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DISEÑO Y PRODUCCIÓN DE SENSORES Y BIOSENSORES SERIGRAFIADOS. APLICACIÓN A LA DETERMINACIÓN DE AMINAS BIÓGENAS

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Memoria de investigación titulada "*Diseño y producción de sensores y biosensores serigrafiados. Aplicación a la determinación de aminas biógenas*" presentada para optar al Grado de Doctor en la Universidad de Burgos.

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INFORMAN FAVORABLEMENTE sobre la presente Tesis Doctoral, titulada "*Diseño y producción de sensores y circuitos serigrafiados. Aplicación a la determinación de aminas biógenas*", realizada en el Área de Química Analítica del Departamento de Química de la Universidad de Burgos por Don. Wilder Henao Escobar

Y para que así conste, firman el presente informe en Burgos, a de 2017.

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ABBREVIATIONS

Abbreviation	Signification
AOs	Amine oxidases
Agm	Agmatine
AuNPs	Gold nanoparticles
BAs	Biogenic amines
BCMSEP	Bias corrected mean square error of prediction
BDD	Boron-doped diamond
BSA	Bovine serum albumin
Cad	Cadaverine
DAO	Diamine oxidase
DCl	Dansyl chloride
GA	Glutaraldehyde
His	Histamine
HMD	Histamine deshydrogenase
HOD	Histamine oxidase
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IS	Internal standard
ITC	Isothermal titration calorimetry
LMS	Least median square regression
MAO	Monoamine oxidase
MSEP	Mean square error prediction
OLS	Ordinary least square regression
OPA	o-phthaladehyde

PAO	Plasma amine oxidase
Pea	Phenyl ethylamine
PLS	Partial least squares
PSAO	Pea seedlings amine oxidase
PUO	Putrescine oxidase
Put	Putrescine
RRMSEP	Relative root square of prediction
RSD	Relative standard deviation
SEP	Standard errors of prediction
SMO	Spermine oxidase
SPAuE	Screen-printed gold electrode
Spm	Spermine
Spd	Spermidine
SPEs	Screen-printed electrodes
SPCEs	Screen-printed carbon electrodes
SPP	Sweet potato peroxidase
SPPtE	Screen-printed platinum electrode
SWV	Square wave voltammetry
TAO	Tyramine oxidase
TCA	Trichloroacetic acid
Tryp	Tryptamine
Tyr	Tyramine
TTF	Tetratiafulvalene

1. INTRODUCCIÓN

En la actualidad, las tendencias en el campo de la nutrición humana muestran un gran interés hacia una alimentación más sana, lo que ha generado una clara evolución hacia el desarrollo y producción de productos alimenticios percibidos como más saludables. Es por ello que en las últimas décadas se ha producido un aumento considerable en la investigación de los efectos que ciertos componentes presentes en los alimentos pueden ejercer en nuestra salud y, que ha dado lugar a una modificación de las metodologías de elaboración y/o conservación encaminadas a limitar la presencia de dichos componentes.

Dentro de los numerosos contaminantes alimentarios, las aminas biógenas (BAs) son uno de los analitos de mayor interés en la actualidad debido al potencial problema de salud pública, que su presencia en los alimentos constituye.

Existen evidencias de que una disminución en la calidad higiénica de un producto alimenticio se encuentra acompañada de un aumento del contenido de BAs en dicho producto. Por lo tanto, es importante el control de los niveles de BAs en este tipo de productos, ya que son buenos indicadores de la frescura de los mismos.

Las BAs desempeñan un papel esencial en numerosas funciones fisiológicas a niveles bajos de concentración. Sin embargo, si estos compuestos son consumidos en grandes cantidades pueden surgir diversos problemas toxicológicos. Los síntomas de intoxicación química transmitidos por los alimentos contaminados con BAs son de tipo alérgico, caracterizados principalmente por erupciones cutáneas. También pueden generar problemas gastrointestinales y neurológicos acompañados de náuseas, vómitos, diarrea, dolores de cabeza, palpitaciones, sofocos, sensación de hormigueo, ardor y picazón, o problemas cardiovasculares como hipotensión y taquicardia. Por lo tanto es sumamente importante en el análisis de calidad de los

alimentos, el desarrollo de procedimientos para la cuantificación e identificación de BAs.

Las BAs generalmente se encuentran presentes en niveles de concentración bajos en matrices complejas, es por ello que la determinación de estos compuestos requiere el empleo de métodos analíticos sensibles y selectivos. Tradicionalmente, la determinación de BAs se ha llevado a cabo fundamentalmente mediante cromatografía líquida de alta resolución (HPLC). Esta metodología analítica presenta una elevada sensibilidad y fiabilidad permitiendo análisis en intervalos de tiempo reducidos; no obstante, la instrumentación utilizada suele ser altamente sofisticada y costosa, por lo que encarece su utilización. Además, su alta especialización la convierte en una metodología analítica poco versátil, sobre todo en aquellos casos en que se requieren análisis en tiempo real.

La gran evolución tecnológica que ha tenido lugar en los campos de la informática y la electrónica, ha permitido el desarrollo de dispositivos analíticos eficientes y confiables que se pueden incorporar fácilmente en determinados procesos automatizados. Además, paralelamente al desarrollo de grandes equipos automatizados de análisis, se ha seguido otra alternativa en la instrumentación analítica: el desarrollo de pequeños dispositivos analíticos que por una parte simplifiquen las etapas que requieren un análisis químico y, que por otra sean competitivos en cuanto a la selectividad y sensibilidad en comparación con los tradicionales métodos instrumentales de análisis. A este pequeño grupo de pequeños dispositivos que permiten simplificar de manera importante el procedimiento analítico, pertenecen los sensores químicos y entre ellos los biosensores electroquímicos. Estos dispositivos han generado una gran atención en los últimos años para la

determinación de BAs debido a su simplicidad, reproducibilidad, bajo coste, reducido tiempo de análisis, y elevada sensibilidad.

En el desarrollo de esta tesis doctoral se ha planteado la determinación de las principales BAs implicadas en intoxicaciones alimentarías (Cadaverina (Cad), Histamina (His), Putrescina (Put) y Tiramina (Tyr)), mediante la utilización de sensores y biosensores basados en electrodos serigrafiados de carbono (SPCEs). Estos electrodos se han modificado, variando la composición de las tintas utilizadas para su elaboración así como mediante la incorporación de nanopartículas metálicas, y/o diferentes amino oxidasas (AOs), dando así lugar a biosensores electroquímicos con características operacionales adecuadas para su aplicación a la resolución de problemas analíticos reales. Con el principal objetivo de lograr obtener metodologías analíticas fiables, rápidas y selectivas, para la determinación tanto individual como colectiva de las BAs anteriormente citadas.

En la Memoria que se presenta, después de enumerar los objetivos del trabajo, el capítulo 3 se dedica a la descripción de la importancia de las BAs en el sector alimentario así como los métodos analíticos utilizados habitualmente para su determinación.

Las diferentes modificaciones de los electrodos de trabajo empleadas en esta tesis se detallan a lo largo de cada uno de los 5 capítulos dedicados a la descripción de la parte experimental. Así, en el capítulo 4 se describe el desarrollo de un biosensor selectivo para la determinación electroquímica de Put, el electrodo de trabajo fue modificado mediante la incorporación del mediador tetratiafulvaleno (TTF) en la pasta de carbono del electrodo de trabajo y la inmovilización del enzima monoamino oxidasa (MAO), permitiendo trabajar a potenciales más bajos de los que se han publicado en la bibliografía para este tipo de biosensores, logrando de este modo

reducir los posibles interferentes que se pudieran presentar en el análisis y, mejorando por tanto la selectividad del mismo. Además, el método desarrollado fue aplicado a la determinación de Put en muestras de anchoas y vegetales.

En el capítulo 5 se detalla el desarrollo de un biosensor electroquímico para la determinación simultánea y selectiva de Put y Cad. En este caso se ha empleado un sistema electródico con una nueva configuración, que incorpora dos electrodos de trabajo diferentes, los cuales son modificados individualmente mediante la inmovilización de diferentes cantidades del enzima MAO, además de la incorporación del mediador TTF y de nanopartículas de oro (AuNPs) con la finalidad de lograr una mayor sensibilidad y selectividad. El biosensor desarrollado fue aplicado a la determinación de estas aminas en muestras de pulpo fresco.

En la actualidad, los esfuerzos en el desarrollo de nuevos biosensores se centran principalmente en la mejora de su selectividad, parámetro que depende en gran medida de la especificidad del enzima utilizado; de este modo en el capítulo 6 se presenta un biosensor altamente selectivo para la determinación de Put gracias a la utilización del enzima putrescina oxidasa (PUO) que presenta una especificidad mucho más elevada hacia la Put que otras AOs. El efecto de la inmovilización por entrecruzamiento sobre la especificidad y la actividad de la PUO fue estudiado mediante la utilización de la técnica de valoración colorimétrica isotérmica (ITC). Finalmente el biosensor se utilizó en la determinación de Put en muestras alimenticias y los resultados fueron comparados mediante HPLC.

En el capítulo 7 se presenta un biosensor bienzimático que permite la determinación simultánea y selectiva de His y Put mediante la utilización de una configuración basada en dos

electrodos de trabajo incorporados en un mismo sistema electródico y modificados con el mediador TTF. Uno de los electrodos de trabajo es modificado mediante la inmovilización del enzima histamina deshidrogenasa (HMD), mientras que en el segundo electrodo de trabajo es el enzima PUO el que es inmovilizado, de este modo se obtiene un dispositivo desechable altamente selectivo para la determinación amperométrica individual y simultánea de estas dos BAs. De modo similar a los casos anteriores en la fase final se demostró la fiabilidad de los biosensores construidos en el analisis de muestras reales.

Finalmente en el capítulo 8 se presenta un sensor para la determinación simultánea de Tyr, His, Cad y Put mediante voltamperometría de onda cuadrada (SWV) y el uso de un electrodo de diamante dopado con boro (BDD). El análisis de estas BAs en una misma muestra es complejo debido a que estas aminas presentan potenciales de oxidación similares, lo que conduce a que las señales se encuentren generalmente solapadas. Así, en este capítulo se describe el empleo de la metodología de calibración multivariante mediante mínimos cuadrados parciales (PLS) para la resolución de las señales superpuestas de estas BAs y su determinación individual y simultánea en una muestra de jamón.

2. OBJECTIVES

This research work presents two main objectives:

- The design and development of biosensors based on screen-printed electrodes (SPEs) for the electrochemical determination of the most common BAs related to intoxication effects.

- The development of electrochemical systems that allow the simultaneous determination of different BAs in the same sample.

The first objective implies the performance of the following research steps:

- Study of different modifications of the electrode surface in order to find the most adequate biosensor for the analysis of the different BAs. These modifications involve the use of nanomaterials, redox mediators, and enzymes, including the study of different immobilization procedures of the biological element.

- Optimization of the experimental variables including pH and working potential with the aim of obtaining a high and sensitive electrochemical signal.

- Characterization of each developed biosensor by determining its sensitivity, capability of detection, linear calibration range and precision. This characterization also includes the analysis of the possible interferences.

- Application of each developed biosensor to the determination of the target BA in different real samples.

The second analytical objective involves additional research stages including the design of dual electrochemical systems or the application of multivariate calibration methods in order to resolve the overlapped electrochemical signals.

3. Theoretical Foundations

3.1. ELECTROCHEMICAL BIOSENSORS

A chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal. Chemical sensors usually contain two basic components connected in series: a chemical (molecular) recognition system (receptor) and a physico-chemical transducer [1].

Biosensors are chemical sensors in which the recognition system utilizes a biochemical receptor (bioreceptor) (Figure 3.1). This bioreceptor is a sensitive biological element (enzyme, DNA probe, antibody, etc.) immobilized on a physical transducer that catalyzes a biochemical reaction. The main purpose of this recognition system is to provide the biosensor with a high degree of selectivity for the analyte to be measured. The transducer is that part of the biosensor that converts the change observed in the system as a consequence of the bioreaction into a measurable signal related to the analyte concentration [2-4].

Among the various possible combinations of biocomponents and transducer techniques, the one involving electrochemical detection has been prominently used in the analysis of different substances. In this case, electrodes fabricated from different materials are frequently used as transducers.

The high use of electrochemical biosensors is due to the effective combination of the sensitivity of electrochemical transducers, as it is indicated by their low detection limits, with the high specificity of biological recognition processes. In fact, some of these sensor devices have reached the commercial stage and are

routinely used in clinical, environmental, industrial, and agricultural applications [5].

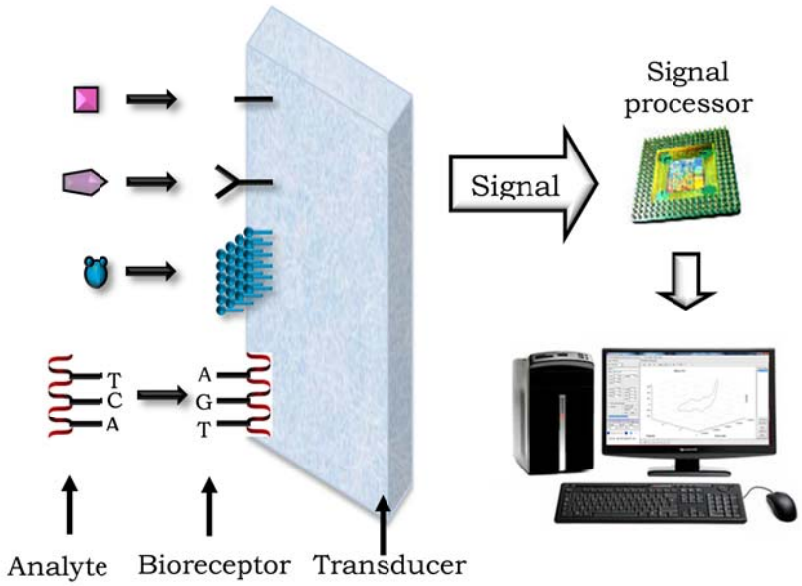


Figure 3.1. *General scheme of a biosensor*

Electrochemical biosensors constitute also an interdisciplinary field of research that is currently one of the most active areas of study due to the important analytical advantages of these devices. These advantages include a low-cost of instrumentation and relatively fast response times eliminating in many cases the need for sample preparation.

The performance of a biosensor is usually evaluated by means of the determination of different variables including, its sensitivity, limit of detection, linear calibration range, reproducibility or precision of the response, selectivity and also its response to interferences. In this way, electrochemical biosensors usually present good values for these figures of merit, which highlights their good performance in many analytical applications [5].

Storage stability, ease of use and portability are other parameters often analyzed in the performance description of a biosensor. For many clinical, food, environmental, and even defense applications, the sensor should be capable of continuously on-line monitoring of the analyte. However, disposable, single-use biosensors are satisfactory for some important other applications [5]. Electrochemical biosensors offer both possibilities, being their possible portable and disposable characteristics especially interesting in many analytical applications.

The main applications for electrochemical biosensors are in food and beverage quality control, security, environmental monitoring, bioprocessing, and most commonly in health care. For instance, determination of glucose in blood continues to be the most dominant and most studied application of electrochemical biosensors, being also the most successful commercial application of enzyme-coupled biosensors [6]. This commercial device is an amperometric glucose sensor, which was first introduced by Clark and Lyons in 1962 [7], and that was the starting point for further impressive advances and improvements.

3.1.1. ENZYMATIC ELECTROCHEMICAL BIOSENSORS

Electrochemical biosensors can be divided into two main categories based on the nature of the biological recognition process i.e. biocatalytic devices and affinity sensors [8]. Biocatalytic devices incorporate enzymes, whole cells or tissue slices that recognize the target analyte and produce electroactive species, while affinity sensors rely on a selective binding interaction between the analyte and a biological component such as an antibody, nucleic acid, or a receptor [5].

Although sensitive biological elements such as antibodies, nucleic acids and receptors have been frequently used in the development of electrochemical biosensors, enzymatic biosensors have emerged as one of the most sensitive and rapid analytical devices in environmental monitoring, quality control systems and notably, in the food industry [9].

Enzyme-catalyzed reactions have been frequently used in the fabrication of biosensors for the determination of a wide variety of substrates. Despite their high extraction, isolation and purification costs, enzymes are the most commonly used biological elements in the construction of electrochemical biosensors due to their fast and effectively formation of selective bonds with the substrate [4, 10].

More than 2000 enzymes are nowadays known. Several hundred have been isolated, many in pure or crystalline form. Over 400 enzymes are commercially available, because of the relatively recent availability of increasing numbers of highly purified and very active enzyme at reasonable cost [11]. However, the ideal enzyme selected for the construction of the electrochemical biosensor should have a high catalytic activity for the corresponding substrate and be fairly stable in the sample matrix. It should also be readily available in a purified and soluble form at a reasonable cost. Moreover, the enzyme should contain surface functional groups that can be used to form conjugates with other molecules as needed without spoiling its catalytic activity or compromising the biorecognition events. The redox active product that is formed by the enzyme catalysis should have a low redox potential to minimize interference from other components in the sample, while the substrate should be electroinactive at the measuring potential to keep the background signal low [5].

Most enzymes are very unstable in solution retaining their activity only for a short period of time. In this way, enzyme immobilization overcomes the instability, poor precision, restricted linear concentration range, and high cost often encountered with soluble enzymes. The main objective of the immobilization process is to achieve an intimate contact between the enzyme and the sensing surface of the transducer without blocking the active site of the enzyme or drastically altering the enzyme geometry [8].

The immobilization of the enzyme must be then carried out very carefully in order to assure the well performance of the biosensor. In selecting the most appropriate method, several factors must be taken into account, including the physical-chemical properties of the analyte, the nature of the biological element, the type of transducer used and the type of sample to be analyzed [4]. The main characteristics features of the most commonly used immobilization methods are described in the next section.

3.1.1.1. Enzyme immobilization methods.

A large number of immobilization methods are described in the literature for the development of electrochemical biosensors with different applications. These immobilization methods are usually classified into two categories; chemical attachment and physical retention (Figure 3.2)[12].

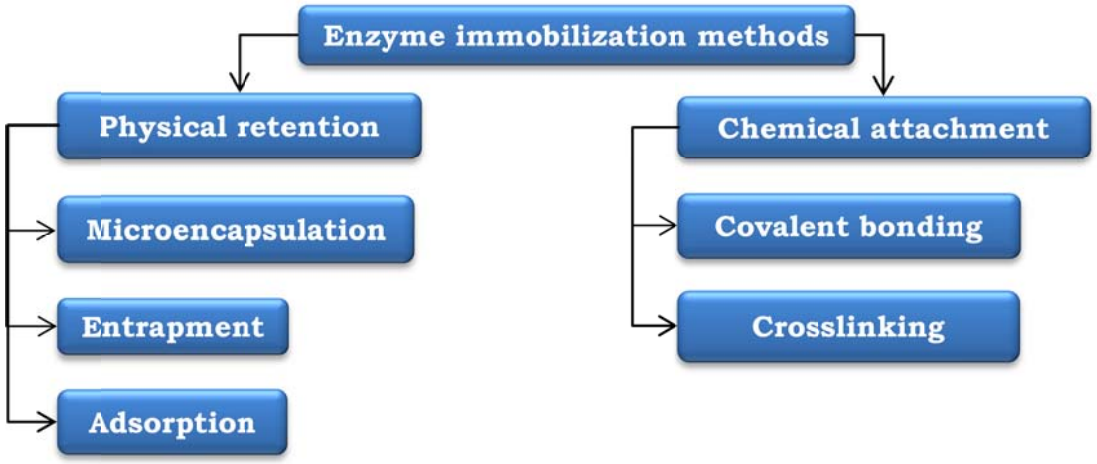


Figure 3.2. *Enzyme immobilization methods*

Adsorption

This procedure is one of the first immobilization methods used in the construction of enzymatic biosensors. The enzyme binds to a non-functionalized support via ionic interactions, van der Waals forces and hydrogen bonds (Figure 3.3). Thus, this is the simplest immobilization method, which involves minimal preparation and no reagents are required. Moreover, there is no clean-up step and there is less disruption to the enzymes. However, the bonding is weak and it is only suitable for exploratory work over a short time-span [13].

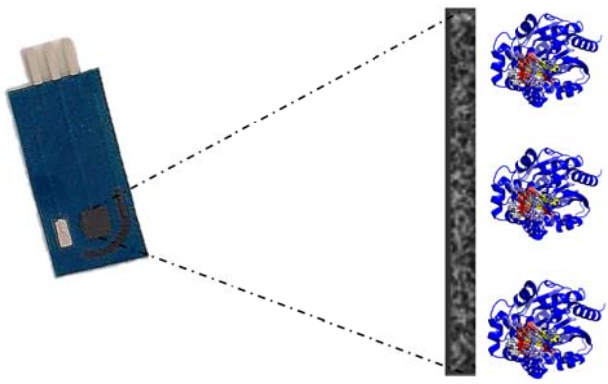


Figure 3.3. *Schematic immobilization by adsorption*

However, the bonding is weak and adsorbed biomaterial is very susceptible to changes in pH, temperature, ionic strength and substrate. Thus, this immobilization method is only satisfactory for short-term investigation and exploratory work [13].

Microencapsulation

The first commercial biosensor developed for the analysis of glucose is an example of the use of this immobilization procedure, based on the utilization of an inert semipermeable membrane to trap the enzyme on the transducer, giving close contact between the biomaterial and the transducer (Figure 3.4). The main advantage of this method is the protection performed by the membrane, which diminishes the contamination and biodegradation. Moreover, the biosensors constructed, using this immobilization method, remain stable towards changes in temperature, pH, ionic strength and chemical composition [13].

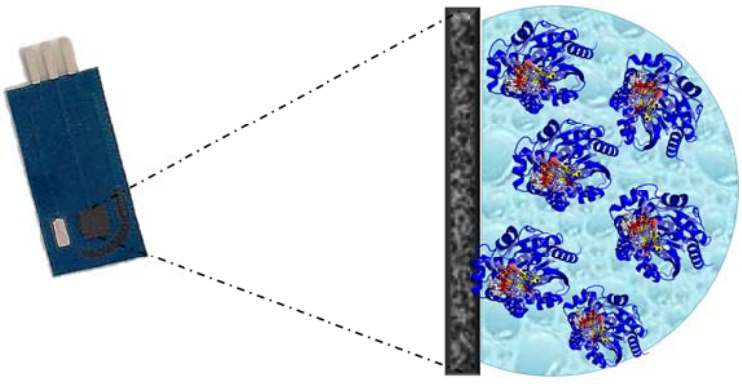


Figure 3.4. *Schematic immobilization by microencapsulation*

The main types of membranes used are the following: Cellulose acetate (dialysis membrane), which excludes proteins and

reduces transport of interfering species such as ascorbate; Polycarbonate (Nucleopore), a synthetic material which is non-permselective; collagen, an natural protein; and polytetrafluoroethylene (Teflon), a synthetic polymer, which is selectively permeable to gases such as oxygen. Other material sometimes used are Nafion and polyurethane [13].

Entrapment

In the entrapment immobilization method the enzyme is mixed with a monomer solution, which is then polymerized to a gel, trapping the enzyme (Figure 3.5). The most common polymer used is polyacrylamide. It is prepared by copolymerization of acrylamide with N,N´-methylenebisacrylamide. Other materials used are starch gel, nylon, silastic gels, and conducting polymers such as polypyrrole [13].

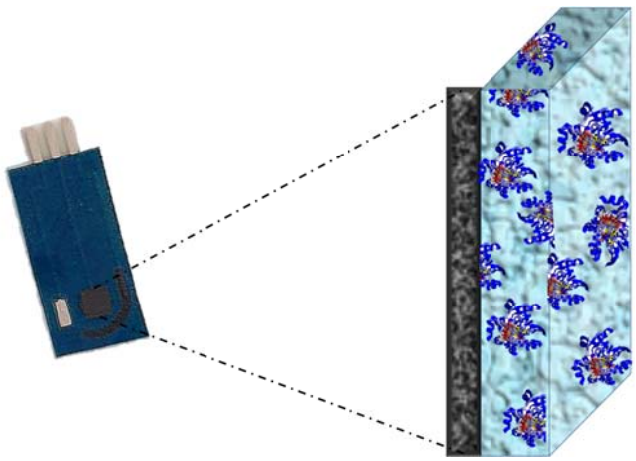


Figure 3.5. *Schematic immobilization by entrapment*

Among the disadvantages related to this immobilization method it must be highlighted the large barriers created, inhibiting

the diffusion of the substrate, which slows the reaction and hence the response time of biosensor. There may also be a loss of enzyme activity through the pores of the gel. However, the entrapment using an adequate matrix can improve enzyme stability, being this immobilization method as simple as physical adsorption [4].

Covalent bonding

This method involves a carefully design bond between a functional group in the enzyme to the support matrix (Figure 3.6). Some functional groups which are not essential for the catalytic activity of an enzyme can be covalently bonded to the support matrix (transducer or membrane). This method uses nucleophilic groups for coupling such as NH_2, CO_2H, OH, C_6H_4OH, SH and imidazole [13].

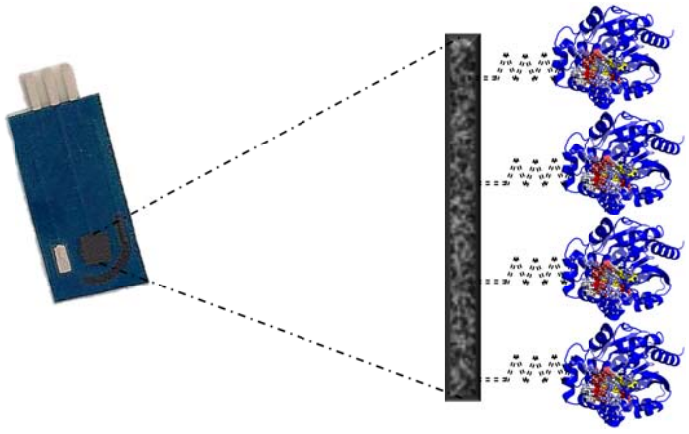


Figure 3.6. *Schematic immobilization by covalent bonding*

Reactions need to be performed under mild conditions such as low temperature, low ionic strength and pH values within the physiological range. The advantage of this immobilization method is that the enzyme will not be released during use.

Crosslinking

In this immobilization method, the biomaterial is chemically bonded to solid supports or to another supporting material such as a gel through the utilization of bifunctional reagents (Figure 3.7). The most commonly used materials are GA, which will react with lysine amino acid residues in the enzyme, hexamethylene diisocyanate and 1,5-dinitro-2,4 difluorobenzene [13]. This is a simple method, which produces irreversible intramolecular bonds between the enzyme molecules and between them and the support, resisting extreme conditions of pH and temperature [14].

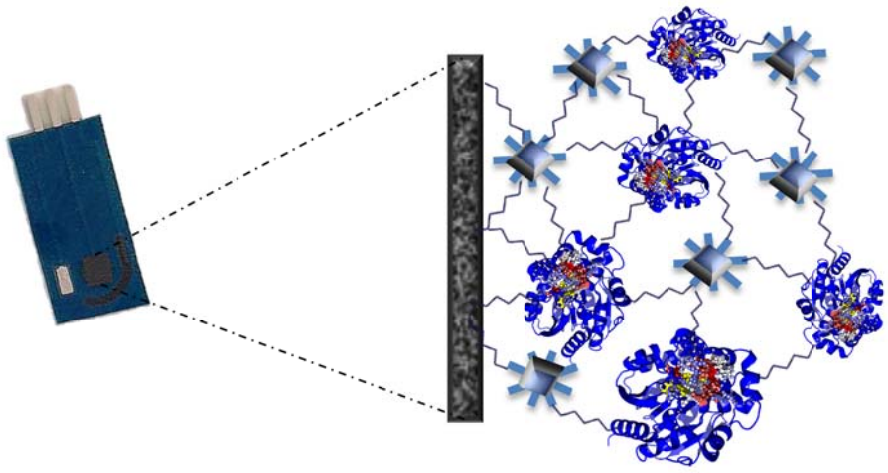


Figure 3.7. *Schematic immobilization by crosslinking*

3.1.1.2. Enzymatic biosensors modified with nanomaterials.

Biosensors can be modified in order to increase their sensitivity or selectivity depending on the purpose for which they are intended. In this way, the modification using nanomaterials plays an

important role in the preparation of enzymatic electrochemical biosensors.

Nowadays, a wide variety of different nanomaterials are used for the preparation of surfaces for biosensing applications (Table 3.1). Depending on the measurement technique, they need to fulfill, special requirements, such as electrical conductivity.

As it can be seen in table 3.1, different nanomaterials have been used in the development of biosensors namely, metallic nanoparticles (NPs), grapheme, and nanotubes and nanowires of carbon. These nanomaterials have advantages in terms of their high surface area, mechanical strength, excellent electrical conductivity and good chemical stability (Figure 3.8). Among these materials, NPs are of great interest due to their numerous possible applications and they have been the subject of intense research due to their important properties.

