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# Enzymatic biosensors for spermidine amperometric determination

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*To my life women,*

*Mum,*

*Sister*

*and Ticha*

# Abstract

Polyamines (PA) detection has been shown of great interest as biomarkers for various analytical purposes, such as freshness of fish, fruit and vegetables and cancer control. Various methods have been developed for separation and quantification of biogenic amines and among then all, electrochemical biosensors. These types of biosensors, particularly the ones involving the screen-printing technology, which combine construction simplicity with biological recognition through enzyme specificity, have been reported as a good and cheap alternative to the traditional techniques. Screen-printing technology offers design flexibility, process automatization and good reproducibility in the transducers fabrication, as well as the possibility of using a wide choice of materials.

In this work, Monoamine Oxidase (MAO) and Diamine Oxidase (DAO) based biosensors using screen-printed carbon electrodes have been attempted for the determination spermidine. The enzymes have been immobilized onto the working electrode by means of glutaraldheyde and chronoamperometric variables (applied potential and solution pH) adjusted for better reply as experimental variables can affect biosensor chronoamperometric response.

One form of screen-printed electrodes (SPEs) modification consists of the incorporation of metallic nanoparticles on the working electrode surface. Due to their reduced size, metallic nanoparticles exhibit important physical and electrical properties which make them very useful for the construction of more sensitive electrochemical biosensors. Silver and gold nanoparticle-modified carbon SPEs show important advantages when they are used as working electrodes in electrochemical techniques. Thus, different experiments were performed using modified nanoparticles DAO/MAO biosensors in order to evaluate nanoparticles influence on detection limit, sensibility, repeatability and reproducibility.

Isothermal titration microcalorimetry (ITC) may be used as a tool for obtaining overall apparent molar enthalpy for catalytic reactions and enzyme kinetic constants. ITC is useful, in this regard, since it directly measures the heat change as catalysis proceeds and this is proportional to the rate of the reaction. ITC is a well established, powerful, versatile and high-sensitivity technique that is widely used for measuring the thermodynamics of equilibrium association reactions. In addition, well designed experiments can yield an approximate value for the equilibrium association constant for the enzyme-substrate complex ($K_A$) when product formation is the limiting step, as well as the reaction stoichiometry (n). Thus, this technique has been tried to study MAO activity when immobilized on a non-modified SPE.

**Key words:** Polyamines; Spermidine; Biosensors; Screen-printted electrodes; Nanoparticles; Isothermal titration microcalorimetry.

# Resumo

Presentemente, diversos trabalhos têm demonstrado a utilidade das poliaminas (PA) como biomarcadores em várias técnicas de análise para a avaliação da qualidade dos alimentos (peixe, fruta e vegetais) e também no controlo do cancro. Vários métodos têm sido desenvolvidos para a separação e quantificação de aminas biogénicas, destacando-se de entre eles, o uso dos biosensores electroquímicos. Este tipo de biosensores, particularmente os que envolvem a tecnologia de electrodos serigrafados, combinam a simplicidade de construção com o reconhecimento biológico através da especificidade enzimática. A tecnologia de electrodos serigrafados oferece ainda flexibilidade de design, processos de produção automatizados, boa reprodutibilidade, e a possibilidade de uso de diversos materiais.

Neste trabalho, usando elétrodos de carvão serigrafados (SCE) imobilizados com oxidase da monoamina (MAO) ou oxidase da diamina (DAO) tentou-se construir um biosensor capaz de detectar espermidina. A imobilização das enzimas sobre o elétrodo de trabalho foi realizada por cross-linking usando glutaraldeído. Para uma melhor resposta eletroquímica, procedeu-se ao ajuste das variáveis cronoamperométricas (potencial aplicado e pH da solução) dado saber-se que estas podem interferir com a resposta electroquímica.

Uma forma de modificação dos SPE consiste na incorporação de nanopartículas metálicas na superfície do elétrodo de trabalho. Devido ao seu reduzido tamanho, as nanopartículas metálicas exibem propriedades elétricas e físicas importantes que as tornam vantajosas para a construção de biosensores eletroquímicos mais sensíveis. SCE modificados com nanopartículas de prata e ouro apresentam vantagens importantes quando são usados como elétrodos de trabalho em técnicas eletroquímicas. Assim, diversas experiências usando biosensores MAO/DAO modificados com nanopartículas, foram realizadas, para avaliar a sua influência no limite de deteção, na repetibilidade e na reprodutibilidade do método.

A técnica de titulação microcalorimétrica (ITC) pode ser usada como uma ferramenta na obtenção da entalpia molar aparente total de reações catalíticas e na obtenção dos parâmetros cinéticos das enzimas. Neste sentido, a ITC é útil, uma vez que mede diretamente a variação de calor durante a catálise, valor esse proporcional à velocidade de reação. A ITC é uma técnica, bem estabelecida, sensível, versátil e poderosa, que é amplamente usada na determinação da termodinâmica de reações associadas ao equilíbrio. Além disso, através de experiencias bem programadas pode-se obter um valor aproximado da constante de equilíbrio de associação para o complexo enzima-substrato ($K_A$) quando o produto formado é o passo limitante, bem como a estequiometria da reação (n). Portanto, tentou-se estudar a atividade enzimática da MAO quando imobilizada num SCE não modificado com nanoparticulas.

**Palavras-Chave:** Poliaminas; Espermidina; Biosensores; Electrodos Serigrafados; Nanopartículas; Titulação microcalorimétrica.

# Índice

# Chapter I – Polyamines

## 1.1 PA characteristics

Putrescine (put) (1,4-diaminobutane), spermidine (spd) (N-(3-aminopropyl)-1,4-diaminobutane) and spermine (spm) (N,N'-bis-(3-aminopropyl)-1,4-diaminobutane) (**Figure 1.1.1**) form a group of polycationic amines referred to as physiological Polyamines (PAs). Traditionally they have been classified within the group of biogenic amines (BA) (Kalac and Krausová, 2005).

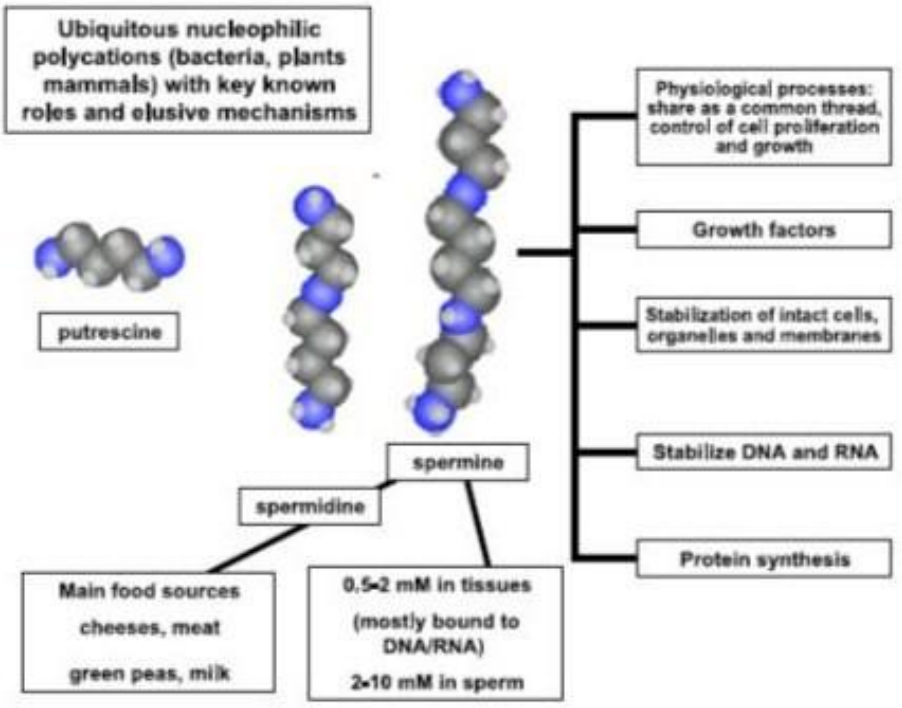



**Figure 1.1.1 -** Poliamines and its functions; adapted from Gucliucci, 2004

This PAs are a group of naturally occurring compounds exerting a large number of biological effects, yet despite several decades of intensive research work, the mode of action of the PAs at the molecular level is largely unknown (Bachrach *et al.*, 2001; Igarashi and Kashiwagi, 2000; Janne *et al.*, 1991; Seiler, 1990; Patocka and Kuehn, 2000; Russel, 1983). They bear unique structural features of regularly spaced positive charges interrupted by hydrophobic methylene bridges, as shown in Figure 1 and with particular chemical aspects as demonstrate in **Table 1.1.1**.

**Table 1.1.1 -** pKs of each polyamine; Gugliucci, 2004

| | $pK_1$ | $pK_2$ | $pK_3$ | $pK_4$ |
|---|---|---|---|---|
| Putrescine | 9.35 | 10.8 | – | – |
| Spermidine | 9.52 | 10.8 | 11.56 | – |
| Spermine | 8.90 | 9.79 | 10.95 | 11.50 |

In 1678, van Leuwenhoek reported crystals in sperm samples when left to dry, which we now know were spm-phosphate crystals. In 1926, Dudley achieved spm synthesis, he also synthesized spd, and proved its existence in tissues (Dudley *et al.*, 1926). Is not hard to understand that put had been isolated from tissues after bacterial decomposition (Brieger, 1879).

PAs like others substances could contribute to several damages in human organism if their intake is in an excessive way, so it is necessary to quantify PA toxicity. Therefore acute and subacute toxicity of the individual PAs was determined in Wistar rats. The acute toxicity was observed to be 2000, 600 and 600 mg/kg body weight for put, spd and spm, respectively. The no-observed-adverse-effect level (NOAEL) was 180, 83 and 19 mg/kg body weight for put, spd and spm, respectively (Til *et al.*, 1997). However, such extreme intakes of dietary amines cannot be supposed.

During the last few years, spd and spm have been investigated as to their conformational behavior (Carvalho *et al.*, 1999; Marques and Carvalho 2000; Marques *et al.*, 2002a, b; Amorim da Costa *et al.*, 2003, 2004; Marques and Carvalho, 2007). The conformational preferences of these linear alkylamines were found to depend on several factors, from steric, dipolar and hyperconjugative effects to the balance between intra- and intermolecular interactions. This kind of systems are characterised by a high conformational freedom (put, for instance, can adopt six different stable conformations), and by an interdependence of the particular effects due to the electronegativity and electron one-pairs of the nitrogen atoms. This constitutes an advantage for an effective interaction with biological receptors and lead to the formation of intra- and intermolecular hydrogen bonds which determine the solid-state conformational behaviour of the amines (Amado *et al.*, 2004).

In addition, the dual hydrophilic–lipophilic character of the PA ligands, comprising cationic amine groups (both primary and secondary) and variable length hydrophobic alkyl linkers, will possibly lead to an enhanced cellular uptake. This hydrophilic–lipophilic balance depends on the relationship between the length of the carbon bridging chains and the number of amine moieties. In addition, the presence of NH groups will favor the molecular recognition of the PAs and PA-based agents by the polyphosphate backbone of DNA and related targets (e.g. purine bases) (Liu *et al.*, 2006a).

The naturally occurring PAs are present in all prokaryotic and eukaryotic cells thus far studied. They stabilize nucleic acids and stimulate their replication. Spd and spm can bridge the major and minor grooves of DNA, acting as a clamp holding together either two different molecules or two distant parts of the same molecule (Feuerstein *et al.*, 1986; Feuerstein *et al.*, 1989; Feuerstein *et al.*, 1990; Feuerstein *et al.*, 1991). However, the exact nature of the PA–DNA interaction is not clearly established and is the subject of great controversy (Agostinelli *et al.*, 2010). Furthermore, amines are known to be suitable chelating ligands for transition metal ions such as Pt(II) or Pd(II), yielding stable and usually water soluble coordination compounds, often active as pharmacological agents (e.g. anticancer drugs) (Agostinelli *et al.*, 2010).

# 1.2 PA synthesis and functions

## 1.2.1 PA synthesis

The only PAs synthesized in mammalian cells are put, spd and spm (Gugliucci, 2004). Adequate cellular PA levels are achieved by a careful balance between biosynthesis, degradation, and uptake of the amines (Russell, 1983; Seiler, 1990; Jänne *et al.*, 1991; Murakami *et al.*, 1992; Tsirka e Coffino, 1992). PAs are bound to macromolecules (mainly nucleic acids) and this pool is in equilibrium with a free PA pool, which accounts for up to 7–10% of the total cell content. Some of the regulatory mechanisms involved in maintaining a balance in the cellular PA pools are truly unique.

The PA biosynthetic pathway consists of two highly regulated enzymes: ornithine decarboxylase (ODC); S-adenosylmethionine decarboxylase (AdoMetDC); and two aminopropyltransferases, spd synthase and spm synthase, both constitutively expressed enzymes (**Figure 1.2.1.1**)

This pathway depends on adequate supply of ornithine, usually the product of the urea cycle arginase, which is then, in a way another key element for adequate PA synthesis. Another important element is the universal aminopropyl donor S-adenosyl methionine, a key coenzyme in this pathway.

Put is formed by the action of the dimeric form of the enzyme ODC (Pegg, 2006). This reaction is mediated by the using pyridoxal phosphate as a coenzyme, lysine residues play an important role in dimer stabilization and in the classic binding to the coenzyme (Osterman *et al.*, 1995a; Osterman *et al.*, 1995b; Osterman *et al.*, 1999). This enzyme is therefore a critical step in maintaining PA levels and is exquisitely regulated. ODC is frequently described as the rate-limiting step in PA synthesis but this is inaccurate. ODC is usually the rate-limiting

factor in the production of put but the supply of the aminopropyl donor decarboxylated S-adenosylmethionine (dcAdoMet) by the action of AdoMetDC also influences the conversion of put into the higher PAs. Once converted to dcAdoMet, S-adenosylmethionine (AdoMet) is exclusively diverted to PA biosynthesis since methyl transferases do not use dcAdoMet as a substrate. The steady state level of dcAdoMet is therefore kept very low (c. 1–2% of the AdoMet content) and its supply limits the conversion of put to the higher PAs (Pegg, 2009). Recent structural and biochemical studies of mammalian AdoMetDC indicate that there is one put-binding site per (αβ) unit and that this site is located at a significant distance from the active site. Put binding to the $(\alpha\beta)_2$ dimer that makes up the enzyme is quite strongly cooperative and binding causes structural and electrostatic alterations at the active site (Bale *et al.*, 2008).

