https://doi.org/10.1016/j.bios.2008.07.057

Jin, M., Zhang, X., Zhen, Q., He, Y., Chen, X., Lyu, W., … Ding, M. (2017). An electrochemical sensor for indole in plasma based on MWCNTs-chitosan modified screen-printed carbon electrode. *Biosensors and Bioelectronics*, *98*, 392–397. https://doi.org/10.1016/j.bios.2017.07.018

Kang, J. H., Aasi, D., & Katayama, Y. (2007). Bisphenol A in the aquatic environment and its endocrine-disruptive effects on aquatic organisms. *Critical Reviews in Toxicology*, *37*(7), 607–625. https://doi.org/10.1080/10408440701493103

Karigar, C. S., & Rao, S. S. (2011). Role of microbial enzymes in the bioremediation of pollutants: A review. *Enzyme Research*, *2011*(1). https://doi.org/10.4061/2011/805187

Kirgöz, Ü. A., Tural, H., Timur, S., Pazarlioğlu, N., Telefoncu, A., & Pilloton, R. (2005).

Laccase biosensors based on mercury thin film electrode. *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology*, *33*(4), 447–456. https://doi.org/10.1080/10731190500290261

Kolomytseva, M., Myasoedova, N., Samoilova, A., Podieiablonskaia, E., Chernykh, A., Classen, T., … Golovleva, L. (2017). Rapid identification of fungal laccases/oxidases with different pH-optimum. *Process Biochemistry*, *62*(July), 174–183. https://doi.org/10.1016/j.procbio.2017.07.027

Kumar, A., Bisht, M., & Venkatesu, P. (2017). Biocompatibility of ionic liquids towards protein stability: A comprehensive overview on the current understanding and their implications. In *International Journal of Biological Macromolecules* (Vol. 96). https://doi.org/10.1016/j.ijbiomac.2016.12.005

Kunene, K., Sabela, M., Kanchi, S., & Bisetty, K. (2018). High-Performance Electrochemical Biosensor for Bisphenol A Using Screen Printed Electrodes Modified with Multiwalled Carbon Nanotubes Functionalized with Silver-Doped Zinc Oxide. *Waste and Biomass Valorization*, *0*(0), 0. https://doi.org/10.1007/s12649-018-0505-5

Lee, G. W., Kim, J., Yoon, J., Bae, J. S., Shin, B. C., Kim, I. S., … Ree, M. (2008). Structural characterization of carboxylated multi-walled carbon nanotubes. *Thin Solid Films*, *516*(17), 5781–5784. https://doi.org/10.1016/j.tsf.2007.10.071

Li, R., Wang, Y., Deng, Y., Liu, G., Hou, X., Huang, Y., & Li, C. (2016). Enhanced Biosensing of Bisphenol A Using a Nano interface Based on Tyrosinase/Reduced Graphene Oxides Functionalized with Ionic Liquid. *Electroanalysis*, *28*(1), 96–102. https://doi.org/10.1002/elan.201500448

Liu, H., & Yu, H. (2019). Ionic liquids for electrochemical energy storage devices applications. *Journal of Materials Science and Technology*, *35*(4), 674–686. https://doi.org/10.1016/j.jmst.2018.10.007

Lizardi-mendoza, J., & Valencia, F. M. G. (2016). *Chitosan Learn more about Chitosan Chemical Characteristics and Function- al Properties of Chitosan Chitosan as an antimicrobial in food products Chitin , Chitosan , and Co-Products : Chemistry , Production , Applications ,*.

Lovrić, M. (2010). Square-wave voltammetry. *Electroanalytical Methods: Guide to*

Experiments and Applications, *0*(0), 121–145. https://doi.org/10.1007/978-3-642-02915-8_6

Lu, S., Song, X., Cao, D., Chen, Y., & Yao, K. (2004). Preparation of water-soluble chitosan. *Journal of Applied Polymer Science*, *91*(6), 3497–3503. https://doi.org/10.1002/app.13537

Lu, X., Wang, X., Jin, J., Zhang, Q., & Chen, J. (2014). Electrochemical biosensing platform based on amino acid ionic liquid functionalized graphene for ultrasensitive biosensing applications. *Biosensors and Bioelectronics*, *62*, 134–139. https://doi.org/10.1016/j.bios.2014.06.036

Luong, J. H. T., Male, K. B., & Glennon, J. D. (2008). Biosensor technology: Technology push versus market pull. *Biotechnology Advances*, *26*(5), 492–500. https://doi.org/10.1016/j.biotechadv.2008.05.007

Miller, J. N., & Miller, J. C. (2004). Statistics and Chemometrics for Analytical Chemistry. In *Technometrics* (Vol. 46). https://doi.org/10.1198/tech.2004.s248