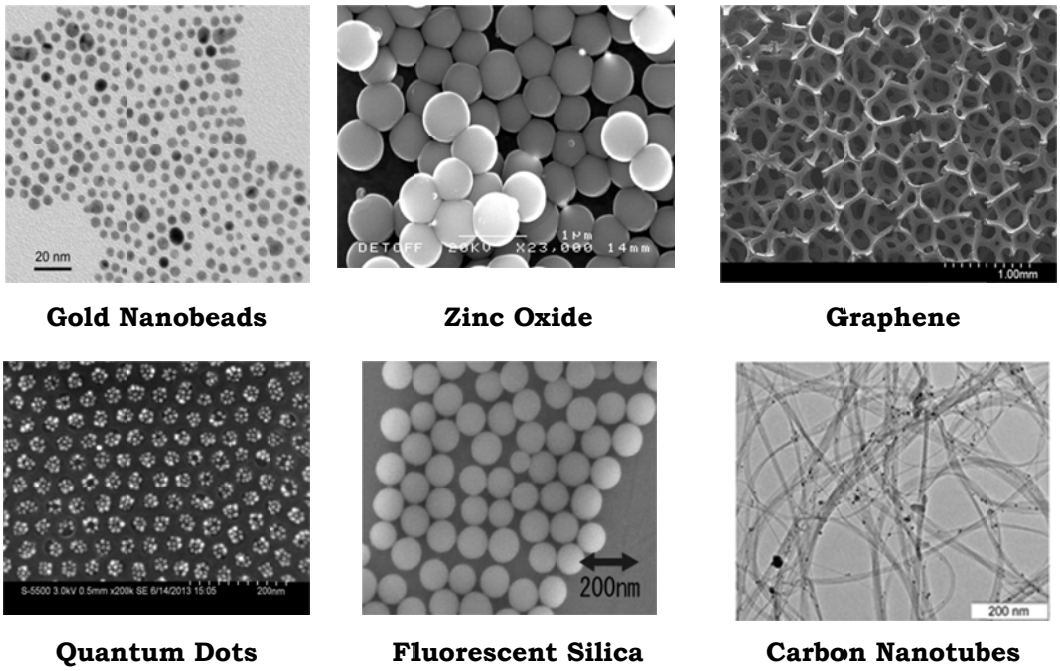


Figure 3.8. *Nanomaterials commonly applied in biosensor fabrication*

Table 3.1 *Properties and functionality of nanomaterials commonly used in biosensor fabrication [15]*

Class of nanomaterial	Material	Properties	Application	Ref.
Transition metals	Gold	Immobilization Label Catalytic	Biorecognition agent immobilization Fluorescent label Catalyst	[16]
	Silver	Immobilization Label Catalytic	Biorecognition agent immobilization Fluorescent label Catalyst	[17]
	Palladium, Copper, Nickel	Catalytic	Direct oxidation	[17]
Carbon	Nanotubes	Thermal conductivity Electrical conductivity Mechanical strength Catalyst	Electrode 3D architecture Immobilization matrix Field-effect-transistors	[18]
	Graphene	Bioaffinity molecule Immobilization Semi-conductor	Enzyme immobilization	[19]
	Nanowires	Semi-conductor Large surface area Mechanical strength	Label-free detection Field-effect-transistors	[19]
Fluorescent	Fluorescent silica	High intensity Encapsulating fluorescent dyes	Labeling Biocompatibility	[20]
	Quantum dots	Semi-conductor Narrow emission spectra Stable	Labeling Electrochemical detection	[20]

Table 3.2. Cont.: *Properties and functionality of nanomaterials commonly used in biosensor fabrication[15]*

Metal oxides	Zinc oxide	Mediator Protein absorption	Biorecognition agent immobilization	[21]
	Titanium oxide	Photocatalytic Protein adsorption	Labels Catalyst	[22]
	Iron oxide	Paramagnetic Ease of fabrication Catalyst	Biorecognition agent immobilization Target concentration Direct electrooxidation of peroxide and glucose	[22]
	Zirconia	Protein adsorption	Biorecognition agent immobilization	[23]
	Nickel oxide	Protein adsorption Semi-conductor	Biorecognition agent immobilization Direct enzyme wiring	[24]
	Manganese oxide	Protein adsorption Catalyst	Bioaffinity agent immobilization Direct oxidation of glucose	[25]

Among the different types of NPs, AuNPs have many advantageous qualities for a variety of biosensing applications using electrochemical biosensors [26]. In this way, AuNPs introduce many advantages to these sensors, encompassing their ability to provide a friendly and efficient loading platform for immobilizing enzymes and further improve electron transfer between the active site and electrode. Frequently, adsorption onto bulk surfaces results in protein denaturation and decreased performance [27]. Because of the high biocompatibility of AuNPs and surface free energy, enzymes retain their bioactivity and enzyme loading increases [28, 29].

The general procedure used in the modification of electrochemical biosensors with AuNPs implies the attachment or deposition of the AuNPs onto the electrode surface. This modified electrode can then host the bio-recognition layer.

The bibliography lists numerous methods describing the synthesis of metallic NPs, including AuNPs, in solution as well as by deposition on solid surfaces. These methods include chemical synthesis by means of reduction with different reagents such as citrate [30, 31], UV light or electron beam irradiation [32] and electrochemical methods [33-38]. The latter methods provide an easy and rapid alternative for the preparation of AuNPs modified electrodes in a short period of time, enhancing the electrode conductivity and facilitating the electron transfer [35].

3.1.1.3. Amperometric biosensors

Amperometric techniques are based on the measurement of the changes in the current intensity generated by the electrochemical oxidation or reduction of the target analyte. These measurements are directly monitored with time while a constant potential is maintained at the working electrode with respect to a reference electrode. The recorded electrochemical signal results proportional to the concentration of the electroactive species in the sample [39].

Amperometric detection is commonly used with biosensors due to its simplicity and low limit of detection. The fixed potential during amperometric detection minimizes the background signal that adversely affects the limit of detection. In addition, hydrodynamic amperometric techniques can provide significantly enhanced mass transport to the electrode surface [40], for example when the working

electrode moves with respect to the solution by rotating or vibrating [41], or in flow conditions where the sample solution passes over the stationary electrodes [40].

During the evolution of the technology of amperometric biosensors three main transduction methods were developed. This can be classified into three types of biosensors: first, second and third generation biosensors.

First-generation amperometric biosensors

These biosensors are based on the electrochemical signal produced by a product or a cofactor of the enzymatic reaction (Figure 3.9).

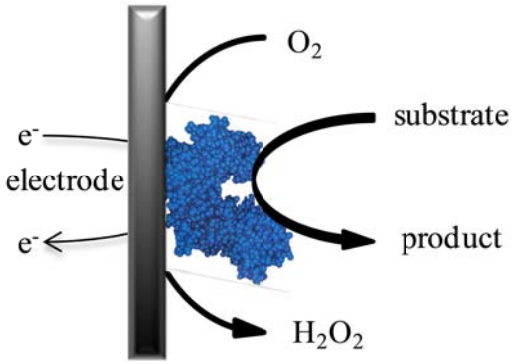


Figure 3.9. *Schematic example of a first-generation biosensor*

The glucose biosensors that monitor the product of the enzymatic reaction (H_2O_2) or the current decrease due to O_2 consumption are an interesting example of this type of biosensors [11].

Second-generation amperometric biosensors

Second-generation biosensors incorporate a redox mediator that produces the electron transfer between the active site of the enzyme and the electrode surface (Figure 3.10). The redox mediator may be incorporated along with the enzyme in the biocatalytic layer. In this way, the biosensor becomes independent of any additional reagent in solution [42].

The first second-generation biosensor was developed by Cass and Davis for the determination of glucose, using ferrocene as mediator [43]. Other mediators used in the development of biosensors are ferri/ferrocyanide couple, 1,4-benzoquinone, tetracyanoquinodimethane (TCNQ), TTF, Meldola blue and methylene blue [44].

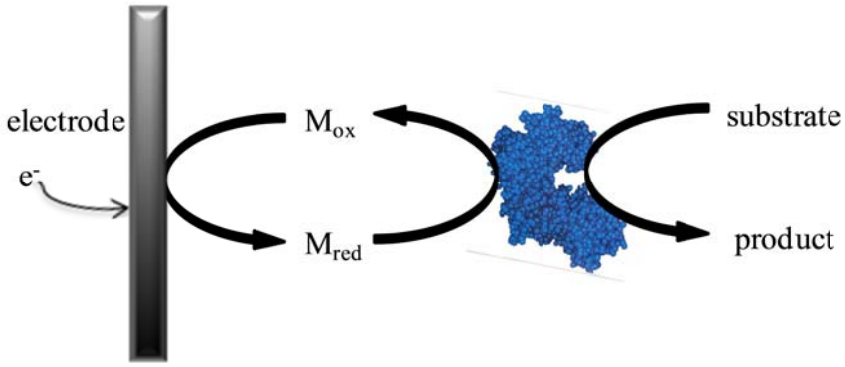


Figure 3.10. *Schematic second-generation biosensor*

Third-generation amperometric biosensors

Third-generation biosensors are based on the direct electron transfer from the active site of the enzyme to the electrode surface. Thus, these biosensors operate without any mediator, offering further options for the creation of advanced bioanalytical systems. Moreover, third-generation biosensors enable working in potential ranges close to the redox potentials of enzymes being less exposed to possible interference (Figure 3.11). Current research is focused on improving

sensor performance by rational use of new materials, particularly nanomaterials [6].

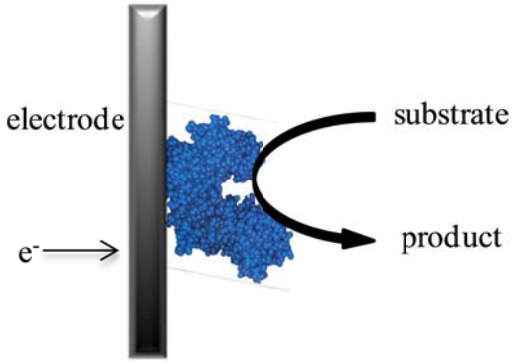


Figure 3.11. *Schematic third-generation biosensor*

3.1.2. SCREEN-PRINTED BIOSENSORS

Nowadays there is a growing trend towards the miniaturization of analytical devices and their components [45]. Miniaturized techniques offer attractive advantages over classic techniques, giving the possibility of making portable and automated analysis devices with lower costs and energy consumption as well as higher speed. These features make them able to respond to the more every day demanded small sample volumes and zero-cost and fast-response analysis [46, 47].

It is also noteworthy that the physical and chemical properties such as capillary forces, surface roughness or chemical interactions are different in small-scale devices. In the case of electrochemical systems, miniaturized devices have a higher surface area to volume ratio than traditional electrochemical systems. This feature may lead to an increased performance and a higher sensitivity [48].

The trend towards electroanalytical devices miniaturization has made that traditional bulky electrochemical cells have started to be replaced by other miniaturized, which can be considered as disposable such as SPEs and microelectrodes (Figure 3.12) [49].

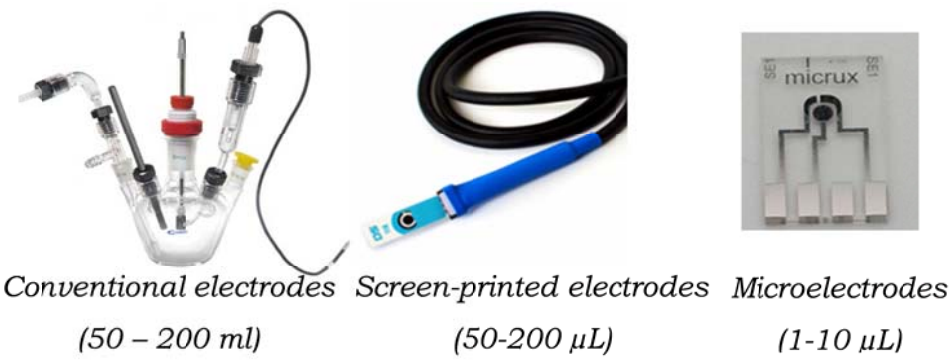


Figure 3.12. *Miniaturization of the electrochemical electrodes*

Screen-printed technology consists of layer-by-layer depositions of different inks upon a solid substrate, through the use of a screen or a mesh, defining the geometry of the sensor [9]. This technology has advantages of design flexibility, process automation, good reproducibility, or a wide choice of materials. The construction of SPEs for the development of disposable sensors has been well documented [50]. It includes a series of basic stages, namely election of the screen, selection and preparation of the inks, selection of the substrate and the printing, drying and curing stages. In this way, several SPEs configurations based on different materials can be built (Figure 3.13). Compared to other technologies that are available for manufacturing electrodes, such as thin-film, SPEs are relatively inexpensive, simple to fabricate and are congruent for mass production [50].

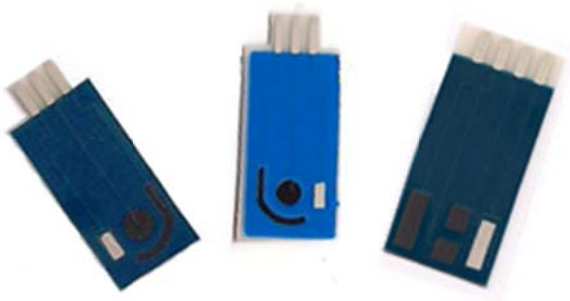


Figure 3.13. *SPEs used in this work for the different analytical applications*

The great versatility presented by the SPEs lies in the wide range of procedures which may be used for the modification of the electrodes. In this way, the composition of the printing inks may be altered by the addition of different substances such as metals, enzymes, polymers, complexing agents, etc. On the other hand, the possibility also exists of modifying the manufactured electrodes by means of depositing various substances on the surface of the electrodes such as metal films, polymers, enzymes, etc [10].

In addition to these very attractive advantages, the technology enables biomolecules to be immobilized onto the electrode surface, in order to fabricate selective and disposable biosensors. In this way, enzymes, microorganisms, antibodies, nucleic acids and receptors have been commonly employed in the construction of screen-printed biosensors. They can be immobilized onto the surface of the working electrode, which can be of different nature such as carbon or gold, through adsorption, entrapment, microencapsulation, crosslinking or covalent attachment [10, 50].

The screen-printing technique is now well-established as a reliable method for the fabrication of electrochemical sensors and biosensors. In this way, many research laboratories have developed prototype devices and applied them to a wide diversity of applications

areas. The potential of this manufacturing method for the production of commercial devices is exemplified by the personal glucose biosensor widely used by diabetics. The large market for this product has provoked numerous efforts in other wide number of diverse areas including pharmaceutical, biomedical, environmental, and industrial analyses, towards the use of this type of biosensors [51].

In this work, screen-printed biosensors have been developed for the determination of analytes of interest in food industry. Thus, disposable biosensors have been developed for the determination of different BAs in different food samples.

3.2. BIOGENIC AMINES

BAs are non-protein nitrogenous compounds of low molecular weight that are naturally present in living organisms and foods. Generally, BAs are classified into two distinctive categories: endogenous and exogenous.

Endogenous BAs are produced in many different tissues during normal metabolic processes in living cells from the degradation of biological molecules like proteins and/or, decarboxylation of certain amino acids by microbial enzymes (Figure 3.14) [52].

Endogenous amines formed from cellular metabolism play several important roles within the cardiovascular, nervous, and digestive systems. For example, His is formed in mast cells and liver and is subsequently transmitted locally or via the blood stream to perform various functions as in the modulation of growth and

proliferation of eukaryotic cells by Spm (Spermine) and Spd (spermidine) [53].

As it has been described above, endogenous formation of BAs may also result from decarboxylation of free amino acids by enzymes of bacterial origin. Amino acid decarboxylation takes place by removal of the α-carboxyl group to give the corresponding amine. In this way, Figure 3.14 shows how arginine is easily converted to agmatine (Agm) or it can be degraded to ornithine from which Put is formed by decarboxylation, as a result of bacterial activity. In Figure 3.14 it can also be seen that lysine can be converted by bacterial action into Cad and histidine may, under certain conditions, be decarboxylated to His. Likewise, Tyr, tryptamine (Tryp), and phenyl ethylamine (Pea) derive from tyrosine, tryptophan, and phenylalanine, respectively [54].

In the formation of BAs there is a great influence of important variables such as pH, temperature and oxygen presence. In this way, the pH level is an important factor influencing amino acids decarboxylase activity being BAs formation by bacteria generally optimum in low pH medium [55]. In the case of temperature influence, it can be said that the production rate generally growths with the increase of this parameter [56]. In the case of temperature influence, it can be said that the production rate generally growths with the increase of this parameter [56]. For example, *Enterobacter cloacae* can produce Put at 20°C but not at 10°C and Cad formation by *Klebsiella pneumonia* was also detected more extensively at 20°C than at 10°C [56]. The rate of His formation was very dependent upon microbial activity being slowed at 10°C and almost terminated at 5°C due to the slow growth of His forming bacteria. The optimum formation of this BA was at 37.8°C [54].[56].

Finally, oxygen appears to have a marked effect on the formation of BAs, which is dependent on the producing species. Thus, for example, *Enterobacter cloacae* produces about twice Put quantity in aerobic compared to anaerobic condition, while *Klebsiella pneumonia* produces considerably less Cad but acquires the ability to produce Put under anaerobic condition [56].

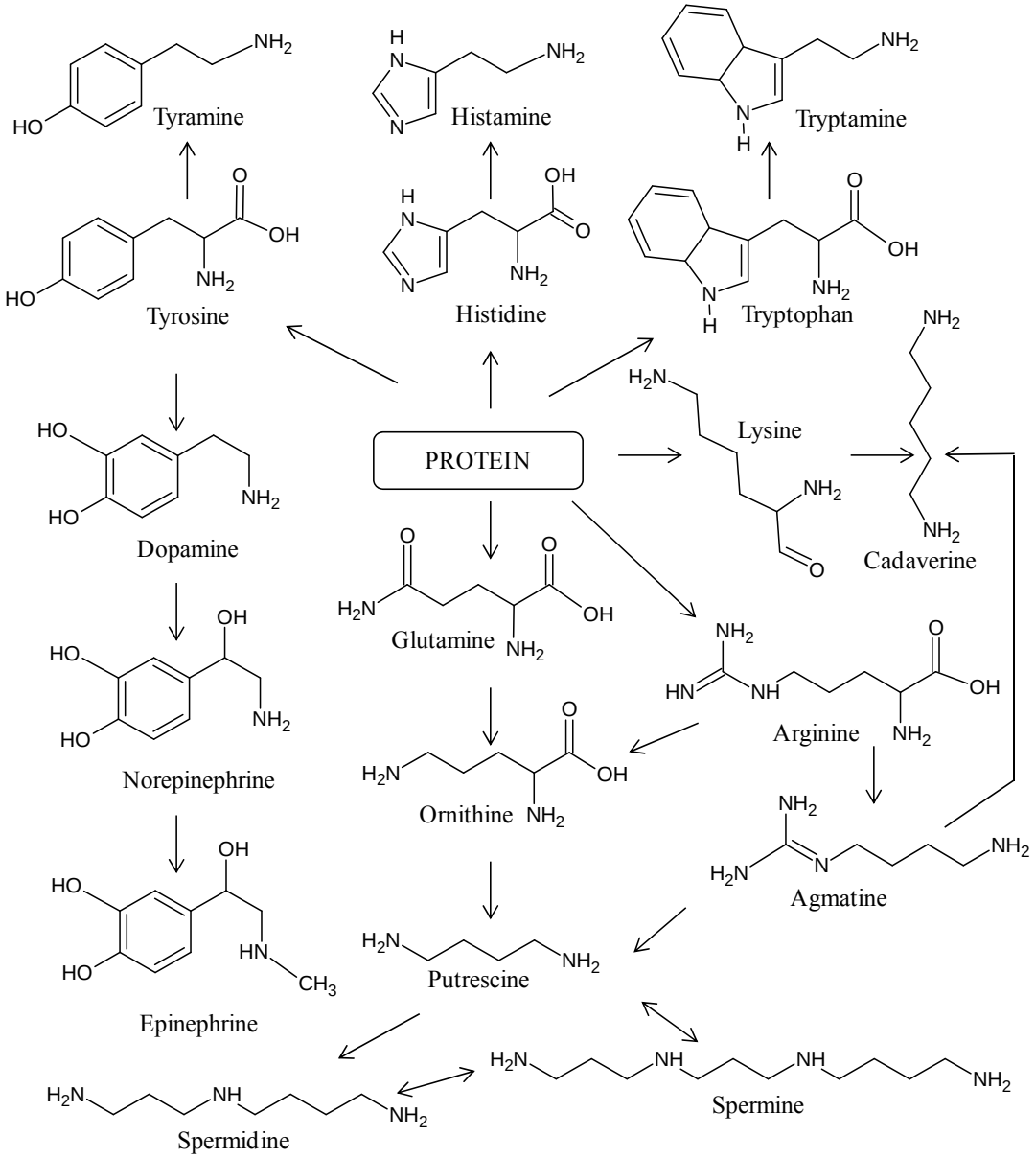


Figure 3.14. *Schematic formation of BAs by degradation of proteins and / or decarboxylation of certain amino acids (adapted from Halász, A. [56])*

BAs may also be formed exogenously as intermediates in the chemical and pharmaceutical industries, or released into the atmosphere from livestock breeding operations, animal feeds, waste treatment/incineration, and in automobile exhaust fumes. The exogenous BAs are directly absorbed in the intestine, a process that is enhanced by intake of alcohol [57].

3.2.1. IMPORTANCE OF BAS

BAs have an important metabolic function in cells, being a nitrogen source and serving as precursors for the synthesis of alkaloids, nucleic acids and hormones, Moreover, BAs may influence some processes in the human body such as regulation of body temperature or increase or decrease in blood pressure [58]. BAs also play important roles as neurotransmitters and are required for essential biological functions such as protein synthesis [59]. However, if these compounds are consumed in large quantities may arise various undesirable health problems since they are related to food poisoning [60]. In this way, the presence of BAs in food products constitutes in some cases a potential public health problem because of their potential toxicological effects (Table 3.2) [61-63]. Food products with a high content of BAs have been associated in some cases with toxicological problems on consumers being Tyr and His are the BAs having the highest toxic effect Other commonly known polyamines (Put, Cad, Agm, Spm, Spd) are highly resistant to degradation during processing and are capable of withstanding procedures like canning, freezing, smoking, and cooking.[53].

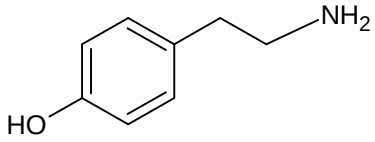
Table 3.3. *Potential physiological and toxicological effects of BAs.*

BAs	Physiological effects	Toxicological effects
Tyr	Neurotransmitter; favored peripheral vasoconstriction; increases blood pressure and respiration; increases blood sugar level (blood glucose); it is involved in the formation of nitrosamines, cause lacrimation and salivation.	Causes Headache, migraine; neurological disorder, nausea, vomiting, disorder respiratory, hypertension and hypertensive crisis (reaction cheese), limb paralysis, tachycardia, causes cerebral and cardiac hemorrhage, etc.
His	Neurotransmitter; psychoactive; controls gastric acid secretion; Excites the smooth muscles of the uterus, the intestine, and the respiratory tract; involved in growth and cell differentiation, circadian rhythm, regulating body temperature, learning and memory, immune response, allergic reactions; liberates adrenaline involved in inflammatory processes, in tissue healing, in the regulation of local circulation, has a vasodilatory effect of blood, capillary and arterial vessels; lowers blood pressure.	Headache, migraine, nasal obstruction, hypotension, arrhythmia, tachycardia, palpitations, flushing, sweating, hives, edema, anaphylaxis, bronchoconstriction, rash, bronchospasm, flushing, itching, gastrointestinal pain, diarrhea, dizziness, vomiting , nausea, difficulty breathing, blood pressure disorders and neurotransmission, etc.
Try and Pea	Promotes peripheral, increases the blood pressure, vasoconstriction.	Hypertension, migraines.
Put and Cad	Neurotransmitter; psychoactive; regulates gene expression; involved in intestinal and maturation absorption, growth and cell differentiation and the formation of nitrosamines; lowers blood pressure and the catabolism of His and Tyr.	Tachycardia, hypertension, carcinogenic effects , jaw stiffness, bradycardia, potentiates the effect of other BAs, causes neurotransmission disorders, etc.

Determination of the exact toxicity threshold of BAs in individuals is extremely difficult, since the toxic dose is strongly dependent on the efficiency of the detoxification mechanisms of each individual [56, 64]. Normally, during the food intake process, low amounts of BAs are metabolised in the human gut to physiologically less active degradation products. This detoxification system includes specific enzymes such as AOs, which are enzymes found in bacteria, fungi, plant and animal cells able to catalyse the oxidative deamination of amines with production of aldehydes, hydrogen peroxide and ammonia [57]. However, upon intake of high loads of BAs in foods, the detoxification system is unable to eliminate these BAs sufficiently. Moreover, in the case of insufficient enzyme activity, caused for example by genetic predisposition, gastrointestinal diseases or inhibition of enzyme activity due to secondary effects of medicines or alcohol, even low amounts of BAs cannot be metabolised efficiently [65].

A deeper description of the characteristics of the main BAs present in food products has been included in the following sections.

3.2.1.1 Tyramine



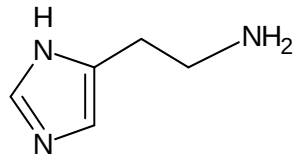
Tyr is an aromatic monoamine, derived from the decarboxylation of tyrosine by the action of the enzyme tyrosine decarboxylase. Tyr acts as a neurotransmitter as it has been demonstrated by the presence of this BA in the human brain, which has been confirmed by postmortem analysis [66]. Additionally, the possibility that Tyr acts directly as a neurotransmitter has been revealed by the discovery of a G protein-coupled receptor with high

affinity for Tyr. Thus Tyr may be involved in modulating a variety of behaviors including mood, cognition, and addiction [67].

Ingested Tyr does not normally cause any direct hazard to human health due to its metabolization to p-hydroxylphenylacetic acid under MAO enzyme catalytic action. However, patients under treatment with MAO inhibitors, such as antidepressants or antitubercular drugs, can suffer a serious hypertensive reaction if they take large amounts of this amine [68].

Poisoning caused by taking high concentrations of Tyr presents symptoms such as hypertension, tachycardia, increased respiration and blood glucose, release of norepinephrine, brain and heart bleeding, among others. These health effects are known as the "cheese reaction" due to originally these symptoms were associated with the consumption of cheese with high concentrations of Tyr [53, 62, 65, 68-72]. Moreover, its production and accumulation in human body have been linked to elevated blood pressure and headaches.

3.2.1.2 Histamine



His is derived from the decarboxylation of the amino acid histidine by a reaction catalyzed by the enzyme L-histidine decarboxylase. His is also a neurotransmitter, produced by basophiles and mast cells, involved in immune response and in the regulation of physiological functions in the gut. His enhances the permeability of capillaries to white blood cells and protein antibodies, which are needed to destroy foreign substances and nullify their harmful effects. It also acts in pro-inflammatory response to tissue

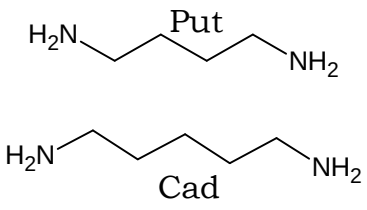
damage or allergic reactions and also enhances the secretion of gastric HCl through His receptors [53]

However, His can be considered toxic at high concentration levels being one of the most common toxic BAs present in food. Its toxic effect is known as histamine poisoning and "scombroid poisoning", as it is related to the consumption of fish of this family, such as tuna, mackerel and sardines containing high concentrations of the amino acid histidine [59, 72]. This toxic effect is due to the accumulation of His in the intestinal tract from food, which causes an increase of His in plasma leading to histaminosis.

The symptoms of His poisoning are of four types (Table 3.2) [73-76]:

- ✓ *At the level of the circulatory system*: headache, hypotension, arrhythmia and anaphylaxis.
- ✓ *Skin:* itching, hives and redness.
- ✓ *The respiratory system*: obstruction nasal and bronchoconstriction.
- ✓ *The gastrointestinal tract*: abdominal pain, diarrhea, nausea and vomiting.

3.2.1.3 Putrescine and Cadaverine



Put and Cad are organic bases with aliphatic structure, which are synthesized and degraded during normal metabolism of living and dead microorganisms, animals, and plants. In humans, Put acts as a precursor for physiological polyamines (i.e. Spd and Spm) being an important constituent of mammalian cells. Put is also essentially

involved in a variety of regulatory steps during normal, adaptive, and even malignant cell proliferation [56]. Because of high proliferation rates, intestinal and colonic mucosa has a special demand for Put [77], being the growth of murine colon tumour cells stimulated by newly incorporated Put [78].

The role of Cad is less known but it could also potentiate the effect toxic of the above mentioned diamines and polyamines. Moreover, it has been reported that in some systems, including bacteria, Cad can replace Put [79].

The pharmacological activities of Put and Cad seem to be less potent than those defined for His and Tyr. Adverse effects described are hypotension, bradycardia, lockjaw and paresis of the extremities (Table 3.2) [54, 56, 80]. Perhaps, the most relevant effect of Put and Cad in relation to food is the potentiation of the toxicity of other amines, especially His and Tyr [65, 76, 81-83].

Finally, these diamines are involved in the formation of nitrosamines, potentially carcinogenic compounds [76, 84]. This phenomenon is especially important in those meat products, which contain nitrates and nitrites and are heat-treated. This process promotes the interaction between BAs and nitrites to form nitrosamines [85]. N-Nitrosamines (NAs), including N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR) and N-nitrosopiperidine (NPIP) are potent carcinogens that can induce tumours in various animal species.

Secondary amine, dimethylamine (DMA), is the most abundant nitrosatable precursors in nitrite-cured fish meat [86]. However, BAs subjected to heat may also form nitrosatable amines. In this way, Put and Cad, which are commonly found in decomposed fish and shellfish, may generate N-nitroso compounds, NPYR and NPIP, respectively, as it is shown in Figure 3.15 [87].

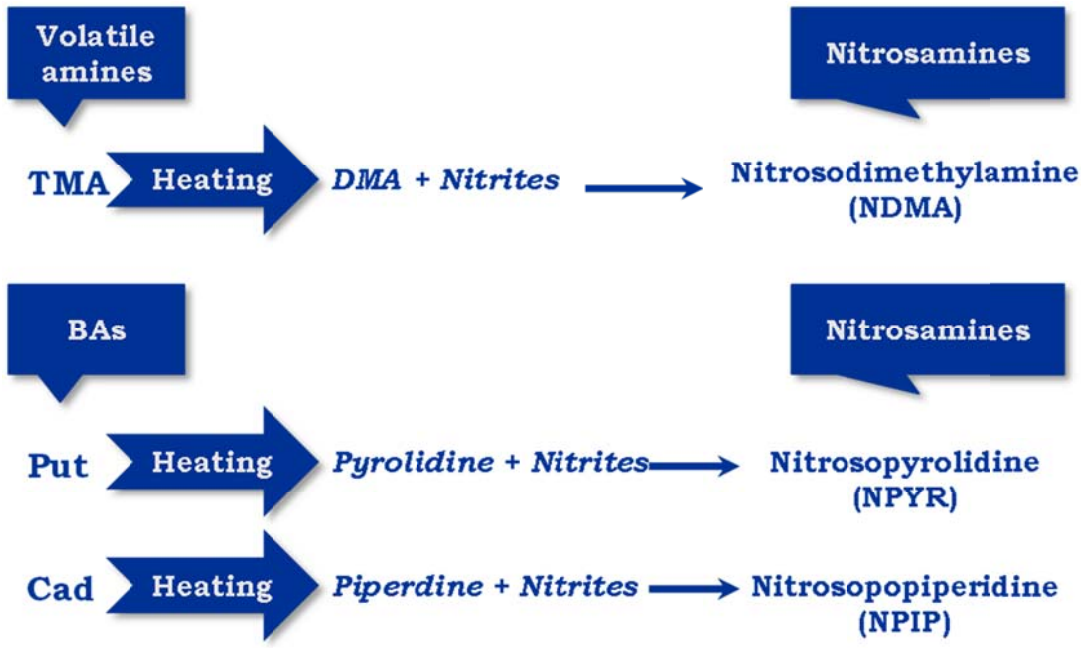
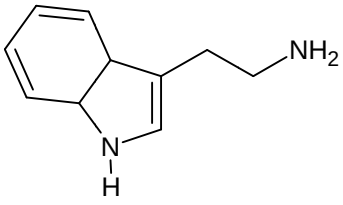


Figure 3.15. *Nitrosation of amines. TMA: trimethylamine, DMA: dimethylamine*

3.2.1.4 Tryptamine



Tryp is an aromatic monoamine, structurally similar to its precursor amino acid tryptophan, which contains in its structure an indole ring. Tryps are a group of alkaloid compounds found in plants, animals, and fungi, known for their psychotropic effects such as psilocybin (from "magic mushrooms") and dimethyltryptamine. Tryp has toxic effects on human beings such as blood pressure increase, therefore it causes hypertension, however there is no regulation on the maximum amount of Tryp consumption [54, 65].