## 1.2.2 PA regulation

With important exception of the activation of AdoMetDC by put, PA content is controlled by changes in content of the key enzymes rather than by alterations in their activities by post-translational modifications or by binding effector molecules of low molecular weight.

***Regulation of ODC***

The regulation of ODC is complex, showing features that do not always fit into the generally accepted rules of molecular biology. ODC activity appears to be regulated solely via changes in the amount of ODC protein rather than modification of its catalytic activity. The regulation of ODC is not mediated by posttranslational or allosteric mechanisms. Instead, its steady-state concentration depends mainly on transcription, mRNA translation, mRNA stability and catabolism (Osterman *et al.*, 1994; Osterman *et al.*, 1999; Pegg, 2006; Shantz and Levin, 2007).

The PA biosynthetic enzyme ODC is degraded by the 26S proteasome via an ubiquitinin-dependent pathway in mammalian cells (Murakami *et al.*, 1992; Tsirka e Coffino, 1992). Its degradation is greatly accelerated by association with the PA-induced regulatory protein antizyme 1 (AZ), which binds to ODC, inhibits its activity, and targets it for proteasome degradation. AZ synthesis is increased in response to high PA levels predominantly via increasing a +1 frameshifting mechanism, which is needed to allow read through of a stop codon that prevents AZ synthesis (Petros *et al.*, 2005; Ivanov and Atkins, 2007). A second protein termed AZIn binds to AZ more tightly than ODC and can displace it and thus prevent the degradation. The structure of AZIn is quite similar to that of the ODC monomer but it lacks catalytic activity and is monomeric (Albeck *et al.*, 2008). This unusual pathway remains an exception only found so far in PA metabolism (Pegg, 2009).

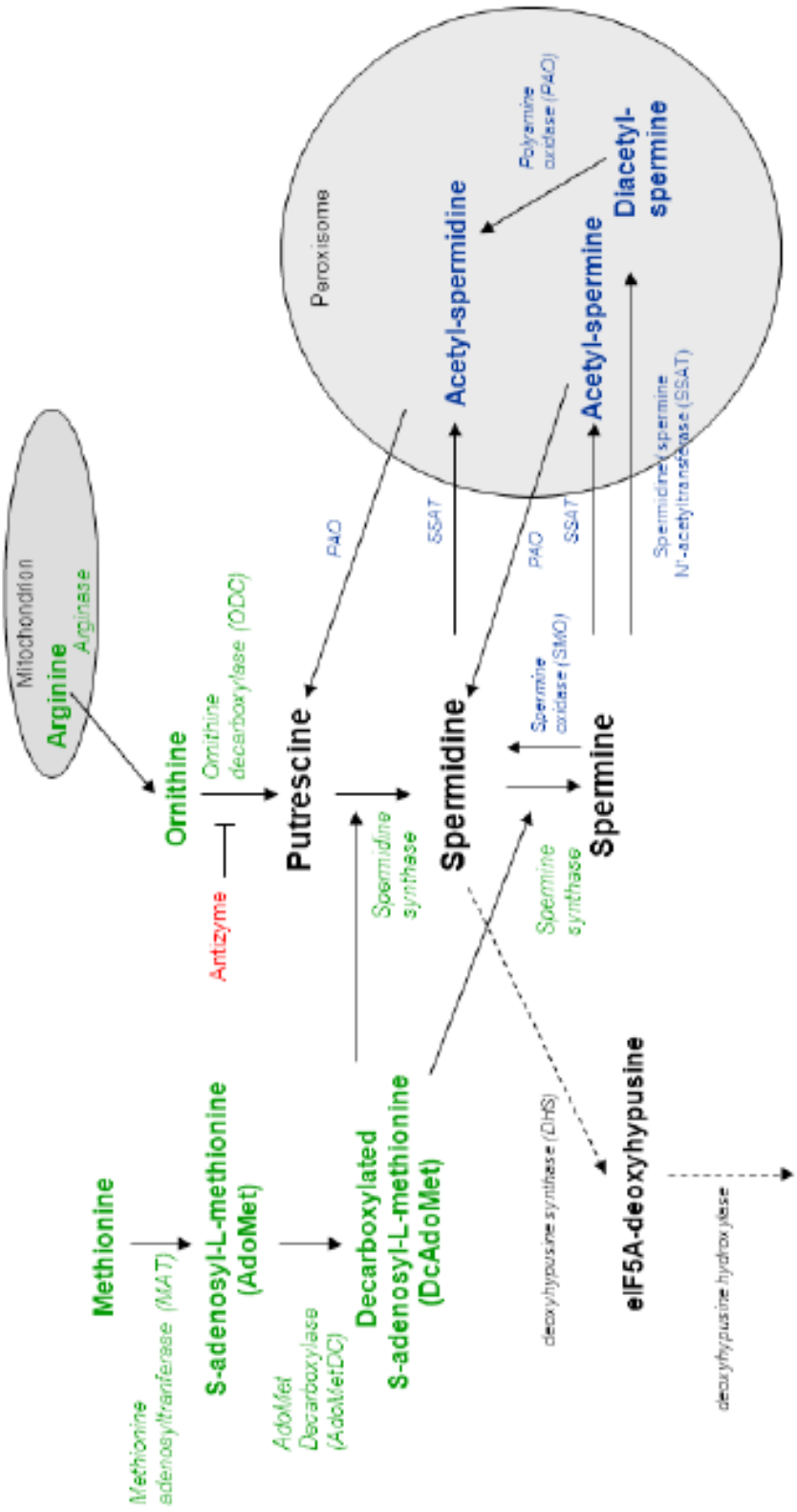



**Figure 1.2.1.1** - Poliamine Metabolism. Minois *et al.*, 2011

***Regulation of AdoMetDC***

In addition to the activation of AdoMetDC proenzyme processing and enzyme activity by put, the amount of AdoMetDC protein is highly regulated to maintain cellular PA levels at multiple steps including transcription, mRNA translation and protein turnover (Stanley, 1995; Pegg *et al.*, 1998). The amount of AdoMetDC is negatively regulated by increased spd/spm content at all three of these steps. Degradation of AdoMetDC requires polyubiquitination (Kahana, 2007). Reduction of PA content in response to various drugs causes a marked increase in the half-life of AdoMetDC protein (Pegg *et al.*, 1998; Kahana, 2007).

***Regulation of Aminopropyltransferases***

Synthesis of the higher PAs is brought about by aminopropyltransferases termed spd synthase and spm synthase (Ikeguchi *et al.*, 2006) (**Figure 1.2.1.1**). Despite the close similarity of their of their reactions, human Spd synthase and Spm synthase are distinct enzymes and show strict substrate specificity (Wu *et al.*, 2007; Wu *et al.*, 2008). Both structures contain two fully conserved key aspartic residues ($Asp^{104}$ and $Asp^{173}$ in Spd synthase; $Asp^{201}$ and $Asp^{276}$ in Spm synthase) that play a key part in the catalytic mechanism.

It is generally accepted that the formation of the products of these enzymes is determined by the availability of the substrates rather than by fluctuations in the levels of spd synthase or spm synthase (Pegg, 2009).

## 1.2.3 PA transport

Polyamine-specific carriers are widely distributed in prokaryotes and eukaryotes. Mammalian polyamine transport activity is also acutely controlled by cell cycle events and hormonal stimulation. Polyamine transport is a saturable, carrier-mediated, and energy-dependent process (Igarashi and Kashiwagi, 1999)

There are transport systems for both the uptake of polyamines and for their efflux. These are currently poorly understood at the biochemical level. The PA transport system is not highly specific and can transport a number of related compounds including paraquat, mepacrine and synthetic drugs conjugated with a polyamine (Rossi *et al.*, 2008; Kaur *et al.*, 2008). These may prove to be useful drugs for cancer chemotherapy when administered together with inhibitors of the biosynthetic pathway (Burns *et al.*, 2009).

Cell surface heparin sulfate proteoglycans have been implicated in PA transport, and uptake of PAs was blocked by a single chain variable fragment anti-heparan sulfate antibody (Welch *et al.*, 2008). Recently, a caveolin-regulated system has been shown to transport PAs in colon cancer cells. Phosphorylation of caveolin-1 at $Tyr^{14}$ increased the activity of this system (Roy

*et al.*, 2008). Such phosphorylation was stimulated by k-Ras providing another step at which this oncogene may increase cellular PAs.

Recent studies have identified SLC3A2, previously known as a glycosylated heavy chain of a cationic amino acid transporter, as a part of a put and acetylpolyamines efflux system. SLC3A2 with its partner y+ LAT light chain was found to bring about arginine uptake and put efflux (Uemura *et al.*, 2008). This efflux was coupled to arginine uptake suggesting that there is a put/arginine exchange reaction. The expression of SLC3A2 was negatively regulated by k-Ras (Uemura *et al.*, 2008). Thus, this oncogene can increase PA content by affecting both influx and efflux. Alternatively, it is thought that PA uptake in mammals could be performed by endocytosis (Minois *et al.*, 2011).

### 1.2.4 PA in gene regulation

PAs are strongly positively charged at physiological pH and bind to acidic sites on cellular macromolecules including proteins, nucleic acids and phospholipid membranes (Pegg, 1988; Childs *et al.*, 2003; Sarkar *et al.*, 2009). All of these interactions are likely to have some physiological effect (**Figure 1.2.4.1**).

It is clear that PAs affect RNA and DNA structure, ribosome function, and the activity of many enzymes including kinases and phosphatases. PA response elements have been identified in some genes (Childs *et al.*, 2003; Casero and Marton, 2007) and the transcription of many genes appears to be influenced through the PA status (Pegg, 1986; Pegg, 1988; Wallace *et al.*, 2003; Childs *et al.*, 2003; Jänne *et al.*, 2004). These genes include transcription factors such as c-Myc and c- Jun (Liu *et al.*, 2006b; Xiao *et al.*, 2007), which can lead to additional signaling pathways. Similarly, PAs selectively influence the translation of many mRNAs; examples include the effects on AZ, AdoMetDC and SSAT synthesis (Pegg, 2009).

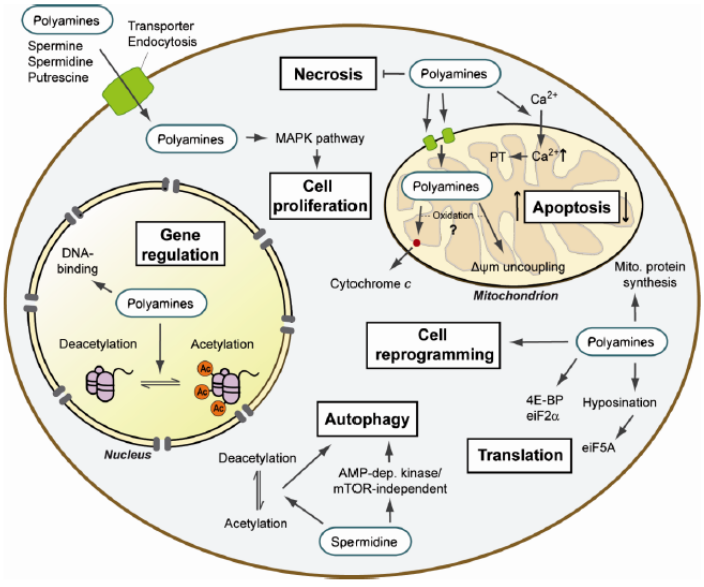



**Figure 1.2.4.1** - Cell functions of polyamines. Minois *et al.*, 2011

PA-related alterations in cell:cell interactions mediated via cadherins (Liu *et al.*, 2009) or Toll-like receptors (Chen *et al.*, 2007), effects on the cytoskeleton mediated by changes in the activity of G-proteins such as Rac1 and RhoA (Vaidya *et al.*, 2005; Ray *et al.*, 2007; Mäkitie *et al.*, 2009), and alterations in the microtubule network (Mechulam *et al.*, 2009) are also possible sites of physiological PA action.

High PA levels alter histone acetylation and histone acetylases and deacetylases activities in proliferative cells (Minois *et al.*, 2011).

Another finding is that PAs facilitate oligomerization of nucleosomal arrays *in vitro*, and that PA-mediated chromosome condensation is inhibited by histone hyperacetylation (Pollard *et al.*, 1999). These results suggest that PAs are repressors of transcription *in vivo*, and that one role of histone hyperacetylation is to antagonize the ability of PAs to stabilize highly condensed states of chromosomal fibers (Igarashi and Kashiwagi, 2010)

A screen for the loss of effect of spd on life span in yeast showed that the hypoacetylation by spd was mainly due to an inhibition of histone acetylases rather than activation of histone deacetylases. Taken together, the results showed that PAs affect acetylation, which in turn will affect gene regulation.

It thus seems that PAs, by modulating acetylation levels and protein synthesis, will trigger varied changes that can potentially lead to complex cellular responses. Such actions of PAs may help explain how they can promote both cell growth and cell death and can show such a complex involvement in aging, stress and diseases (Minois *et al.*, 2011).

### 1.2.5 PA in autophagy

Autophagy is the mechanism responsible for the degradation of intracellular macromolecules and organelles. Autophagy is an essential function in development and survival as it will dispose of unwanted molecules such as damaged molecules during stress, aging or diseases, or during developmental remodeling. Autophagy is involved in many diseases (Levine and Kroemer, 2008; Mizushima *et al.*, 2008; Fleming *et al.*, 2011). Many genetic and non genetic manipulations increasing life span induce autophagy (Madeo *et al.*, 2010a) and it appears as a point of convergence of many genetic pathways involved in aging (Markaki *et al.*, 2011). It was demonstrate that induction of autophagy was probably the main mechanism of action of spd to trigger its beneficial effects on life span and aging (Eisenberg *et al.*, 2009; Morselli *et al.*, 2011; Madeo *et al.*, 2010b).