Mirceski, V., Laborda, E., Guziejewski, D., & Compton, R. G. (2013). New approach to electrode kinetic measurements in square-wave voltammetry: Amplitude-based quasireversible maximum. *Analytical Chemistry*, *85*(11), 5586–5594. https://doi.org/10.1021/ac4008573

Montereali, M. R., Seta, L. Della, Vastarella, W., & Pilloton, R. (2010). A disposable Laccase-Tyrosinase based biosensor for amperometric detection of phenolic compounds in must and wine. *Journal of Molecular Catalysis B: Enzymatic*, *64*(3–4), 189–194. https://doi.org/10.1016/j.molcatb.2009.07.014

Mousty, C., Vieille, L., & Cosnier, S. (2007). Laccase immobilization in redox active layered double hydroxides: A reagentless amperometric biosensor. *Biosensors and Bioelectronics*, *22*(8), 1733–1738. https://doi.org/10.1016/j.bios.2006.08.020

Naghdi, M., Taheran, M., Brar, S. K., Kermanshahi-pour, A., Verma, M., & Surampalli, R. Y. (2019). Fabrication of nanobiocatalyst using encapsulated laccase onto chitosan-nanobiochar composite. *International Journal of Biological Macromolecules*, *124*, 530–536. https://doi.org/10.1016/j.ijbiomac.2018.11.234

Nakao, K., Hattori, Y., & Suto, H. (2017). Numerical investigation of a spatially

developing turbulent natural convection boundary layer along a vertical heated plate. *International Journal of Heat and Fluid Flow*, *63*, 128–138. https://doi.org/10.1016/j.ijheatfluidflow.2016.09.006

Oliveira, T. M. B. F., Barroso, M. F., Morais, S., Araújo, M., Freire, C., de Lima-Neto, P., … Delerue-Matos, C. (2014). Sensitive bi-enzymatic biosensor based on polyphenoloxidases-gold nanoparticles-chitosan hybrid film-graphene doped carbon paste electrode for carbamates detection. *Bioelectrochemistry*, *98*, 20–29. https://doi.org/10.1016/j.bioelechem.2014.02.003

Pang, Q., Li, Y., Meng, L., Li, G., Luo, Z., & Fan, R. (2019). Neurotoxicity of BPA, BPS, and BPB for the hippocampal cell line (HT-22): An implication for the replacement of BPA in plastics. *Chemosphere*, *226*, 545–552. https://doi.org/10.1016/j.chemosphere.2019.03.177

Peniche, C., Argüelles-Monal, W., Peniche, H., & Acosta, N. (2003). Chitosan: An Attractive Biocompatible Polymer for Microencapsulation. *Macromolecular Bioscience*, *3*(10), 511–520. https://doi.org/10.1002/mabi.200300019

Petrakis, D., Vassilopoulou, L., Mamoulakis, C., Psycharakis, C., Anifantaki, A., Sifakis, S., … Tsatsakis, A. M. (2017). Endocrine disruptors leading to obesity and related diseases. *International Journal of Environmental Research and Public Health*, *14*(10), 1–18. https://doi.org/10.3390/ijerph14101282

Portaccio, M., Di Martino, S., Maiuri, P., Durante, D., De Luca, P., Lepore, M., … Mita, D. G. (2006). Biosensors for phenolic compounds: The catechol as a substrate model. *Journal of Molecular Catalysis B: Enzymatic*, *41*(3–4), 97–102. https://doi.org/10.1016/j.molcatb.2006.05.002

Quan, D., Kim, Y., & Shin, W. (2004a). Characterization of an amperometric laccase electrode covalently immobilized on platinum surface. *Journal of Electroanalytical Chemistry*, *561*(SUPPL. 1), 181–189. https://doi.org/10.1016/j.jelechem.2003.08.003

Quan, D., Kim, Y., & Shin, W. (2004b). Sensing characteristics of tyrosinase immobilized and tyrosinase, laccase co-immobilized platinum electrodes. *Bulletin of the Korean Chemical Society*, *25*(8), 1195–1201. https://doi.org/10.5012/bkcs.2004.25.8.1195

Quan, D., & Shin, W. (2004a). Amperometric detection of catechol and catecholamines by immobilized laccase from Denilite. *Electroanalysis*, *16*(19), 1576–1582. https://doi.org/10.1002/elan.200302988

Quan, D., & Shin, W. (2004b). Amperometric detection of hydroquinone and homogentisic acid with laccase immobilized platinum electrode. *Bulletin of the Korean Chemical Society*, *25*(6), 833–837. https://doi.org/10.5012/bkcs.2004.25.6.833

Quan, D., & Shin, W. (2004c). Modification of electrode surface for covalent immobilization of laccase. *Materials Science and Engineering C*, *24*(1–2), 113–115. https://doi.org/10.1016/j.msec.2003.09.036