3.2.2. OCCURRENCE OF BAS IN FOOD PRODUCTS

BAs formation in food products is either a result of endogenous amino acids decarboxylase activity in raw food material or the growth of decarboxylase positive microorganisms under favorable conditions for enzyme activity. Therefore, prerequisites for BAs formation in foods are the availability of free amino acids, the presence of decarboxylase active microorganisms and of an environmental condition enabling microorganisms growth [55]. In this way, BAs can be found at low concentrations in non-fermented food such as fruits, vegetables, meat, fish, chocolate, or milk and, at high concentrations, in fermented foods as cheese, beer, wine and traditional sauces, as a result of a contaminating microflora exhibiting amino acid decarboxylase activity [53-55, 57, 64, 65].

Fermentation is a biological method of processing foodstuff to preserve its quality or to transform foods into stable and useful forms. The fermentation process produces finished products with characteristic flavors and textural properties. The process is potentiated mostly by enzymes and microbial activity and metabolism. In this way, several different microorganisms including *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Pediococcus* species have been shown to participate in various food fermentations. These microorganisms may be naturally present in the food material or added to foods as starter culture. They excrete their enzymes (including various decarboxylases) in foods for their transformation, which results in an accumulation of large amounts of BAs in fermented foods through the decarboxylation of amino acids by microbial decarboxylases [53].

3.2.2.1. Fresh meat

Fresh meat such as beef, pork, and poultry are well-known food products which have a high content of BAs. Their high proteinaceous nature makes them disposed to proteolysis in order to form free amino acids that may subsequently be decarboxylated by microbial enzymes into different BAs. Thus, Spm and Spd are believed to be present in most of fresh meats at fairly constant levels [53]. Other predominant BAs in fresh meat are Put and Cad, together with relatively lower levels of His, Tryp, and Tyr [28]. Thus, for beef, Tyr or a combination of the three most prevalent BAs, Tyr, Put, and Cad, have been recommended for use as index of quality or spoilage [29]. Thus although the concentration of His is usually quantitatively lower than that found, for example in fish [69], the potential for forming carcinogenic nitrosamines in some meat products, with high BAs levels and added nitrates and nitrites as preservatives, is particularly important [69].

Fresh poultry meat may have significant amounts of BAs including, Spd, Put, Cad, His, and Tyr. Moreover, the accumulation of these compounds tends to increase during storage. For example, in poultry, Put and Cad can be considered as the predominant BAs achieving total levels of 50–100 mg kg^{-1} when stored over extended periods. BAs also accumulate more rapidly in raw poultry meat because of the higher susceptibility of white muscle fibers to proteolysis versus the darker muscle fibers of red meats, beef, or pork [88].

Zee *et al.* [89] quantified the levels of BAs found in samples of fresh and processed pork meat. Both products contained high levels of adrenaline, Spd and Spm (up to 581, 280 and 685 mg kg^{-1}, respectively), but low levels of noradrenaline, Put, His, Cad and Tyr.

The investigation of the BAs content in pork meat during storage at different temperatures showed that Put and Cad contents increased during storage, while Spd and Spm contents decreased. This phenomenon could be due to the differences in the reaction rates of amine synthesis and amine deamination. Put, Cad, Spd and Spm levels changed less during storage at -20°C than at 5°C [90].

In the case of beef samples, an increase of Put content has been observed after storage, while no changes in Spd and Spm contents during storage were observed even after long periods of 39 days in vacuum-packed [91, 92].

In fresh meats, parameters such as temperature, salt content, and pH play an important role in BAs production. However, the effect of these parameters varies from species to species. For example, low pH values enhance the production of His, Tyr, and Tryp in poultry meats by lactic acid, while *Enterobacteriaceae* produces less Cad at low pH medium. A High salt concentration level has a great inhibitory effect on the formation of BAs, due to inhibition of microbial growth. In the case of the temperature value, it has been found that high values of this parameter lead to a great formation of different BAs in beef, pork, and poultry samples, being this formation process inhibited at frozen temperatures. Thus, a high salt content or a low storage temperature (or both) is recommended for limiting BAs formation and extending the shelf life of meat products [93].

3.2.2.2 Fermented Meat products

As it has been described above, raw meats tend to have lower amounts of BAs, such as Spm and Spd, than fermented products, and some industry-derived commercial products tend to have relatively higher amounts of BAs than their non-industry produced traditional products.

Examples of fermented meats include ham, salami, pepperoni, chorizo, bologna, and sausages. His, Tyr, Tryp, Cad, Put, Spd, and Spm are common BAs frequently present in these products. The levels and distribution of these molecules in fermented meat products vary depending on factors such as quality of the raw material and the availability of precursor molecules to serve as substrates [53, 59].

The presence of BAs in fermented food products can be associated to microbial activity. The microorganisms responsible for BAs production in fermented meats include *Enterobacteriaceae*, *Enterococci*, *Lactobacilli*, *Pediococcus*, and *Pseudomonads*. The lactic acid bacteria strain, *Lactobacilli* and *Enterococci*, are more associated with Tyr production, while *Enterobacteriaceae* is more connected with high Put and Cad levels in fermented meat products [57].

Although it is possible to keep the microbial load associated with fermented meat products at a low level, the enzymes (proteases and decarboxylases) released by these microorganisms into the products may survive the fermentation process and cause continued production of BAs in the final products. Storage of the raw materials intended for fermentation at low temperatures is a good practice to control both microbial proliferation and enzymatic activity that lead to BAs formation in the products. In general, high temperature, high pH, and low salt content can enhance BAs formation [53]. For instance, the amount of BAs content is higher in products prepared at 15^{o}C compared with similar products made at 4^{o}C, and more His is formed during ripening at 18^{o}C as compared with processing at 7^{o}C;

BAs formation is also less at lower pHs, because the decarboxylation of histidine tends to be relatively low at acidic pH. A high salt content also generally inhibits microflora growth because the reduced water activity makes the product less conducive for the growth and proliferation of microbes; consequently, low levels of BAs are formed [53].

3.2.2.3 Fresh fish products

Fresh fish products are particularly known to contain different BAs due to their high protein and free amino acid contents and their high perishability. The levels of BAs in fish tissues vary greatly and are influenced by various factors including muscle type, the native microflora, harvesting, and postharvest management practices including handling, processing, transportation and storage. The relative amounts of BAs and their distribution in fish tissues depend to a large extent on the types of microorganisms associated with the raw material and their capacity to produce decarboxylases and hydrolytic enzymes like proteases and lipases. Generally, when the microbial load is high, the BAs levels also tend to be high.

His usually tends to be the most abundant BA in fresh fish products, followed by Put and Cad in that order. As it has been already descried, in the case of His, this BA is produced in species of the *Scombroidae* family such as tuna and mackerel, mahimahi, blue fish, and sardine. High His levels in these species have been explained by naturally high free histidine contents. Thus, His has been associated with scombroid poisoning in many studies [76].

3.2.2.4 Fermented fish products

Fish may also be fermented into products such as fish paste (e.g., bagoong, patis, and shrimp paste), and fish sauce (e.g., joetgal, shottsuru, garum, nuoc mam, and nam pla). These products also may contain different kinds of BAs. In this way, His, Put, Cad, Tyr, Spd and Spm have all been detected in fermented fish products being their levels increased with storage [27]. However, the amounts of BAs found in these products (0–70 mg kg^{-1} sample) are considered as safe for human consumption, if they are handled and stored properly [27].

BAs content of fermented fish products are predominated by His, Put, Cad and Tyr [27, 94]. His, Put and Cad are in fact the most abundant BAs in fermented products, such as fish sauce with maximum reported values of 1220, 1257 and 1429 ppm, respectively. Tyr is present in a minor amount with a maximum reported value of 1178 ppm [55]. Other BAs, including Tryp, Pea, Spm, Spd and Agm are also present in this kind of food products but at lower concentration level [27, 55, 94].

In the case of fish sauces the type of BA can be related to the presence of different types of microorganisms due to their different decarboxylase activity. Table 3.3 shows different examples of microorganisms that have been attributed to the formation of BAs in fish sauce and its related products. Since His is the most abundant BA in fish sauce and mostly related to the poisoning incidence, its microbial producers are of particular interest being *Enterobacteriaceae*, *Micrococcaceae* and *Lactobacilli* the most active microorganisms in the formation of His in fish sauce [55].

Table 3.4. *Bacteria responsible for BAs formation in fish sauces.*

BAs	Microorganism	
His	*Tetragenococcus muriaticus*	[95]
	Tetragenococcus halophilus	[96]
	Enterobacter cloacae, Pantoea sp., Pantoea agglomerans	[97]
	Bacillus coagulans, Bacillus megaterium	[98]
	Morganella morganii, Klebsiella pneumoniae, Hafnia alvei Morganella psychrotolerans	[56]
	Lactobacillus sp., Lactobacillus sakei,	[99]
	Lactobacillus mesenteroides	[96]
	Staphylococcus epidermidis, Staphylococcus capitis	[100]
Put	*Enterobacter cloacae, Pantoea agglomerans*	[97] [98]
	Bacillus megaterium	
Cad	*Enterobacter cloacae, Pantoea agglomerans*	[97] [98]
	Bacillus megaterium	
Tyr	*Paenibacillus tyramiegenes*	[101]
	Lactobacillus brevis	[102]
Spm and Spd	*Pantoaea sp., Pantoea agglomerans*	[98]

3.2.2.5 Fermented Vegetal products

BAs content in fresh vegetables is very low; however, the concentration increases during the fermentation process and throughout storage. Fermented vegetable products include pickles,

ripe, olives, sauerkraut, miso, tempeh, soy sauce, cider, sake, and vinegar; being produced from sources such as beans, grains, cucumbers, lettuce, olives, cabbage, turnips, fruits, and rice. [53].

Compounds like Tyr, Put, and Cad are commonly found in fermented vegetable products. And, in this case, there does not appear to be a significant correlation between BAs levels and the spoilage [103, 104].

The amounts of BAs present in fermented vegetable products vary and depend on factors such as temperature, salt content, and pH, as well as starter culture type. These factors all affect the growth and metabolism of the microorganism, which influence the formation of the BAs. Low temperatures generally minimize microbial growth and proliferation, and enzyme activities to reduce BAs formation, while elevated temperatures tend to do the reverse [53].

3.2.2.6 Cheese

Cheese is a commonly consumed fermented food product that usually contents BAs formed during fermentation and ripening processes. During these processes, milk casein is hydrolyzed by proteases and peptidases into a mixture of smaller peptides and free amino acids. Some of the free amino acids produced become substrates for the decarboxylases of microorganisms associated with cheeses to form BAs (mainly, Put, Cad, His, and Tyr) in the final products. As well, free amino acids from proteolysis may form heterocyclic BAs with creatinine. Some microorganisms have been implicated in the formation of His, Tyr, Put, and Cad in cheeses. For example, *Lactobacillus buchneri* is known to produce vast amounts of His in cheeses, which can potentially provoke histamine poisoning in

consumers, while *L. brevis* and *Enterococcus faecalis* have also been implicated in the formation of Tyr in certain cheese products [54].

The BAs content in cheese depends on the type and time of fermenting process. Thus, processed cheeses with longer ripening times and higher degrees of proteolysis may have higher amounts of BAs. The use of high temperatures during cheese making and/or storage may also increase BAs levels in the products.

As it has been described above, His is one of the most common BA present in cheese, thus, after fish, cheese is the next most commonly implicated food product associated with histamine poisoning [59]. Reported outbreaks include cheeses made from raw as well as from pasteurized milk, such as Gouda, Swiss cheese, Cheddar, Gruyere and Cheshire [54, 105]. The His concentrations in cheeses that were implicated in outbreaks range between 850 and 1870 mg kg^{-1} [59].

Tyr is other BA frequently present in cheese samples. In this way, high concentrations of Tyr in cheese have been associated to a serious hypertensive reaction known as the "cheese reaction" [68].

3.2.2.7 Beer

Alcoholic beverages, including beer, produced from plant materials by microbial fermentation process, are common sources of BAs [62]. During the fermentation process, microorganisms such as *Saccharomyces cerevisiae* and *S. ellipsoideus* hydrolyze carbohydrates into simpler sugars that are further degraded into ethanol and a variety of flavor compounds. The beverages are then stored and matured for various time periods. During this time of storage, Tyr

and His levels can increase considerably in insufficiently pasteurized bottled beers [106].

His, Tyr, Put, and Cad have been found in certain beers and because alcohol enhances the absorption of BAs in humans, a legal upper limit for BAs content of 2 mg per alcoholic beverage has been established as guide for manufacturers of the products [107].

The formation and accumulation of BAs in beer is due to several factors, such as the quality of the raw material and microbial contaminations. In this way, lactic acid bacteria produce Put, Cad, His, and Tyr during fermentations; storage and aging may lead to further increases in the levels of these compounds in beers [53]. Thus, the formation and/or accumulation of BAs in beer may be controlled by adopting proper good sanitary and hygienic practices during food handling and processing, to control microbial contaminations and prevent temperature abuse during storage and transportation [53].

3.2.2.8 Wine

As it has been described above, BAs can be present in alcoholic beverages. In this way, BAs have also been detected in different kinds of wines being the amount content in red wines generally larger than white in wines, due to the difference of the vinification processes. [26].

The formation of BAs in wines is mainly considered an indicator of lack of hygiene during the winemaking process. Moreover the formation of BAs causes a taste in the wine that is disliked for the consumers. Thus, the reduction of BAs amounts is an important challenge for the wine producers.

There may be three possible origins of BAs in wines: (1) they are already present in the must; (2) they are formed by the yeast during alcoholic fermentation; and/or (3) formed in wine by the action of bacteria involved in malolactic fermentation [108-110]. The fermentation conditions such as temperature, pH changes, oxygen access or sodium chloride content of wine are important factors that affect the amount of BAs in the finished product [63]. The influence of other factors during the vinification process has also been cited, which includes must treatment, length of fermentation in the presence of pulp and skin, alcohol content, sulphur dioxide concentration, added nutrients, pH, temperature, and quantity and type of finings and clarification agents [63].

According to several research works, more than 20 BAs have been identified in wine such as: butylamine, Cad, 1,3-diaminopropane, dimethylamine, ethanolamine, ethylamine, hexylamine, His, Tyr, isopropylamine, isopentylamine, methylamine, 2-methylbutylamine, morpholine, pentylamine, Pea, piperidine, propylamine, pyrrolidine, Put, serotonine, Spd, Spm, and 2-pyrrolidone [63], being His, Tyr, and Put the main BAs present in wines [83, 111].

Put and Spd are usually abundant in grapes, whereas Agm, Cad, Spm, His, Tyr, and Pea have been found in small amounts in grapes [112, 113]. Although Tryp and Cad are also found in wine, they are in much lower concentrations than the others mentioned [114]. The amount of BAs in wine must be controlled. In this way, the recommended upper limit for His in wine is 2 mg L^{-1} in Germany, 5 to 6 mg L^{-1} in Belgium, 8 mg L^{-1} in France, and 10 mg L^{-1} in Switzerland [63].

3.3 ANALYTICAL METHODS FOR DETECTION OF BAS

As it has been already described, the development of different methods for the determination of BAs content in food products is an interesting field of research. In fact, there are many analytical systems described in bibliography based on different techniques for the determination of different BAs in different samples. [65, 115-117].

3.3.1 BIOLOGICAL METHODS

The original analytical methods for the analysis of BAs in food are based on *in vivo* detecting of biological activity (AOAC, 1995) [118]. For example, elevated levels of His in raw and canned sardines and mackerel using contraction of guinea pig ileum were reported by Geiger 1944 [119]. However, these methods have now been superseded by in vitro methods largely based on chromatography or fluorometry.

3.3.1.1. Immunoassays

Immunoassay kits for His are commercially available from a variety of manufacturers. These assays are predominantly validated for application to seafood, with minimal extraction. These qualitative or quantitative methods are usually relatively rapid and available at different levels of sensitivity (usually with a detection limit of around 0.5 mg kg^{-1}). However, there are no reported commercially available assays for determining of other BAs in foods.

Immunoassays have the advantage of providing semi-quantitative data and can simultaneously analyze multiple samples within 20 minutes to 6 hours. This format provides a suitable alternative for screening His (but not other BAs) [120].

3.3.1.2. Colorimetric method

A colorimetric assay for His has been described based on the formation of a red complex between His and copper ions (Patange *et al.* [121]). The assay provides a detection limit of around 5 mg kg^{-1} in mackerel and tuna samples. The method allows the quantification of His content either visually or by measuring the absorbance using a spectrophotometer at 496 nm. The assay is rapid (about 45 minutes).

3.3.2. FLUOROMETRIC METHODS

A fluorometric assay, as the AOAC Official Method [122], has been widely used for the determination of His in foods (usually fishery products). The method comprises an organic extraction from fish using methanol. His is then derivatised with o-phthaladehyde, to produce a fluorometric product which can be quantitatively determined on a spectrofluorometer. This method has a detection limit around 0.5 mg kg^{-1}, but it is not suitable for the determination of BAs other than His, moreover, there is very limited validation data on its use for matrices other than fish. Other reported method based on the fluorimetric determination of BAs is the described by Azab *et al.* [123]. In this work, a chameleon dye (Py-1) is placed to the bottom of the wells of a standard microplate that enables parallel and rapid

detection of BAs (96 samples in 2 min) with standard fluorescence equipment. Upon reaction with BAs, the blue and virtually non-fluorescent dye Py-1 is converted into a red conjugate which is strongly fluorescent (λ_{em} = 620 nm). The close similarity of the linear ranges of all amines suggests determination of the total amount of BAs in real life samples. The method is applied to the quantification of BAs in seafood by standard addition method of His, using a previous extraction step using methanol.

3.3.3 CHROMATOGRAPHIC METHODS

Several chromatographic methods have been reported for determination of BAs in foods, most of them based on a previous derivatization step before Ultraviolet (UV) or fluorescence (FL) detection, being FL based methods more sensitive than UV detection methods [124]. These methods include thin layer chromatography, gas chromatography, and HPLC, being probably HPLC the most common chromatographic technique used for BAs determination in food [63]. In this way, the reference method specified in the European Commission Regulation (EC) No 2073/2005 (microbiological criteria for foodstuffs) for determination of His in fresh and treated fishery products is based on the use of HPLC after dansyl-derivatisation and UV detection [125].

Derivatization methods can be mainly divided into two categories: pre-column (the derivatization is carried out before the chromatographic separation) and post-column (the derivatization is carried out after the chromatographic separation) derivatization methods, being pre-column derivatization most frequently used because of providing more sensitive detection. Various reagents for derivatization have been used in the determination of BAs by means

of HPLC (either UV or FL detection) including dansyl and dansyl chloride (DCl), benzoly chloride, fluoresceine, 9-fluorenylmethyl chlorormate, o-phthaladehyde (OPA), naphthalene-2,3-dicarboxaldehyde, N-acetylcyteine, 2-mercaptoethanol. DCl is probably the most widely used reagent for pre-column derivatisation, while OPA is mostly used for post-column derivatisation [124].

Chromatographic methods coupled with mass spectrometry (MS) detection are very popular among many researchers, because of its higher sensitivity than conventional methods. It provides the ability to identify the structure of the analytes and also has high resolution. However reported applications of these methods for food measurements are limited due to their high cost and specialist operation requirements [124].

Thus, although HPLC methods show low detection limits (around 0.1 mg kg^{-1}), and may be used in the quantification of most of BAs, they are time-consuming, requiring specialized equipment and skills.

3.4. ELECTROCHEMICAL DETECTION FOR BAS

As it has been described above, the analytical determination of BAs is not simple due to their chemical structures variety and because they are usually present at low concentrations in complex matrices. Thus, the determination of these compounds requires the use of sensitive and selective analytical methods, although the spectroscopic and chromatographic instruments can offer good selectivity and low limit of detection, they often require cost and complex pretreatment steps and expensive instrumentations, so its

applicability to routine determinations is reduced [124, 126-128]. Thus, alternative approaches for BAs detection are necessary.

Electrochemical sensors provide a crucial analytical tool for the determination of BAs. In this way, several BAs including ethylenediamine, Put, Cad, Spm and Spd have been successfully analyzed using a BDD electrode [129].

Within electrochemical methods, electrochemical biosensors have attracted much attention in last years due to their simplicity, reproducibility, low cost, short time of analysis, and sensitivity. Unlike spectroscopic and chromatographic instruments, electrochemical biosensors can be easily adapted for detecting a wide range of analytes, while remaining inexpensive. Additionally, these sensors are capable of being incorporated into robust, portable, or miniaturized devices, for particular applications. Thus, the application of electrochemical technology in the development of novel sensors has been very useful for the fast and sensitive determination of BAs [128].

Several electrochemical biosensors have been described in the literature for the determination of different BAs. These methods are based on the immobilization of different AOs and peroxidases including MAO [130], diamine oxidase (DAO) [131-139], plasma amine oxidase (PAO) [134], PUO [130, 135, 140-149], tyramine oxidase (TAO)[150], AO from pea seedlings (PSAO) [128, 151-154], sweet potato peroxidase (SPP) [155], Histamine oxidase (HOD)[156] and AO from soybean seedlings [157], on different electrode surfaces.

As it has been previously described, among the different transducers, SPEs offer important advantages, which make them an interesting alternative in the construction of electrochemical biosensors for the determination of different analytes including BAs.

A description of different disposable systems used in the determination of BAs has been then included as an additional section.

3.5. DETERMINATION OF BAS USING SPES.

As it has been described above, the analysis of the different BAs has been frequently performed by means of chromatographic techniques. However, the use of enzymatic disposable electrochemical biosensors has been presented as an interesting, simple and low cost alternative by several authors. Most of these disposable biosensors are based on the use of AO enzymes being MAO [60, 158-160], DAO [133, 150, 161-166], PAO [68, 150, 167], and, less frequently PUO [168] and spermine oxidase (SMO) [167], the most used enzymes in the development of this kind of biosensors.

These AO based amperometric biosensors work according with the reaction mechanism described in figure 3.16. As it can be seen in this figure, the enzyme AO catalyzes the oxidation of the corresponding BA towards aldehyde, with the subsequent release of ammonia and hydrogen peroxide. At the adequate working potential the oxidation of the produced hydrogen peroxide takes place generating the analytical signal, which can be related to the concentration of the BA present in the sample [150].

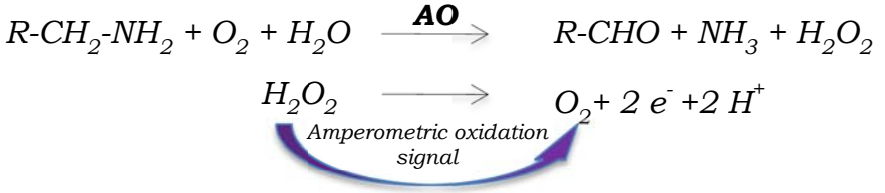


Figure 3.16. *Mechanism of an AO based electrochemical biosensor*

AOs based disposable biosensors have been used in the determination of different BAs in different samples. Thus, Chemnitius *et al.* [158] have performed the determination of Put, Cad and His in fish samples using platinum screen-printed electrodes (SPPtEs). These electrochemical biosensors were constructed by means of the joint immobilization of two different enzymes, PUO and MAO, by crosslinking. In the same way, Di Fusco *et al.* [164] have been developed an analytical method for the total BAs content determination in wine and beer samples. In this case, the enzyme DAO was immobilized on the surface of two different disposable electrodes, SPPtEs and gold screen-printed electrodes (SPAuEs). SPPtEs have been also used in the determination of His, Put and Tyr by using PAO, DAO and TAO respectively [150]. In this case, the three different developed biosensors showed a lack of selectivity in the analysis of the corresponding BA.

The described AO electrochemical biosensors present a common problem related to the high working potential (≥ + 600 mV) necessary for obtaining the analytical signal. This high potential considerably reduces the selectivity of this kind of sensors since other electroactive species present in the ample can be oxidized, including ascorbic and uric acids, among others [126, 128, 169-171].

The working potential of AO based biosensors can be reduced by the utilization of redox mediators. Figure 3.17 shows the operation scheme of this type of electrochemical biosensors. As it can be seen in this figure, the observed amperometric signal is often related to the electrochemical oxidation of the mediator on the electrode. This signal can be associated to the concentration of the corresponding BA present in the sample [60, 68, 159, 160, 166, 168, 172].

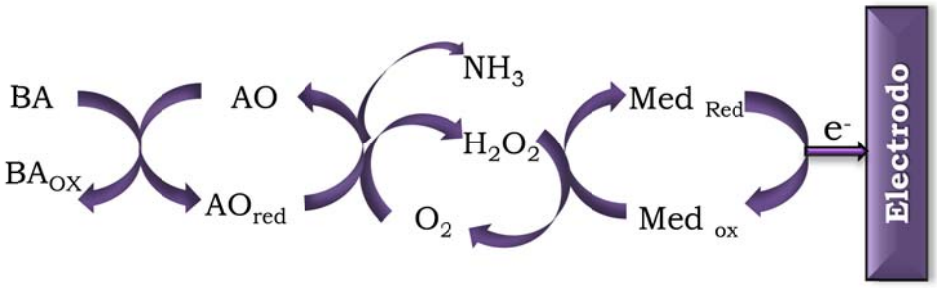


Figure 3.17. *Mechanism of an AO based electrochemical biosensor using a redox mediator*

Several mediators have been described in the literature for the development of AO based disposable biosensors including, hydroxymethylferrocene. Thus, in the work described by Calvo-Pérez *et al.* [68] a PAO based electrochemical biosensor has been developed using the enzyme immobilized on SPCEs using hydroxymethylferrocene as a redox mediator. This biosensor allowed the determination of Tyr in cheese samples using a working potential of + 260 mV. The same mediator has been used in the development of a bienzymatic using a mixture of DAO and horseradish peroxidase (HRP) for the determination of total BAs content in fish samples. The enzymes were covalently immobilized onto the carbon working electrode, previously modified by an aryl diazonium salt, using hydroxysuccinimide and carbodiimide [159]. Likely, Pérez *et al.* [165] have also developed a bienzymatic biosensor, but in this case the mixture DAO/HRP was immobilized on SPCEs using a polysulfone/carbon nanotubes which, allowed the analysis of His in fish samples at a low potential value of – 50 mV.

Potassium hexacyanoferrate (III) and imidazole acetaldehyde have also been used as redox mediators in the development of electrochemical biosensors for the determination of BAs. In this way Keow *et al.* [161] have described a disposable biosensor for the

determination of His in tiger prawn samples. In this case the DAO enzyme was immobilized on SPCEs by entrapment in a poly(2-hydroxyethyl methacrylate) (photoHEMA) film.

Leonardo *et al.* [166] describe the development of three electrochemical biosensor using Co(II)phthalocyanine, Prussian Blue, and Os-wired as mediators for the determination of His, Put, Cad in naturally-spoiled fish. In this work the enzyme DAO was mixed with magnetic beads which were trapped on SPCEs with a magnetic support at the back side. The three developed sensors have low working potentials (+400 mV, −100 mV and −50 mV vs Ag/AgCl, respectively).

The analysis of BAs has also been performed by means of electrochemical biosensors modified with MnO_2 as a redox mediator. Thus, in the work described by Telsning *et al.* [151] PSAO enzyme was immobilized on a SPCE using a Nafion solution. In this case, the measurements were performed by flow injection analysis using a working potential of + 400 mV allowing the analysis of different BAs in chicken meat samples.

Finally, Piermarini *et al.* [163] have developed a DAO based disposable biosensor using Prussian Blue as the redox mediator. The bio-component was immobilized by crosslinking and the measurements were performed at a working potential of – 50 mV. The biosensor was applied to the analysis of BAs in human saliva samples.

Most of the mediator modified biosensors described above led to good values of sensitivity, limit of detection and stability. Moreover, the incorporation of the mediator produced an important decrease of the working potential, which reduced the influence of other electroactive species present in the sample. However, most of these developed biosensors present a low specificity to a particular BA,

which led to these systems to estimate the total content of BAs in a sample.

Nowadays, the main efforts in the development of biosensors for BAs are focused on the improvement of their sensitivity, but also of their selectivity, residing this parameter in the specificity of the enzyme used. Thus, novel selective and specific enzymes have been started to be used for the analysis of different BAs. In this way, the SMO enzyme has been used in the development of disposable biosensors for specific determination of Spm [167]. The biosensor was prepared by entrapment of the enzyme in poly(vinyl alcohol) bearing styrylpyridinium groups (PVA-SbQ), a photocrosslinkable gel, onto an SPE surface. Prussiam blue was used as the redox mediator, which allowed working at a low potential of − 100 mV. Biosensor performance was evaluated by means of flow injection amperometric measurements and the proposed device was then applied to the determination of BAs in blood samples. The results obtained with this biosensor confirmed that SMO was able to selectively detect Spm. In fact, as an example, the response towards Spmd was ≤3 % with respect to the response towards Spm.

An specific enzyme has also been used by Rosini *et al.* [156] for the selective determination of His in fish samples. The biosensor is based on the immobilization of the enzyme HOD by crosslinking on a platinum microelectrode. A novel PUO and HRP based amperometric biosensor has been described by Bóka *et al.* [142]. The developed biosensor selectively measured Put, which can be considered as an indicator of microbial spoilage.

Recently, Veseli *et al.* [173] have developed an electrochemical sensor for the determination of His in fish sauce using SPCEs modified with rhenium(IV) oxide as mediator, which allowed working at a low potential of (-100 mV), However, this sensor does not show

an adequately selectivity for His since other amines such Ty, Tryp and serotonine cause some notable amperometric response up to about 20% of the histamine peak current.

Thus, the development of selective sensors towards a specific BA has not been properly performed being still necessary the construction of analytical systems which allow not only the sensitive determination of total BAs content, but also the simultaneous specific determination of each BAs present in a sample.

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4. A Screen-Printed Disposable Biosensor for Selective Determination of Putrescine

La Put es una de las BAs cuya presencia es frecuente en productos alimentarios. Debido a sus importantes efectos tóxicos para el ser humano, su determinación resulta interesante desde el punto de vista analítico.

En este capítulo se describe el desarrollo de un nuevo método para la determinación electroquímica de Put empleando un biosensor selectivo utilizando SPCEs modificados con el enzima MAO. En la fabricación del biosensor se incorpora un mediador que permite de manera simple trabajar con potenciales de medida más bajos, reduciendo los posibles interferentes que se pudieran presentar en el análisis, logrando así un importante avance en comparación de los trabajos publicados en la bibliografía.

El biosensor desarrollado resulta muy reproducible con una buena capacidad de detección y selectividad hacia la Put. Además, puede ser aplicado con éxito en la determinación de este compuesto en pescados y en muestras vegetales.

El resultado experimental de este trabajo ha sido publicado en: *Microchimica Acta 180 (2013) 687-693*. Además, ha sido presentado en la *XXXIII Reunión del Grupo de Electroquímica de la Real Sociedad Española de Química*, celebrada en Miraflores de la Sierra (Madrid) del 1 al 4 de Julio de 2012.