## 1.2.6 PA effect on ion channels

***Glutamate receptors***

PAs influence glutamate receptors mediating slow voltage-dependent responses such as N-methyl-D-aspartate (NMDA) receptors, and those producing fast responses at excitatory synapses, such as a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainate receptors (Dingledine *et al.*, 1999).

The NMDA receptors are involved in synaptic plasticity and may also play a role in seizure activity. Spm (and, to a lesser extent, spd) has multiple effects on these receptors including stimulation and a weak voltage-dependent inhibition representing an open-channel block. Spm stimulation, which occurs at 1 M concentrations, causes an enhancement of the current gated by glutamate and glycine. There is a 'glycine-dependent stimulation', which produces an increase in the affinity of the receptor for glycine. Binding of spm can also produce a 'glycine-independent stimulation' at saturating concentrations of glycine.

The AMPA-type glutamate receptors are responsible for fast excitatory neurotransmission in the CNS. They are heteromeric ligand-gated channels composed of four possible subunits (GluR1-4) whose properties depend on the presence of GluR2. Those lacking the GluR2 subunit are permeable to $Ca^{2+}$ ions, possess a high single-channel conductance, and are subject to a block by endogenous intracellular PAs (predominantly spm) that confers profound rectification on the responses and influences frequency-dependent facilitation at synapses expressing these receptors. Thus, PAs may regulate the amount of $Ca^{2+}$ flux and the excitability threshold at developing synapses (Pegg, 2009). PAs may also affect the activity via interactions with protein kinase C (Shin *et al.*, 2007).

***Kir Channels***

The inward rectifier potassium channel gene family consists of seven subfamilies (Kir1-7). Voltage-dependent block by intracellular PAs is the common mechanism underlying the inward rectification in all the Kir channels. All of the natural PAs can bind and have some effects in experimental conditions but the affinity increases from put to spd to spm (Stanfield and Sutcliffe, 2003; Guo and Lu, 2003).

## 1.2.7 PA catabolism

Catabolism of PAs is less well characterized (Seiler, 1975; Seiler, 1985; Kusche *et al.*, 1985; Seiler, 1990).

Several enzymes are involved in the reversal of the aminopropyltransferase reactions, which like the decarboxylases are effectively irreversible (Seiler, 2004; Wang and Casero, 2006; Pegg *et al.*, 2008). Spermine oxidase (SMO) converts spm to spd plus 3-aminopropanaldehyde but does not act significantly on spd (Wang and Casero, 2006). Acetylpolyamine oxidase (APAO) converts N1-acetylspermidine into put plus N-acetyl-3-aminopropanaldehyde (Wang *et al.*, 2005; Takao *et al.*, 2009; Henderson Pozzi *et al.*, 2009). APAO also acts on efficiently on N1-acetylspermine to form spd (Pegg, 2009). The substrates for the APAO reaction are synthesized by the action of spd/spm-N1-acetyltransferase (SSAT) (Pegg, 2008). SSAT acetylates both spm and spd. Acetylated spm and spd then move into the peroxisome where they are oxidized by Polyamine oxidase (PAO) (FADdependent). By-products of this oxidation include hydrogen peroxide ($H_2O_2$) and acetaminopropanal. (Nadège Minois *et al.*, 2011). The net results of the SMO and APAO/SSAT reactions are to convert the higher PAs to put, which is more readily excreted from the cell and can also be degraded by diamine oxidase. Therefore, activation of these pathways can reduce PA content. Studies with transgenic mice that overexpress SSAT have provided convincing demonstrations that this enzyme is an important regulatory step in maintaining PA content (Jänne *et al.*, 2006; Niiranen *et al.*, 2006; Jell *et al.*, 2007). In addition cytosolic SSAT is the rate-limiting enzyme of PA catabolism (**Figure 1.2.1.1**).

Both APAO and SMO are flavoproteins that generate reactive aldehydes and $H_2O_2$ and may cause oxidative damage. This may be a more serious problem with SMO since APAO is located in peroxisomes (Pegg, 2009). Products from both the SMO and APAO reactions, as well as acrolein, a highly toxic product of spm oxidation formed after cell damage by extracellular copper-dependent oxidases (Yoshida *et al.*, 2009), have been linked with renal failure (Igarashi *et al.*, 2006) and stroke (Tomitori *et al.*, 2005).

# 1.3 PAs in food

PAs are present in various types of food such as: vegetables, meat and its products, fish and milk as well as its derivatives.

## 1.3.1 PAs in foods of plant origin

Put, spm and spd universally occurring in plant organs are involved in a wide array of processes, ranging from triggering organogenesis to protecting against stressPut contents are commonly the highest among PAs. Some of the tested foods (**Table 1.3.1.1** and **1.3.1.2**) have a considerably high mean put level (above 40 mg $kg^{-1}$), namely oranges, orange juice, mandarins, grapefruit juice and the processed foods sauerkraut, ketchup, frozen green peas and fermented soy products. Spd contents in plant foods are commonly higher than spm levels. Legumes, mainly soybean, pear, cauliflower and broccoli belong to food items with the highest spd content, usually above 30 mg $kg^{-1}$. The same foods, mainly legumes, also have the highest spm levelA proportion of PAs leaches to cooking water. For put, approximately 20–25% leaches from broccoli and celery and about 40% from cauliflower and asparagus. Similarly for spd, 10–20% leaches from broccoli, savoy and celery, and 20–30% from cauliflower and asparagus (Ziegler *et al.*, 1994). PA contents also may change during the storage of fresh vegetables. Simon-Sarkadi et al. (1994) observed changes in PAs during the storage of fresh Chinese cabbage, endive, iceberg lettuce and radicchio at 5 ºC over five days. The put content increased 3–8-fold during this period, while spd and spm levels did not change significantly.

**Table 1.3.1.1** - Content of polyamines (mg $kg^{-1}$) in potato, vegetables and fruits. Adapted from Kalac and Krausová, 2005

| Product | *n* | Putrescine | | | | Spermidine | | | | Spermine | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $x$ | $S_x$ | $x_{min}$ | $x_{max}$ | $x$ | $S_x$ | $x_{min}$ | $x_{max}$ | $x$ | $S_x$ | $x_{min}$ | $x_{max}$ | |
| *Potato*, fresh | 3 | 9.7 | – | – | – | 11.2 | – | – | – | 3.0 | – | – | – | Bardócz et al. (1993) |
| | 3 | 17.6 | – | – | – | 13.5 | – | – | – | ND | – | – | – | Okamoto et al. (1997) |
| | 6 | 9.7 | 2.1 | 5.8 | 12.8 | 11.3 | 1.7 | 8.3 | 13.6 | 2.6 | 1.2 | 0.8 | 4.0 | Eliassen et al. (2002) |
| Cooked | 3 | 21.6 | – | – | – | 15.2 | – | – | – | 5.2 | – | – | – | Bardócz et al. (1993) |
| | 4 | 3.9 | – | ND | 6.9 | 23.5 | – | 13.6 | 35 | – | – | – | – | Ziegler, Hahn, and Wallnöfer (1994) |
| | 4 | 8.5 | 2.3 | 5.6 | 12.4 | 10.9 | 2.2 | 9.1 | 15.7 | 2.2 | 1.2 | ND | 3.4 | Eliassen et al. (2002) |
| Chips | 4 | 21.6 | – | – | – | 24.8 | – | – | – | 2.6 | – | – | – | Bardócz et al. (1995) |
| Potato crisps | 3 | – | – | 38.4 | 41.9 | – | – | 35.2 | 39.9 | – | – | 4.2 | 5.1 | Bardócz et al. (1995) |
| *Vegetables* | | | | | | | | | | | | | | |
| Cauliflower, fresh | 3 | – | – | 3.1 | 4.5 | – | – | 21.7 | 27.8 | – | – | 2.0 | 2.8 | Bardócz et al. (1993) |
| | 7 | 4.9 | – | 2.2 | 7.6 | 31.2 | – | 17.1 | 42.8 | – | – | – | – | Ziegler et al. (1994) |
| | 5 | 5.3 | 2.1 | 3.3 | 8.9 | 28.3 | 6.5 | 21.3 | 39.3 | 6.1 | 1.6 | 4.6 | 8.9 | Eliassen et al. (2002) |
| Cooked | 4 | 4.0 | 1.2 | 2.6 | 5.9 | 26.2 | 10.6 | 19.0 | 45.2 | 6.3 | 2.8 | 4.4 | 11.3 | Eliassen et al. (2002) |
| Broccoli, fresh | 4 | 9.0 | – | 7.0 | 10.5 | 33.2 | – | 31.8 | 36.0 | – | – | – | – | Ziegler et al. (1994) |
| | 5 | 6.4 | 2.9 | 3.4 | 10.8 | 41.3 | 9.1 | 24.5 | 51.8 | 9.9 | 3.2 | 5.8 | 15.9 | Eliassen et al. (2002) |
| Cooked | 4 | 5.6 | 2.9 | 2.5 | 8.9 | 27.3 | 6.4 | 17.3 | 33.1 | 7.1 | 1.4 | 5.3 | 8.9 | Eliassen et al. (2002) |
| Cabbage | 3 | – | – | 0.4 | 1.6 | – | – | 3.2 | 5.1 | – | – | 3.2 | 3.6 | Bardócz et al. (1993) |
| | 4 | – | – | – | – | 14.4 | – | 13.2 | 16.6 | – | – | – | – | Ziegler et al. (1994) |
| Sauerkraut | 121 | 146 | 99.0 | 2.8 | 529 | 8.2 | 6.6 | ND | 47.0 | – | – | – | – | Kalač, Špička, Křížek, Steidlová, and Pelikánová (1999) |
| Savoy | 4 | – | – | – | – | 11.6 | – | 10.6 | 13.0 | – | – | – | – | Ziegler et al. (1994) |
| Spinach, frozen purée | 32 | 12.9 | – | ND | 119 | 7.3 | 3.8 | 1.3 | 15.4 | 2.2 | 1.8 | ND | 3.8 | Kalač, Švecová, and Pelikánová (2002) |
| Cucumber | 3 | 3.2 | – | – | | 1.5 | – | – | – | 0.4 | – | – | – | Bardócz et al. (1993) |
| | 5 | 6.9 | 1.4 | 5.5 | 8.7 | 7.4 | 1.6 | 5.4 | 10.3 | 1.2 | 0.8 | ND | 2.8 | Eliassen et al. (2002) |
| Carrot | 3 | – | – | 1.2 | 1.8 | – | – | 7.7 | 8.3 | – | – | 2.0 | 2.8 | Bardócz et al. (1993) |
| | 4 | 2.8 | – | 2.0 | 3.9 | 4.5 | – | 4.3 | 4.7 | – | – | – | – | Ziegler et al. (1994) |
| | 2 | 3.5 | – | – | – | 8.0 | – | – | – | ND | – | – | – | Okamoto et al. (1997) |
| | 6 | 1.5 | 0.7 | 0.7 | 2.7 | 6.7 | 2.3 | 3.6 | 11.9 | 0.6 | 1.2 | ND | 3.8 | Eliassen et al. (2002) |
| Tomato | 3 | – | – | 9.3 | 122 | – | – | 1.6 | 2.5 | ND | – | – | – | Bardócz et al. (1993) |
| | 2 | 10.6 | – | – | – | 1.7 | – | – | – | ND | – | – | – | Okamoto et al. (1997) |
| Concentrated tomato pasta | 19 | 25.9 | 8.2 | 7.9 | 41.1 | 8.4 | 3.7 | ND | 15.8 | – | – | ND | 2.9 | Kalač et al. (2002) |
| Ketchup | 24 | 52.5 | 54.1 | ND | 165 | 6.1 | 9.0 | ND | 33.4 | – | – | ND | 12.1 | Kalač et al. (2002) |
| Onion | 3 | – | – | 5.5 | 7.2 | – | – | 5.5 | 8.1 | – | – | 0.8 | 1.2 | Bardócz et al. (1993) |
| Lettuce | 3 | – | – | 3.3 | 4.8 | – | – | 4.2 | 8.3 | ND | – | – | – | Bardócz et al. (1993) |
| | 3 | 5.6 | 1.3 | 4.5 | 7.3 | 9.1 | 1.5 | 7.4 | 10.3 | 0.8 | 0.8 | ND | 1.8 | Eliassen et al. (2002) |
| Celeriac | 3 | 6.1 | – | 3.7 | 7.7 | 26.7 | – | 19.7 | 34.7 | – | – | – | – | Ziegler et al. (1994) |
| Asparagus | 3 | 2.9 | – | 2.0 | 3.8 | 10.3 | – | 9.2 | 10.9 | – | – | – | – | Ziegler et al. (1994) |
| *Fruits* | | | | | | | | | | | | | | |
| Apple | 3 | – | – | 0.4 | 1.7 | – | – | 2.2 | 2.8 | ND | – | – | – | Bardócz et al. (1993) |
| | 2 | ND | – | – | – | 1.0 | – | – | – | ND | – | – | – | Okamoto et al. (1997) |
| Pears | 3 | – | – | 23.6 | 24.2 | – | – | 30.2 | 76.0 | – | – | 8.1 | 49.3 | Bardócz et al. (1993) |
| Orange | 3 | – | – | 95.1 | 140 | – | – | 8.8 | 9.7 | ND | – | – | – | Bardócz et al. (1993) |
| | 2 | 117 | – | – | – | 1.9 | – | – | – | 1.6 | – | – | – | Okamoto et al. (1997) |
| | 5 | 137 | 11.3 | 119 | 153 | 4.1 | 4.0 | 0.4 | 11.6 | 0.2 | 0.2 | ND | 1.4 | Eliassen et al. (2002) |
| Orange, canned | 3 | – | – | 27.0 | 30.0 | – | – | 0.7 | 1.0 | ND | – | – | – | Bardócz et al. (1993) |