Rahman, M. A., Noh, H. B., & Shim, Y. B. (2008). Direct electrochemistry of laccase immobilized on Au nanoparticles encapsulated-dendrimer bonded conducting polymer: Application for a catechin sensor. *Analytical Chemistry*, *80*(21), 8020–8027. https://doi.org/10.1021/ac801033s

Riaz, N., Wolden, S. L., Gelblum, D. Y., & Eric, J. (2016). *Chitin and Chitosan: Production and Application of Versatile Biomedical Nanomaterials*. *118*(24), 6072–6078. https://doi.org/10.1002/cncr.27633.Percutaneous

Ribeiro, F. W. P., Barroso, M. F., Morais, S., Viswanathan, S., de Lima-Neto, P., Correia, A. N., … Delerue-Matos, C. (2014). Simple laccase-based biosensor for formetanate hydrochloride quantification in fruits. *Bioelectrochemistry*, *95*, 7–14. https://doi.org/10.1016/j.bioelechem.2013.09.005

Rodríguez-delgado, M. M., Alemán-nava, G. S., Rodríguez-delgado, J. M., Dieck-assad, G., Martínez-chapa, S. O., Barceló, D., & Parra, R. (2015). Trends in Analytical Chemistry Laccase-based biosensors for detection of phenolic compounds. *Trends in Analytical Chemistry*, *74*, 21–45. https://doi.org/10.1016/j.trac.2015.05.008

Rodríguez-Delgado, M. M., Alemán-Nava, G. S., Rodríguez-Delgado, J. M., Dieck-Assad, G., Martínez-Chapa, S. O., Barceló, D., & Parra, R. (2015). Laccase-based biosensors for detection of phenolic compounds. *TrAC - Trends in Analytical Chemistry*, *74*, 21–45. https://doi.org/10.1016/j.trac.2015.05.008

Santos, L. H. M. L. M., Araújo, A. N., Fachini, A., Pena, A., Delerue-Matos, C., & Montenegro, M. C. B. S. M. (2010). Ecotoxicological aspects related to the presence

of pharmaceuticals in the aquatic environment. *Journal of Hazardous Materials*, *175*(1–3), 45–95. https://doi.org/10.1016/j.jhazmat.2009.10.100

Scognamiglio, V., Pezzotti, I., Pezzotti, G., Cano, J., Manfredonia, I., Buonasera, K., … Giardi, M. T. (2012). Towards an integrated biosensor array for simultaneous and rapid multi-analysis of endocrine disrupting chemicals. *Analytica Chimica Acta*, *751*, 161–170. https://doi.org/10.1016/j.aca.2012.09.010

Seguro, I. B. (2017). *Interacción de nanoestructuras de carbono o metálicas con (bio)moléculas y su aplicación al desarrollo de sensores*.

Shah, A. H., Zaid, W., Shah, A., Rana, U. A., Hussain, H., Ashiq, M. N., … Kraatz, H.-B. (2015). pH Dependent Electrochemical Characterization, Computational Studies and Evaluation of Thermodynamic, Kinetic and Analytical Parameters of Two Phenazines. *Journal of The Electrochemical Society*, *162*(3), H115–H123. https://doi.org/10.1149/2.0481503jes

Shah, K. A., Najar, F. A., Sharda, T., & Sreenivas, K. (2018). Synthesis of multi-walled carbon nanotubes by thermal CVD technique on Pt–W–MgO catalyst. *Journal of Taibah University for Science*, *12*(2), 230–234. https://doi.org/10.1080/16583655.2018.1451114

Sheng, Z., Wang, C., Ren, F., Liu, Y., & Zhu, B. (2018). ScienceDirect Molecular mechanism of endocrine-disruptive effects induced by Bisphenol A : The role of transmembrane G-protein estrogen receptor 1 and integrin α v β 3. *Journal of Environmental Sciences*, 1–13. https://doi.org/10.1016/j.jes.2018.05.002

Sheng, Z., Wang, C., Ren, F., Liu, Y., & Zhu, B. (2019). Molecular mechanism of endocrine-disruptive effects induced by Bisphenol A: The role of transmembrane G-protein estrogen receptor 1 and integrin αvβ3. *Journal of Environmental Sciences (China)*, *75*, 1–13. https://doi.org/10.1016/j.jes.2018.05.002

Shleev, S., Persson, P., Shumakovich, G., Mazhugo, Y., Yaropolov, A., Ruzgas, T., & Gorton, L. (2006). Laccase-based biosensors for monitoring lignin. *Enzyme and Microbial Technology*, *39*(4), 835–840. https://doi.org/10.1016/j.enzmictec.2006.01.009