4.1. INTRODUCTION

BAs are natural contaminants, synthesized and degraded during normal metabolism of animals, plants and microorganisms. Among them, His, Put, Cad, Tyr, Tryp, Spm and Spd are found to be the most common BAs present in food [1].

Formation of BAs can occur during food processing and storage as a result of bacterial activities. In fact, an increasing BAs content corresponds to a decrease in food product hygienic quality. Therefore, BAs have been defined as chemical indicators to estimate bacterial spoilage in fast-perishable foods, especially in fermented and rich in amino acids products, such as fish, meat, fruit juice, wine, beer, cocoa, milk and cheese [2]. BAs are neutralized under normal conditions by the action of specific enzymes in the gut. Like this, DAO metabolizes His, while MAO metabolizes Tyr. However, some foods in decomposition, rich in Cad and Put, may disrupt this mechanism [2]. For these reasons, monitoring of BAs amount present in food and beverages is becoming increasingly demanded by regulatory commissions as the Commission Regulation (EC 2073/2005) [2].

Analytical methods used for measurement of BAs are mainly based on different chromatographic procedures [3]. These techniques often require time-consuming pretreatments and long measurement times. Additionally, these instrumental systems are sophisticated and therefore very expensive [4]. Fluorescence methods have been also used for the analysis of different BAs including Put. These methods are often based on the synthesis of complex molecules that selectively react with BAs [5-7]. These last analytical methods fail on simplicity of fabrication procedure. Moreover, they lack of the applicability

characteristics of other analytical systems such as electrochemical biosensors. In this way, electrochemical biosensors offer a simple, rapid and cost-effective analysis alternative [8].

AOs are a class of enzymes that catalyse the oxidation of primary amines in aldehydes, in the presence of molecular oxygen as electron acceptor, with the formation of ammonia (NH_3) and hydrogen peroxide (H_2O_2). This enzymatic reaction can be then followed by amperometric detection of the electrochemical oxidation of H_2O_2 [2].

In fact, in recent years, the use of AOs in combination with various amperometric transducers has been widely described in the literature, either in a mono-enzyme [9-16] or bi-enzyme [1, 17-19]. The use of a combination of AOs and HRP allows working at a low operational potential and thus, reducing the probability of interference in the analysis of real samples.

Among the different transducers used, SPEs ones, offer additional advantages related to their disposable character and great versatility, which lies in the wide range of possible methods of modification of this kind of electrodes. The composition of the printing inks may be altered by the addition of different substances such as metals, enzymes, polymers, mediators, etc. [4].

In this way, SPEs have been used in the construction of amperometric biosensors based on AOs for the determination of BAs (Table 4.1). A common problem of the monoenzymatic biosensors is the lack of selectivity due to the high operational potential that is applied (higher than + 600 mV) [2, 10, 13].

Table 4.5. *Figures of merit of disposable biosensor for determination of Put*

Enzyme	Working electrode	E (mV)	pH	Capability of detection	Biosensor selectivity	Sample	Ref.
DAO	SPPtE, SPAuE	+600	7.4	0.2 mg L^{-1}	Total BAs	Wine, Beer	[2]
PUO MAO	SPPtE	+600	8.5	0.06 µM Put 0.1 µM Cad-Spm-Tyr 1.5 µM Agm	Combination Put-Cad-His	Fish	[10]
PAO TAO DAO	SPPtE	+700	7.5	100 mg kg^{-1} His 5 mg kg^{-1} Put	100% Tyr, <1% other BAs 100 % His, 90 % Tyr, 13% Spd, <5 % other BAs 100% Put, 50% < 8% other BAs	Salmon, Beer, Cheese , Wine.	[13]
MAO/HRP DAO/HRP	SPCE	+250	9.3	0.18 µM His 0.40 µM His	100% Put, 95% Cad, 80% His, 73% Tyr, Spm, Spd, 23% Tryp	Anchovy	[17]
PSAO	SPCE	+400	7.5	0.3 µM Put-Cad	100% Cad, 72 % Put, < 8% other	Chicken	[20]
PAO	SPCE	+260	6.7	2 µM Tyr	100% Cad, 87% Put, 84% Tyr, 74% His. < 55% other BAs	Cheese	[21]
DAO	SPCE	+250	7.4	0.65 ppm His	-	Prawn	[22]
DAO	SPCE	-50	7.4	0.1 µM Put	100% Put, 90% Cad, 72% Spd, 70% Spm, 92% His, 85% Pea	Human Saliva	[25]
DAO	SPCE	+350	7.4	70 µM His	100% His, 5.7% Put and Cad	Prawn	[23]
MAO	SPCE	+250	11	17,2 µM Put	Put	Zucchini anchovies	This wor

In order to solve this problem, mediators [20-25] as well as bienzymatic systems [17], have been used. However, the developed biosensors present a low specificity to a particular BA, which led to estimate the total content of BAs in a sample.

In this work, a simple biosensor has been built for the selective determination of Put using SPCEs in which the mediator, TTF, has been also screen-printed (TTF/SPCEs). MAO enzyme was then immobilized on the TTF/SPCE by crosslinking in presence of glutaraldehyde (GA) and Bovine serum albumin (BSA). These biosensors (MAO/TTF/SPCEs) have been successfully applied to the determination of Put in anchovies and zucchini samples.

4.2. EXPERIMENTAL

4.2.1. REAGENTS

Different inks were used in the fabrication of TTF/SPCEs, namely C10903P14 (carbon ink) and D2071120D1 (dielectric ink) (Gwent Electronic Materials, Torfaen, UK), Electrodag 418 (Ag ink) and Electrodag 6037 SS (Ag/AgCl ink) (Acheson Colloiden, Scheemda, The Netherlands).

All solutions were prepared using Milli-Q water (Millipore, Bedford, USA). All reagents used were of analytical grade. The supporting electrolyte used for the measurements was a 50 mM KH_2PO_4 buffer (Sigma-Aldrich, Steinheim, Germany) and 100 mM KCl (Merck, Darmstadt, Germany) solution. 1 M NaOH solution (J.T. Baker, Deventer, The Netherlands) was used to adjust the pH value.

TTF was provided by Sigma-Aldrich (Steinheim, Germany). MAO (EC 1.4.3.4; activity, 92 U mg^{-1}) was obtained from Sigma–Aldrich, (Steinheim, Germany), BSA (Sigma–Aldrich, Steinheim, Germany), which was prepared by dissolving the appropriate amount of this reagent in a 50 mM KH_2PO_4 buffer (pH 6) solution, and GA aqueous solution (Sigma–Aldrich, Steinheim, Germany) were used in the enzyme immobilization process.

For the study of amines, Put, Cad, Tyr, His (Sigma–Aldrich, Steinheim, Germany) and Spd, Spm and Tryp (Acros Organics, Geel, Belgium) solutions were used. $HClO_4$ (Panreac, Barcelona, Spain), ethyl acetate, NaCl and butanol from Fluka (Steinheim, Germany), ammonia solution, acetone, Na_2SO_4 and HCl from Merck (Darmstadt, Germany) were used for the Put extraction in real samples. Salted anchovies and zucchini samples were obtained from a local market.

4.2.2. APPARATUS

The TTF/SPCEs were produced on a DEK 248 printing machine (DEK, Weymouth, UK) using screen polyester mesh and polyurethane squeegees. Electrochemical measurements were made with a PalmSens electrochemical system (Palm Instrument BV, Houten, The Netherlands). pH of the buffer solutions were measured with a HI 221 pHmeter (HANNA Instruments, USA).

4.2.3. MANUFACTURING OF SPCES WITH TTF 5 %

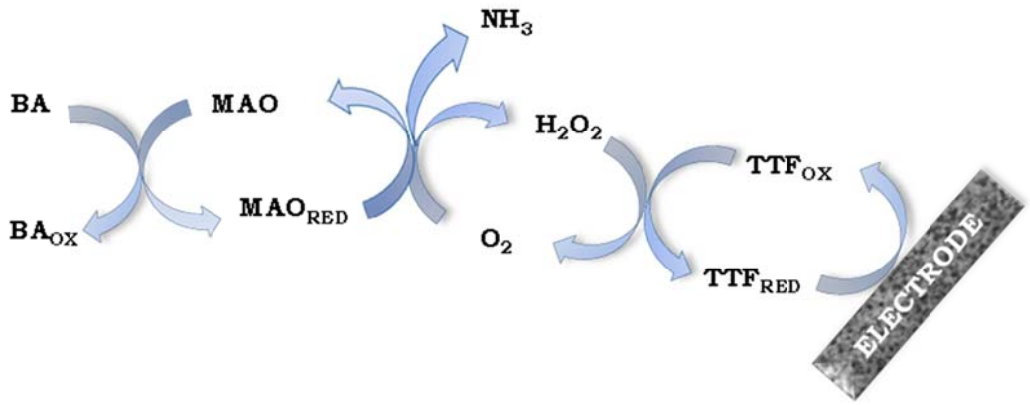
The electrodes were prepared in five steps by a known method [24, 26]. In this case TTF was incorporated directly into the carbon ink for printing the working electrode.

4.2.4. BIOSENSORS PREPARATION

MAO enzyme immobilization on the working electrode surface was performed using a crosslinking process with GA and BSA. This process was carried out by dropping on the surface of the working electrode 5 µL of a mixture containing BSA (6 % w/v):GA (2.5 % v/v):MAO (0.56 units μL^{-1}) in the volume ratio 4:5:1. Once deposited, this mixture was allowed to dry for 90 min at 4 °C.

4.2.5. ELECTROCHEMICAL MEASUREMENTS

All measurements were made at room temperature in a cell containing 5 mL of a supporting electrolyte solution of the desired pH, under constant stirring. The amperometric detection (Scheme 4.1) has been performed by measuring the amperometric current due to the oxidation of the mediator TTF at a potential of + 250 mV vs. screen-printed Ag/AgCl reference electrode (Ag/AgCl SPE). The corresponding sample was added after reaching a stable baseline. The characterization of the biosensors, except in the section of substrate specificity determinations, was done using Put.



Scheme 4.1. *Enzymatic mechanism of MAO/TTF/SPCEs biosensors*

4.2.6. SAMPLES PREPARATION

Zucchini samples were extracted with a simple preparation method, involving direct sample extraction with 0.1 M HCl and neutralization with NaOH according to the procedure described by Moret *et al.* [27].

In the case of salted anchovies, the extraction of Put was carried out using an ethyl acetate–acetone mixture, according to the procedure described by Yigit *et al.* [28]. Thus, 3 g of the previously homogenized sample and 10 mL of a 5% $HClO_4$ solution were mixed in a vortex mixer for 1 min. The mixture was placed in an ultrasonic bath for 10 min and then, centrifuged for 10 min at 7000 rpm. The supernatant was recovered and neutralized with a 3 % ammonia solution, saturated with NaCl. Then, it was extracted with 10 mL of an ethyl acetate–acetone (2:1) solvent system in a vortex mixer for 1 min. Next, the mixture was briefly centrifuged. Finally, the organic phase was separated and dried with anhydrous Na_2SO_4, and the solvent was evaporated.

4.3. RESULTS AND DISCUSSION

The response of the developed MAO biosensors to Put is based on the mechanism shown in Scheme 4.1. Put is oxidized to aldehyde through the action of MAO enzyme. The hydrogen peroxide generated is then oxidized by the mediator TTF. The TTF_{red} produced is finally oxidized on the electrode surface. The oxidation current obtained is proportional to the concentration of Put allowing its quantitative determination.

4.3.1. BIOSENSOR CHARACTERIZATION

Any analytical procedure should be characterized establishing its precision, as well as its capability of detection. In order to set the optimal experimental conditions for the evaluation of these parameters, some previous experiments were carried out.

Since the electrochemical redox reaction of the mediator TTF has a maximum response at + 250 mV, this value was established as the applied potential. A series of experiments were carried out between 7 and 11 pH values, in order to determine the optimal one. The highest current intensities were recorded when pH values between 9 and 11 were used.

Different quantities of MAO were immobilized onto the electrode surface with the aim of determining the influence of the concentration of enzyme. The study was performed at pH 9 and 11, in the presence of a 59 µM solution of Put (Table 4.2). A pH of 11 and 1.1 units of MAO were selected as the optimal values. Then,

MAO/TTF/SPCEs were analytically characterized under these conditions.

Table 4.2. *Influence of MAO concentration in determination of Put using MAO/TTF/SPCEs.*

MAO units	pH	Current (nA)
0.56	9	0
	11	10
1.10	9	6
	11	11
1.70	9	4
	11	1

Calibration parameters were calculated using the program Progress [29] which evaluates the presence of anomalous points using a least median square regression (LMS). Once those points are removed from the calibration set, a calibration curve is built with the remaining points by ordinary least square regression (OLS). This OLS regression provides a proper assessment of the slope (sensitivity) and the independent term of calibration. Both terms are important for judging the quality of calibration and, indeed, the analytical performance [30]. In this way, a linear response in the range from 16 to 101 μM was obtained.

Reproducibility was then evaluated in terms of sensitivity using the slopes of regressions built using different MAO/TTF/SPCEs. The sensors showed an acceptable relative standard deviation (RSD) value of 9.6 % (n=4). The capability of detection was calculated for a probability of false positive (α) and

negative (β) equal to 0.05, according to [31, 32]. The average capability of detection found was of 17.2 ± 4.6 µM (n=4, α=β=0.05).

4.3.2. TTF/SPCEs CHARACTERIZATION

Once the electrodes were printed, the presence of the mediator TTF was verified by cyclic voltammetry. Figure 4.1 shows the cyclic voltammogram recorded between + 800 and - 400 mV in KCl 0.1 M using a TTF/SPCE. The voltammogram showed the typical behaviour of TTF with two oxidation peaks and their two corresponding reduction peaks ($TTF \rightarrow TTF^{+} \rightarrow TTF^{2+}$)[33].

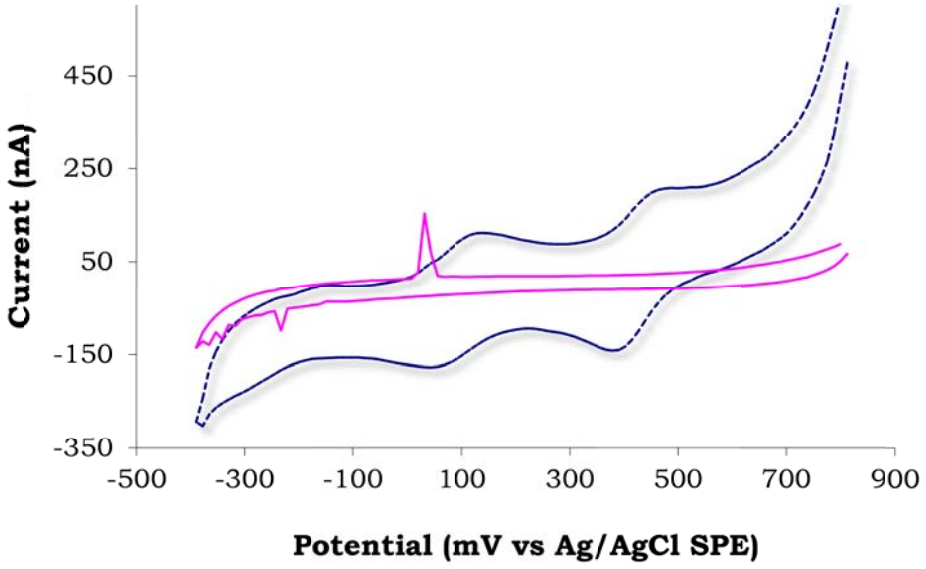


Figure 4.1. *Cyclic voltammograms recorded in a 0.1 M KCl solution using a SPCE (▬) and a TTF/SPCE (-----). Scan rate; 0.1 V s^{-1}*

4.3.3. SUBSTRATE SPECIFICITY DETERMINATION

The possible interference from other BAs was evaluated. The BAs selected in this work were those most commonly found in food samples, namely, Tyr, His, Cad, Tryp, Spd, Spm and Put. Under the optimal operational conditions previously optimized, only Cad and Tyr interfered in the determination of Put using MAO/TTF/SPCEs at the level concentration of 60 µM. The interference of Tyr is attributed to direct oxidation of the phenol group of this molecule at the applied potential. In the case of Cad, the interference is due to the lack of specificity of MAO enzyme towards the oxidation of BAs. Thus, a kinetic enzymatic study, using Michaelis-Menten equation and Lineweaver-Burk plot, was carried out in order to explain the interference level of Cad, Spm and Spd. These BAs were chosen due to their similar lineal molecular structure.

Since not analytical response was observed for Spm and Spd using MAO/TTF/SPCEs, this study was performed using SPCEs modified with MAO by crosslinking (MAO/SPCEs). Table 4.3 shows the kinetic parameters obtained in the concentration range from 99 µM to 1.5 mM. In this table it can be seen the higher affinity (low values of K_m) showed by the MAO biosensor for Put than for Cad, Spm and Spd.

Table 4.3. *Kinetic parameters obtained using MAO/SPCEs, in a 50 mM KH_2PO_4 and 100 mM KCl solution, pH 11, at an applied potential of + 0.9 V*

Substrate	V_{max} (s^{-1})	K_m (mM)
Put	3.3×10^{-3}	0.43
Spd	1.0×10^{-3}	1.50
Spm	1.6×10^{-2}	2.50
Cad	1.4×10^{-2}	1.20

With the aim of reaching the limit where the oxidation rate of Cad was not significant, a decrease in the amount of immobilized enzyme was attempted. It was observed that an enzyme concentration lower than 0.28 units led to a biosensor sensitive to Put but not to Cad.

4.3.4. APPLICATION IN SALTED ANCHOVIES SAMPLES

The higher affinity of MAO for Put allows the selectively determination of this molecule, even in presence of other BAs, in food samples by means of the described MAO/TTF/SPCE based biosensor. In this way, the developed biosensors were applied to the determination of Put in salted anchovy samples using the standard addition method. The extraction of Put was performed according to the procedure described above. The extract obtained was dissolved in 2 mL of water. A volume of 100 µL of this extract was placed into the electrochemical cell, containing 5 mL of buffer solution pH 11, and following by successive additions of 50 µL of a 1 mM Put solution (Figure. 4.2). The concentration of Put found was 34 ± 3 µg g^{-1} (n=4, α=β=0.05) with a RSD of 5.8 % (Table 4.4). This result agrees with previously described works where similar values of Put in anchovies have been reported [12].

In order to test the viability of this procedure in the determination of Put in anchovy samples, a recovery study was also performed. The total amount of Put in a spiked sample, 69.4 µg g^{-1}, was determined by standard addition in quadruplicate.

Table 4.4. *Recovery (%), confidence interval for mean and RSD values for extracted Put from anchovies and zucchini samples, using MAO/TTF/SPCEs based biosensors.*

Anchovies sample				
Put spiked level (μg/g)	Put detected (μg/g)	Recovery (%)	95% Confidence interval for mean	RSD
—	36.2	—	34.0 ± 3.0	5.8 %
	33.4	—		
	31.6	—		
	34.9	—		
35.4	71.9	104	69.3 ± 4.4	4.0 %
	70.9	102		
	68.8	99		
	65.6	94		
Zucchini sample				
Put spiked Level (mg/100g)	Put detected (mg/100g)	Recovery (%)	95% Confidence interval for mean	RSD
—	3.6	—	3.7 ± 0.4	7.2 %
	3.5	—		
	3.7	—		
	4.1	—		
4.2	8.2	103	8.1 ± 0.3	2.7 %
	8.2	103		
	7.8	98		
	8.3	104		

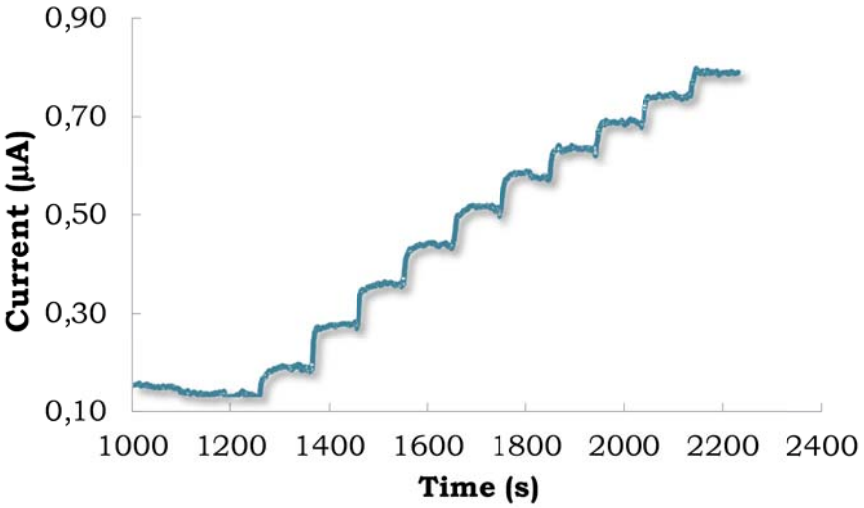


Figure 4.2. *Chronoamperogram recorded for the determination of Put in anchovies samples using a MAO/TTF/SPCE based biosensor. The first addition (1300 s) corresponds to 100 µL of sample extract and then successive additions of 50 µL of a 1 mM Put solution were made*

The concentration of Put found was 69.3 ± 4.4 µg g^{-1} (n=4, α=0.05, RSD=4 %), with an average recovery of 99.8 % (Table 4.4). On the basis of these data the method can be considered appropriate to the determination of Put in anchovy samples

4.3.3. APPLICATION IN ZUCCHINI SAMPLES

The determination of Put in zucchini samples was performed as well, following the described procedure (Table 4.4). The concentration of Put found, 3.7 ± 0.4 mg/100 g (n=4, α=β=0.05), matches with previously described data of Put content in zucchini samples [27]. Recovery studies were also performed with this sample. The total amount of Put in a spiked sample, 8 mg/100 g, was determined by standard addition in quadruplicate. The concentration of Put found, 8.1 ± 0.3 mg/100 g (n=4, α=0.05, RSD=4 %), with an average recovery

of 102 %, highlights the suitability for the determination of Put in these vegetables samples.

4.4. CONCLUSIONS

A selective and reagentless method for the determination of Put was developed. The screen-printed TTF mediator reagent allows the simpler manufacture as well as decreasing the operational potential to + 250 mV. This fact represents an important advantage from previously described monoenzymatic screen-printed biosensors, which used potentials higher than + 600 mV. Moreover, the choice of an appropriate amount of immobilized enzyme in the electrode makes this new biosensor selective to the determination of Put even in the presence of similar BAs.

The biosensor construction is highly reproducible, allowing to obtain sensors with very similar sensitivities (RSD = 9.6 %). The biosensors present a linear response range from 16 to 101 µM and a capability of detection of 17.2 ± 4.6 µM (n=4, $\alpha=\beta=0.05$).

The biosensor was successfully used as an efficient screening tool to quantify Put in complex samples, such as salted anchovies and zucchini samples.

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5. Simultaneous Determination of Cadaverine and Putrescine Using Disposable Mono Amine Oxidase Based Biosensors

En el capítulo anterior se observó que es posible llevar a cabo la determinación de Put utilizando biosensores basados en la utilización del enzima MAO como elemento biológico. No obstante, el biosensor desarrollado presentó una pequeña respuesta electroquímica hacia la Cad. En base a estos resultados, en este capítulo se propone el desarrollo de un biosensor electroquímico para la determinación simultánea de Put y Cad basado en el diseño de un dispositivo con una nueva configuración que incorpora dos electrodos de trabajo diferentes en el mismo dispositivo.

El biosensor amperométrico desarrollado en este capítulo se basa en la diferente especificidad observada por el enzima MAO hacia la Put y la Cad. En este sentido, el empleo de diferentes cantidades del enzima MAO conduce a una selectividad diferenciada hacia dichas BAs. Para la impresión de los dos electrodos de trabajo se empleó una tinta de carbono modificada con un 3 % del mediador TTF (TTF/SPCE) con la finalidad de reducir el potencial de trabajo y evitar así posibles interferentes que se podrían presentar a potenciales más elevados. Uno de los electrodos fue modificado mediante la electrodeposición de AuNPs, con el objetivo de incrementar la sensibilidad del sensor, y a continuación 0.92 unidades del enzima MAO fueron inmovilizadas mediante entrecruzamiento con GA y BSA sobre la superficie de dicho electrodo (MAO/AuNPs/TTF/SPCEs). Este electrodo resulta sensible a la presencia tanto de Put como de Cad. El otro electrodo de trabajo fue modificado mediante la inmovilización de 0.46 unidades de MAO, cantidad optimizada para la determinación selectiva de Put (MAO/TTF/SPCEs). El biosensor desarrollado generó buenos resultados en términos de selectividad, reproducibilidad y capacidad de detección, de manera conjunta e individualmente para cada compuesto.

Una parte de este trabajo ha sido presentada en la *XXXVI Reunión del Grupo de Electroquímica de la Real Sociedad Española de Química y XV Encontro Ibérico*, celebrada en Valencia del 15 al 17 de Julio de 2013, así como en el *VIII J Annual CISCS Symposium*, celebrado en Covilhã (Portugal) del 1 al 2 de Julio de 2013. Además, el trabajo experimental desarrollado ha dado lugar a una publicación científica en la revista *Talanta 117 (2013) 405-411.*

5.1. INTRODUCTION

BAs are organic bases with aliphatic (Put, Cad, Spm and Spd); aromatic (Tyr and Pea) or heterocyclic (His and Tryp) structures [1]. These natural contaminants are synthesized and degraded during normal metabolism of animals, plants and microorganisms [2]. High amounts of certain amines may be present in a wide range of food products including fish, meat, wine, beer, vegetables, fruits, nuts and chocolate [2], as a consequence of microbial contamination and inappropriate conditions during processing and storage.

Therefore, the content of BAs, especially Put, Cad, His and Tyr can be considered as freshness markers and could be used as indicator of microbial spoilage [3]. Ingestion of food contaminated with BAs, can lead to several health problems, such as headache, blushing, itching, skin irritation, impaired breathing, tachycardia, hypertension, hypotension and vomit [4]. Moreover, it has been reported that certain types of cancer produce an increase of Put and Cad concentration in some human tissues. In this way, Put is often accumulated in blood, serum and mucous of cancer patients. Thus, Put and Cad are listed as tumour markers and their determination in clinical samples can be important for diagnosis of malignancy and, even for monitoring the efficiency of treatments, such radio or chemotherapy [5]. For these reasons, monitoring of BAs amount present in food and beverages is becoming increasingly demanded by regulatory commissions as the Commission Regulation (EC 2073/2005) [6].

Since BAs are usually present at low levels in complex matrices, the determination of these compounds requires the use of sensitive and selective analytical methods. Traditionally, BAs are

determined using chromatographic methods [7, 8], which are time consuming and require special instrumentation. Contrary to the above mentioned methods, biosensors offer simple, rapid and cost-effective solution for the determination of BAs [9]. Amperometric enzymatic sensors hold a leading position among the presently available biosensor systems. These devices combine the selectivity of the enzyme for the recognition of a given target analyte with the direct transduction of the rate of the biocatalytic reaction into a current signal, allowing a rapid, simple and direct determination of numerous compounds [10]. Among the different transducers used, SPEs offer additional advantages related to their disposable character and great versatility. This versatility lies in the wide range of possible methods of modification of this kind of electrodes, since the composition of the printing inks may be altered by the addition of different substances such as metals, enzymes, polymers or mediators [11].

Therefore, fast determination of BAs with different enzymatic disposable biosensors has become increasingly popular. Recently, electrochemical biosensors [2, 3, 5, 6, 9, 12-35] or bioreactors [36, 37] based on commercial or home-purified AOs have been proposed in the literature for the detection of BAs. An over view of different biosensors used for the detection of BAs is given in Table 5.1. The main problems of the described biosensors come from the low selectivity of enzymes and the high working potentials needed, leading to an increase in interferences.

The complexity of food matrix, the presence of potential interferences and the simultaneous occurrence of several BAs are therefore typical problems encountered in the analysis of BAs in food when using this kind of biosensors [38].

At present, efforts in the development of biosensors for BAs are mainly focused on the improvement of their selectivity and sensitivity as well as their miniaturization, driven by the growing need for rapid in situ analyses to secure the imposed safety standards of food [9]. Within this context, a new method has been developed in this work for the simultaneous and selective determination of Put and Cad in food samples. The novelty of this work lies in the use of an innovative electrochemical disposable screen-printed device, which includes two different modified working electrodes connected in array mode.

5.2. EXPERIMENTAL

5.2.1. REAGENTS

Different inks were used in the fabrication of SPCEs, namely C10903P14 (carbon ink) and D2071120D1 (dielectric ink) (Gwent Electronic Materials, Torfaen, UK), Electrodag 418 (Ag ink) and Electrodag 6037SS (Ag/AgCl ink) (Acheson Colloiden, Scheemda, The Netherlands). All solutions were prepared using Milli-Q water (Millipore, Bedford, USA). All reagents used were of analytical grade. The supporting electrolyte used for the measurements was a 100 mM KH_2PO_4 buffer solution (Fluka, Steinheim, Germany), containing KCl 100 mM (Merck, Darmstadt, Germany). A 1 M NaOH solution (J.T. Baker, Deventer, The Netherlands) was used to adjust the pH. TTF was provided by Sigma-Aldrich (Steinheim, Germany). A 1 mM hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4.3H_2O$, Sigma-Aldrich, Steinheim, Germany) and 0.5 M sulfuric acid (Merck,

Darmstadt, Germany) solution was used for the electrodeposition of AuNPs.

A human recombinant MAO (EC1.4.3.4; activity, 92 U mg^{-1}) was obtained from Sigma-Aldrich, (Steinheim, Germany). BSA (Sigma-Aldrich, Steinheim, Germany) solution, which was prepared by dissolving the appropriate amount of this reagent in a 50 mM KH_2PO_4 buffer (pH 6) solution (Fluka, Steinheim, Germany), and GA (Sigma-Aldrich, Steinheim, Germany) aqueous solution were used in the enzyme immobilization process.

Put, Cad (Sigma-Aldrich, Steinheim, Germany), Tyr, His (Fluka, Steinheim, Germany), Spd, Spm and Tryp (Across Organics, Geel, Belgium) solutions were used.

$HClO_4$ (Panreac, Barcelona, Spain), ethyl acetate, NaCl and butanol (Fluka, Steinheim, Germany), ammonia solution, acetone and Na_2SO_4 (Merck, Darmstadt, Germany) were used for Put extraction in real samples.

Fresh octopus samples were obtained from a local market.

5.2.2. APPARATUS

The different screen-printed electrodic systems were produced on a DEK 248 printing machine (DEK, Weymouth, UK), using several screen polyester mesh and polyurethane squeegees. Electrochemical measurements were made with a PalmSens Bipotentiostat electrochemical system (Palm Instrument BV, Houten, The Netherlands). The pH of the buffer solutions was measured with a HI 221 pH meter (HANNA Instruments, USA).

5.2.3. ELECTRODIC SYSTEMS

Two different home-made electrodic systems, including one or two working electrodes, have been used.