**Table 1.3.1.2** – Content of polyamines (mg $kg^{-1}$) in cereals and legumes. Adapted from Kalac and Krausová, 2005

| Product | *n* | Putrescine | | | | Spermidine | | | | Spermine | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $x$ | $S_x$ | $x_{min}$ | $x_{max}$ | $x$ | $S_x$ | $x_{min}$ | $x_{max}$ | $x$ | $S_x$ | $x_{min}$ | $x_{max}$ | |
| *Cereals* | | | | | | | | | | | | | | |
| Wheat flour | 2 | 1.5 | – | – | – | 9.6 | – | – | – | 5.3 | – | – | – | Okamoto et al. (1997) |
| Bread, white | 3 | – | – | 1.5 | 1.8 | – | – | 5.0 | 5.2 | – | – | 3.4 | 3.8 | Bardócz et al. (1993) |
| Whole grain | 3 | – | – | 0.5 | 0.9 | – | – | 21.3 | 27.4 | – | – | 7.1 | 9.1 | Bardócz et al. (1993) |
| | 5 | 3.4 | 0.5 | 2.5 | 4.0 | 13.1 | 1.5 | 10.2 | 14.8 | 6.3 | 2.0 | 3.4 | 8.7 | Eliassen et al. (2002) |
| Pasta, cooked | 3 | – | – | 1.0 | 1.1 | – | – | 7.0 | 7.3 | – | – | 10.5 | 12.9 | Bardócz et al. (1993) |
| Breakfast cereals, mixed | 10 | – | – | 2.0 | 2.2 | – | – | 24.1 | 24.4 | – | – | 6.1 | 6.7 | Bardócz et al. (1995) |
| Rice, polished | 2 | <0.9 | – | – | – | 3.9 | – | – | – | <4.1 | – | – | – | Okamoto et al. (1997) |
| Cooked | 3 | – | – | 1.0 | 1.3 | – | – | 1.3 | 1.6 | – | – | 8.1 | 10.1 | Bardócz et al. (1993) |
| *Legumes* | | | | | | | | | | | | | | |
| Green peas, frozen | 14 | 46.3 | 27.0 | 11.7 | 107 | 46.6 | 23.5 | 2.9 | 88.4 | 3.8 | 2.0 | ND | 8.5 | Kalač et al. (2002) |
| Cooked | 3 | – | – | 5.4 | 5.9 | – | – | 62.1 | 68.2 | – | – | 33.5 | 71.7 | Bardócz et al. (1993) |
| Green beans, cooked | 3 | – | – | 4.3 | 5.4 | – | – | 7.7 | 8.8 | – | – | 4.6 | 5.5 | Bardócz et al. (1993) |
| Red kidney bean | 3 | – | – | 0.3 | 0.4 | – | – | 19.0 | 20.0 | – | – | 22.8 | 25.7 | Bardócz et al. (1993) |
| Soybean, dried | 3 | – | – | 1.6 | 6.5 | – | – | 33.2 | 62.1 | – | – | 29.7 | 34.3 | Bardócz et al. (1993) |
| | 1 | 17.0 | – | – | – | 128 | – | – | – | – | – | – | – | Ziegler et al. (1994) |
| | 2 | 41 | – | – | – | 207 | – | – | – | 69 | – | – | – | Okamoto et al. (1997) |
| Soybean miso | 11 | 51.1 | 40.7 | 9.8 | 143.1 | – | – | – | – | – | – | – | – | Yen (1986) |
| | 2 | 20.2 | – | – | – | 11.7 | – | – | – | 2.0 | – | – | – | Okamoto et al. (1997) |
| Soy sauce | 22 | 88.1 | 129 | ND | 514 | – | – | – | – | – | – | – | – | Yen (1986) |

## 1.3.2 PAs in meat, fish and meat products

In contrast to foods of plant origin, low levels of put are typical for well-treated foods of animal origin (**Table 1.3.2.1**). Fish sauces, cod roe and canned crab are the reported exceptions. High spm contents, usually between 20 and 60 mg/kg, are usual in meat and meat products of warm-blooded animals. Lower spm contents, commonly below 10 mg $kg^{-1}$, were reported in fish. Spd levels in meat and fish rarely exceed 10 mg $kg^{-1}$. Thus, an opposite relation between spd and spm contents is typical for foods of animal origin as compared with plant products. Silva and Glória (2002) explain higher spd than spm contents in their samples of chicken-based meat products by the incorporation of a considerable proportion of vegetable components.

Yano *et al.* (1995) observed a considerable increase of put content over 13 days of storage, while no changes in spd and spm contents during storage up to 39 days in vacuum-packed beef sirloin at 0, 5 or 10 ºC. In a classical work, Mietz and Karmas (1978) included PAs as indicators of seafood decomposition. They observed that spd and spm contents decreased during storage, while put content increased. In recent years, similar changes were observed in Mediterranean hake. Put content increased significantly during storage for 29 days, being more intensive at 6–8 ºC than at 0 ºC (in ice). Spd and spm contents somewhat decreased from the initial levels 4 and 10 mg $kg^{-1}$, respectively (Baixas-Nogueras *et al.*, 2002). Similarly, an extensive formation of put and a slight decrease of spd content were determined in carp

meat, while spm content remained stable during storage at 3 or 15 ºC until spoilage (Krízek *et al.*, 2002).

**Table 1.3.2.1 -** Content of polyamines (mg $kg^{-1}$) in beef, pork and chicken meat, meat products and in fish and fish products. Adapted from Kalac and Krausová, 2005.

| Product | *n* | Putrescine | | | | Spermidine | | | | Spermine | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $x$ | $S_x$ | $x_{min}$ | $x_{max}$ | $x$ | $S_x$ | $x_{min}$ | $x_{max}$ | $x$ | $S_x$ | $x_{min}$ | $x_{max}$ | |
| *Beef* | | | | | | | | | | | | | | |
| Raw, lean | 3 | – | – | 5.5 | 5.9 | – | – | 18.3 | 19.7 | – | – | 30.7 | 42.0 | Bardócz et al. (1993) |
| | 2 | 0.5 | – | – | – | 2.6 | – | – | – | 28.3 | – | – | – | Okamoto et al. (1997) |
| | 6 | – | – | – | – | 3.1 | 0.8 | 1.9 | 4.2 | 39.8 | 5.8 | 28.7 | 44.6 | Hernández-Jover et al. (1997) |
| | 5 | 10.1 | 14.3 | 0.8 | 38.5 | 5.5 | 3.2 | 2.6 | 12.0 | 27.3 | 4.4 | 16.8 | 26.0 | Eliassen et al. (2002) |
| Ground | 3 | 8.8 | – | – | – | – | – | 70.6 | 72.9 | – | – | 46.3 | 47.5 | Bardócz et al. (1993) |
| | 8 | 4.0 | 5.7 | 0.8 | 18.7 | 3.0 | 0.7 | 2.2 | 4.8 | 20.8 | 3.6 | 13.3 | 26.7 | Eliassen et al. (2002) |
| Cooked | 3 | – | – | 1.9 | 2.8 | – | – | 5.7 | 6.8 | – | – | 22.8 | 33.3 | Bardócz et al. (1993) |
| Fried | 2 | – | – | 1.4 | 30.2 | – | – | 2.6 | 5.7 | – | – | 26.1 | 36.2 | Eliassen et al. (2002) |
| Sirloin, raw | 6 | – | – | – | – | 1.5 | – | – | – | 30 | – | – | – | Yano, Kataho, Watanabe, Nakamura, and Asano (1995) |
| | 7 | 2.1 | 3.2 | 0.6 | 12.8 | 2.2 | 0.6 | 1.0 | 3.0 | 17.0 | 6.7 | 6.1 | 29.1 | Eliassen et al. (2002) |
| *Pork* | | | | | | | | | | | | | | |
| Raw, lean | 3 | 3.1 | – | – | – | – | – | 2.9 | 4.9 | – | – | 30.1 | 70.3 | Bardócz et al. (1993) |
| | 2 | 1.1 | – | – | – | 4.6 | – | – | – | 28.3 | – | – | – | Okamoto et al. (1997) |
| | 13 | – | – | – | – | 3.0 | 1.0 | 0.8 | 4.5 | 33.5 | 4.4 | 27.3 | 40.6 | Hernández-Jover et al. (1997) |
| Chops, raw | 5 | 0.2 | 0.3 | ND | 0.7 | 2.8 | 0.7 | 2.0 | 4.1 | 22.4 | 7.5 | 14.5 | 34.5 | Eliassen et al. (2002) |
| *Meat products* | | | | | | | | | | | | | | |
| Sausage | 3 | – | – | 13.8 | 14.5 | – | – | 5.8 | 6.4 | – | – | 24.0 | 25.9 | Bardócz et al. (1993) |
| Sausage, wiener | 5 | 0.9 | 0.3 | 0.4 | 1.1 | 2.3 | 0.7 | 1.2 | 3.5 | 9.9 | 2.0 | 5.0 | 12.5 | Eliassen et al. (2002) |
| Mortadella (cooked salami) | 20 | – | – | ND | 5.7 | 4.0 | 2.3 | 1.0 | 8.9 | 17.2 | 7.5 | 7.6 | 32.2 | Hernández-Jover et al. (1997) |
| Pork ham, smoked | 3 | – | – | 4.0 | 4.3 | – | – | 2.0 | 8.8 | – | – | 40.2 | 50.3 | Bardócz et al. (1993) |
| Roasted | 3 | 9.0 | – | – | – | 6.1 | – | – | – | – | – | 40.2 | 60.4 | Bardócz et al. (1993) |
| Cooked | 20 | – | – | ND | 12.4 | 2.1 | 0.6 | 1.4 | 3.5 | 21.4 | 8.4 | 6.4 | 35.7 | Hernández-Jover et al. (1997) |
| Dry-cured | 23 | – | – | ND | 17.4 | 5.6 | 0.9 | 4.4 | 7.3 | 35.7 | 8.2 | 24.9 | 62.1 | Hernández-Jover et al. (1997) |
| Ripened dry fermented Spanish sausage "chorizo" | 20 | – | – | 2.6 | 416 | 4.1 | 2.5 | 1.9 | 10.0 | 26.1 | 8.1 | 13.8 | 43.5 | Hernández-Jover et al. (1997) |
| | 3 | – | – | 0.8 | 185 | – | – | 6.7 | 8.2 | – | – | 39.1 | 58.8 | Ruiz-Capillas and Jiménez-Colmenero (2004) |
| Different Spanish meat products | 17 | – | – | 0.2 | 10 | – | – | 1.7 | 7.6 | – | – | 17.8 | 59.3 | Ruiz-Capillas and Jiménez-Colmenero (2004) |
| *Game (stored at 4 °C for 7 days)* | | | | | | | | | | | | | | |
| Roe deer | 3 | 19.3 | 17.4 | – | – | 14.7 | 2.9 | – | – | 54.3 | 7.4 | – | – | Dičáková et al. (2003) |
| Red deer | 3 | 9.0 | 7.6 | – | – | 17.0 | 7.0 | – | – | 59.3 | 2.3 | – | – | Dičáková et al. (2003) |
| Fallow deer | 3 | 38.0 | 17.8 | – | – | 14.7 | 2.7 | – | – | 60.7 | 0.7 | – | – | Dičáková et al. (2003) |
| Pheasant | 3 | ND | – | – | – | 21.0 | 1.0 | – | – | 83.0 | 6.9 | – | – | Dičáková et al. (2003) |
| *Chicken* | | | | | | | | | | | | | | |
| Raw | 3 | 2.9 | – | – | – | 9.3 | – | – | – | 59.2 | – | – | – | Bardócz et al. (1993) |
| | 2 | <0.4 | – | – | – | 2.9 | – | – | – | 62.6 | – | – | – | Okamoto et al. (1997) |
| Grilled | 5 | 2.0 | 0.5 | 1.3 | 2.7 | 17.3 | 3.9 | 13.2 | 25.7 | 44.4 | 6.1 | 33.7 | 53.1 | Eliassen et al. (2002) |
| Chicken breast, raw | 4 | <0.8 | – | – | – | 7.3 | 0.8 | – | – | 17.9 | 1.3 | – | – | Silva and Glória (2002) |
| Chicken thigh, raw | 4 | <0.8 | – | – | – | 7.2 | 1.8 | – | – | 16.2 | 0.9 | – | – | Silva and Glória (2002) |
| Chicken based frankfurter | 10 | 0.6 | – | ND | 1.4 | 15.8 | – | 11.9 | 26.6 | 10.8 | – | 6.0 | 17.1 | Silva and Glória (2002) |
| Mortadella | 10 | 2.6 | – | ND | 19.2 | 10.8 | – | 4.9 | 24.3 | 10.1 | – | 6.4 | 15.9 | Silva and Glória (2002) |
| Hamburger | 10 | 0.6 | – | ND | 1.9 | 12.6 | – | 4.2 | 24.4 | 9.2 | – | 4.5 | 15.6 | Silva and Glória (2002) |

### 1.3.3 PAs in milk and its derivatives, human breast milk and eggs

Levels of all PAs are very low in cow milk, yoghurt, human milk and hen eggs. However, contents of all PAs can reach an extremely high level in cheeses, mainly in matured types.

The PA contents in human milk were reviewed by Löser (2000). Content of PAs varies during the suckling period. During the first week postpartum, put levels remained very low, while spd and spm contents rose markedly during the initial three days, reaching plateau levels that were 12 and 8 times higher, respectively, than the values determined on the initial day of lactation. After four months of lactation, put content slightly increased, whereas spm and spd contents remained almost stable (Romain *et al.*, 1992). Mothers seem consistently to have relatively high or relatively low contents of spd and spm in their milk. These individual variations may be due to diet, lifestyle or genetic background (Dandrifosse *et al.*, 2000). Spd and spm contents in infant powdered formulas were observed to be considerably lower than values usual in breast milk (Buts *et al.*, 1995; Romain *et al.*, 1992).

## 1.4 PAs in health and diseases

### 1.4.1 Aging

PA levels decrease with age in many organisms (Scalabrino and Ferioli, 1984). For instance, Nishimura *et al.* (2006) measured PA levels in 14 different tissues in 3, 10 and 26 week-old female mice and found that spd levels decreased in 11 out of the 14 tissues. In contrast, spm decreased only in skin, heart and muscles. Put levels were very low in all tissues at all ages. Vivó et al. (2001) reported a negative correlation between spd content and age in several areas of the basal ganglia in human brains and a similar trend for spm.