Soares, J. C. (2011). *Biossensores eletroquímicos fabricados a partir da imobilização da urease em filmes de polipirrol*. https://doi.org/10.11606/T.88.2011.tde-21042011-

155326

Solná, R., & Skládal, P. (2005). Amperometric flow-injection determination of phenolic compounds using a biosensor with immobilized laccase, peroxidase and tyrosinase. *Electroanalysis*, *17*(23), 2137–2146. https://doi.org/10.1002/elan.200403343

Tang, H., Zhang, W., Geng, P., Wang, Q., Jin, L., Wu, Z., & Lou, M. (2006). A new amperometric method for rapid detection of Escherichia coli density using a self-assembled monolayer-based bienzyme biosensor. *Analytica Chimica Acta*, *562*(2), 190–196. https://doi.org/10.1016/j.aca.2006.01.061

The, D. R. (1999). ELECTROCHEMICAL BIOSENSORS : RECOMMENDED Electrochemical biosensors : Recommended de ® nitions and classi ® cation (Technical Report). *Electroanalytical Chemistry*, *71*(12), 2333–2348. https://doi.org/10.1351/pac199971122333

Thostenson, E. T., Ren, Z., & Chou, T. (2001). Advances in the science and technology of carbon nanotubes and their composites: a review. *Composites Science and Technology*, *61*, 1899–1912. https://doi.org/10.1016/S0266-3538(01)00094-X

Timur, S., Pazarloğlu, N., Pilloton, R., & Telefoncu, A. (2004). Thick film sensors based on laccases from different sources immobilized in polyaniline matrix. *Sensors and Actuators, B: Chemical*, *97*(1), 132–136. https://doi.org/10.1016/j.snb.2003.07.018

Vandermeersch, G., Lourenço, H. M., Alvarez-Muñoz, D., Cunha, S., Diogène, J., Cano-Sancho, G., … Robbens, J. (2015). Environmental contaminants of emerging concern in seafood - European database on contaminant levels. *Environmental Research*, *143*, 29–45. https://doi.org/10.1016/j.envres.2015.06.011

Vianello, F., Cambria, A., Ragusa, S., Cambria, M. T., Zennaro, L., & Rigo, A. (2004). A high sensitivity amperometric biosensor using a monomolecular layer of laccase as biorecognition element. *Biosensors and Bioelectronics*, *20*(2), 315–321. https://doi.org/10.1016/j.bios.2004.01.022

Vianello, F., Ragusa, S., Cambria, M. T., & Rigo, A. (2006). A high sensitivity amperometric biosensor using laccase as biorecognition element. *Biosensors and Bioelectronics*, *21*(11), 2155–2160. https://doi.org/10.1016/j.bios.2005.10.006

Wang, X., & Hao, J. (2016). Recent advances in ionic liquid-based electrochemical

biosensors. *Science Bulletin*, *61*(16), 1281–1295. https://doi.org/10.1007/s11434-016-1151-6

Wolf, K. P. W., & Groves, W. (1994). *SURFACE-MODIFIED ELECTROCHEMICAL BIOSENSOR*. (19).

Xu, X., Lu, P., Zhou, Y., Zhao, Z., & Guo, M. (2009). Laccase immobilized on methylene blue modified mesoporous silica MCM-41/PVA. *Materials Science and Engineering C*, *29*(7), 2160–2164. https://doi.org/10.1016/j.msec.2009.04.019

Yaropolov, A. I., Shleev, S. V., Morozova, O. V., Zaitseva, E. A., Marko-Varga, G., Emneus, J., & Gorton, L. (2005). An amperometric biosensor based on laccase immobilized in polymer matrices for determining phenolic compounds. *Journal of Analytical Chemistry*, *60*(6), 624–628. https://doi.org/10.1007/s10809-005-0138-2

Yu, X., Chen, Y., Chang, L., Zhou, L., Tang, F., & Wu, X. (2013). β-cyclodextrin non-covalently modified ionic liquid-based carbon paste electrode as a novel voltammetric sensor for specific detection of bisphenol A. *Sensors and Actuators, B: Chemical*, *186*, 648–656. https://doi.org/10.1016/j.snb.2013.06.089

Zhang, Y., Zeng, G. M., Tang, L., Huang, D. L., Jiang, X. Y., & Chen, Y. N. (2007). A hydroquinone biosensor using modified core-shell magnetic nanoparticles supported on carbon paste electrode. *Biosensors and Bioelectronics*, *22*(9–10), 2121–2126. https://doi.org/10.1016/j.bios.2006.09.030

Zhu, C., Du, D., Lin, Y., Zhang, T., Liu, J., Wang, C., … Mazzei, F. (2017). Beyond graphene: Electrochemical sensors and biosensors for biomarkers detection. *Biosensors and Bioelectronics*, *89*(2), 886–898. https://doi.org/10.1155/2016/2981931