The first one served for the optimization of experimental variables in the separate determination of Cad and Put, and has been constructed according to our previous works [39-41]. This system consisted of three electrodes: a Ag/AgCl-SPE, a carbon counter electrode and a working electrode modified with TTF (TTF/SPCEs). The working electrode was modified with MAO (MAO/TTF/SPCEs) for determination of Put, and with MAO and AuNPs (MAO/AuNPs/TTF/SPCEs) for both Put and Cad determination.

The second electrodic system represented a novel device (array-TTF/SPCEs) for the simultaneous analysis of both analytes, Put and Cad. A home-made screen-printed electrochemical design with two different working electrodes connected in array mode has been designed (Figure. 5.1).

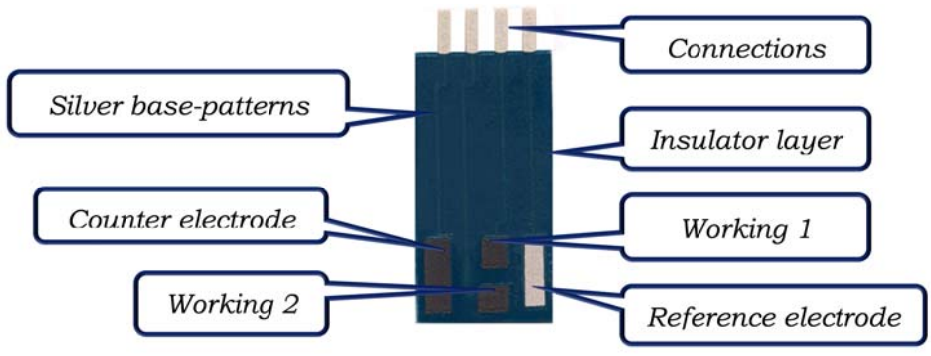

Figure 5.1. *Home-made array-TTF/SPCE (2.8 cm x 1.8 cm)*

The manufacturing of the electrodic system was carried out in five steps, through which the different electrodes were printed following a similar procedure as described in previous works [39-41]. Figure. 5.2 shows a schematic diagram of these steps. In this case TTF in a 3 % proportion has been incorporated directly into the carbon ink for printing the working electrode (step 3).

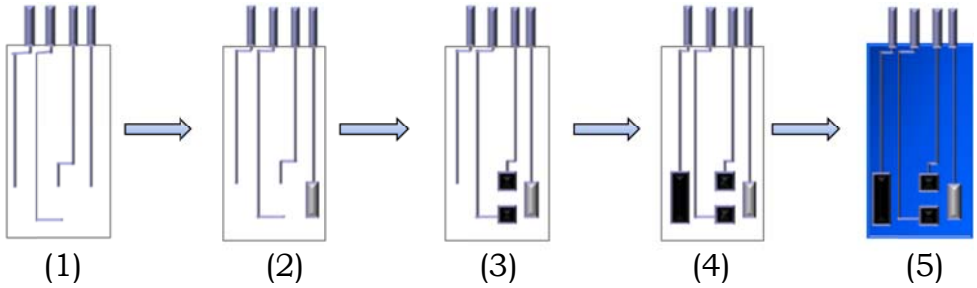

Figure 5.2 *Schematic diagram of the array-TTF/SPCEs preparation procedure: (1) Silver base-patterns, (2) reference electrode (Ag/AgCl), (3) working electrode (carbon ink + TTF 3 % with respect to the mass of the carbon ink), (4) counter electrode (carbon ink) and (5) insulator layer*

5.2.3.1. Deposition of gold nanoparticles in SPCEs

The modification of the working electrodes with AuNPs was carried out by electrochemical deposition. A volume of 100 µL of a 1 mM $HAuCl_4$, prepared in 0.5 M H_2SO_4, solution, was deposited onto the electrodic system and a potential of + 0.18 V *vs* Ag/AgCl-SPE was applied during 10 seconds [39]. The electrodic system was finally washed with Milli-Q water.

5.2.3.2. Immobilization of MAO onto the screen-printed working electrode

MAO enzyme was immobilized on the working electrodes surface using a crosslinking process with GA and BSA. First, a mixture of 1 µL of a 6 % (w/v) BSA solution prepared in 50 mM phosphate buffer pH 6, and 2 µL of a 2.5 % (v/v) GA aqueous solution was deposited onto the working electrode. Next, 0.46 or 0.92 units of MAO solution were deposited onto the surface of working electrodes by adding 1 µL and 2 µL of a 0.46 units/µL enzyme solution, respectively. Once deposited, the electrodic system was allowed to dry for 90 min at 4 °C.

5.2.4. CHARACTERIZATION OF TTF/SPCEs

Once the electrodes were printed by the procedure described in previous sections, the presence of the mediator TTF was verified by cyclic voltammetry. Figure 5.3 shows the cyclic voltammograms recorded between + 800 and – 400 mV in KCl 0.1 M using a TTF/SPCE. The voltammograms corresponded to the typical behaviour of TTF with two oxidation peaks and their two corresponding reduction peaks ($TTF \rightarrow TTF^{+} \rightarrow TTF^{2+}$) [42].

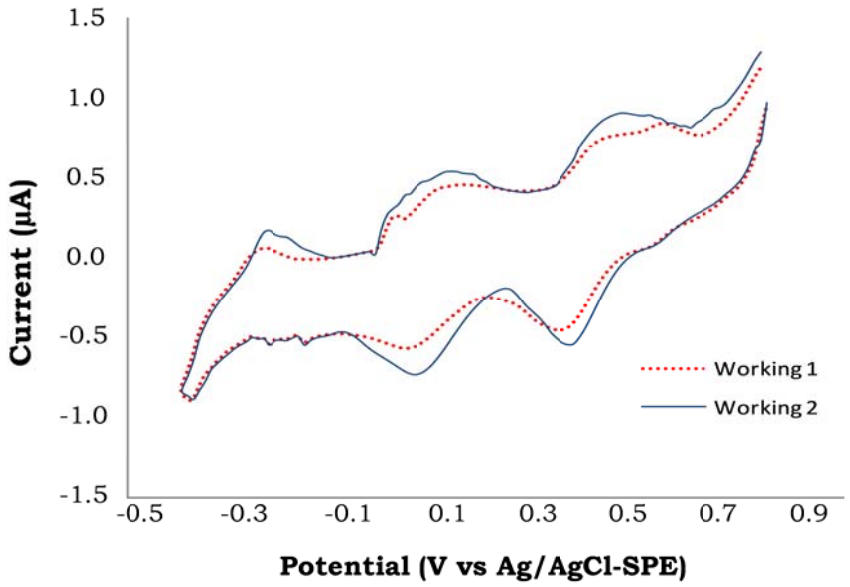

Figure 5.3. *Cyclic voltammograms recorded in a 0.1 M KCl solution using an array-TTF/SPCE*

5.2.5. ELECTROCHEMICAL MEASUREMENTS

All measurements were made at room temperature in a cell containing 5 mL of a supporting electrolyte solution of the desired pH, under constant stirring. The amperometric detection was performed by measuring the oxidation of TTF_{RED} at a potential of + 250 mV *vs.* Ag/AgCl-SPE (Scheme 4.1). The sample to be analyzed was added after reaching a stable baseline.

5.2.6. SAMPLE PREPARATION

Fresh octopus samples were extracted with a simple preparation method, using an ethyl acetate–acetone mixture, according to the procedure described by Yigit *et al.* [43]. Thus, 5 g of

the previously homogenized sample and 10 mL of a 5 % $HClO_4$ solution were mixed in a vortex mixer for 1 min. The mixture was placed in an ultrasonic bath for 10 min and then, centrifuged for 10 min at 7000 rpm. The supernatant was recovered and neutralized with a 3 % ammonia solution, saturated with NaCl. Next, it was extracted with 10 mL of an ethyl acetate–acetone (2:1) solvent system in a vortex mixer for 1 min. Subsequently, the mixture was briefly centrifuged. Finally, the organic phase was separated and dried with anhydrous Na_2SO_4, and the solvent was evaporated.

5.3. RESULT AND DISCUSSION

It is well known that MAO catalyses the oxidation process of both Put and Cad. However, previous experiments showed that the selectivity of MAO towards these molecules is different, being the K_m value for Put, 0.43 mM and for Cad 1.2 mM [44]. The efficiency of an enzyme catalyzed reaction is related to substrate selectivity. Differences in substrate selectivity for an enzyme toward different substrates may affect substrate sensitivity [45]. Thus, when both substrates, Put and Cad, are present in the same medium, they will compete for reaction with the enzyme. The amount of enzyme immobilized on the electrode could therefore affect the selectivity and sensitivity of a developed biosensor. In this way, a detailed study of the influence of the quantity of MAO immobilized on the working electrode surface has been carried out. At the developed biosensor the BA was oxidized to aldehyde (BA_{OX}) through the action of the enzyme MAO. The hydrogen peroxide enzymatically generated is then oxidized by the mediator TTF_{OX}. The recorder signal corresponded to the electro oxidation of $TTF_{RED.}$

Several biosensors have been developed by immobilization of different amounts of MAO ranging from 0.1 to 2 units onto the TTF/SPCEs surface and their amperometric response was then measured. This study has revealed that the developed biosensor responded to the presence of Put for amounts of MAO higher than 0.46 units. In contrast, Cad detection was only possible for quantities of MAO higher than 0.92 units. The amperometric response obtained for the developed biosensors, however, was relatively poor for Cad. The problem was solved by depositing AuNPs onto the working electrode. In this way, an electrode modified with AuNPs and 0.92 units of MAO, responded quantitatively to the presence of both Cad and Put.

Bearing in mind the above results, one of the working electrodes (working 1) of the developed array-TTF/SPCE system has been modified immobilizing 0.46 units of MAO, resulting in a sensitive response for Put only. The second working electrode (working 2) has been modified with AuNPs and 0.92 units of MAO being, therefore sensitive to both molecules. With this device, the simultaneous determination of both molecules in the same sample could be carried out using a single calibration set. Previous to the simultaneous determination of Cad and Put, an optimization process of the experimental parameters has been carried out separately.

5.3.1.-DETERMINATION OF PUT AND CAD WITH MAO/AUNPS /TTF/SPCES

As described above, the enzymatic oxidation of Cad or Put, in the presence of TTF, allowed the quantitative determination of these amines following the amperometric oxidation of the mediator TTF. An

applied potential of + 250 mV *vs*. Ag/AgCl-SPE was selected as the optimum value of this parameter for the amperometric analysis of Put and Cad at the MAO/AuNPs/TTF/SPCEs (Figure. 5.2). The best amperometric signals were obtained at pH 11.

Thus this value was chosen as the optimum supporting electrolyte pH. With the aim of carrying out the characterization of these processes, several calibrations sets were performed for each amine.

Calibration parameters were calculated using the program PROGRESS [46], which evaluated the presence of anomalous points using a LMS. Once those points were moved from the calibration set, a calibration curve was built with the remaining points by OLS. This OLS regression provided a proper assessment of the slope (sensitivity) and the independent term of calibration. Both terms were important for judging the quality of calibration and, indeed, the analytical method. In this way, a linear response in the range from 19.6 till 107.1 μM was obtained for Cad and from 9.9 till 74.1 μM for Put.

Reproducibility was then evaluated in terms of sensitivity using the slope of regression built using different MAO/AuNPs/TTF/SPCEs. The sensors showed an acceptable RSD value of 4.9 % and 10.3 % (n=4) for Put and Cad, respectively.

Table 5.2. *Characterization of biosensor for Cad and Put determination*

Biosensor	Analyte	Sensitivity (nA/µM)	Reproducibility RSD (%)	Capability of detection (µM)	Linear range (µM)
MAO/AuNPs/TTF/SPCEs	Put	1.36	4.9	9.9	9.9- 74.1
		1.27			
		1.15			
		1.16			
	Cad	1.05	10.3	19.9	19.6-107.1
		1.22			
		1.26			
		10.3			
MAO/TTF/SPCEs	Put	1.65	8.0	12.6	11.9–77.0
		1.41			
		1.47			
		1.65			

The capability of detection was calculated for a probability of false positive (α) and negative (β) equal to 0.05, according to the literature [47, 48]. The average capability of detection found was 19.9 ± 0.9 μM for Cad and 9.9 μM for Put (α=β=0.05) (n=4). Table 5.2 summarizes these results.

5.3.2. DETERMINATION OF PUT WITH MAO/TTF/SPCES

As it has been explained above, when the working electrode surface was modified with 0.46 units of MAO, only Put gave an analytical amperometric response. Thus, this electrode was characterized for Put. The MAO/TTF/SPCE biosensor presented a linear response in the Put concentration range from 11.9 till 77.0 μM at pH 11. This biosensor showed a capability of detection of 13 ± 2 μM (α=β=0.05) and a precision of 8% (n=4) in terms of RSD (Table 5.2).

5.3.3. SUBSTRATE SELECTIVE DETERMINATION

The possible interference from other BAs was evaluated. The BAs selected in this work were those most commonly found in food samples, namely Tyr, His, Tryp, Spd, and Spm. Under the optimal operational conditions, only Tyr resulted interference at a concentration of 60 μM in the determination of Cad and Put when using the MAO/AuNPs/TTF/SPCE biosensor by giving a well-defined amperometric response. Tyr interfered also in the analysis of Put when using MAO/TTF/SPCE biosensors.

A deep study of the possible interference caused by the presence of other BAs was performed, using His as the selected interference. In this way, different calibration sets for Put and Cad were carried out using the MAO (0.92 units)/AuNPs/TTF/SPCE in the presence of two different levels of His (9 and 77 μM). The sensitivity (slope) of these calibrations was not altered by the presence of His. Similarly, the sensitivity of MAO (0.46 units)/TTF/SPCEs biosensor towards Put was not altered by the presence of His even at a high level of concentration.

5.3.4. APPLICATION IN OCTOPUS SAMPLES ANALYSIS

The simultaneous determination of Put and Cad in octopus samples was performed using the standard addition method with array-TTF/SPCEs. The extraction of amines was performed according to the procedure described in Section 5.2.6. The extract obtained was dissolved in 2 mL of water. A volume of 500 μL of this extract was placed into the electrochemical cell, containing 5 mL of buffer solution pH 11, and following by successive additions of 100 μL of Put 5 mM solution (Figure. 5.4). The concentration of Put and Cad found was 22.1 ± 1.6 and 15.6 ± 1.1 mg kg^{-1} (n=3 α=0.05), respectively. This result agrees with previously described works [49].

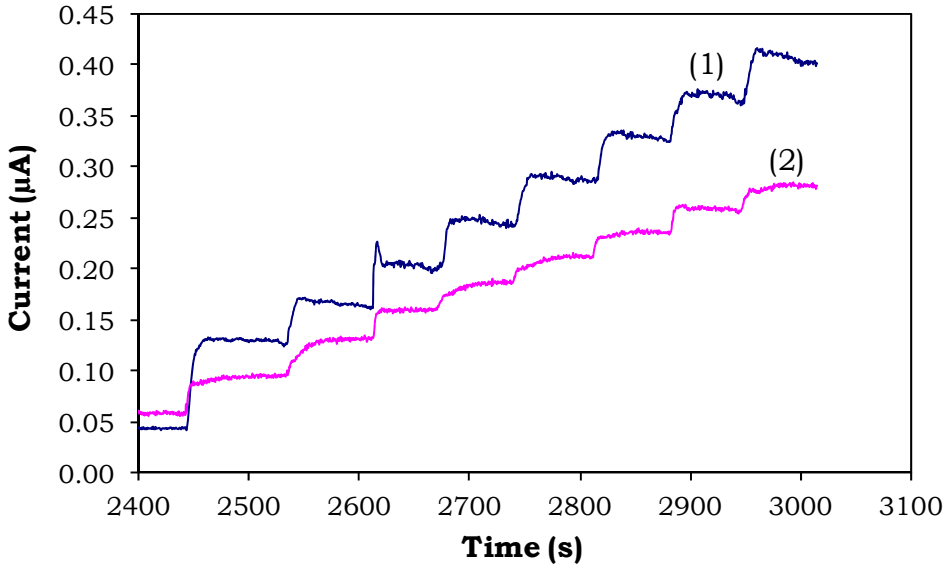

Figure 5.4. *Chronoamperogram recorded for the simultaneous determination of Put and Cad in octopus samples in the array-TTF/SPCEs biosensor. (1) Working 1: MAO/TTF/SPCE and (2) working 2: MAO/AuNPs/TTF/SPCE. The first addition (2450 s) corresponds to 500 mL of sample extract and then successive additions of 100 mL of a 5 mM Put solution were made. pH 11,* E_{ap} *= + 250 mV vs. Ag/AgCl-SPE*

In order to test the viability of this procedure for the simultaneous determination of Put and Cad in octopus samples, a recovery study was also performed. The total amount of Put and Cad in a spiked octopus sample was determined by standard addition in quadruplicate. The concentration of Put and Cad found was 44 ± 3 and 29 ± 1 mg kg^{-1} (n=4 α=0.05), respectively. The average recovery found was 100 ± 6 % and 95 ± 3 % for Put and Cad, respectively (Table 5.3). On the basis of these data the method can be considered appropriate to be applied to simultaneous determination of Put and Cad in these samples.

Table 5.3. *Recovery (%), confidence interval for mean and RSD values for extracted Put and Cad from spiked octopus samples, using MAO/TTF/SPCEs and MAO/AuNPs/TTF/SPCEs based biosensors (n = 4).*

Analyte	Spiked concentration (mg kg^{-1})	Concentration found (mg kg^{-1})	Recovery (%)
Put	-	22 ± 2	-
Cad	-	16 ± 1	-
Put	21.9	44 ± 3	100 ± 6
Cad	15.4	29 ± 1	95 ± 3

5.3. CONCLUSIONS

A new and disposable, screen-printed array electrodic system including two working electrodes, an auxiliary and a reference electrode has been developed, in order to perform the simultaneous determination of Cad and Put.

The screen-printed TTF mediator reagent allows taking measures at a low operational potential of + 250 mV, thus reducing the possible interfering by substances present in the complex sample. The biosensors construction is reproducible, allowing sensors with very similar sensitivities to be obtain. The biosensors present a linear response range from 9.9 to 74.1 µM for Put and 19.6 to 107.1 µM for Cad with a capability of detection of 19.9 µM and 9.9 µM for Cad and Put, respectively.

The biosensor was successfully used as an efficient screening tool to simultaneously quantify Put and Cad in cephalopod mollusc samples.

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6. Characterization of a Disposable Electrochemical Biosensor Based on PUO from *Micrococcus rubens* for the Determination of PUT

Como se ha descrito anteriormente uno de los principales inconvenientes que presentan los biosensores electroquímicos enzimáticos descritos en la bibliografía es su falta de selectividad, es por ello que en este capítulo se ha desarrollado un biosensor desechable para la determinación de Put basado en la incorporación del enzima PUO, un enzima altamente específico y selectivo hacia la Put. La actividad y especificidad de la PUO inmovilizada en los SPCEs hacia la Put es evaluada mediante ITC. De igual modo se demostró su idoneidad para determinar el contenido de Put en muestras complejas mediante la comparación con los resultados obtenidos mediante HPLC.

Este trabajo ha dado lugar a una publicación en una revista científica (*Electroanalysis* 27 (2015) 368-377), y ha sido en parte presentado en el *XXXV Meeting of electrochemistry of Spanish Royal Society of Chemistry – 1St E3 Mediterranean symposium: Electrochemistry for environment and energy c*elebrada en Burgos del 14 al 16 de Julio 2014.

6.1. INTRODUCTION

BAs are a group of low-molecular-mass organic bases that contain at least one amine group. BAs are produced through naturally occurring decarboxylation of amino acids by substrate-specific amino acid decarboxylases [1]. With the exception of BAs of endogenous origin, present at low concentrations, higher concentrations of BAs can be detected in raw and processed foods as a result of degradation [2]. There is evidence that a decrease in the product hygienic quality leads to an increase of BAs content. As a result, it is important the monitoring of BAs levels in foodstuffs as they are good indicators of food freshness [3]. Also, the presence of BAs in food constitutes a potential public health concern. At low concentrations, BAs are essential for many physiological functions, conversely if these compounds are consumed in high quantities, several toxicological problems arise [2]. Therefore, it is extremely important, in food quality analysis, the development of procedures for BAs quantification and identification.

It is very difficult to establish the BAs toxicological level because it depends on individual characteristics and on the presence of other amines in food. Nevertheless, a BAs maximum content level of 750–900 mg kg^{-1} has been proposed as a safety limit [4]. Furthermore, it has been also reported that certain types of cancer induce an increase of Put and Cad concentration in human tissues.

Moreover, Put is often accumulated in blood, serum and mucous of cancer patients. Accordingly, Put and Cad are listed as tumour markers, being their determination in clinical samples important for diagnosis of malignancy and for monitoring cancer treatments efficiency [5].

Analytical determination of BAs is not simple due to their chemical structures variety and because they are usually present at low concentrations in complex matrices. Thus, the determination of these compounds requires the use of sensitive and selective analytical methods, being traditionally determined using chromatographic methods [2, 6, 7]. However, cost and complexity of these methods reduce their applicability for routine determinations.

Electrochemical biosensors for the determination of BAs have attracted much attention in the last years due to their simplicity, reproducibility, low cost, short time analysis, and sensitivity. Among the different transducers used, SPEs offer additional advantages related to their disposable character and great versatility [8]. This versatility lies in the wide range of possible methods used to modify this kind of electrodes, the printing inks composition may be altered by the addition of different substances such as metals, polymers, mediators or enzymes. Several disposable electrochemical biosensors have been recently developed based on the use of commercial AOs as biological recognition elements for detecting BAs. PAO [9, 10], DAO [3, 10-15], and MAO [13, 15-17] have been successfully applied. The main problem of the described biosensors come from the low enzyme selectivity and the high working potentials needed for operation, leading to an increase in interferences [6].

The selectivity of a biosensor resides in the specificity of the enzyme used. In this way, PUO is a pure active enzyme much more specific to Put than other AOs [18]. This enzyme catalyzes the oxidation of Put according to the following reaction:

$$H_2N(CH_2)_4NH_2 + O_2 + H_2O \xrightarrow{PUO} 4\ H_2N(CH_2)_3\ CHO + NH_3 + H_2O_2$$

The enzymatic reaction can be followed using an amperometric biosensor based on the electrochemical oxidation of enzymatically generated H_2O_2. This enzyme has been used for the determination of BAs using different kinds of electrodes (Table 6.1). In this work, PUO enzyme has been used for the analysis of Put content in different samples. SPCEs have been used as electrochemical transducers and TTF as electrochemical mediator in order to decrease the applied potential in the amperometric measurements. TTF was screen-printed by incorporating it into the carbon ink. PUO enzyme was immobilized by crosslinking with BSA and GA on the TTF modified SPCEs (TTF/SPCEs). GA is a bifunctional crosslinking agent which reacts with lysine residues on the exterior of the proteins; addition of BSA accelerates the crosslinking process due to the lysine groups present in its structure.

This approach leads to more efficient catalysis than with direct enzyme immobilization, probably because biological activity losses are prevented owing to the friendly environment for enzyme immobilization. With BSA molecules the enzyme maintains its catalytic sites more accessible for redox reactions, since crosslinking does not affect the enzyme molecules significantly so there can be higher enzyme activity and greater stability [9, 19].

One of the main stages in the design and production of enzymatic biosensors is the immobilization of the enzyme on the electrochemical transducer. Features as important as their sensitivity depend in a great extent on the immobilization methodology used. The biological material must be immobilized on the electrode surface in an effective manner, leaching out of biomolecules during reaction

and workup should be prevented. In addition, enzyme biological activity must be preserved [20, 21]. Consequently, the activity value of an immobilized enzyme can be considered as an excellent measure of the immobilization process effectiveness. Most of the quantitative studies that evaluate enzyme activities are based on photometric methods. The main problem with these methods is that, for each enzyme, a suitable, low-cost and easy to label chromophore or fluorphore must be found, limiting the general application of these approaches [21].

Calorimetry offers an alternative to the photometric methodology to quantify an enzyme activity. The heat released during an enzymatic process represents a suitable indication of enzymatic activity, which can be used to follow and analyze the kinetics of an enzymatic reaction. ITC is known as one of the useful methods to study biological interactions thermodynamics [22]. ITC has been successfully applied in the activity analysis of different enzymes both in the free state [22-27] and immobilized on solid supports [21]. In the present work, the activity of PUO immobilized on SPCEs was evaluated by ITC. To our knowledge, this is the first time that this technique is applied to enzymes immobilized on SPCEs.

Table 6.1. *PUO based electrochemical biosensors.*

Enzyme	Working electrode	Immobilization	E (mV)	pH	Capability of detection (µM of Put)	Biosensor selectivity	Sample	Ref.
PUO (*Micrococcus rubens*)-*HRP*	Graphite	Crosslinking (PEGDGE)	+ 50	8.0	5.00	100% Put, 11% Cad, 5% Spd, 2% Tyr	Pork, Fish	[28]
PUO (*Micrococcus rubens*)-*HRP*	Graphite	Crosslinking (PEGDGE)	+ 50	8.0	5.00	100% Put, 10.7% Cad, 5.2% Tryp, 5% Spd, 2% Tyr, 1.7% His	Beer	[29]
PUO (*Micrococcus roseus*)	Glassy carbon (MWCNTs/APTES)	Mixture MWCNTS-PDDA-APTES	- 450	7.0	5.00	100% Put, 83% Cad, 71% Spd, Spm < 6.8%	Mouse plasma	[30]
PUO (*Micrococcus roseus*)	Glassy Carbon (MWCNTs/APTES)	Crosslinking (GA-BSA)	- 250	8.4	0.50	100% Put, 35.5 % Spd, 32.8 % Cad, 2.7% Spm	---	[31]
PUO (*Cellulomonas sp*)	Glassy Carbon	Crosslinking (GA-BSA)	+ 50	7.0	5.00	100% Put, 123% Spd, 4.7% Cad, 27.5 % Spm	Fish	[32]
PUO (*Micrococcus roseus*)	Pt - (film photolithography)	Crosslinking (membrane + GA)	+ 600	8.5	0.50	---	Human blood and plasma	[5]
PUO	Pt wire	Chitosan porous beads + GA	+ 500	7.8	100	100% Put, 97% Cad, 96 Spd, 8% Spm	Chicken	[33]
PUO (*Micrococcus roseus*)	Pt wire	Pore glass beads + GA	+ 600	7.0	0.07	100% Put, 12% Cad, 10% Agm and Tyr, 9% Spm, 8% Tryp, 1% > His	Fish	[34]
PUO (*Micrococcus roseus*)	Au-Pt wire	Crosslinking (GA-BSA)	+ 500	8.5	0.50	100% Put, 40% Cad, 20% Spm, interfering Tyr	Vaginal discharge	[18]
PUO (*Micrococcus rubens*)	TTF/SPCEs	Crosslinking (GA-BSA)	+ 300	10	10	100% Put, Tyr: interference at 150 µM	Octopus	This work

6.2. EXPERIMENTAL

6.2.1. REAGENTS

All reagents used were of analytical-reagent grade. Ultrapure water obtained from a Milli-Q water purifier (Millipore, Bedford, MA, USA) was used for the preparation of all solutions.

PUO from *Micrococcus rubens* (EC 1.4.3.10, K_m = 38 µM at 20 ºC for Put) was obtained from Inbiotec (León, España). BSA and GA, used in the immobilization procedure, were purchase from Sigma-Aldrich (Steinheim, Germany). Put and Cad were obtained, as well, from Sigma-Aldrich (Steinheim, Germany). Tyr and His were acquired from Fluka (Steinheim, Germany). Other BAs, including (Spd and Spm were purchased from Across (Organics, Geel, Belgium).

Different inks were used to construct the TTF/SPCEs. C200802P2 carbon ink and D2071120D1 dielectric ink were obtained from Gwent Electronic Materials (Torfaen UK). Silver 5029 conductor paste was purchased from Dupont Limited (Bristol, UK). Electrodag 6037 SS (Ag/AgCl ink) from Acheson Colloiden (Scheemda, The Netherlands) was also used.

TTF was provided by Sigma-Aldrich (Steinheim, Germany). The supporting electrolyte solution used for the amperometric measurements was a 100 mM KH_2PO_4 (Fluka, Steinheim, Germany) and 100 mM KCl (Merck, Darmstadt, Germany). The pH value was adjusted with a 1M NaOH solution (J.T. Baker, Deventer, The Netherlands).

Fresh octopus and zucchini samples were obtained from a local market. $HClO_4$ (Panreac, Barcelona, Spain), ethyl acetate, NaCl and butanol from Fluka (Steinheim, Germany) and, ammonia solution, acetone and Na_2SO_4 from Merck (Darmstadt, Germany) were used for the extraction of Put from real samples.

$NaHCO_3$ (Panreac, Barcelona, Spain), DCl (Sigma-Aldrich, Madrid, Spain) reagent (5 mg mL^{-1} in acetone), proline (Sigma-Aldrich, Madrid, Spain) solution (100 mg mL^{-1}), diethyl ether (Fisher Chemical, Loughborough, UK) and acetonitrile (Sigma-Aldrich, Madrid, Spain) were used in the derivatization procedure of the samples for HPLC analysis.

6.2.1. APPARATUS

The TTF/SPCEs were produced on a DEK 248 printing machine (DEK, Weymouth, UK) using screen polyester mesh and polyurethane squeegees.

Calorimetric measurements were made using a Flow Microcalorimeter (Microscal FMC 4 Vi, Microscal Limited, London, UK) operated in the titration mode.

Electrochemical measurements were performed with a PalmSens potentiostat driven by the PS Trace program (PalmSens® Instruments BV, Houten, The Netherlands). pH of buffer solutions was measured with a HI 221 pHmeter (HANNA Instruments, USA).

Quantitative analysis of BAs was carried out using an HPLC system (1260) from Agilent Technologies (Waldbronn, Germany) equipped with an auto-sampler, a quaternary pump and a diode array detector. Separation was achieved using a reversed phase

column (Chromolith (R) High Resolution RP-18 endcapped 100-4.6 mm (Merck KGaA, Darmstadt, Germany)). The detection was performed with a UV-Vis system set at 240 nm.

6.2.3. Manufacturing of screen-printed electrodes modified with TTF

Construction of the TTF/SPCEs was carried out in five steps according to previously described works [8, 16, 35, 36]. These devices involved a conventional three-electrode configuration using a pseudo reference electrode based on Ag/AgCl ink, a carbon ink counter electrode and a mixture of 3 % of TTF with carbon ink for the working electrode.

6.2.4. Immobilization of PUO on TTF/SPCEs

PUO enzyme was immobilized on TTF/SPCEs (PUO/TTF/SPCEs) using a crosslinking process with GA and BSA. A volume of 5 µL of a solution containing PUO (1.25 U μL^{-1}): BSA (3% w/v): GA (2.5% v/v) in the volume ratio 1:2:2 was deposited on the working electrode surface. Once deposited, the mixture was allowed to dry for 90 minutes at 4 ºC [16, 35].