Soda et al. (2009) fed male mice a low, normal or high-PA chow. They showed that mortality in mice fed a high-PA chow was lower in the first 88 weeks. The authors also reported a lower incidence of age-related kidney glomerular atrophy kept on high-PA diet. Finally, they observed that old mice on high-PA chow kept a thicker coat with age and appeared more active. These results are promising and further studies in rodents are urgently required.

Spd increased chronological life span in wild-type yeast as well as remaining replicative life span in old yeast cells. Was also showed that spd supplementation increased life span in the nematode worm *Caenorhabditis elegans* by 15% and in the fly *Drosophila melanogaster* by up to 30%. At the cellular level, spd increased survival of human peripheral blood mononuclear cells after 2 days from 15% in the controls to 50% by preventing death from necrosis. These results strongly suggest that spd could represent a new preventive agent in our fight against

aging (Minois *et al.*, 2011). However, so far research has mainly focused on the effect of spd on life span and not on the importance of its effect.

### 1.4.2 PAs and stress

Various studies showed that PAs had important roles in and generally correlate with stress resistance. PAs are particularly important for adaptation and resistance to cold stress (Alcázar *et al.*, 2011) and PA levels increase in plants during abiotic stress such as salinity, extreme temperature, paraquat or heavy metals. This regulation of PAs under stress is achieved by differential expression of PA biosynthesis enzymes, such as arginine decarboxylase, spd and spm synthase or AdoMetDC. Exogenous application of PAs led to, in varying degrees, preserved membrane integrity and lower growth inhibition during stress, reduced accumulation of ROS and increased activity of antioxidant enzymes such as catalase. In contrast, PA synthesis inhibitors triggered decreased stress resistance.

### 1.4.3 PAs and diseases

As regulators of cell growth and death, it is likely that PAs may affect the severity and process of diseases. PA levels increase in many diseases. As PAs also regulate growth in pathogens, they may have an impact on infectious and parasitic diseases as well. However, as for aging and stress resistance, PAs have different effects in different models and on different diseases (Minois *et al.*, 2011).

More than 90% of circulating spd and over 70% of spm are associated with red blood cells. Gomes-Trolin et al. (2002) observed that in red blood cells, put levels decreased in Parkinson's disease and amyotrophic lateral sclerosis patients. In contrast, spd and spm increased in both sets of patients. There was no correlation between the levels measured and the severity of the disease. Earlier, Yatin et al. (1999) suggested a possible involvement of PA metabolism in Alzheimer's disease.

For instance, spm exacerbated ischemic neuronal injury in rodent models of ischemia (Duan *et al.*, 2011). However, no such damaging effect was observed with put or spd. Spm also increased neuronal damage in culture rat hippocampal neurons induced by oxygen and glucose deprivation. In contrast, PAs can also be neuroprotective (Bell *et al.*, 2011).

PAs are also important in diseases such as pancreatitis. The pancreas is the organ where the highest levels of spd are observed in mammals. Transgenic rats overexpressing SSAT exhibited a depletion of spd and spm and developed pancreatitis. Furthermore, these rats failed to initiate liver regeneration after partial hepatectomy. Liver regeneration could only begin once the spd levels were restored because of ODC activation.

Many diseases are associated with inflammation and PAs have been involved in inflammatory responses. PA levels generally increase with inflammation (Minois *et al.*, 2011).

Put and spm induced the accumulation of tumor necrosis factor (TNF) and mixed glial cultures. Spd did not have such an effect. The authors concluded that ODC expression was an early response to inflammation and that the increased PA levels resulting from ODC activation could lead to pro- or anti-inflammatory roles depending on the microenvironment. The potential anti-inflammatory role of PAs, which also lead to the production of nitric oxide, has led Soda to hypothesize that PA uptake may help with cardiovascular diseases (Soda, 2010). Recently, spd was shown to be beneficial against two age-related diseases: cataract formation (Lentini *et al.*, 2011) and multiple sclerosis (Guo *et al.*, 2011). To conclude, PAs have a complex relationship with diseases. They may be harmful, neutral or beneficial, depending on the specific PA and disease. However, it seems that spd showed the most positive effects, which would be in line with its beneficial effects reported on life span and stress.

### 1.4.4 PAs and cancer

The PAs metabolism is of much interest as they are required for cell growth and proliferation. High levels of PAs are therefore observed in rapidly divided cells and tissues such as tumour cells (Russell, 1983; Gerner and Meyskens, 2004; Erdman *et al.*, 1999). Gene expression and activity of enzymes involved in PA biosynthesis, especially ODC, are higher in cancer tissues than in normal surrounding tissues (Kingsnorth *et al.*, 1984; LaMuraglia *et al.*, 1986; Becciolini *et al.*, 1991; Canizares *et al.*, 1999; Linsalata *et al.*, 2002).

A strategy to study the involvement of PAs in cancer has been to use PA biosynthesis pathway inhibitors or PA analogues. Drugs interfering with PA biosynthesis or their biological role thus have considerable potential as therapeutic agents. Investigated chemopreventive or anti-neoplastic agents include ODC inhibitors (Seiler, 2003a) and PA structural analogues and derivatives (Seiler, 2003b). However, tumour cells have the ability to uptake extracellular PAs, both dietary and produced by gastrointestinal bacteria, and compensate effects of the mentioned therapeutic agents. Many reviews and papers have gathered the knowledge on this field (Wallace *et al.*, 2003; Wallace and Fraser, 2004; Casero and Marton, 2007; Amendola *et al.*, 2009; Senanayake *et al.*, 2009; Szumilak *et al.*, 2010; Babbar and Gerner, 2011). The ODC inhibitor DL-α-difluoromethylornithine (DFMO) was the first one synthesized and studied. This compound showed no effect during clinical trials, and is currently being studied as a chemopreventive agent rather than a chemotherapeutic one (Casero and Marton, 2007). DFMO and other compounds like methylglyoxal-bis guanylhydrazone (MGBG), an inhibitor of S-Adenosylmethionine (SAM), became key tools in the elucidation of the PA metabolic system, but only few of them were efficient as inhibitors of tumor growth (Tabor and Tabor, 1984;

Herr *et al.*, 1986; Erdman, 1990; Wang *et al.*, 1993; Davis *et al.*, 2001). It has been claimed that DFMO may improve the efficacy of some of the existing cytotoxic drugs.

Another approach thus started in the 1990s – deprivation of exogenous PAs (Quemener *et al.*, 1994). PA deprivation, combining the inhibition of PA synthesis in tumour cells and reduction of the main exogenous sources including food and microflora-derived PAs, has shown to be a promising therapeutic strategy. Stimulation of the antitumoural immune response is an additional effect of PA deprivation (Catros-Quemener *et al.*, 1999). However, it has not yet been experimentally proved that altering of the dietary PA intake can help cancer patients (Kalac and Krausová, 2004).

Numerous reports have shown that both blood and urine PA concentrations (**Table 1.4.4.1**) are often increased in cancer patients (Uehara *et al.*, 1980; Kubota *et al.*, 1985; Weiss *et al.*, 2002; Linsalata *et al.*, 2002). Moreover, these levels decrease after tumor eradication and increase after relapse (Loser *et al.*, 1990; Chatel *et al.*, 1987; Kubota *et al.*, 1985; Uehara *et al.*, 1980; Kingsnorth *et al.*, 1984).

A number of studies have indicated higher concentration of Put, Spd and Spm or total PA contents (free and acetylated) in cancer patients compared to healthy subjects (Matthews, 1993; Russell, 1971; Tang *et al.*, 1994; Nishioka *et al.*, 1995; Chanda and Ganguly, 1995; Chowdhury *et al.*, 1995; Suh *et al.*, 1995). Lee *et al.* (2008) observed higher mean levels of PAs in serum of uterine cancer patients than those in normal serum. Suh et al. reported significant differences in urinary PAs in advanced gastric carcinoma, ovarian cancer, acute myelocytic leukemia, non-Hodgkins lymphoma as compared to healthy subjects. In solid tumors the activity of ODC and AdoMetDC is higher compared to normal tissue (Russel *et al.*, l983; La Muraglia *et al.*, 1986; Heby and Person, *et al.*, 1990; Porter *et al.*, 1987). Consequently, the concentrations of Put and Spd are also enhanced considerably (Upp *et al.*, 1988). Peng *et al.* reported that total PAs, especially the contents of Cad and Spd in malignant ascites were significantly higher as compared to the patients with cirrhosis and tuberculosis suggesting that ascite levels of PA can be regarded as one of the cancer markers. Chowdhury et al. explored the relation between PA spectrum and the degree of malignancy in human breast cancer patients. The results showed that PA levels varied with clinical staging of the disease and bore a direct relationship to the degree of the disease.

In the responding patients, PA levels are shown to normalize when the patient is in remission. On the other hand, patients with a recurrent tumor or metastatic disease show higher PA excretion, thus the response of an individual can be monitored (Wallance, 1996; Russel *et al.*, l983; Russell, 1977; Wan *et al.*, 1990). Nonresponders of the chemotherapy tended to show elevated Put without any significant increase in Spd. The plot of post to pretreatment Spd ratio over post to pre Put ratio had value of 1.4 for complete responders, 1.2 for partial

responders and 0.4 for nonresponders (Russel et al., l983). Bakowski et al. observed that patients undergoing remission induction chemotherapy showed a significant elevated plasma Spd level within 48 h and suggested that tumor response to chemotherapy can be predicted on the basis of plasma PAs.

**Table 1.4.4.1 -** Determination of polyamines in urine from cancer patients. Adapted from Khuhawar and Qureshi, 2001

| Age (yr) | Sex (M/F) | Diagnosis | Polyamines (μg/mg creatinine) | | |
|---|---|---|---|---|---|
| | | | Put | Spd | Spm |
| 27 | F | Breast cancer | 2.910 | 1.521 | 1.056 |
| 49 | M | Lung cancer | 8.063 | 1.312 | + |
| 53 | F | Melanoma | 5.509 | 1.809 | + |
| 44 | M | Ovary tumour | 10.27 | 1.092 | + |
| 53 | M | Lung cancer | 12.49 | 4.632 | 10.45 |
| 73 | M | Lung cancer | 40.88 | 5.862 | + |
| 58 | M | Lung cancer | 8.80 | 2.62 | + |
| 63 | F | Lung cancer | 10.15 | 2.84 | 6.33 |
| 60 | F | Lung cancer | 288.0 | 8.80 | + |
| 56 | M | Lung cancer | 2.10 | + | + |
| 66 | M | Lung cancer | 1.70 | 1.46 | + |
| 29 | M | Lung cancer | 1.90 | 0.19 | + |
| 51 | M | Lung cancer | 29.0 | 13.0 | 8.71 |
| 64 | M | Lung cancer | 9.29 | + | + |
| 53 | M | Lung cancer | 21.60 | 43.8 | + |
| 50 | M | Lung cancer | 4.86 | + | + |
| Average ($n$=16) | | | 28.60 | 5.56 | 1.66 |
| SD | | | 70.0 | 10.7 | 3.3 |

+ Under the level of determination.

***PAs and cancer spread***

Cancer cells maintain their proliferative capacity by the interaction of PAs with oncogenes (Gerner and Meyskens, 2004). Growth-associated genes, such as c-fos and c-myc proto-oncogenes, are activated during cellular proliferative processes (Davis et al., 2001). Likewise, malignant transformation leads to an increase in PA biosynthesis, deregulation of ODC, and the amplification of proto-oncogenes (Erdman, 1990; Herr *et al.*, 1986; Davis *et al.*, 2001; Wang *et al.*, 1993; Tabor and Tabor, 1984).

Patients with increased PA levels either in the blood or urine are reported to have more advanced disease and worse prognosis compared to those with low levels, regardless of the type of malignancy (Kubota *et al.*, 1985; Uehara *et al.*, 1980; Weiss *et al.*, 2002; Linsalata *et al.*, 2002). Therefore, inhibition of PA synthesis and availability by cancer cells could retard cancer cell growth.

**a) The role of PAs on separation of cancer cells from the tumor cluster**

This separation is initiated by decreased cell adhesion (**Figure 1.4.4.1**). Hypoxia, a common condition in cancer tissues, exerts a strong pressure on cells to separate from the tumor cluster and migrate into circulation (Klymkowsky and Savagner, 2009; Pouyssegur *et al.*, 2006). The cellular response to hypoxia involves the stabilization and resultant increase in

levels of hypoxia inducible factor-1 (HIF-1), a transcription factor that enhances gene expression to promote angiogenesis, anaerobic metabolism, cell survival, and invasion (Harris, 2002). Among these, suppression of adhesion molecules induced by hypoxia-induced HIF-1 stabilization is a strong selective pressure that enhances outgrowth of cells with high-grade malignancy. CD44 and E-cadherin are adhesion molecules whose expression decreases in response to hypoxia (Beavon, 1999; Hasan *et al.*, 1998). Reduced CD44 expression is reported to promote cancer metastasis and invasion, allowing detachment of cancer cells from the primary tumor cluster and seems to contribute to the increased migration capacity of hypoxic HT-29 cells (De Marzo *et al.*, 1998; Kallakury *et al.*, 1996).

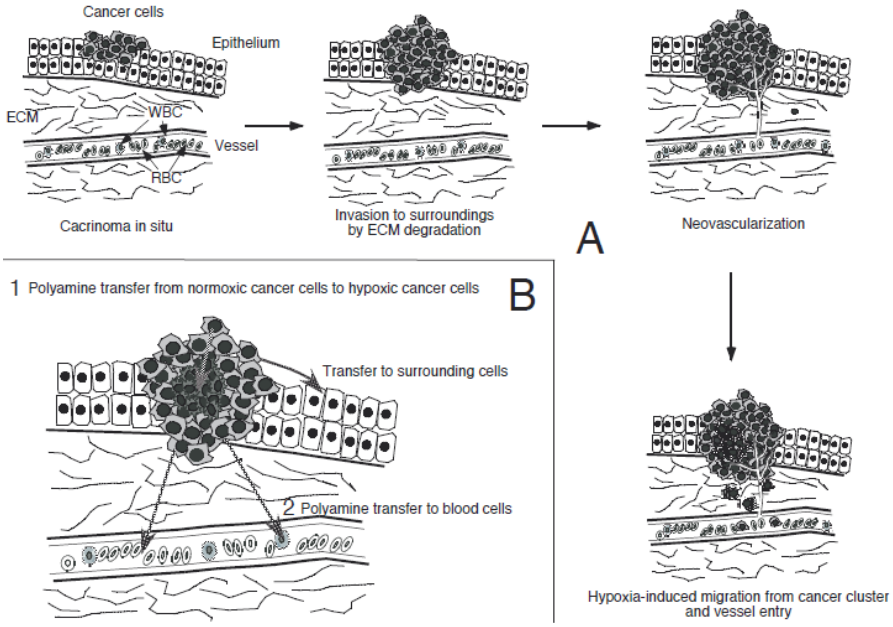



**Figure 1.4.4.1** - Mechanism of cancer metastasis. A) Progression and development of metastasis; B) Cancer cells exchange polyamines to the surroundings to stimulate other cancer cells under hypoxia. Adapted from Soda, 2011

In cells exposed to chronic hypoxia, PA synthesis is decreased, while the ability to take up PAs from the surroundings is increased (Tantini *et al.*, 2006; Aziz *et al.*, 1994). It has been reported that cancer cells under hypoxia lose regulation of PA homeostasis and have increased PA uptake from surrounding tissues (**Figure 1.4.4.1**) (Tsujinaka *et al.*, 2011). Several experiments indicated a possible role for PAs in the invasive potential of cancer cells (Jun *et al.*, 2008; Manni *et al.*, 2005; Sunkara and Rosenberger, 1987).

**b) Role of PAs in cancer cell transmigration to the circulation**

Cancer invasion is the process in which cancer cells migrate through surrounding tissues and enter into a blood vessel, which enables cancer cells to be transported throughout the body and establish secondary tumors (Soda, 2011).

Cancer cells have the ability to create new blood vessels in the tumor, i.e. angiogenesis, so that cancer cells can obtain supplies of blood and oxygen (Dvorak *et al.*, 2011). Increased PA synthesis appears to be accompanied by cancer invasiveness as ODC overexpression enhances the invasive characteristics of cancer cells (Kubota *et al.*, 1997). In contrast, inhibition of PA synthesis by the ODC inhibitor DFMO attenuates the invasive characteristics of cancer cells (Jun *et al.*, 2008; Manni *et al.*, 2005; Ashida *et al.*, 1992), and supplementation with PA reverses the DFMO-induced decrease in invasive qualities (Ashida *et al.*, 1992). The close correlation between increased PA synthesis and increased matrix metalloproteinases (MMP) synthesis has also been shown using DFMO, which caused decreases in cancer cell expression and concentrations of MMPs, such as matrilysin, meprin, and MMP-7 (Wallon *et al.*, 1994; Matters *et al.*, 2005).

**c) Possible role of PAs on cell rooting and colonization at secondary tumor sites**

Cancer cells that invade blood vessels and escape from immune system detection in circulation anchor to endothelial vasculature to establish new sites of growth. Upon vessel entry, cancer cells have access to abundant oxygen supplies that could enable cancer cells to restore their original activities such as increased gene expression that translates to enhanced enzymatic activities for PA synthesis, proteinase, and angiogenesis factors. The expression of CD44 of normoxic cancer cells is higher than that of hypoxic cells (Tsujinaka *et al.*, 2011), suggesting that the circulating cancer cells possibly recover their original adhesion characteristics. They invade and rapidly grow because of their increased capacity to synthesize PAs indispensable for cell growth and proteins that degrade the tissue matrix and create new vessels (Soda, 2011).

**d) PAs help cancer cells escape immune system detection**

Immune suppression, often observed in cancer patients, accelerates cancer spread. Various defects in cellular functions indicative of immune suppression have been reported, including attenuated adhesion properties of peripheral blood mononuclear cells (PBMCs) (Hersh *et al.*, 1982; Grosser *et al.*, 1976; MacFarlane *et al.*, 1982), impaired production of tumoricidal cytokines and chemokines (Heriot *et al.*, 2000; Rampone *et al.*, 2001; Monson *et al.*, 1986), and decreased cytotoxic activity of killer cells, especially lymphokine activated killer (LAK) cells (Wood *et al.*, 1990; Herman *et al.*, 1990; Funk *et al.*, 2005; Balch *et al.*, 1985). Animal experiments have shown that PA deprivation prevents the development of tumor-induced immunosuppression (Chamaillard *et al.*, 1997).

The time- and dose-dependent decrease in adhesion produced by PAs was accompanied by decreases in the expression of lymphocyte function-associated antigen-1 (LFA-1), which consists of an integrin alpha L (CD11a) and beta 2 (CD18) chain (Soda *et al.*, 2005). PAs in particular decrease the number of cells expressing bright CD11a. In addition, the number of CD56 bright cells was decreased by PAs in vitro. LFA-1 and CD56 contribute to the induction

of tumoricidal cell activities, especially lymphokine activated killer (LAK) activity (Ellis and Fisher, 1989; Weil-Hillman, 1989). LAK cells, which have tumoricidal activities against established tumors, are induced by co-culture with IL-2 (Mule *et al.*, 1984; Rosenberg *et al.*, 1985). In animal experiments, PA deprivation reversed the tumor inoculation-induced suppression of IL-2 production without decreasing the number of T lymphocytes (Chamaillard *et al.*, 1997). In addition, spm and spd inhibit the production of tumoricidal cytokines, such as tumor necrosis factor (TNF), and chemokines in vitro, while they do not inhibit production of transforming growth factor beta, which has immunosuppressive properties (Soda *et al.*, 2003; Zhang *et al.*, 1997; Hasko *et al.*, 2000).

***PAs' limitations as cancer markers***

Variations in the PA levels in diseases other than cancer have also been reported. Patients with cystic fibrosis (Russell *et al.*, 1979), muscular dystrophy (Kaminska *et al.*, 1981; Russell and Stern, 1980) psoriasis (Voorhus, 1979), hepatic failure (Desser *et al.*, 1980), diabetics (Seghieri *et al.*, 1997) and uremic (Gumprecht *et al.*, 1995) indicate higher levels of PAs in biological fluids.

Increase in urinary PAs has also been reported during normal pregnancy (Russell *et al.*, 1978). PA synthesis could be stimulated in response to stressful procondition such as electrical stimulation, traumatic injuries, neurotoxins and ischemia (Pajumen *et al.*, 1978; Dienel and Cruz, 1984; Desiderio *et al.*, 1988; Vera *et al.*, 1991; Porcella *et al.*, 1991; Paschen *et al.*, 1988). However, a high protein diet did not increase urinary PA excretion (Marko *et al.*, 1998). Loser et al. observed a significant increase in PA levels in serum and urine of colorectal cancer as compared to healthy controls (Loser *et al.*, 1990). However, nonmalignant gastrointestinal diseases partly showed similar tendency (Bachrach, 1992) (**Tables 1.4.4.2** and **1.4.4.3**).

Since PA concentrations in serum and in urine normalized in patients after curative operations while these levels were further elevated in patients with proven tumor relapse and metastases, these substances may play a clinical role in predicting therapeutic success or indicating relapse of the tumor (Khuhawara and Qureshi, 2001).

In conclusion, PA levels could be high in others diseases besides cancer or high in different cancers which contributes to its non specificity to mark this type of disease. In addition, there are several normal conditions like pregnancy that could contribute to the increase of PAs production; therefore high levels of PA not always correspond to a disease.

**Table 1.4.4.2** – Total polyamine concentration (X ± S.E.M) in the serum (nmol/ml) and urine (nmol/mg creatinine), of parents with colon cancer, healthy volunteers, and patients with nonmalignant gastrointestinal and the corresponding sensitivity and specificiy. Adapted from Khuhawar and Qureshi, 2001.

| | | No. | Age (yr) | Serum (nmol/ml) | | | | Urine (nmol/mg of creatinine) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | PUT | CAD | SPD | SPM | PUT | CAD | SPD | SPM |
| Normal patients | $X$±S.E.M. | 30 | 51.6 | 0.488 | 0.32 | 0.286 | 0.175 | 13.90 | 3.4 | 6.00 | 0.54 |
| | | | 1.8 | 0.03 | 0.07 | 0.01 | 0.01 | 1.0 | 0.5 | 0.5 | 0.08 |
| Nonmalignant disease patients | $X$±S.E.M. | 40 | 49.8 | 0.710 | 0.470 | 0.589 | 0.110 | 28.23 | 12.21 | 12.18 | 1.67 |
| | | | 1.7 | 0.08 | 0.05 | 0.06 | 0.02 | 2.5 | 1.8 | 0.9 | 0.2 |
| Colon cancer patients | $X$±S.E.M. | 50 | 58.1 | 0.765 | 0.380 | 0.640 | 0.180 | 37.3 | 15.7 | 20.62 | 1.48 |
| | | | 1.9 | 0.04 | 0.04 | 0.03 | 0.03 | 2.89 | 1.6 | 1.45 | 0.23 |
| Significance III/I | | | | 0.01 | NS | 0.001 | NS | 0.001 | 0.001 | 0.001 | 0.005 |
| Significance III/II | | | | NS | NS | NS | NS | 0.01 | NS | 0.005 | NS |
| Sensitivity | (%) | | | 60.25 | 50.50 | 89.15 | 34.30 | 84.00 | 66.3 | 0.92.1 | 74.3 |
| Specificity | (%) | | | 54.2 | 67.6 | 27.2 | 89.0 | 58.6 | 43.6 | 40.0 | 13.1 |

S.E.M.: Standard error of the mean; NS: not significant.
Data reproduced from Ref. [15], with permission.

**Table 1.4.4.3** – Free and acetylated polyamine concentration (X ± S.E.M.) in the urine (nmol/mg of creatinine) of patients with colon cancer, healthy volunteers, and patients with nonmalignant gastrointestinal diseases, and the corresponding sensitivity and specificity. Adapted from Khuhawar and Qureshi, 2001.

| | | No. | Urine (nmol/mg of creatinine) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | AcPUT | PUT | CAD | $N^1$-Acspd | $N^8$-Acspd/ SPD | $N^1$-Acspd/ $N^8$-Acspd | SPD | SPM |
| Normal patients | $X$±S.E.M. | 30 | 10.7 | 0.68 | 0.52 | 3.40 | 2.00 | 1.70 | 0.24 | 0.38 |
| | | | 0.7 | 0.1 | 0.06 | 0.3 | 0.18 | 0.11 | 0.02 | 0.08 |
| Nonmalignant disease patients | $X$±S.E.M. | 40 | 21.75 | 2.19 | 1.96 | 6.52 | 4.87 | 1.69 | 0.76 | 1.04 |
| | | | 2.2 | 0.17 | 0.17 | 0.57 | 0.43 | 0.19 | 0.07 | 0.16 |
| Colon cancer patients | $X$±S.E.M. | 50 | 31.93 | 2.75 | 2.03 | 11.92 | 6.28 | 2.29 | 0.72 | 0.65 |
| | | | 2.7 | 0.33 | 0.28 | 0.7 | 0.48 | 0.13 | 0.09 | 0.11 |
| Significance III/I | | | 0.001 | 0.005 | 0.005 | 0.001 | 0.001 | NS | 0.01 | NS |
| Significance III/II | | | 0.05 | NS | NS | 0.05 | NS | NS | NS | NS |
| Sensitivity | (%) | | 84.5 | 78.2 | 53.9 | 79.3 | 78.6 | 17.4 | 65.25 | 34.9 |
| Specificity | (%) | | 53.6 | 50.5 | 40.4 | 65.5 | 61.5 | 92.2 | 30.0 | 44.5 |

S.E.M.: Standard error of mean.
Data reproduced from Ref. [15], with permission.

# Chapter II - Analytical methods for PA determination

## Introduction

Polyamines (Put, Spd and Spm) and their acetyl conjugates do not contain a suitable chromophore or fluorophore group. Hence, they cannot be determined with adequate sensitivity by spectrophotometric or fluorescence detection. Therefore, most of the analytical procedures require different derivatizing reagents to increase the sensitivity of the method. The compounds have similar structural features, and for their selective separation they involved chromatographic (Villanueva *et al.*, 1998), electrophoretic (Ma *et al.*, 1999) radioimmunoassay (Bartos *et al.*, 1975) or enzymatic assay (Mashige *et al.*, 1988; Yoneda *et al.*, 1988) procedures. In biological samples the polyamines are present in low concentrations and most of the derivatizing reagents react with amino acids which are present at higher concentrations. Therefore, effective separation methods are required for polyamine determinations in the presence of amino acids in biological samples based on pre- or post-derivatization with spectrophotometric, fluorometric or electrochemical detection. For the separation of the polyamines all types of chromatographic methods have been used mainly, liquid, gas and planar chromatography, as well capillary electrophoresis. However, as we will see, all these methods involve complicated procedures or the use of expensive equipment.

## 2.1 Planar chromatography

Paper chromatography is one of the oldest methods for the analysis of polyamines where ninhydrin has been used as a locating reagent. Semi-quantitative assay is possible after extraction of the ninhydrin spots by 75% ethanol (Bachrach, 1973). Wiesner (Wiesner, 1979) reported $R_F$ values for 19 polyamines and three monoamines using PC. The method is rarely reported in recent literature and has been replaced by thin-layer chromatography (TLC). TLC is the simplest analytical technique. Cellulose or silicic acid plates are used to analyse free polyamines or their coloured or fluorescent derivatives (Simon-Sarkadi *et al.*, 1994; Shalaby, 1996). The commercialization of high-performance thin-layer plates (Merck, Whatman) has contributed towards excellent separations of amines by this method especially when bidimensional techniques are employed (Abdul-Monem *et al.*, 1978). Seiler and Knodgen (Seiler and Knodgen, 1977) determined Put, Spd and Spm as dansyl derivatives from mouse liver tissue, using this technique developed with a solvent mixture of cyclohexane-ethyl acetate (1:1).