6.2.5. Enzyme activity monitored by Isothermal Titration Calorimetric measurements

ITC involving continuous rate measurements following a single injection of substrate was used to assess kinetic parameters. The working electrode (with immobilized enzyme or without it) was introduced into the microcalorimeter cell (171 µL). This cell is interfaced with two highly sensitive thermistors capable of detecting small temperature changes within the cell. The heat evolution or absorption during a reaction is indicated by changes in potential (imbalance in the thermistor bridge in which the two thermistors measure temperature changes in the cell). Thus, when an exothermic reaction occurs, the calorimeter will sense an increase in energy and a positive signal will appear in the thermogram. The opposite is observed for an endothermic reaction.

After the introduction of the working electrode onto the microcalorimeter cell, a volume of 100 µL of phosphate buffer solution pH 10 was added. The system was allowed to equilibrate for 3 hours before the titration with substrate. Then, for calibration, a current is supplied, which generates an energy increase followed by a return to the original baseline value (exothermic process). 10 µL of the substrate solution (Put) was then injected after a time of 4000 s. A change in energy occurs followed again by a return to the original base line when the substrate is completely consumed after a time of approximately 3500 s. Several experiments were made using different substrate concentration.

The use of ITC to measure the amount of heat (Q) generated during an enzymatic reaction is well established. The rate of a reaction is directly proportional to the thermal power, defined as the heat produced as a function of time [22]:

$$Power = \frac{dQ}{dt} \qquad (1)$$

The amount of Q associated with n moles of substrate conversion is given by:

$$Q = n \cdot \Delta H_{app} = [P]_{Total} \cdot V \cdot \Delta H_{app} \qquad (2)$$

Where V is the volume of the solution in the reaction cell, $[P]_{Total}$ is the total molar concentration of generated product, and ΔH_{app} is the experimentally determined molar reaction enthalpy [27]. According to equations (1) and (2), the measured amount of heat as a function of time is given by the following equation:

$$Power = \frac{dQ}{dt} = \frac{d[P]}{dt} \cdot V \cdot \Delta H_{app} \qquad (3)$$

Therefore, reaction rate can be determined by the measured amount of heat generated during an enzymatic reaction:

$$Rate = \frac{d[P]}{dt} = \frac{1}{V \cdot \Delta H_{app}} \cdot \frac{dQ}{dt} \qquad (4)$$

According to Equation 4, it is clear that in order to obtain a Michaelis-Menten plot it is necessary to use a calorimeter to measure two parameters (dQ/dt and ΔH_{app}): The total molar enthalpy must be determined in experiments where there is sufficient enzyme in the cell to convert all the injected substrate into product in a given time period. The power generated (dQ/dt) must be determined at different substrate concentrations (above K_m). The following equation is used to determine ΔH_{app}:

$$\Delta H_{app} = \frac{1}{[S]_{Total} \cdot V} \int_{t=0}^{t=\infty} \frac{dQ(t)}{dt} dt \quad (5)$$

The substrate concentration for a specific time ($[S]_t$) is calculated according to:

$$[S]_t = [S]_0 - \frac{\int_{t=0}^{t} dQ/dt}{\Delta H_{app} V} \quad (6)$$

where $[S_0]$ is the initial concentration of the substrate (t = 0) [22].

6.2.6. Electrochemical Measurements of Put with PUO/TTF/SPCEs

All measurements were made at room temperature (20ºC approximately) in a cell containing 5 mL of the supporting electrolyte solution of the desired pH, under constant stirring. The amperometric detection has been performed by measuring the anodic current due to the oxidation of the mediator TTF operated at a potential of + 300 mV vs. a pseudo Ag/AgCl-SPE. During optimization procedure different potentials were tested. The characterizations of the biosensors, with the exception of specificity determinations, were done using Put.

6.2.7. Extraction of Put in Octopus and Zucchini Samples

Fresh octopus and zucchini samples were extracted with a simple preparation method, using an ethyl acetate-acetone mixture, according to the procedure described by Yigit *et al.* [37]. 5 g of the previously homogenized sample and 10 mL of a 5 % $HClO_4$ solution were mixed in a vortex mixer for 1 min. The mixture was placed in an ultrasonic bath for 10 min and then, centrifuged for 10 min at 7000 rpm. The supernatant was recovered and neutralized with a 3 % ammonia solution, saturated with NaCl. Next, it was extracted with 10 mL of an ethyl acetate-acetone (2:1) solvent system in a vortex mixer for 1 min. Subsequently, the mixture was briefly centrifuged. The organic phase was separated and, finally, the solvent was evaporated. For the sample to be used in HPLC analysis, before extraction a known amount of 1,7 diaminoheptane (internal standard, IS) was added.

6.2.8. DETERMINATION OF PUT WITH HPLC

HPLC analysis of extracts was adapted from the method described by Moret *et al.* [38], where derivatization with DCl is used. Briefly, 0.5 mL of saturated $NaHCO_3$ and 1 mL of DCl reagent (5 mg mL^{-1} in acetone) were added to 1 mL aliquot of the extract in a test tube. The mixture was carefully mixed using a vortex for 1 min and left in the dark for 1 h at 40°C, with occasional shaking. In order to eliminate the excess of DCl, the mixture was treated with 200 mL of a proline solution (100 mg mL^{-1}), vortexed for 1 min and left to react in the dark for 15 min at room temperature (20°C approximately). The sample was then extracted twice with 1 mL of diethyl ether. The combined extracts were dried under nitrogen flow and the residue was re-dissolved in acetonitrile for injection.

6.3. RESULTS AND DISCUSSION

One of the main focuses of this study is the development of a sensitive disposable biosensor for the selective determination of Put. For this purpose, PUO enzyme was immobilized on SPCEs modified with TTF. This immobilization was performed following the crosslinking procedure described above.

6.3.1. IMMOBILIZED ENZYME KINETIC PARAMETERS

A study of the biosensor in terms of immobilized enzyme kinetics was performed. The aim of this study was the determination of the enzymatic activity of immobilized PUO on the disposable electrode surface. The determination of different kinetic parameters was carried out using Put as substrate. As mentioned before, continuous rate measurements following a single injection of substrate were made by monitoring the chemical thermal power until substrate is completely consumed.

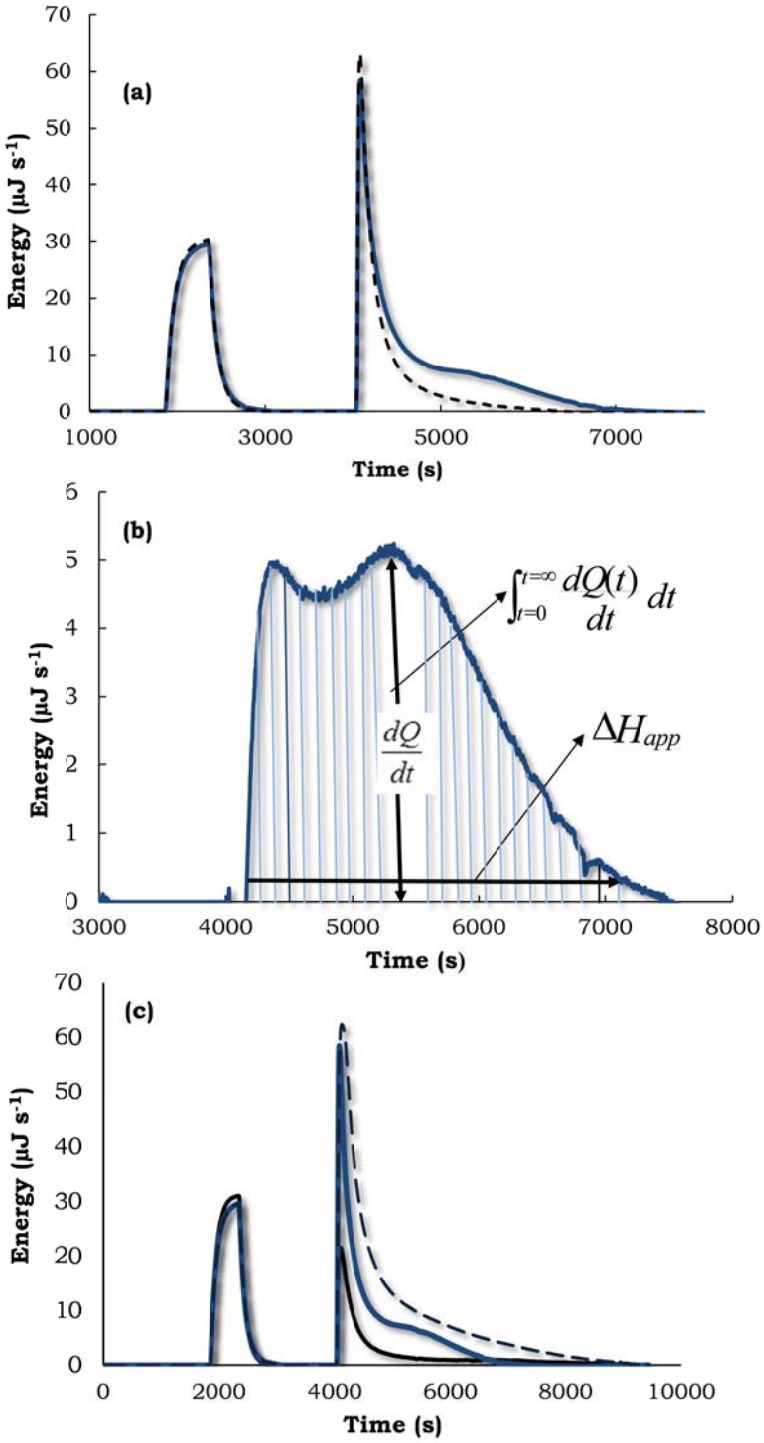


Figure 6.1. *Thermal power registered for (**a**) a TTF/SPCE modified with BSA and GA (—) and a TTF/SPCE modified with PUO immobilized by crosslinking using BSA and GA (—). (**b**) Change in thermal power for PUO catalyzed oxidation of Put. Injections of 10 mL of Put 40 mM (4000 s) in phosphate buffer pH 10. (**c**) Thermal power registered using a PUO/TTF/SPCE for injections of 10 mL of Put (—), 25 mM (—) 40 mM and (- -) 50 mM*

The change in the thermal power was collected from different experiments (in absence or presence of enzyme and using different initial substrate concentrations (Figure 6.1c)). In Figure 6.1a it can be seen the change in the thermal power as function of time using a TTF/SPCE, in absence of enzyme (dash line) and PUO/TTF/SPCE with immobilized enzyme (solid line). The first signal observed at 2000 s corresponds to the energy increase caused by the application of an initial current for instrumental system calibration. The change in thermal power that occurred after substrate injection (4000 s) can be attributed to heat of dilution and adsorption (in absence of enzyme, dashed line) plus heat of enzymatic reaction (in the presence of enzyme, solid line). Separated experiments revealed a major contribution from dilution (data not shown). The difference between both curves illustrates the calorimetric response of Put oxidation catalyzed by PUO (Figure 6.1b).

After an initial baseline a rapid increase in instrumental thermal power occurred because Put oxidation is an exothermic process. The thermal power remained constant for a while, as under these conditions the reaction was preceding at nearly maximum rate (V_{max}). Put depletion causes a decrease in rate resulting in a return of the power to the initial baseline when all substrate is consumed.

Raw data in Figure 6.1b were integrated to give ΔH_{app}, according to Equation 5, it was not necessary the correction of raw data by the instrument time constant, once the time course over which the enzyme reaction rate decays to zero is much larger than the instrument time response (<3 s). The initial substrate concentrations were well chosen above the K_m (the K_m value for the free enzyme was used as estimate) so that the substrate concentrations obtained from the change in thermal power curve (Figure. 6.1b) can be used to yield a complete Michaelis-Menten curve

with some points of the curve below K_m and some above it. Care should be taken when chosen the highest substrate concentration, so that oxygen does not become the limiting reagent during kinetics. Finally the continuous kinetic curve (Figure 6.2) was constructed taking into account Equations 4 and 6.

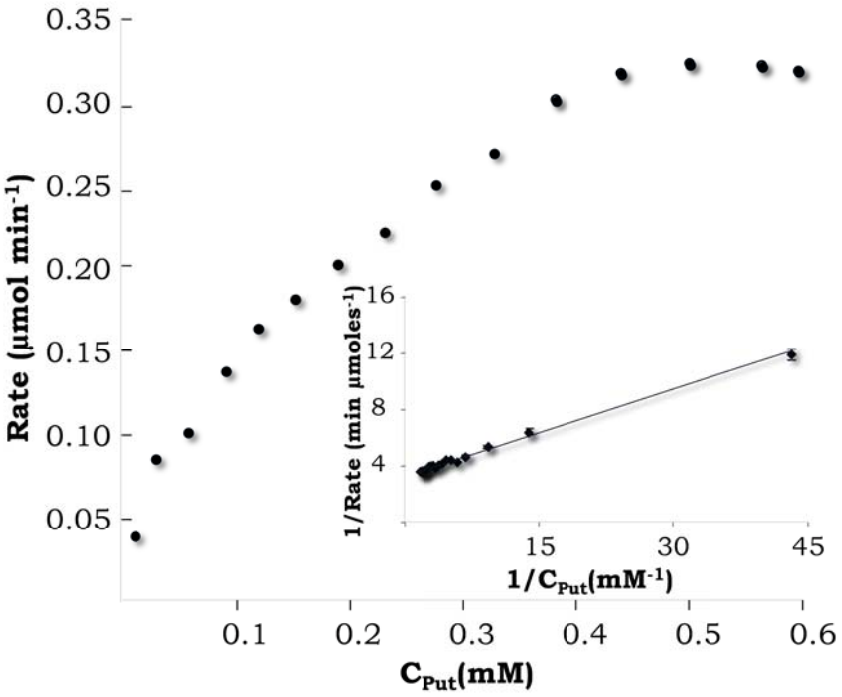


Figure 6.2. *Michaelis-Menten representation of immobilized PUO kinetic. Insert: Lineweaver-Burk plot*

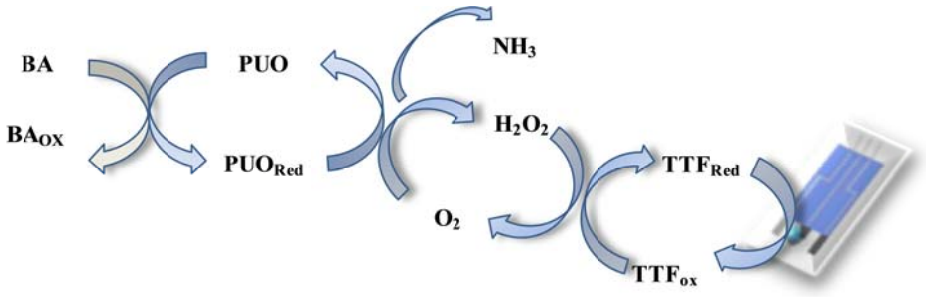
Kinetic parameters were obtained by Lineweaver-Burk linearization of the classical Michaelis-Menten kinetics (Figure 6.2). The K_m value for PUO found was 1.0 ± 0.2 x 10^{-4} M with a V_{max} of 5.9 ± 0.2 x 10^{-7} molmin^{-1}. Therefore, the affinity of the immobilized PUO (correlated to K_m) is lower than the one presented by the free enzyme in solution (K_m = 3.8 x 10^{-5} M). This loss of affinity due to the

immobilization procedure has been also observed in other electrochemical biosensors using a MAO enzyme [39].

Nevertheless, since the relationship between reaction rate and substrate concentration is fairly linear only for substrate concentrations below the K_m, its value (100 μM, for the immobilized enzyme) sets an upper limit for the linear response range of the biosensor, which was in fact below the K_m value (PUO/TTF/SPCEs showed a linear response range from 10 a 74 μM). Enzyme activity has been also reduced, yet the activity of the immobilized enzyme was enough to fully convert the substrate, producing a reproducible electrochemical signal.

6.3.2. AMPEROMETRIC DETERMINATION OF PUT

The developed PUO/TTF/SPCE biosensor produced an amperometric signal related to the mediator oxidation (Figure 6.3), which can be associated to the concentration of added Put according to the mechanism shown in Scheme 6.1 [16, 35]. Briefly, the BA is oxidized to aldehyde (BA_{ox}) through the action of the enzyme PUO at the developed biosensor. The hydrogen peroxide enzymatically generated is then oxidized by the mediator TTF_{ox}. The recorded signal corresponds to the electro oxidation of TTF_{RED} on the electrode surface.



Scheme 6.1. *Reaction occurring at surface of PUO/TTF/SPCEs biosensors during determination of Put*

The amperometric response obtained by means of PUO/TTF/SPCEs biosensors depends on several experimental parameters, which can define the sensitivity but also the selectivity of the developed biosensors. Therefore, an optimization procedure of these experimental variables was carried out. Applied potential and pH values were optimized by means of a 2^2 central composite design [40, 41], taking as response variable the intensity of oxidation corresponding to a 99 μM Put solution. To carry out this experimental design, a high and a low level were chosen for each of the factors to be optimized. Then 11 experiments derived from all the possible combinations of each factor were carried out and evaluated in terms of analysis of variance. The values which correspond to the high (+) and low (-) levels and to the central point for each factor were the following,

pH (+) = 11 $\quad E_{app}$ (+) = + 400 mV

pH (−) = 9 $\quad E_{app}$ (−) = + 100 mV

pH (0) = 10 $\quad E_{app}$ (0) = + 250 mV

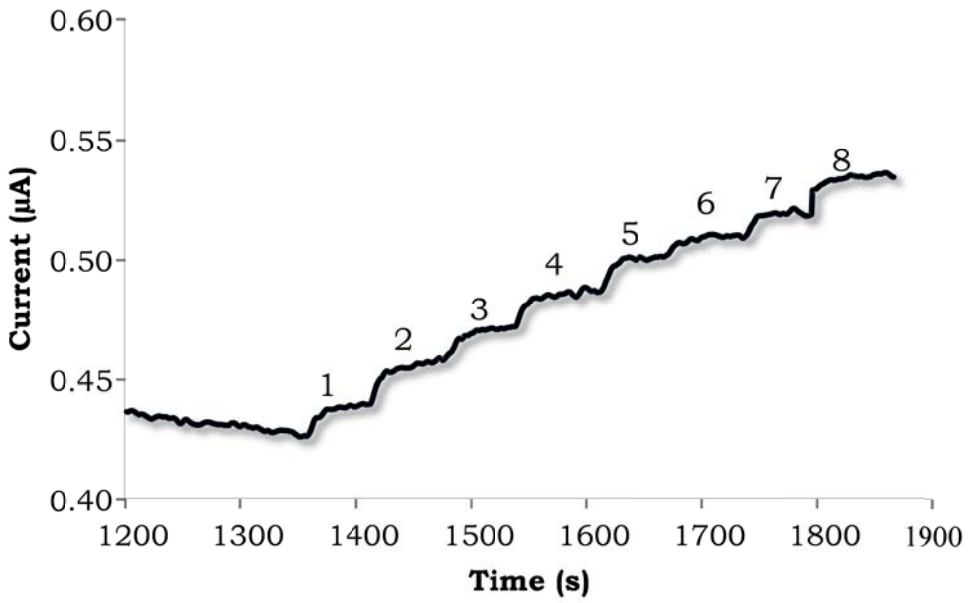


Figure 6.3. *Chronoamperogram recorded for determination of Put using a PUO/TTF/SPCE based biosensor. 1-8, additions of 50 µL of a 1 mM Put solution. pH, 10; E_{app}, + 300 mV vs Ag/AgCl- SPE*

These values were selected bearing in mind the results obtained in experiments prior to the optimization procedure. The optimum values obtained for the experimental variables were a pH value of 10 and an applied potential of + 300 mV vs. Ag/AgCl SPE as it can be seen in Figure 6.4. Once the optimum parameters for the analysis of Put using PUO/TTF/SPCEs biosensors were selected, the developed analytical procedure was characterized establishing its precision, as well as its capability of detection. In this way, the linear range of the developed biosensors was established from a concentration interval ranging from 10 up to 74 µM. Precision of the method was also calculated in terms of reproducibility. Thus, the RSD of the slopes of the calibration curves recorded using different biosensors was calculated. As it can be seen in Figure 6.5, the developed biosensor shows an acceptable reproducibility with an RSD value of 6.7%.

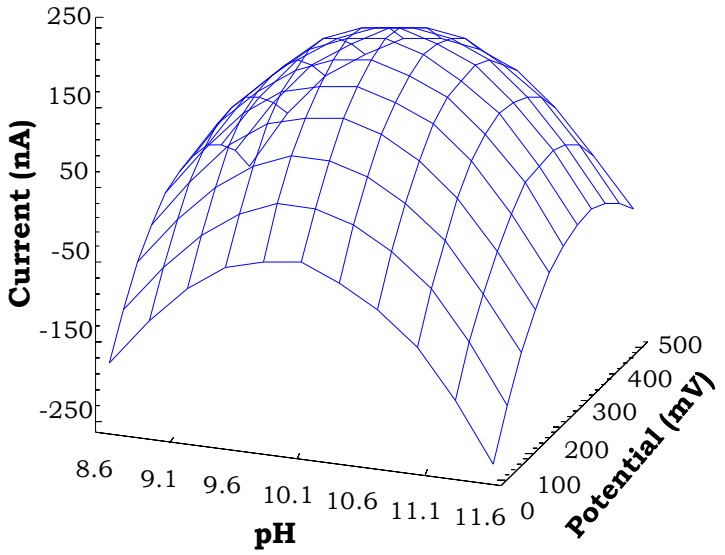


Figure 6.4. *Response surface for the 2^2 central composite design performed for the optimization of the experimental variables for the analysis of Put using biosensor PUO/TTF/SPCEs*

Finally, the capability of detection was calculate for a probability of false positive (α) and negative (β) equal to 0.05, according to the literature [42, 43]. This biosensor showed a capability of detection of 10.1 ± 0.6 μM.

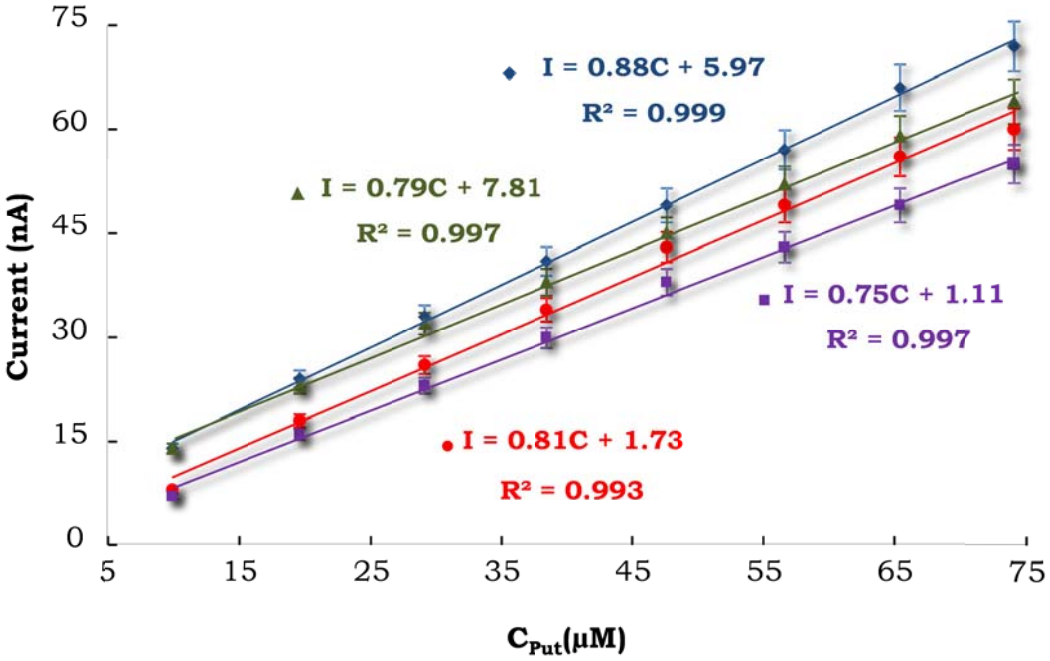


Figure 6.5. *Experimental points and regressions for the different calibration curves carried out to evaluate the reproducibility in the determination of Put using PUO/TTF/SPCEs.*

6.3.3. SELECTIVITY ANALYSIS OF PUO/TTF/SPCES BIOSENSORS

In order to study the selectivity of the developed biosensor, the amperometric response presented by other similar BAs to Put, was recorded using the PUO/TTF/SPCEs. The selected BAs in this work were those most commonly found in food samples, namely, Tyr, His, Cad, Spd, and Spm. Thus, the amperometric response obtained in the concentration range from 10 up to 200 µM was evaluated for each BA. Under the optimal operational conditions only Tyr interfered in Put determination at the concentration level of 150 µM. The interference of Tyr is attributed to direct oxidation of its phenol group at the applied potential. Thus the interference caused by Tyr takes place at concentration levels high enough to avoid problems in analysis of real samples. For samples with high content of Tyr, a

simple dilution step would be appropriate to eliminate the interference caused by such species.

6.3.4. APPLICATION OF PUO/TTF/SPCES BIOSENSORS IN FOOD SAMPLES

The developed biosensor was applied to the determination of Put in octopus and zucchini samples. The extraction of Put from these samples was performed according to the procedure previously described in the experimental section. The obtained extracts were dissolved in 4 mL of water. A volume of 200 and 50 µL of, respectively, octopus and zucchini extract solutions was placed into the electrochemical cell, containing 5 mL of buffer solution pH 10. Next, successive additions of 50 and 100 µL of a 1 mM Put solution were performed for octopus and zucchini analysis, respectively. The concentration of Put found was 14. 3 ± 0.4 mg kg^{-1} (n = 5, α = 0.05) for octopus and 25.5 ± 6.3 mg kg^{-1} (n = 3, α = 0.05) for zucchini samples. Finally the results, on these food samples, were compared with a reference method based on a HPLC analysis.

6.3.5. HPLC ANALYSIS OF FOOD SAMPLES

DCl-derivatives obtained following the procedure described in the experimental section were analyzed by HPLC. This analysis was performed using a mobile phase consisted of trichloroacetic acid (TCA) (solvent A) and acetonitrile (solvent B). The gradient elution program was held at 40% of B for 1 min, ramped at 70% (15 min), 100% of B (17 min) and held until the end of the run (23 min) with a flow rate of 0.8 mL min^{-1}. The column was thermostatized at 32 °C. As mentioned, 1,7-diaminoheptane was used as IS.

Detector response was linear in the concentration range from 1.0 up to 125.0 mg L^{-1}. Linearity was verified by the calculation of the RSD associated with response factors (peak area/concentration) calibration curves slopes. The RSD of response factors was less than 5% in all cases, ranging from 0.3 % to 4.9 %. The chromatograms obtained for a standard solution (10.0 mg L^{-1}) of different BAs and for octopus and zucchini samples are shown in Figure 6.6 a, b and c.

Under the stated experimental conditions, baseline separation of the six BAs was achieved in less than 23 min. No interfering peaks appeared at the retention times of the analytes. Amine identification was made on the basis of retention time by comparison with standard solution. The chromatograms presented low variability of the retention time and peak area, facilitating amine quantification. Food samples tested and the levels of the BAs found are shown in Table 6.2 In the analysis of octopus samples, a t-test for the means shows that the mean obtained by electrochemistry is different to the one obtained by HPLC at $\alpha = 0.05$.

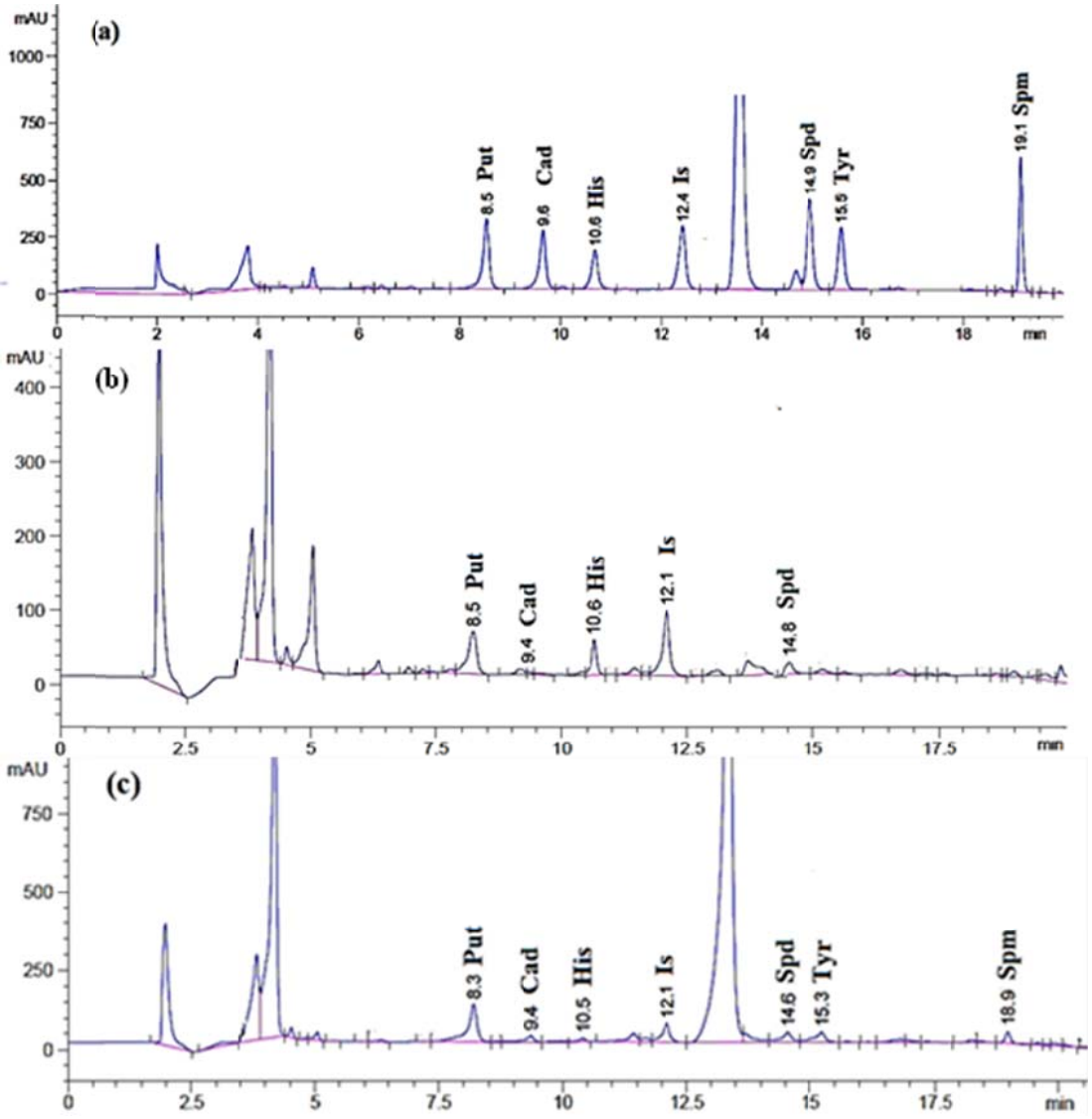


Figure 6.6. *HPLC chromatograms of (a) BAs standard solution (10 mg L^{-1} each), (b) octopus sample extracts and (c) zucchini samples extracts.*

However, it can be considered that the concentration of Put quantified in octopus samples using the developed biosensor (14.3 ± 0.4 mg kg^{-1}) matches the value obtained by HPLC (15.7 ± 1.5 mg kg^{-1}) considering the associated uncertainties. In the case of zucchini samples, both the mean obtained using PUO/TTF/SPCEs (25.5 ± 6.3 mg kg^{-1}) and that obtained by HPLC (26.6 ± 0.4 mg kg^{-1}) are shown to be equal since the null hypothesis of the t-test cannot be rejected at

the 95.0% confidence level. Thus, the proposed method is both accurate and suitable for the analysis of Put in complex samples.