## 2.2 Gas chromatography

The use of gas chromatography (GC) for the analysis of polyamines in biological samples is gradually increasing. Enormous abilities of high-resolution capillary column GC together with selective detection systems have enabled GC for quantification of polyamines at high sensitivity and selectivity (Dorhost et al., 1997; Shipe et al., 1997). GC of the polyamines without derivatization is possible (Berinali et al., 1978), but derivatization with suitable reagents improves GC elution and resolution with considerable enhancement in the sensitivity of the detection system. Bakowski et al. analysed Spd, Spm and Put as isobutyloxycarbonyl derivatives, using an N-sensitive glass bed detector and helium as a carrier gas. The method was applied for the determination of plasma polyamines and its application in tumor has been shown (Bakowski et al., 1981).

## 2.3 Liquid chromatography

Among the analytical methods for the determination of the polyamines and their conjugates, more procedures are available based on high-performance liquid chromatography (HPLC). For detection purposes, spectrophotometric and spectrofluorimetric detection devices are frequently used, followed by electrochemical or enzymatic post-column detection. The common derivatizing reagents reported for the purpose are ninhydrin (Chen *et al.*, 1979), orthophthaldehyde (Corbin *et al.*, 1989), *N*-succinimidyl-3-ferrocenylpropionate (Shimada *et al.*, 1989), 9-fluoroenylmethyl chloroformate (Gilbert *et al.*, 1991), benzoyl chloride (Talbe *et al.*, 1993) 3,5-dinitrobenzyl chloride (Wongyai et al., 1988), dansyl chloride (Minchin et al., 1984; Kabra et al., 1986; Walter et al., 1987), dabsyl chloride (Krause et al., 1995), 4-(2-phthalimidyl)benzoyl chloride (Zheng *et al.*, 1993), 1-phenylsulfonyl-3,3,3-trifluoropropene (Nakaijma *et al.*, 1990), 1-naphthylacetic anhydride (Wu and Gaind, 1992) and 2-chloroethylnitrourea (Vandenabeele *et al.*, 1998).

Dansyl chloride (5-dimethylaminonapthalene-1-sulfonyl chloride) used by us as a derivatizing agent reacts with primary as well secondary amino groups with the result that Put, Spd and Spm form fluorescent di-, tri- and tetradansyl derivatives, respectively (Brown and Stricker, 1982; Bontemps *et al.*, 1984). Put, Spd and Spm derivatives separated on a μBondapak $C_{18}$ column with 1-heptanesulfonic acid and acetonitrile as mobile phases, using programmed solvent gradient system separating within 30 min giving detection limit of 1 pmol (Brown *et al.*, 1979). A complete separation of 42 amines is shown in **Figure 2.3.1** (Price *et al.*, 1992).

# 2.4 Capillary electrophoresis

CE has developed enormously in terms of analytical technique, detection devices, sample introduction techniques and application to the analysis of biological samples. Mattausch et al. (Mattusch *et al.*, 1995) applied capillary zone electrophoresis (CZE) for the separation and quantification of Put, Cadaverine, Spd and Spm after their derivatization with fluorescin isothiocyanate. The detection limits were within 0.7–3.2 nmol/ l with Residual Standard Deviations (RSDs) of 4.6 to 12%. CE indicated better detection limits than GC or HPLC for polyamines analysis, but RSD is slightly on higher side.

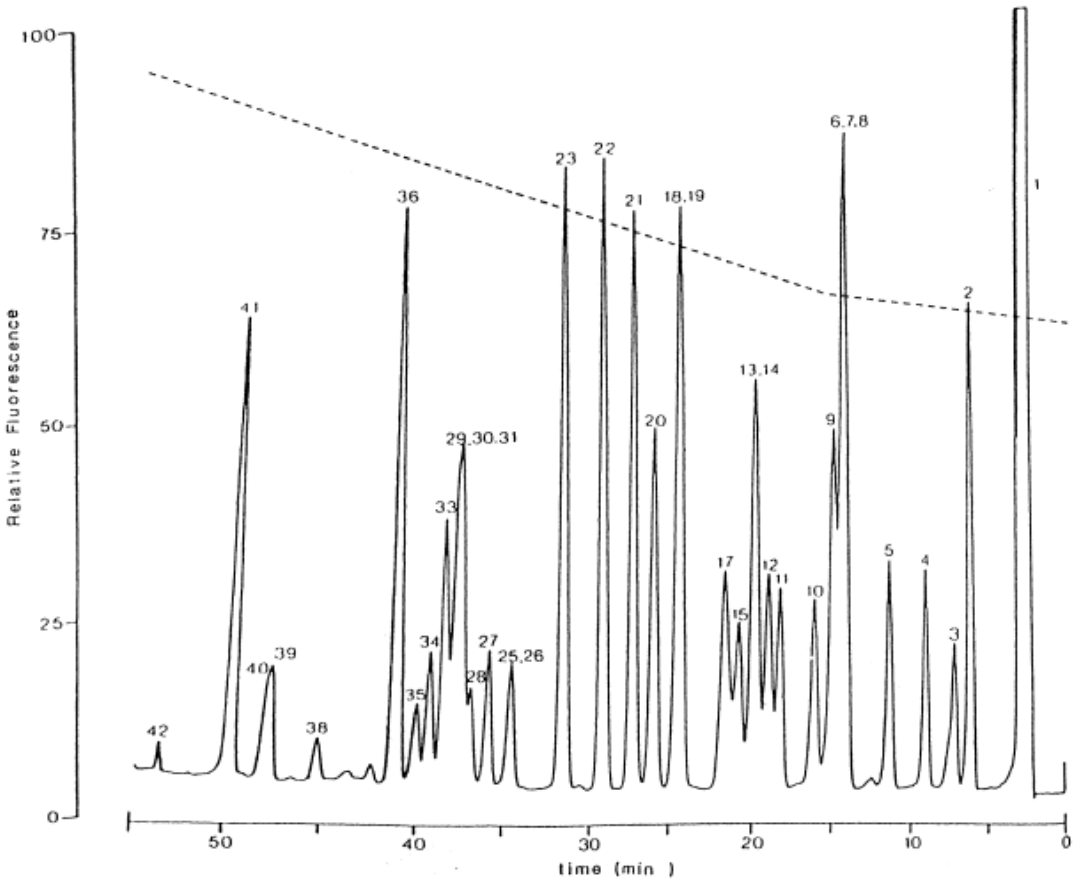



**Figure 2.3.1** – HPLC of dansylated standards separated on a Spheri-5RP-18, 5 µm reversed-phase column. Methanol-water gradient elution with flow rate 1 ml/min and fluorometric detection at excitation 340 nm and emission 540 nm. Each peak represents 0.9 nmol of amine. Peaks: (1) dansyl-OH, (2) ammonia, (3) ethanolamine, (4) methylamine, (5) ethylamine, (6) dimethylamine, (7) isopropylamine, (8) dansyl-Cl, (9) *n*-propylamine, (10) phenylethanolamine, (11) norephedrine, (12) isobutylamine, (13) *n*-butylamine, (14) benzylamine, (15) tryptamine, (16) agmatine, (17) L-ephedrine, (18) isoamylamine, (19) 2-phenylethylamine, (20) 1,3-diaminopropane, (21) Put, (22) Cadaverine, (23) 1,6-diamino-hexane, (24) *N*-methyl-putrescine, (25) DL-octopamine, (26) histamine, (27) 3-methyoxy-4-hydroxybenzylamine, (28) serotonin, (29) metanephrine, (30) 3-hydroxy-4-methoxyphenylethylamine, (31) 3-methyoxy-*p*-htyramine, (32) 1,7-diaminoheptane, (33) DL-synephrine, (34) *p*-tyramine, (35) *o*-tyramine, (36) Spd, (37) homospermidine, (38) norepinephrine, (39) DL-epinephrine, (40) dopamine, (41) Spm, (42) 5-dydroxydopamine. Adapted from Khuhawar and Qureshi, 2001.

# 2.5 Biosensor

As previously presented, many techniques have been developed and improved for detection and quantification of biogenic amines in biological fluids and nervous tissues, including fluorimetry (Palop *et al.*, 2002), thin layer chromatography with fiber optic detection (Aponte *et al.*, 1996) and capillary electrophoresis (Chen *et al.*, 2001; Maruszak *et al.*, 2001). Nowadays, high-performance liquid chromatography (HPLC) with fluorimetric detection (Fotopoulou and Ioannou *et al.*, 2002; Chan *et al.*, 2000), electrochemical detection (McKenzie *et al.*, 2002; Xu *et al.*, 2001) or combined fluorimetric and electrochemical detection, has been applied for neurotransmitter determinations. However, all these methods require sample pre-treatment, long analysis times, and some of these present poor selectivity and/or sensitivity, making them inadequate for routine work (Grossi *et al.*, 1990). Thus, the development of quick, efficient, sensitive, rapid and lowcost methodology for these very important analytes is still important. Electrochemical determination of biogenic amines using chemically modified electrodes (CME), such as carbon paste electrodes have been reported in the literature, as a good and cheap alternative for the traditional methods, in which the carbon paste is a suitable matrix for enzyme immobilization, principally due to the simplicity of the bulk modification, allowing the stabilization of enzymes in the paste, and the possibility of surface renovation (Gorton, 1995; Kalcher *et al.*, 1995; Motta and Guadalupe, 1994). Based on this, carbon paste amperometric biosensors become, in potential, a practical tool for rapid and cheap biogenic amine determinations.

The definition for a biosensor is generally accepted in the literature as a self contained integrated device consisting of a biological recognition element (enzyme, antibody, receptor or microorganism) which is interfaced to a chemical sensor (i.e., analytical device) that together reversibly respond in a concentration-dependent manner to a chemical species (Rodriguez-Mozaz *et al.*, 2005). Biosensors, especially the amperometric ones, have been the most successful and still have the most promising future for practical application (Castilho *et al.*, 2005). There are many different types of biosensors that can be classified according to the transducing system used (Patel, 2002; Mello and Kubota, 2002) (**Figure 2.5.1**):

1) **Potentiometric devices** measure changes in pH and ion concentration when an analyte in a sample interacts with a biomolecule immobilized on an electrode. The potential difference between the electrode bearing the biomolecule and a reference electrode is a function of the concentration of analyte in the sample.

2) **An amperometric biosensor** measures the current produced when an electroactive species is oxidized or reduced at a biomolecule-coated electrode to which an analyte interacts specifically.

3) **Conductimetric and capacitive biosensors** measure the alteration of the electrical conductivity in a solution at constant voltage, caused by biochemical reactions that specifically generate or consume ions. As these transducers are usually non-specific and have a poor signal/noise ratio, they have been little used.

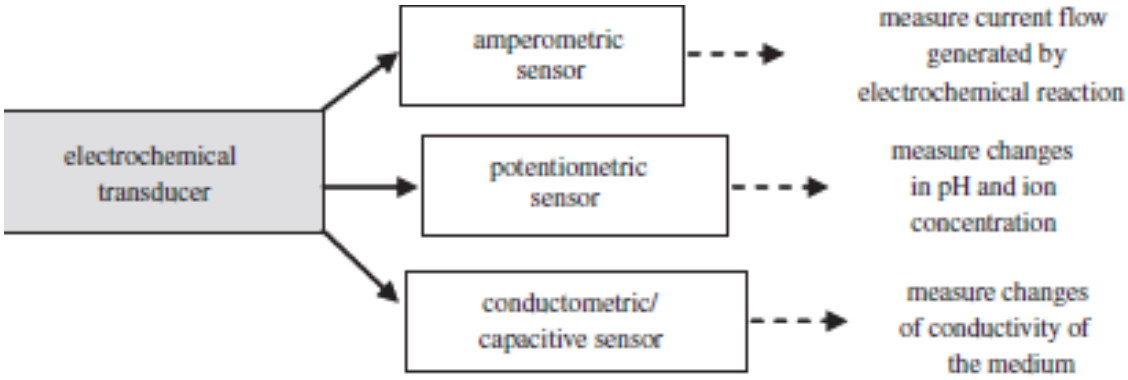



**Figure 2.5.1** – Classification of diferente electrochemical sensors. Adapted from Huet *et al.*, 2011.

## 2.5.1 Thick-film biosensor

Since the 1990s, screen-printing technology, adapted from the microelectronics industry, has offered high-volume production of extremely inexpensive, and yet highly reproducible and reliable single-use sensors; a technique which holds great promise for on-site monitoring. Therefore, the use of screen-printing technology in the serial production of disposable low-cost electrodes for the electrochemical determination of a wide range of substances is undergoing a widespread growth.

The advent of screen-printed (thick-film) technology has made it possible to mass-produce inexpensive electrodes for use with electrochemical instruments (Wang *et al.*, 1993; Yarnitzky *et al.*, 2000; Ugo *et al.*, 1998; Desmond *et al.*, 1998; Jasinski *et al.*, 2001). Planar configuration of a thick-film electrode can be classified into three groups: **multiple-layer deposition** (biological deposition by hand or electrochemically), **biological deposition by screen-printing** (using two or more steps), and the **one-step layer-deposition** or **biocomposite strategy** (Albareda-Sirvent *et al.*, 2000).

**Multi-layer deposition** (**Figure 2.5.1.1**.) entails sequential deposition of layers of the materials used to manufacture the electrode. The layer of the biological component of the sensor is deposited on to previously formed conducting tracks by simple physical adsorption, electropolymerization, cross-linked immobilization, or entrapment. Cagnini et al. (1995) developed a multi-layer biosensor for organophosphorus pesticides and carbamates based on choline enzyme deposited, by adsorption or electrochemical procedures, after previous deposition of a ruthenium layer.