Table 6.2. *BAs content in octopus and zucchini samples*

Sample	Method						
	PUO/TTF/SPCE biosensor	HPLC (mgkg-1, n =3, α =0.05)					
	Put (mg kg^{-1})	Put	Cad	His	Spd	Tr	Spm
Zucchini	25.5 ± 6.3 (n =3, a =0.05)	26.6 ± 5.4	4.3 ± 0.5	4.2 ± 2.2	3.2 ± 0.2	2.1 ± 0.2	7.4 ± 1.2
Octopus	14.3 ± 0.4 (n =5, a =0.05)	15.7 ± 1.5	2.6 ± 0.2	13.2 ± 1.9	1.2 ± 0.2	----	----

6.4. CONCLUSIONS

This work reports a disposable enzymatic biosensor for Put determination, using the enzyme PUO as biological element and SPCEs as electrochemical transducers. The main advantage of the developed biosensor is its greater specificity of the used enzyme towards this analyte than other previously described biosensors being its sensitivity similar to other PUO-based biosensors (Table 6.1). The suitability of the chronoamperometric method for monitoring Put content in food samples has been also demonstrated. Moreover, simple instrumentation of the electrochemical system makes this approach a viable alternative as analytical method to determine the Put content in complex samples.

The activity of immobilized PUO onto TTF/SPCEs has been monitored by ITC. The value of *Km* obtained for the immobilized enzyme differs slightly from the one related to the free enzyme. This fact is expected due to the immobilization procedure. Enzyme activity was also reduced, yet the activity of the immobilized enzyme was enough to fully convert the substrate, producing a reproducible electrochemical signal. ITC has been demonstrated to be a potential practical application in the analysis

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7. Dual Enzymatic Biosensor for Simultaneous Amperometric Determination of His and Put

Como se ha demostrado anteriormente utilizar un enzima altamente específico logra mejorar considerablemente la selectividad del biosensor desarrollado, es por ello que en este capítulo se propone el desarrollo de un biosensor altamente selectivo hacia la His mediante la inmovilización del enzima HMD, un enzima altamente específico y selectivo hacia la His, en el electrodo de trabajo. Además la incorporación de un mediador como TTF permite trabajar a potenciales bajos, y minimizar el riesgo de interferencias por la presencia de otros analitos en la muestra. Es aplicado satisfactoriamente en la cuantificación de His en muestras de vino tinto certificadas.

Además tal como se observó en el capítulo 5, es posible utilizar un biosensor dual para la determinación conjunta de dos analitos simultáneamente, aplicando este principio se propone la construcción de un biosensor para la determinación conjunta de His y Put basado en la combinación de dos biosensores en un mismo dispositivo electródico, uno para la determinación selectiva de Put utilizando el enzima PUO (PUO/TTF/SPCEs), y el otro utilizando el enzima HMD para la determinación selectiva de histamina (HMD/TTF/SPCEs). El dispositivo así desarrollado permite la determinación simultánea y altamente selectiva de las dos BAs en muestras complejas de diferentes alimentos.

Parte de este trabajo ha sido presentado en la *XXXVI Reunión del Grupo de Electroquímica de la Real Sociedad Española de Química y XV Encontro Ibérico*, celebrada en Valencia del 15 al 17 de Julio de 2013. Finalmente este trabajo ha sido publicado en *Food Chemistry* 190 (2016) 818–823.

7.1. INTRODUCTION

BAs are organic bases with different structures that are mainly produced by microbial decarboxylation of their precursor amino acids [1]. These compounds are considered indicators of an earlier microbial decomposition in fish or shellfish, being also present in a great variety of food samples [2, 3] and beverages [4-7] such as meat, beer, chocolate, cheese or wine. The most important BAs related with spoilage in food are His, Put and Cad; the former being one of the most biologically active compounds [8]. The toxicological level of BAs is very difficult to establish. Nevertheless, a maximum total BAs (the sum of Tyr, His, Put, and Cad) level of 750 – 900 mg kg^{-1} has been proposed as a safety limit [9]. At low concentration levels, BAs may be considered essential for many physiological functions [10]. However, if these compounds are consumed in high quantities, several toxicological problems arise. The symptoms of this foodborne chemical intoxication are allergy-like, characterized by cutaneous rash, gastrointestinal and neurological aspects such as nausea, vomiting, diarrhea, head-aches, palpitations, flushing, tingling, burning and itching, or by hypertension [11].

Analytical determination of BAs in food samples is not simple due to the great variety of chemical structures and the level of concentration at which these compounds are present in such complex matrices. HPLC is one of the most often techniques used to determine these amines due to its high resolution and sensitivity [10, 12]. However, this technique usually requires sample preparation including a time-consuming pre-column derivatization. Electrochemical systems may be then presented as an interesting alternative for detecting BAs due to their simplicity, low cost,

effectiveness and short time in the analysis of these kinds of compounds. In this way, electrochemical sensors based on the use of imprinted polymers have been developed for the selective determination of different BAs [13, 14]. Electrochemical biosensors have been described as more simple devices in the determination of BAs in different samples. Thus, enzyme modified biosensors have attracted enormous attention in the electroanalysis of BAs during the recent years [1, 8, 15-23]. As can be seen in Table 7.1, the main problem of the described biosensors comes from the low enzyme selectivity and the high working potentials needed for operation, leading to an increase in the effect caused by the presence of different interfering species.

Nowadays, the main efforts in the development of biosensors for BAs are focused on the improvement of their sensitivity, but also of their selectivity, based upon the specificity of the enzyme used. In this work, two highly selective and specific enzymes were used for the analysis of Put and His, namely PUO from *Micrococcus rubens* and HMD, respectively. These selected enzymes are pure active enzymes much more specific than the commonly used AOs in the construction of biosensors for the determination of BAs [7, 24].

Table 7.6. *Electrochemical biosensors for the determination of BAs based on SPEs*

Enzyme	Electrode	Mediator	E (mV)	Capability of detection	Selectivity	Sample	Ref.
PAO	SPCE	Ferrocene	+260	2.0 ± 0.18µM (Tyr)	Total BAs	Brie Cheese	[2]
PAO TAO DAO	Array of SPPtE	-	+700	10 (His,Tyr) 5 (Put) mg7Kg	Total BAs	Fish and meat	[10]
DAO	SPAuE	-	+600	0.2 mg/L (Put)	Total BAs	Wine and beer	[13]
DAO and HRP	SPCE	Ferrocene	-50	0.17 µM (His)	-	Fish	[17]
MAO	SPCE	TTF	+250	17.2 ± 4.6 µM(Put)	Interference Cad and Put	Zucchini and anchovies	[20]
PSAO	SPCE	MnO_2	+400	0.3 µM(Cad and Put)	Total BAs	Chicken	[21]
MAO	Array of SPCE	TTF	+250	9.9 µM (Put) 19.9 ± 0.9 µM (Cad)	Interfernece Tyr	Octopus	[22]
DAO and HRP	SPCE	Ferrocene	+250	0.4 µM (His)	Total BAs	Anchovies	[25]
DAO	SPCE	Potassium ferricyanide	+350	0.65ppm (His)	-	Prawn	[23]
DAO	SPCE	PB	-50	10 µM (Put)	Total BAs	Human saliva	This work

SPCEs have been used as electrochemical transducers, since they increase the above described advantages of the electrochemical biosensors in the determination of BAs such as His and Put [1, 2, 4, 8, 18, 20, 22, 25-29]. TTF has been used as electrochemical mediator in order to decrease the working potential in the amperometric measurements. TTF was screen-printed by incorporating it into the carbon ink. PUO and HMD enzymes were immobilized on the TTF modified SPCEs (TTF/SPCEs) by crosslinking with GA and BSA. The main novelty of this work resides in the employment of a dual electrode system consisting of two different working electrodes (PUO/TTF/SPCE and HMD/TTF/SPCE), which allowed the simultaneous determination of Put and His. This dual system represents, not only a simple method for the determination of Put and His but also, offers a rapid simultaneous determination of both BAs reducing the time needed for analysis. This last feature makes the developed method suitable for application in routine analysis in food control.

7.2. EXPERIMENTAL

7.2.1. REAGENTS

All reagents used were of analytical-reagent grade. Ultrapure water obtained from a Milli-Q water purifier (Millipore, Bedford, MA, USA) was used for the preparation of all solutions. The supporting electrolyte used for the measurements was a 100 mM KH_2PO_4 buffer solution (Sigma-Aldrich, Steinheim, Germany) containing 100 mM

KCl (Merck, Darmstadt, Germany). The pH value was adjusted with a 1M NaOH solution (J.T. Baker, Deventer, The Netherlands).

HMD (EC: HMD-IFO12069, activity 0.84 U/μL) was obtained from Biolan Microbiosensores (Scientific and Technological Park of Bizkaia, Zamudio, Spain), PUO from *M. rubens* (EC 1.4.3.10, activity 1.25 U/μL) was obtained from Inbiotec (León, Spain). BSA and GA, used in the immobilization procedure, were purchased from Sigma-Aldrich (Steinheim, Germany). Put and Cad were obtained from Sigma-Aldrich (Steinheim, Germany) as well. Tyr and His were acquired from Fluka (Steinheim, Germany). Other BAs, including spermidine (Spd), spermine (Spm) and Tryp were purchased from Acros Organics (Geel, Belgium).

Different inks were used to construct the TTF/SPCEs. C200802P2 carbon ink and D2071120D1 dielectric ink were obtained from Gwent Electronic Materials (Torfaen, UK). Silver 5029 conductor paste was purchased from Dupont Limited (Bristol, UK). Electrodag 6037 SS (Ag/AgCl ink) from Acheson Colloiden (Scheemda, The Netherlands) was also used. TTF was provided by Sigma-Aldrich (Steinheim, Germany).

$HClO_4$ from Panreac (Barcelona, Spain), ethyl acetate and NaCl from Fluka (Steinheim, Germany), ammonia solution, acetone, HCl and Na_2SO_4 from Merck (Darmstadt, Germany) were used for BAs extraction in food samples.

$NaHCO_3$ (Panreac, Barcelona, Spain), dansyl chloride (Sigma-Aldrich, Madrid, Spain) reagent (5 mg mL^{-1} in acetone), proline (Sigma-Aldrich, Madrid, Spain) solution (100 mg mL^{-1}), diethyl ether (Fisher Chemical, Loughborough, UK) and acetonitrile (Sigma-Aldrich, Madrid, Spain) were used in the derivatization procedure of the samples for HPLC analysis.

Fresh octopus and red wine samples were obtained from a local market. Certified canned fish aqueous extracts samples from FAPAS® (8.7 mg kg^{-1}) were also analysed.

7.2.2. APPARATUS

Electrochemical measurements were performed with a PalmSens potentiostat driven by the PS Trace program (PalmSens® Instruments BV, Houten, The Netherlands). pH of buffer solutions were measured with a HI 221 pHmeter (HANNA Instruments, USA). The TTF/SPCEs were produced on a DEK 248 printing machine (DEK, Weymouth, UK) using screen polyester mesh and polyurethane squeegees.

Quantitative analysis of BAs was carried out using an HPLC system (1260) from Agilent Technologies (Waldbronn, Germany) equipped with an auto-sampler, a quaternary pump and a diode array detector. Separation was achieved using a reversed phase column (Chromolith (R) high Resolution RP-18 endcapped 100-4.6 mm (Merck KGaA, Darmstadt, Germany)). The detection was performed with a UV-Vis system set at 240 nm.

7.2.3. BIOSENSOR PREPARATION

Two different electrodic systems have been used in this work: The first one (System I) was used in the His determination and the second one (System II) in the His and Put simultaneous determination.

(System I): A three SPEs configuration system was used for the determination of His consisting of a Ag/AgCl SPE, a carbon ink

(counter electrode), and a carbon ink modified with 5% TTF (working electrode). This electrodic system was constructed following previously described procedures [22, 30-33]. HMD was then immobilized on TTF/SPCEs, obtaining HMD/TTF/SPCEs for the determination of His. The immobilization of the enzyme was performed using a crosslinking process with GA and BSA. A volume of 5 µL of a solution containing HMD (0.84 U/µL); BSA (3% w/v prepared in 100 mM pH 6 phosphate buffer) and GA (2.5% v/v prepared in water), in the volume ratio 1:1:2 was deposited on the working electrode surface. Once deposited, the mixture was allowed to dry for 90 minutes at 4 ºC.

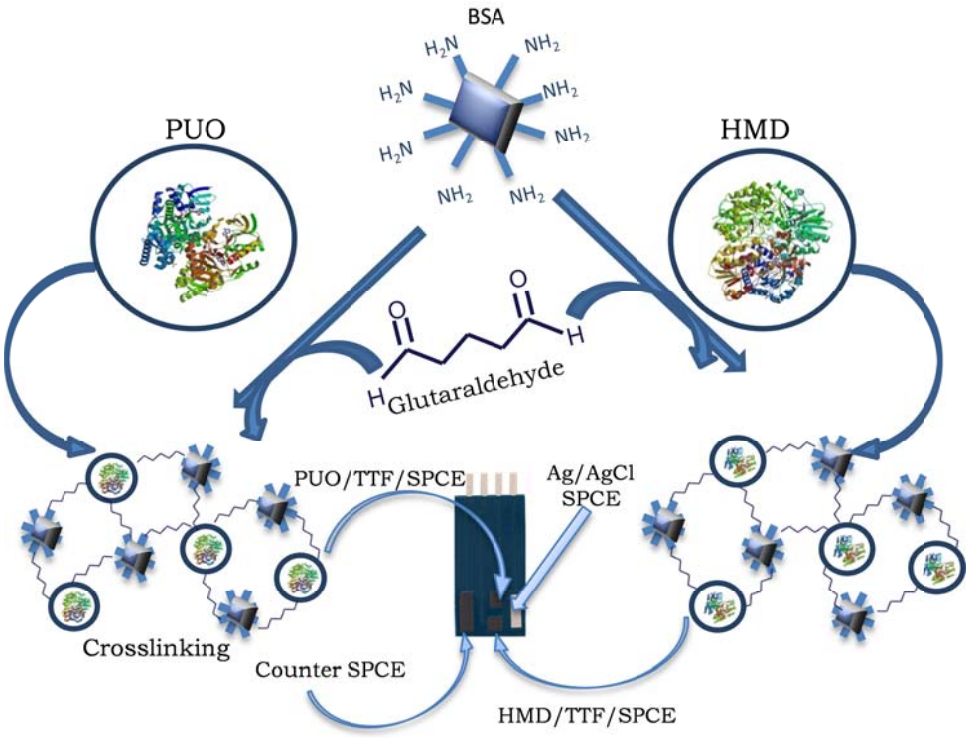


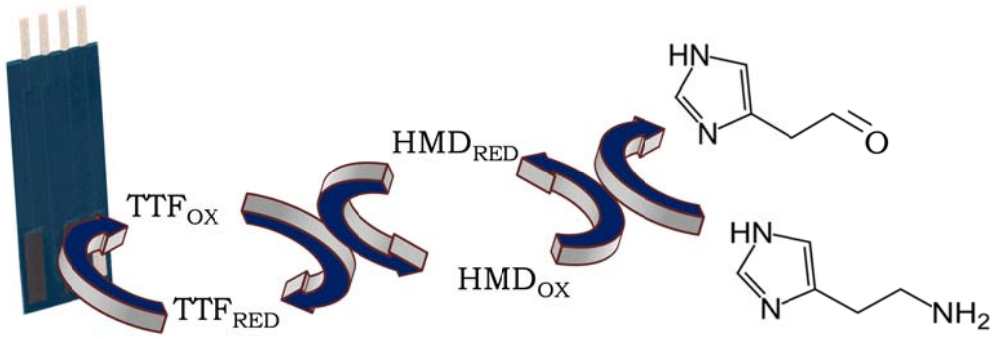
Figure 7.1. *Scheme of a homemade dual-TTF/SPCE (1.3 cm x 2.8 cm)*

(System II): A four SPEs configuration system was used for the simultaneous determination of His and Put consisting of two different working electrodes (dual-TTF/SPCEs) following the procedure

described in previous works [22, 34, 35]. This fabrication procedure is similar to the one used in the conventional three-electrode configuration development, including a second screen-printed carbon working electrode. One working electrode was modified with HMD (HMD/TTF/SPCEs) and the other one with PUO (PUO/TTF/SPCEs) by crosslinking using GA and BSA (Figure 7.1). HMD immobilization was performed by depositing a volume of 5 µL of a solution containing HMD (0.84 U/µL); BSA (3% w/v prepared in 100 mM pH 6 phosphate buffer) and GA (2.5% v/v prepared in water) in the volume ratio 1:2:2, on one working electrode surface. In the same way, PUO immobilization was carried out by depositing a volume of 5 µL of a solution containing PUO (1.25 U/µL):BSA (3% w/v prepared in 100 mM pH 6 phosphate buffer):GA (2.5% v/v prepared in water) in the volume ratio 1:2:2, on the other working electrode surface. Once deposited, the mixture was allowed to dry for 90 minutes at 4 ºC.

7.2.4 ELECTROCHEMICAL MEASUREMENTS

Amperometric measurements using system I, for the determination of His, were made at room temperature (aprox. 20 ºC) in a cell containing 5 mL of the supporting electrolyte solution (100 mM phosphate buffer and 100 mM KCl) of the desired pH, under constant stirring. The amperometric detection was performed by measuring the anodic current due to the oxidation of the mediator TTF (Scheme 7.1), operated at a potential of +130 mV *vs* Ag/AgCl SPE, except for the experimental variables optimization process.



Scheme 7.1. *Enzymatic mechanism of HMD/TTF/SPCEs biosensors*

His and Put were simultaneously determined using a dual-TTF/SPE (system II) based on the amperometric measurement of the anodic current due to the oxidation of the mediator TTF in both working electrodes. The above-described experimental conditions for HMD/TTF/SPCEs in system I, were also used for the amperometric determination of His using the dual system. In the case of amperometric measurements of Put, the working potential was fixed at +300 mV, as it has been described in a previous work for the single analysis of this compound [36].

7.2.5 SAMPLE PREPARATION

Extraction of His and Put in fresh octopus samples was carried out using an ethyl acetate-acetone mixture, according to procedure described for Yigit *et al.* [37]. In this way, 10 g of the previously homogenized sample and 10 mL of a 5 % $HClO_4$ solution were mixed in a vortex mixer for 1 min. The mixture was placed in an ultrasonic bath for 10 min and then, centrifuged for 10 min at 7000 rpm. The supernatant was recovered and neutralized with a 3 % ammonia solution. Then, it was saturated with NaCl and extracted with 10 mL of an ethyl acetate–acetone (2:1) solvent system in a

vortex mixer for 1 min. Then the mixture was briefly centrifuged, and the organic phase was separated. Finally, the solvent was evaporated.

In the case of wine samples a previous pre-treatment step with activated carbon was necessary. In this way, 50 mg of this compound were added to 1 mL of the wine sample. The mixture was then centrifuged at 13000 rpm for 2 minutes. The supernatant was finally analysed using the developed biosensors.

7.3. RESULTS AND DISCUSSION

7.3.1. DETERMINATION OF HISTAMINE USING SYSTEM I

7.3.1.1 Optimization and characterization process

The developed HMD/TTF/SPCEs biosensors produced an amperometric signal related to the mediator oxidation (Figure 7.2 (a) and (b)), which can be associated with the concentration of His.

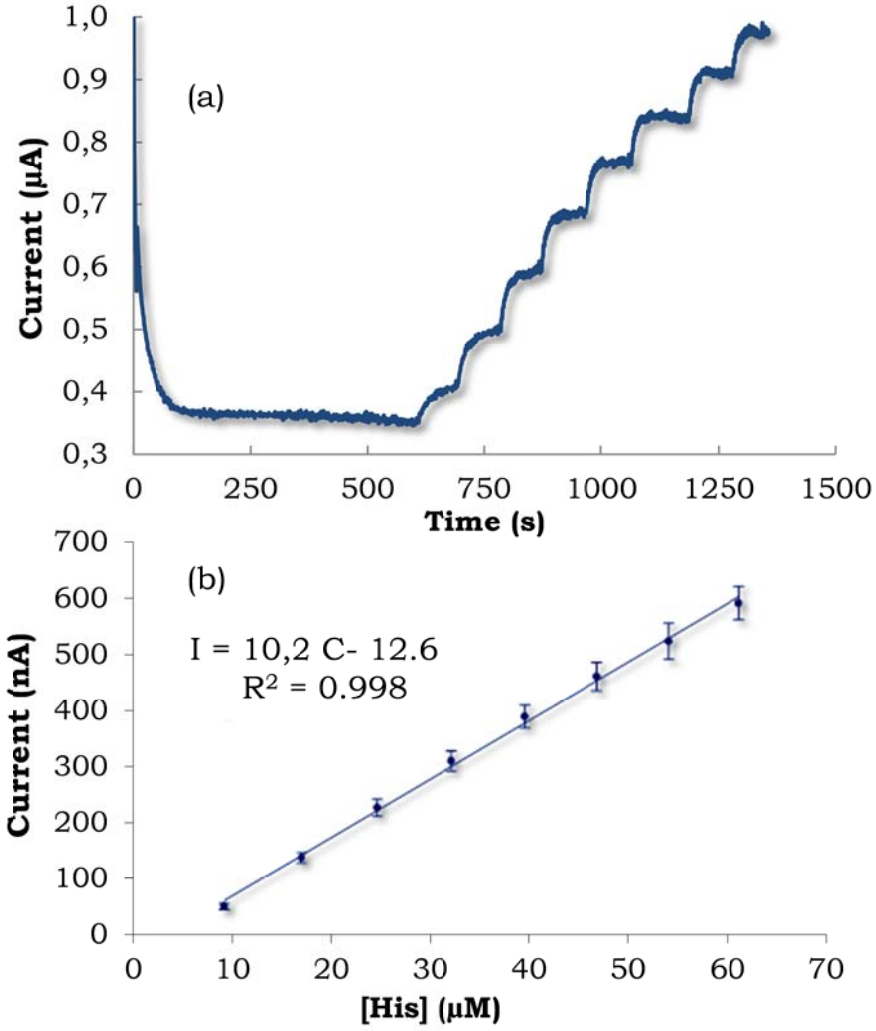


Figure 7.2. ***(a)*** *Chronoamperogram recorded on HMD/TTF/SPCEs for successive additions of 40 µL of a 1 mM His solution. (pH, 6.8; working potential, + 130 mV vs Ag/AgCl SPE).* ***(b)*** *Relation between amperometric current intensity and concentration of His in HMD/TTF/SPCEs biosensors (pH, 6.8; working potential, + 130 mV vs Ag/AgCl SPE)*

The amperometric response obtained by means of these biosensors depends on several experimental parameters, which can define the sensitivity but also the selectivity of these systems. The most influence experimental factors are pH value of the buffer solution and the working potential. Therefore, an optimization procedure of these experimental variables was carried out. These factors were optimized by means of a 2^2 central composite design,

taking as response variable the intensity of oxidation obtained for a 100 μM His solution. The values which correspond to the high (+) and low (-) levels and to the central point (0) for each factor were:

Working potential (+) = + 300 mV pH (+) = 9

Working potential (-) = -100 mV pH (-) = 5

Working potential (0) = 100 mV pH (0) = 7

Then 11 experiments derived from all the possible combinations of each factor were carried out and evaluated in terms of analysis of variance. The combination of factors levels that maximizes the response over the experimental region were a pH value of 6.8 with a working potential of +130 mV *vs* a Ag/AgCl SPE for the determination of His using HMD/TTF/SPCEs.

Under these optimized conditions, calibration parameters were calculated by the robust regression LMS using the program Progress [38]. LMS presents the advantage of being able to detect anomalous points. Once those anomalous points are removed from the calibration set, a regression based on the least squared criterion was performed in order to obtain a correct evaluation of the slope (sensitivity) and the independent term of calibration. Both terms are important for judging the quality of calibration and, indeed, the analytical performance [39]. In this way, a linear response in the range from 8 to 60 μM was obtained for His. The precision of the method was also calculated in terms of reproducibility and repeatability using the slopes of three calibration sets constructed using different biosensors and the same biosensor, respectively. The sensors showed an acceptable RSD value of 3.5 % (n= 3) and 2.5 % (n=3) for reproducibility and repeatability, respectively. The capability

of detection was calculated for a probability of false positive (α) and negative (β) equal to 0.05, according to the literature [40]. This biosensor showed a capability of detection of 8.1 ± 0.7 μM for His.

7.3.1.2 Analytical application

The developed HMD/TTF/SPCEs biosensors were applied in the analysis of His content in fish and red wine samples. In this way, the viability of the HMD/TTF/SPCE biosensor was tested in the determination of the content of His in samples of red wine (9.3 mg L^{-1} (83.7 μM) of His, tested by HPLC), using the method of standard additions.

The presence of possible interferents in wine samples was eliminated by using activated carbon as it has been described in section 7.2.5. Different quantities of this compound were tested in order to find the best possible analytical conditions. In this way, 4, 8, 16, 50 and 100 mg of activated carbon were added to 1 mL of red wine and, a 50 mg treatment was found to be adequate.

Chronoamperograms were then recorded under the optimum conditions by means of an addition of a volume of 100 μL of pretreated wine into the electrochemical cell, containing 5 mL of buffer solution at pH 6.8. Next, successive additions of 40 μL of a 1 mM His solution were performed. The concentration of His found was 9.0 ± 0.7 mg L^{-1} (α=0.05, n=3). This value is in good agreement with the one obtained by HPLC.

A similar procedure was applied for the analysis of a certified Canned Fish sample (FAPAS®, 8.7 mg kg^{-1}). The standard addition method was performed by means of the construction of calibration sets based on a first addition of 100 μL of FAPAS® into the

electrochemical cell, containing 5 mL of buffer solution pH 6.8. Then, successive additions of 40 μL of a 1 mM His solution, were carried out. The concentration reported for this sample was 8.9 ± 0.4 mg kg^{-1} (α=0.05, n=4), which agrees with the value certified by the supplier. Therefore, the developed HMD/TTF/SPCE is suitable for use in the determination of His in these types of complex samples, considering the associated uncertainties.

7.3.2. SIMULTANEOUS DETERMINATION OF HISTAMINE AND PUTRESCINE USING A DUAL-TTF/SPCEs BIOSENSOR (SYSTEM II)

The main objectives of this work has been the development of an electrochemical method for the simultaneous determination of His and Put. In this way, the dual-TTF/SPCEs system constructed as it has been described in section 7.2.3 allowed the selective analysis of both BAs in a same sample.

As it has been described above, the optimum pH value for the analysis of His using HMD/TTF/SPCEs is 6.8. However, the determination of Put using PUO/TTF/SPCEs implies working at pH 10 values. Thus, a study of the optimum conditions for the simultaneous determination of both BAs was necessary. In this way, a pH 8 value was found to be adequate to obtain chronoamperometric responses with the necessary analytical quality

The dual-TTF/SPCEs system resulted very selective towards His and Put determination. In this way, the response of both working electrodes (HMD/TTF/SPCE and PUO/TTF/SPCE) towards different BAs was analyzed. In the case of HMD/TTF/SPCE, the analysis was performed in the concentration range from 10 up to 200 μM for each BA. The BAs selected in this work were those most commonly found

in food samples, namely Tyr, Cad, Put, Spd, Tryp and Spm. Under optimal operational conditions, the HMD/TTF/SPCE biosensor presented a slight signal for Put at concentration levels higher than 190 µM, this signal corresponds to 3 % of that recorded for His in the same concentration (Figure 7.3). In the same way the PUO/TTF/SPCE resulted very selective for the analysis of Put being only Tyr considered as an interferent at the concentration level of 150 µM.

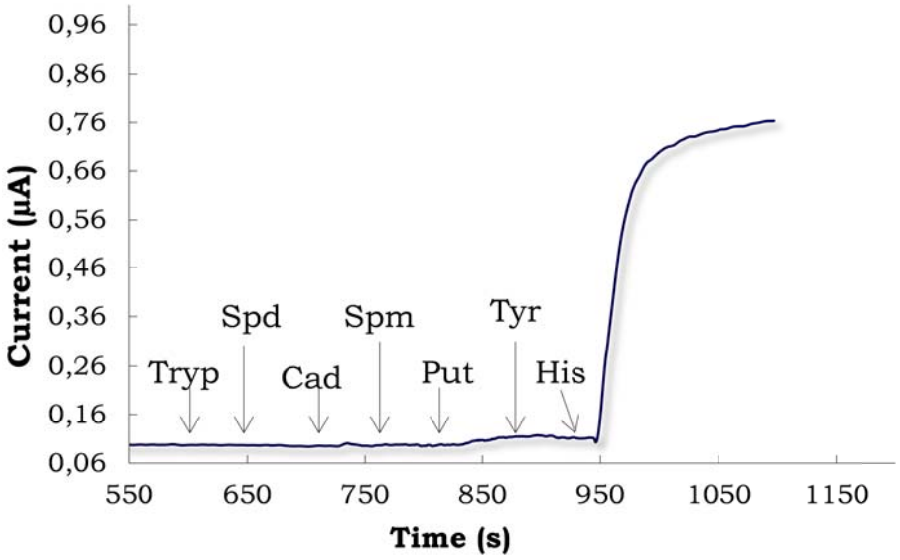


Figure 7.3. *Chronoampeometric response obtained on HMD/TTF/SPCEs for different BAs at a190 µM concentration level (pH, 6.8; working potential, +130 mV vs Ag/AgCl SPE)*

The performance of the dual system has been also demonstrated by means of its application in the simultaneous analysis of His and Put in a complex matrix such as an octopus sample.

Figure 7.4 shows the amperometric response recorded using the dual-TTF/SPCE system II developed biosensor for the simultaneous determination of His and Put by standard addition in

octopus samples. The octopus samples analyzed were previously treated according the procedure described in section 7.2.5. The extract obtained was dissolved in 2 mL of water. A volume of 200 µL of this extract was placed into the electrochemical cell, containing 5 mL of buffer solution pH 8, and following by successive additions of 20 µL of a 10 mM Put and 15 mM His solution.

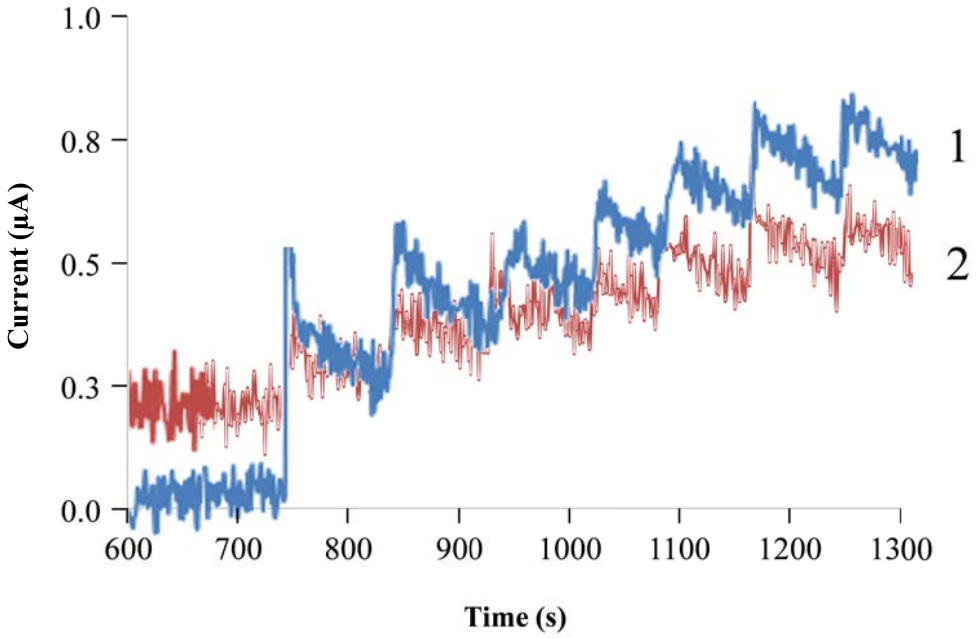


Figure 7.4. *Chronoamperograms recorded for the simultaneous determination of His and Put in octopus samples using a dual-TTF/SPCEs biosensor. (1) Amperometric response of Put on PUO//TTF/SPCE (pH, 8.0; working potential, +300 mV vs Ag/AgCl SPE) and (2) Amperometric response of His on HMD/TTF/SPCE. (pH 8.0; working potential, +130 mV vs Ag/AgCl SPE). The first addition (720 s) corresponds to 200 µL of the sample. Successive additions correspond to a volume of 20 µL of a 10 mM Put and 15 mM His solution*

The levels of Put and His found in octopus samples are shown in Table 7.2. These values are in good agreement with those obtained by HPLC. In this context, the concentration values obtained using dual-TTF/SPCEs and those obtained by HPLC are shown to be equal since the null hypothesis of the t-test cannot be rejected at the 95%

confidence level. Thus, the developed dual biosensor is both accurate and suitable for the simultaneous determination of Put and His in complex samples.

Table 2. Content of His and Put in octopus samples.

Biogenic Amine	Analytical Method	
	dual-TTF/SPCEs (mg kg^{-1}; α=0.05, n=3)	**HPLC (mg kg^{-1}; α=0.05, n=3)**
Put	16 ± 1	16 ± 1
His	15 ± 1	13 ± 2

7.4. CONCLUSIONS

This work reports the development of a novel and very sensitive and selective biosensor for the determination of His based on the modification of SPCEs with HMD. The developed biosensor shows two important advantages: On one hand its disposable character, which makes it very useful in routine analysis of His. On the other hand the selectivity is sufficiently high to meet all analytical requirements for food samples. This selectivity is based on the employment of TTF as mediator but also in the very selective enzyme (HMD) used. The suitability of the disposable biosensor has been also demonstrated in the successful determination of His in samples of wine and fish requiring a very simple preparation of the sample.

Moreover the combination of two different biosensors in a dual electrodic system has permitted the simultaneous determination of two different BAs, Put and His, even in complex samples such as

wine or fish. The described electrochemical dual biosensor allows a very novel, sensitive and selective determination of both compounds using a very simple methodology, which would permit a rapid screening and monitoring of both analytes in food industry.

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8. Resolution of Quaternary Mixtures of Cadaverine, Histamine, Putrescine and Tyramine by Square Wave Voltammetry and Partial Least Squares Method

Los biosensores desarrollados en los capítulos anteriores han permitido llevar a cabo con éxito la determinación sensible de BAs en diversas muestras. Además se ha logrado la determinación selectiva y simultánea de dos BAs Put y Cad y His y Put. En este capítulo se ha desarrollado un sensor que permite la determinación simultánea de cuatro BAs en una misma muestra (Cad, His, Put y Tyr). Dicho sensor se basa en la utilización de un electrodo de diamante dopado con boro empleando como técnica de análisis la voltamperometría de onda cuadrada (SVW) y empleando la metodología de calibración multivariante mediante mínimos cuadrados parciales (PLS). El método desarrollado permite el análisis sencillo y simultáneo de dichas BAs en muestras de alimentos como atún, jamón y calabacín.

El trabajo recogido en este capítulo ha sido publicado en *Talanta* 143 (2015) 97–100.

8.1-. INTRODUCTION

BAs are organic, basic nitrogenous compounds of low molecular weight, usually formed by the removal of the alpha-carboxyl group from a proteinogenic amino acid as a result of normal metabolic activities in humans, animals, plants and microorganisms. They are frequently present in many foods and beverages including cheese, sausages, soy sauces, fish, fermented fish, wine, and beer, being His, Tyr, Put, Cad, Spm and Spd the most important BAs occurring in foods [1, 2]. The intake of foods or beverages containing a high concentration of BAs may cause food intoxication with different symptoms [3]. In this way, His is the causing agent of the food-borne chemical hazard called scombroid poisoning. Scombroid poisoning is usually a mild illness with a variety of symptoms, including rash, urticaria, nausea, vomiting, diarrhea, flushing, and tingling and itching of the skin [4]. Tyr is a potent vasoconstrictor, which may induce hypertension, migraines, brain hemorrhage and heart failure when high concentrations are present in an organism [5]. Put and Cad can react with nitrite to form carcinogenic nitrosamines [6]. Moreover, a high level of BAs indicates poor quality of raw materials, contamination with micro-organisms or improper processing, and storage in food industry [2]. Therefore, the development of sensitive methods for their analysis is of great importance.

Numerous authors have dealt with the detection of BAs content in different types of foodstuffs, under different conditions of treatment and storage, by means of HPLC methods, thin layer chromatography, gas chromatography, and capillary electrophoresis [7-10]. The above-cited techniques require in most cases tedious pre-treatment and derivatization steps of the sample, as well as expensive

and complex instrumentation. Consequently, these techniques result not suitable for in situ analysis. Electrochemical biosensors have been often developed for the determination of BAs. These electrochemical systems are based on the use of commercial AOs enzymes as biological recognition elements for detecting BAs. In this way, PAO [2, 11], DAO [11-17] and MAO [15, 17-19] have been successfully applied in the determination of different BAs. The main problem of the described biosensors come from the poor enzyme selectivity towards the different BAs and/or the high working potentials needed for operation, leading to an increase in interferences [20]. In order to improve the selectivity of biosensors more specific enzymatic systems have been used in the construction of these electrochemical biosensors [21-25]. However, these systems present a higher cost associated with their manufacture, and limit the analysis to only one amine.

The determination of BAs by means of voltammetric techniques has been limited by the high oxidation potentials of these compounds. However, diamond thin-film and BDD electrodes have been successfully applied to the voltammetric determination of different polyamines [26]. The attractive features of conductive BDD include a wide electrochemical potential window in aqueous [27, 28] and non-aqueous [29], very low capacitance [30], and extreme electrochemical stability media [31]. These properties have made BBD electrodes promising electrochemical systems for the electrochemical analysis of different compounds [26, 32-35].

In this work a BDD electrode has been used for the simultaneous determination of His, Tyr, Put and Cad by SWV. The analysis of these BAs in the same sample is complex due to their close oxidation potentials, which lead to high ovelapped signals. Thus, multivariate calibration techniques are then neccesary in order

to achieve the simultaneous analysis of these compounds. In this way, a PLS regression has been performed, which has been succesfuly proven in the resolution of overlapped signals in electrochemical techniques [36-38].

8.2. EXPERIMENTAL

8.2.1 REAGENTS

All reagents used were of analytical-reagent grade. Ultrapure water obtained from a Milli-Q water purifier (Millipore, Bedford, MA, USA) was used for the preparation of all solutions.

Put and Cad were obtained from Sigma-Aldrich (Steinheim, Germany). Tyr and His were acquired from Fluka (Steinheim, Germany).

The supporting electrolyte used for the electrochemical measurements was a 10 mM Na_2CO_3 and 100 mM $NaClO_4$ (Fluka, Steinheim, Germany) solution. The pH value was adjusted with a 5% $HClO_4$ solution (Panreac, Barcelona, Spain).

Ham samples were obtained from a local market. $HClO_4$ (Panreac, Barcelona, Spain), ethyl acetate, NaCl and butanol from Fluka (Steinheim, Germany) and, ammonia solution, acetone and Na_2SO_4 from Merck (Darmstadt, Germany) were used for the extraction of BAs from real samples.

8.2.2. APPARATUS AND MEASUREMENTS

Voltammetric measurements were taken using a μAutolab (Eco Chemie, Utrecht, The Netherlands). A conventional three-electrode configuration system was used for the determination of BAs, consisting of a Ag/AgCl electrode (reference electrode), a platinum electrode (counter electrode) and a BBD electrode, used as working electrode.

All measurements were made at room temperature in a cell containing 15 mL of the supporting electrolyte solution (10 mM Na_2CO_3 and 100 mM $NaClO_4$) pH 10. Square wave voltammograms were acquired and processed by using the general purpose electrochemical system (GPES) software, making an anodic scan from + 250 mV (initial potential) to + 1900 mV (final potential). The experimental parameters were: scan rate 0.12 V s^{-1}, amplitude 20 mV, frequency 25 Hz. Data analysis and experimental designs were performed using Statgraphics statistical software package [39].

pH of buffer solutions was measured with a HI 221 pHmeter (HANNA Instruments, USA).

Chromatographic measurements of BAs were carried out using an HPLC system (Flexar) from PerkinElmer, Inc (Connecticut, U.S.A.) equipped with a binary LC pump and a solvent manager 3-CH degasser. Separation was achieved using a reversed phase column (Col Kromophase C-18 125 x 4.0 mm (Scharlab, S.L.,Barcelona, Spain)). The detection was performed with an UV-Vis system set at 240 nm.

8.2.3 SAMPLE PREPARATION

Extraction of BAs in ham samples was carried out using an ethyl acetate-acetone mixture, according to the procedure described by Yigit *et al.* [40]. In this way, 8 g of the previously homogenized ham and 10 mL of a 5 % $HClO_4$ solution were mixed in a vortex mixer for 1 min. The mixture was placed in an ultrasonic bath for 10 min and then centrifuged for 10 min at 7000 rpm. The supernatant was recovered and neutralized with a 3 % ammonia solution. Then, it was saturated with NaCl and extracted with 10 mL of an ethyl acetate–acetone (2:1) solvent system in a vortex mixer for 1 min. Then the mixture was briefly centrifuged, and the organic phase was separated. Finally, the solvent was evaporated.

8.3. RESULTS AND DISCUSSION

8.3.1 SQUARE WAVE VOLTAMMETRIC ANALYSIS OF BAS

The electrochemical determination of Put, His, Tyr and Cad using a BDD electrode was possible due to the wide potential window of this kind of electrodes. In this way, the analysis of these compounds by BDD electrodes is suitable using the experimental conditions described by Koppang *et al.* [26]. Figure 8.1 shows the square-wave voltammograms obtained for these compounds in a 10 mM Na_2CO_3 and 100 mM $NaClO_4$ solution (pH 10) using a BDD electrode. These voltammograms exhibit well-defined oxidation peaks for the different BAs in the potential range from + 250 mV to + 1900 mV.

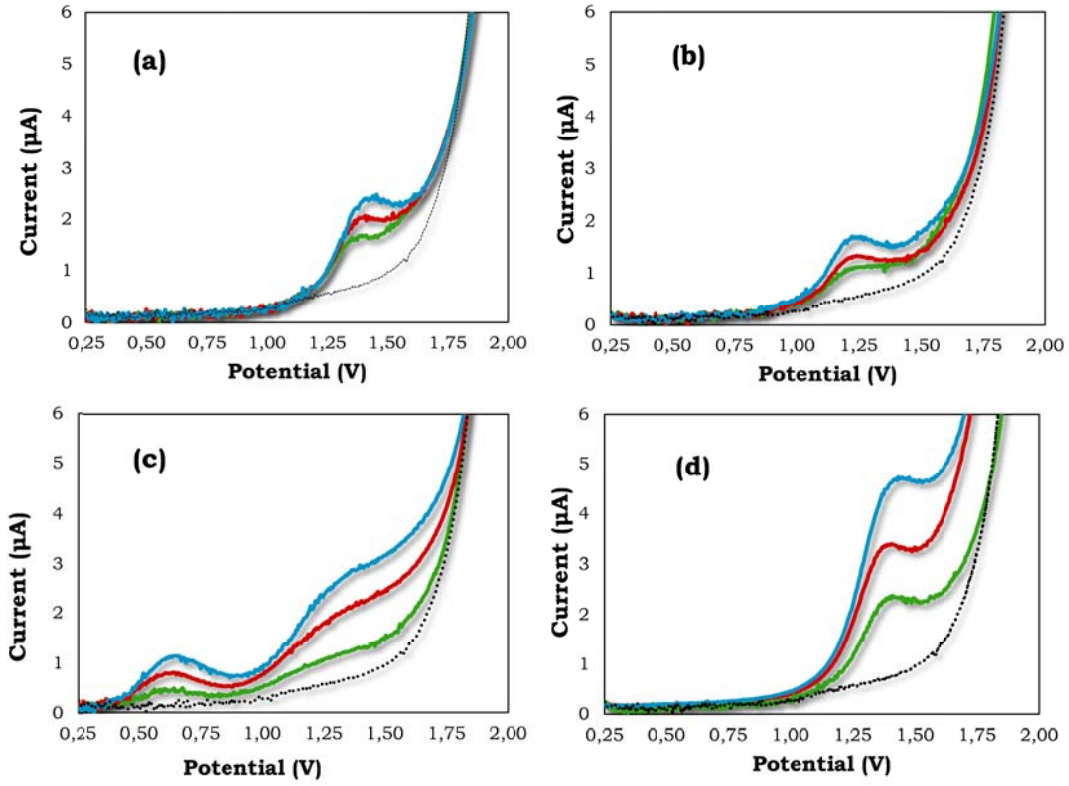

Figure 8.1. *Square-wave voltammograms obtained using a BDD electrode in 10 mM Na_2CO_3 and 100 mM $NaClO_4$ (pH 10) for (···) 0; (—) 25; (—) 50 and (—) 75 µM of (a) Put; (b) His; (c) Tyr and (d) Cad. Potentials vs Ag/AgCl reference electrode*

8.3.2 PARTIAL LEAST SQUARES REGRESSIONS

The electrochemical determination by SWV of the described BAs in a same sample leads to the overlapped voltammograms shown in Figure 8.2. Therefore, the simultaneous determination of these compounds by means of direct measuring of peak current is not possible. Thus, a multivariate calibration analysis by PLS was carried out, which has been demonstrated to be very useful in the resolution of overlapped electrochemical signals [36, 41-43].

The first step in this analysis by means of PLS regression involved a calibration step in which the relation between current

signals and the concentration of the different analytes was established from a set of standard samples (calibration set). The calibration set for quantitative analysis of the four analytes in a mixture consisted of 81 samples, which concentrations correspond to a three-level factorial design with four factors. The range of concentration used for Put, His, Cad and Tyr varied from 22 to 66 µM. A calibration model was constructed for each BA with original variables using 307 potentials ranging from + 400 to + 1900 mV.

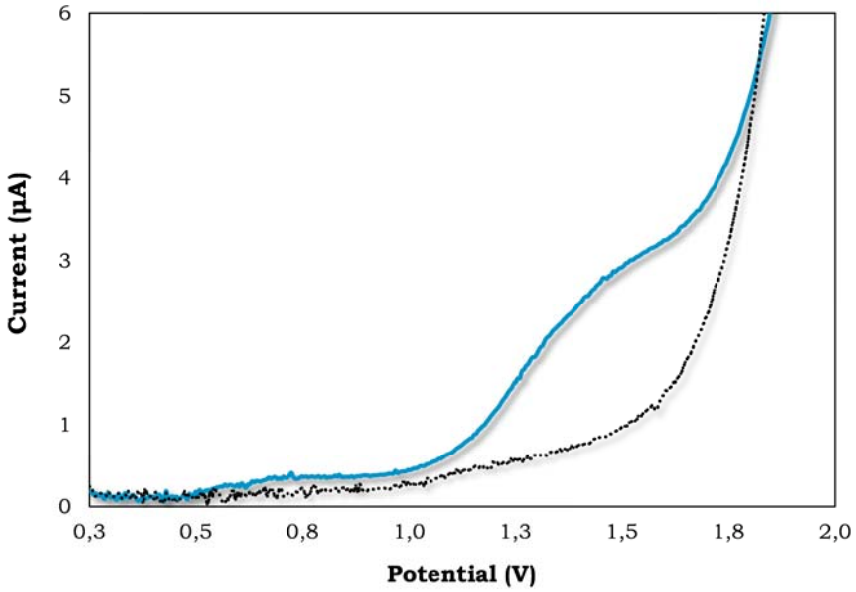

Figure 8.2. *Square-wave voltammograms obtained using a BDD electrode in 10 mM Na_2CO_3 and 100 mM $NaClO_4$ (pH 10) for (- - -) blank and (—) [His] = 25 µM; [Cad] = 25 µM; [Tyr] = 25 µM and [Put] = 50 µM. Potentials vs Ag/AgCl reference electrode*

The predicted concentrations with the different models were compared with the known concentrations and the prediction error sum of squares (PRESS) was calculated. The number of latent variables was then chosen in order to minimize PRESS by cross-validation [44].

The PLS models constructed for each analyte with the entire set of 81 samples led to poor analytical results. Thus, different models were constructed using different samples of the whole set. The best results were obtained for PLS models constructed in the concentration range from 22 to 65 μM for Cad, His and Put, and from 44 to 65 μM for Tyr. The calibration matrix consisted of the 48 samples is shown in table 8.1. The number of latent variables selected according cross-validation criterion was 5, 5, 10 and 6 for the construction of models for Cad, His, Put and Tyr, respectively, being the crossvalidated variance higher than 95 % for all of them.

The PLS models constructed with these 48 calibration samples were applied to a set of 4 additional samples considering as test samples (Table 8.1), in order to evaluate the real prediction capability of the constructed models. The PLS models allowed the simultaneous determination of Cad, His, Put and Tyr with predictions in this external validation set lower than 9 %. Different statistical parameters were then calculated. In this way, the models were validated in terms of standard errors of prediction (SEP). Mean square error or prediction (MSEP) and the relative root square of prediction (RRMSEP) were also calculated in order to estimate the accuracy of the predictions. Trueness was evaluated through the joint confidence interval test (JCIT) of the slope and intercept of the real concentrations versus predicted concentration. Precision was finally assessed by the calculation of the bias corrected MSEP (BCMSEP) [36, 44].

Table 8.7. *Set of calibration samples (1–48) and test samples (t_1–t_4) of Cad, His, Put and Tyr.*

Sample	Cad (µM)	His (µM)	Put (µM)	Tyr (µM)
1	22	22	22	44
2	22	66	44	44
3	44	22	22	44
4	44	65	44	44
5	66	44	44	44
6	66	22	66	44
7	22	44	22	66
8	22	22	44	66
9	44	22	66	66
10	66	44	22	66
11	66	44	44	66
12	66	43	65	66
13	22	44	22	44
14	22	65	66	44
15	44	65	66	44
16	66	44	66	44
17	22	66	22	66
18	65	65	22	66
19	66	22	65	66
20	22	22	44	44
21	22	44	44	44
22	22	22	66	44
23	44	44	22	44
24	44	44	44	44
25	66	22	22	44
26	66	22	44	44
27	66	65	66	44
28	22	65	44	66
29	22	22	66	66
30	44	65	44	66
31	66	22	22	66
32	22	44	66	44
33	44	66	22	44
34	66	65	22	44
35	66	65	44	44
36	22	44	44	66
37	44	44	22	66
38	44	65	66	66
39	44	44	66	44
40	66	44	22	44
41	22	44	66	66
42	44	22	22	66
43	44	22	44	66
44	66	22	44	66
45	66	65	65	66
46	44	22	44	44
47	44	22	66	44
48	22	65	66	66

t_1	66	65	44	66
t_2	44	43	66	66
t_3	44	44	44	66
t_4	44	65	22	66

As it can be seen in Table 8.2 these parameters are acceptable for the four compounds indicating that the PLS regression is appropriate to model the complex system under study.

Table 8.2. *Predictions for the PLS models constructed for the determination of Cad, His, Put and Tyr by SWV*

Model	Cad	His	Put	Tyr
Sensor range (μM)	22 to 65	22 to 65	22 to 65	44 to 65
Number of latent variables	5	5	10	6
SEP	2.8	3.8	4.0	4.2
MSEP	7.6	14.1	16.0	17.5
RRMSEP	6.3	6.9	9.1	6.4
BCMSEP	7.6	14.1	16.0	17.5

8.3.3 ANALYSIS OF HAM SAMPLES

The constructed models were applied to the determination of Cad, His, Put, and Tyr in ham samples. The extraction of BAs in these samples was performed according to the procedure described above. The obtained extracts were dissolved in 3 mL of buffer pH 10 (10 mM Na_2CO_3 and 100 mM $NaClO_4$). A volume of 700 μL of ham extract solutions was placed into the electrochemical cell, containing 15 mL of buffer solution pH 10. Six different organic extracts were analyzed following the described procedure. The concentrations found

for the different BAs using the proposed model are shown in Table 8.3. Finally the results were compared with a reference method based on an HPLC analysis on these food samples [21, 45]. A t-test for the means shows that the mean obtained using the electrochemical method is equal to the one obtained by the HPLC method in all cases since the null hypothesis of the test cannot be rejected at the 95 % confidence level. Thus, the proposed method is both accurate and suitable for the simultaneous determination of Cad, His, Put and Tyr in complex samples.

Table 8.3. *Content of Cad, His, Put and Tyr in ham samples.*

Biogenic Amine	Analytical Method	
	SWV with PLS using a BDD electrode (mg/100 g; n = 6)	HPLC (mg/100 g; n = 3)
Cad	16 ± 5	16 ± 3
His	22 ± 3	24 ± 2
Put	17 ± 1	17 ± 1
Tyr	9 ± 1	8 ± 1

8.4. CONCLUSIONS

This work shows a simple and rapid voltammetric method for the simultaneous determination of Cad, His, Put and Tyr in food samples. In this way, it has been verified that it is possible to resolve strongly overlapped voltammetric signals of quaternary mixtures of

these BAs using a BDD electrode through the application of the PLS regression method.

Good quality values for typical parameters namely, SEP, MSEP, RRMSEP and BCMSEP have been obtained, showing the good performance of the different PLS models for the determination of the different BAs, in terms of accuracy and precision.

The optimized multivariate calibration models allowed the simultaneous determination of the described BAs with prediction errors in external validation samples lower than 9 %.

The developed method has been also applied in the simultaneous determination of the four described BAs in ham samples, obtaining results in good agreement with those achieved with an HPLC reference method. Thus, the described method results highly effective in the analysis of complex mixtures in also complex matrices, avoiding the tedious and time consuming derivatization processes needed in HPLC analysis.

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9. CONCLUSIONES

De los resultados experimentales de los capítulos precedentes se extraen las siguientes conclusiones:

1. La gran versatilidad de los electrodos serigrafiados ha permitido la construcción de biosensores electroquímicos con características operacionales adecuadas para la determinación de BAs.

2. Las enzimas AOs catalizan la oxidación de BAs a sus correspondientes aldehídos produciendo amoniaco y peróxido de hidrogeno. Esta reacción enzimática pudo ser detectada amperométricamente a través de la oxidación electroquímica del peróxido de hidrogeno generado.

3. El biosensor construido mediante la inmovilización del enzima MAO por entrecruzamiento sobre la superficie de un SPCE modificado con TTF (MAO/TTF/SPCEs) permitió la determinación de Put con un valor de reproducibilidad en términos de RSD de las pendientes de las rectas de calibrado de 9.6 % (n=4). Siendo la capacidad de detección del método de 17.2 ± 4.6 μM (n=4, α=β=0.05). El biosensor demostró su viabilidad para realizar análisis cuantitativos de Put en muestras complejas como anchoas saladas y calabacín, en la determinación de Put con MAO/TTF/SPCEs únicamente resultaron como interferentes Cad y Tyr.

4. La combinación de las variables experimentales que maximizan la intensidad de oxidación en la determinación de Put, mediante cronoamperometría utilizando MAO/TTF/SPCEs fueron un potencial de medida de + 250 mV *vs* Ag/AgCl-SPE empleando una disolución de tampón fosfato a pH 11 como electrolito de soporte.

5. La incorporación del mediador TTF en la composición del electrodo de trabajo en los sistemas MAO/TTF/SPCEs, permitió llevar a cabo la determinación de Put empleando potenciales de trabajo menores que otros biosensores descritos en bibliografía.

6. La determinación simultanea de Put y Cad pudo llevarse a cabo empleando un biosensor dual basado en el empleo de dos electrodos de trabajo en el mismo dispositivo. En uno de ellos se inmovilizaron 0.46 unidades del enzima MAO sobre un TTF/SPCE (MAO/TTF/SPCE) y, en el otro se inmovilizaron 0.92 unidades de dicho enzima sobre un TTF/SPCE modificado con AuNPs (MAO/AuNPs/TTF/SPCE).

7. El electrodo MAO/AuNPs/TTF/SPCE resultó sensible a la presencia de Cad y Put mientras que el electrodo MAO/TTF/SPCE únicamente proporcionó respuesta electroquímica para la Put.

8. El biosensor dual descrito anteriormente presentó una respuesta lineal para la Cad en el intervalo de concentraciones comprendido entre 19.6 a 107.1 µM y entre 9.9 a 74.1 µM para la Put.

9. La capacidad de detección del biosensor dual fue de 9.9 y 19.9 ± 0.9 µM ($n = 4$ $\alpha = \beta = 0.05$) con una precisión de 4.9 % y 10.3% en términos de RSD para Put y Cad respetivamente, y se aplicó con éxito en la determinación simultanea de Put y Cad en muestras de pulpo fresco.

10. La Tyr resultó ser interferente a concentraciones superiores a 60 µM en la determinación de Cad y Put con MAO/AuNPs/TTF/SPCE y, en la determinación de Put con MAO/TTF/SPCEs.

11. El biosensor PUO/TTF/SPCE fue construido mediante la inmovilización de 6.25 unidades del enzima PUO por entrecruzamiento sobre un SPCE modificado con un 3% de mediador TTF.

12. La actividad enzimática de la PUO hacia la Put fue analizada empleando la técnica ITC. Dicha actividad resultó ser menor cuando el enzima es inmovilizado sobre TTF/SPCEs que cuando se encuentra en estado libre. Sin embargo la actividad enzimática del enzima inmovilizado resultó suficiente para convertir todo el sustrato y producir una señal electroquímica reproducible.

13. Los factores experimentales influyentes en la sensibilidad y selectividad de los biosensores PUO/TTF/SPCE desarrollados fueron optimizados con un diseño experimental central compuesto 2^2, obteniendo como valores óptimos: pH (Tampón fosfato), 10 y E_{ap}, + 300 mV *vs* Ag/AgCl-SPE.

14. La reproducibilidad del método propuesto para la determinación de Put mediante cronoamperometría usando PUO/TTF/SPCEs es 6.7% (n = 4), en términos de RSD de las pendientes asociadas a las rectas de calibración, en el intervalo de concentraciones de 10 a 74 μM y con una capacidad de detección de 10 ± 0.6 μM.

15. El biosensor PUO/TTF/SPCEs presentó una alta selectividad en el análisis de posibles interferencias, soló la Tyr en concentraciones superiores 150 μM resultó ser interferente en la determinación de Put.

16. El biosensor fue satisfactoriamente validado mediante la comparación de los resultados obtenidos en la determinación

de Put en muestras de pulpo y calabacín, con los obtenidos mediante HPLC.

17. La determinación selectiva de His puede llevarse a cabo empleando TTF/SPCEs en los que el enzima HMD es inmovilizado mediante entrecruzamiento (HMD/TTF/SPCEs).

18. La respuesta amperométrica del biosensor HMD/TTF/SPCE varía linealmente con la concentración de His en el intervalo de concentraciones comprendido entre 8 y 60 μM, con una capacidad de detección de 8.1 ± 0.7 μM. Los valores de reproducibilidad y repetibilidad calculados para este biosensor fueron 3.5% y 2.5% respectivamente en términos de RSD.

19. El biosensor HMD/TTF/SPCEs desarrollado es viable para realizar análisis cuantitativos de His en muestras complejas como atún enlatado y vino.

20. La determinación selectiva y simultánea de His y Put puede llevarse a cabo empleando un biosensor bienzimático basado en un dispositivo serigrafiado con dos TTF/SPCEs como electrodos de trabajo, uno de ellos modificado con 6.25 unidades del enzima PUO y, el otro con 0.42 unidades del enzima HMD.

21. Las valores óptimos de las variables experimentales para la determinación simultanea de His y Put empleando el sistema dual descrito anteriormente fueron: tampón fosfato pH 8 y un potencial de trabajo de +130 mV *vs.* Ag/AgCl-SPE para el electrodo HMD/TTF/SPCE y +300 mV. *vs.* Ag/AgCl-SPE para el PUO/TTF/SPCE.

22. La combinación de los dos biosensores (PUO/TTF/SPCE y HMD/TTF/SPCE) en un mismo sistema electródico permitió la

determinación simultánea de His y Put en muestras complejas como vino y pescados.

23. La determinación simultánea de Put, Cad, His y Tyr fue posible, mediante voltamperometría de onda cuadrada con un electrodo de BDD y empleando un modelo de regresión basado en mínimos cuadrados parciales a pesar del elevado grado de solapamiento de las señales de oxidación de las BAs citadas. pudo realizarse mediante SVW empleando un electrodo de BDD como electrodo de trabajo. Los mejores resultados se obtuvieron en el intervalo de concentraciones comprendido entre 22 y 65 M para Cad, His y Put y, entre 44 y 65 M para Tyr.

24. Las condiciones experimentales óptimas para la determinación simultánea de Put, Cad, His y Tyr con electrodos de BDD y SVW implicaron la utilización de tampón pH 10 (10 mM Na_2CO_3 y 100 mM $NaClO_4$) como electrolito de soporte y un barrido de potencial entre + 250 mV y + 1900 mV.

25. El método multivariante desarrollado se aplicó con éxito en la determinación simultánea de Put, Cad, His y Tyr en muestras de jamón y los resultados resultaron acordes con los obtenidos con un método de referencia HPLC.