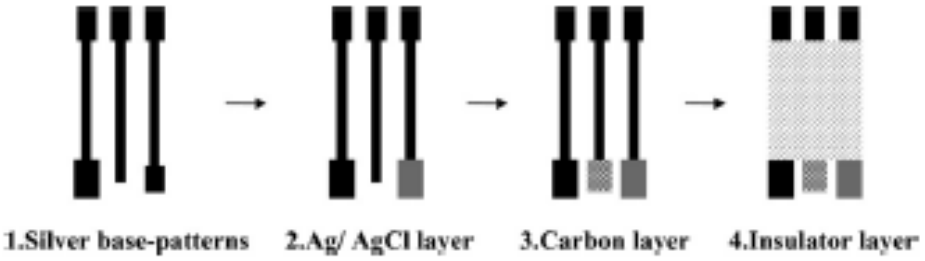



**Figure 2.5.1.1 -** Schematic diagram of the sensor preparation procedure. Adapted from Domínguez-Renedo and Arcos-Martínez, 2006.

**Biological deposition by screen-printing** consists in printing enzyme-containing inks onto electrode surface. The biological material of the biosensor can be printed on the electrode surface, instead of manual deposition as in the previous examples. The SPE is prepared in two or more steps, i.e. a layer of conducting paste (e.g. graphitic carbon) followed by successive screen-printed layers with mediator, enzyme, stabilizers, cross-linker and/or cofactor. Yoon et al. developed a three electrode screen-printed biosensor for L-lactate by printing a pre-mixed ink of enzyme and cofactor on the working electrode pad (i.e. polyester sheet coated with three silver conducting tracks, two with graphite ink and one with Ag/AgCl pseudo-reference electrode ink printed at the end).

A new technique for fabrication of SPEs is based on the **one-step deposition layer – biocomposite strategy**. In this technique enzymes and other materials, for example graphite, mediators, catalysts, stabilizers, and polymers, are mixed, forming the so-called biocomposite ink or paste which is screen-printed onto the working electrode, producing biosensors in a single, **one-step procedure**. This is currently the simplest configuration, and results in greater rapidity and simplicity in the manufacturing process. It is, however, also the technique requiring the most complex optimization, because of the need to maintain the initial properties of materials of different nature and characteristics. Wang *et al*. have reported the preparation of an electrochemical immunosensor based on this **biocomposite strategy**.

Ideally, a thick-film biosensor configuration comprises materials defining the basic structure of the electrode (i.e. **non-biological materials**) and the **biorecognition element**, which are the biological transducer of the biosensor, with their related compounds (e.g. mediator, cofactor) and additives and/or cross-linkers necessary for developing the electrochemical signal (**Figure 2.5.1.2**). In thick-film technology, **nonbiological materials** include substrates such as alumina, ceramics, PVC, gold, iron, etc., and the conducting pad of the electrode consisting of carbon ink/paste, or platinum or other metal paste. The **biorecognition element** traditionally used in the classical thick-film biosensor structure includes enzymes, antibodies, DNA, RNA, cells, etc., mediators (e.g. Meldola blue, Prussian blue), cofactors (e.g. NADH, PQQ), stabilizers, immobilization matrixes and/or additives (e.g. cellulose

acetate, Nafion), and **cross-**linkers (e.g. **glutaraldehyde**) are also often involved in the **biosensors structures** to improve its **sensitivity**, **selectivity**, **stability** and **reproducibility**.

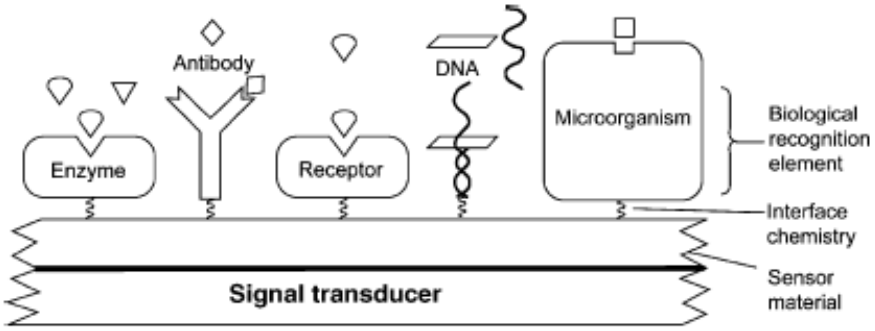



**Figure 2.5.1.2 -** Schematic representation of biosensors. Adapted form Rogers, 2006.

## 2.5.2 Non-biological components

SPEs substrates, are the base on which the functional sections of the sensor are printed and its composition is of extreme importance. Alumina, ceramics (Rantala *et al.*, 1993; Bilitewski *et al.*, 1992), PVC (Hart and Hartley, 1994), cardboard coated with acrylic paint (Weetall and Hotaling, 1987/88), and polycarbonate (Koopal *et al.*, 1994) are very well known substrates. Other substrate materials used for electrode support include gold (Silber et al., 1994), iron (Bilitewski *et al.*, 1992), and fiber glass (Galan-Vidal *et al.*, 1997). The pastes usually contain a **binding agent**, for example different kinds of glass powder, resins, or cellulose acetate, and solvents such as terpineol, ethyl cellosolve, cyclohexanone, or ethylene glycol, and additives that provide functional characteristics. If a **conductive paste** is required, it might contain powdered metals, for example gold (de Mattos *et al.*, 2003), platinum (de Mattos *et al.*, 2003), silver (Tu *et al.*, 2001), or palladium, or even nonmetallic conductors, for example graphite. These pastes are also used as bonding pads for electrical connections (Schuhmann *et al.*, 1992). **Pastes containing graphite** are being reported with increasing frequency, owing to their **electrochemical advantages** and improvements in **response**, **detection limit**, **cost**, etc. (Wring and Hart, 1992; O'Halloran *et al.*, 2001; Ricci *et al.*, 2003). When the carbon paste has the appropriate viscosity and thixotropy the resulting bulk electrodes have several advantages, for example **close proximity of the biocatalytic** and **sensing sites**, the possibility of producing reagent-free biosensors, renewing of the surface, **economy of fabrication**, and **stability** (Prodromidis and Karayannis, 2002). The active parts of the sensors (**biorecognition element**) must be connected to different conductive pads for transducing and conversing signals. For compatibility with the thick-film technology conductive pastes are usually used, and are applied by use of screen-printing techniques, resulting in planar devices named SPEs.

SPEs have enabled the **production of modern sensors** which can be incorporated in **portable systems**, an important requirement of analytical methods for direct analysis of a sample in its "environment" without alteration of the "**natural environmental conditions**" (**Table 2.5.2.1**). The recent possibility of designing and fabricating SPEs has **increased industrial, clinical, and environmental interest** in this field, expanding the possibility of direct implementation of laboratory-developed, screen-printed electrodes in real life applications, with important benefits. The most notable success is the **personal glucose biosensor used by diabetics** (Mathews *et al.*, 1987).

SPEs present important advantages such us the **elimination of memory effects** in the analysis at trace levels, appear to be particularly **attractive for *in situ* determinations**, and have a **low cost production**, **versatility**, and **miniaturization** (Vidal *et al.*, 2003). The **great versatility of SPEs** resides in their **wide range of possible modifications**. In fact, the composition of the inks used in the printing process can be modified by the **addition of substances of a very different nature**, such as metals, polymers, complexing agents etc. Moreover, the possibility also exists of modifying the electrodes once they have been fabricated through the **deposition of films** containing those substances (Domínguez-Renedo and Arcos-Martínez, 2006; Domínguez-Renedo *et al.*, 2008; Hart *et al.*, 2004; Eggings, 2002).

An interesting form of SPEs modification consists of the incorporation of **metallic nanoparticles** on the working electrode surface. Due to their reduced size, **metallic nanoparticles** exhibit important physical and electrical properties which make them very useful for the construction of more sensitive electrochemical sensors and biosensors (Ren *et al.*, 2005; Starowicz *et al.*, 2006; Welch and Compton, 2006). **Silver** and gold **nanoparticle-modified carbon SPEs** show important advantages when they are used as working electrodes in electrochemical techniques (Domínguez-Renedo *et al.*, 2008). Dominguez and Arcos (2007) have fine-tuned a novel, user friendly and rapid method of incorporating **Ag nanoparticles** onto the surface of SPCEs. This method is based on the direct **electrodeposition** of these **nanoparticles**. The modification of SPCEs with silver **nanoparticles** increases the already well known performance of these kinds of disposable electrodes. In order to demonstrate their practical applications, they were used to analyze Sb(III), a significant pollutant of priority interest (Domínguez and Arcos, 2007).

**Table 2.5.2.1 -** Most important applications of SPEs. Adapted from Domínguez-Renedo *et al.*, 2007.

| Analyte | Working SPE | References |
|---|---|---|
| Metals | SPCE | [4,13–15] |
| | Metal-based SPEs | [16,18] |
| | Hg-film-modified SPCE | [2,19-26,28,29,217] |
| | Bi-coated SPCE | [33–37] |
| | Au-coated SPCE | [38–40] |
| | Ni-coated SPCE | [41] |
| | Metallic nanoparticle-modified SPE | [51,52] |
| | Enzyme-modified SPE | [75–79] |
| $H_2O_2$ | SPCE | [5,56,57] |
| | Metallic nanoparticle-modified SPE | [50] |
| | Enzyme-modified SPE | [123–126] |
| Procaine | SPCE | [6] |
| Aurothiomalate | SPCE | [7] |
| Creatinine | SPCE | [8] |
| Cysteine and tyrosine | SPCE | [9] |
| Vitamin B2 | SPCE | [10] |
| Phloroglucinol derivatives | SPCE | [11] |
| Chlorophyll | SPCE | [12] |
| Dopamine and uric acid | SPCE | [218] |
| Pesticides and herbicides | Enzyme-modified SPE | [61–74] |
| | SPE immunosensor | [195–214] |
| Cholesterol | Enzyme-modified SPE | [80–84] |
| Glucose | Enzyme-modified SPE | [87–115,117–122] |
| Ethanol | Enzyme-modified SPE | [104,118,120,127–131] |
| Phenolic compounds | Enzyme-modified SPE | [132–137,139–142] |
| Hormones | SPE immunosensor | [153–163] |
| DNA | SPE immunosensor | [164] |
| Human cytomegalovirus | SPE immunosensor | [156] |
| Pneumococcal pneumonia | SPE immunosensor | [165,166] |
| Mycobacerium tuberculosis | SPE immunosensor | [167] |
| Salmonella | SPE immunosensor | [170] |
| Allergy antibody (IgE) | SPE immunosensor | [173] |
| *Listeria monocytogenes* | SPE immunosensor | [174] |
| *Vibrio cholerae* | SPE immunosensor | [175] |

## 2.5.3 Biorecognition elements

The **biorecognition elements** can be categorized on the basis of **structural** (e.g. enzymes, antibodies, or microorganisms) or **functional** (e.g. catalytic, affinity, or complex cellular functions) characteristics (Rogers, 2006) (**Table 2.5.3.1**).

1) **Enzymes** were the first biocatalysts used in biosensors and remain by far the most commonly employed. **Clark and Lyons** (1962) were the pioneers who showed that an **enzyme** could be integrated into an electrode, thus making a biosensor for the determination of glucose. Use of **enzymes** in biosensors has several advantages, for example the stable source of (primarily biorenewable) material, the possibility of modifying the catalytic properties or substrate specificity by genetic engineering, and catalytic amplification of biosensor response by modulation of enzyme activity in respect of the target analyte (Rogers, 2006). However, biosensors have also some limitations, namely, the limited number of substrates for which enzymes have evolved, the limited interaction between analytes of interest and specific enzymes, high extraction, isolation and purification costs, the lack of specificity in differentiating among compounds of similar classes, for example nerve agents and organophosphate and carbamate pesticides, etc. (Andreescu *et al.*, 2001; Wang *et al.*, 2006; Sapelnikova *et al.*, 2003). General examples of enzyme biosensors based on SPEs can be showed in **Table 2.5.3.1**. Disposable uric acid sensor was reported in 1992 by Gilmartin and Hart. This was followed by several others, including biosensors based on glucose, lactose, and/or sucrose-degrading enzymes for determination of carbohydrates in food (Bilitewski *et al.*, 1993), a tyrosinase sensor for detection of phenol (Kotte *et al.*, 1995), a thick-film biosensor for organophosphates (Mulchandani *et al.*, 1999), a parathion hydrolase biosensor for direct measurement of parathion (Sacks *et al.*, 2000), and, later, nitratemonitoring biosensors (Quan *et al.*, 2005) and an enzyme thick-film electrode for detection of V-type nerve agents (Joshi *et al.*, 2006).

2) **Antibodies** (ABs) also are used for construction of the biosensors based on SPEs. One example is an electrochemical immunosensor based on a disposable SPCE designed for analysis of thyrotropin (Athey *et al.*, 1993). The high affinity of the **ABs** used as recognition element for target analytes results in low detection limits in immunosensor assays (Zhang and Heller, 2005; Micheli *et al.*, 2005). The disadvantage of this, however, is that the antigen is not easily released from the **AB** after the measurement has been made. There are, however, several limitations to the use of imunosensors, including the complexity of the assays, especially the number of specialized reagents (e.g. **ABs**, antigens, tracers, etc.) that must be developed and characterized for each compound, and the limited number of compounds typically determined in an individual assay.

3) **Nucleic acids** (e.g. DNA and RNA) have been successfully incorporated in the SPEs surfaces of biosensors because of their wide range of physical, chemical, and biological activity. Graphite SPEs have been used for accumulation of **nucleic acids** and their behavior was similar to that observed for graphite-paste electrodes (Wang *et al.*, 1996; Marrazza *et al.*, 1999).

4) **Bacteria**, **yeast** and **tissue-culture cells** have been used as biorecognition element in **microbial biosensors** (Rogers, 2006) by immobilizing the cells on a suitable measuring device, for example an $O_2$ electrode. The cells can be directly immobilized on the electrode or mounted separately from the electrode. Because a variety of the enzymes is present in whole cells, all substances affecting the turnover of one of the enzymes could also affect the cellular metabolic activity measured (Heim *et al.*, 1999). These sensors are useful for **detection of complex pollution**, as the quality of a soil or the extent of pollution of water (Riedel, 1994; Skladal *et al.*, 2002; Lanyon *et al.*, 2006). Clinical analysis is another field in which these biosensors have been used (Kelso *et al.*, 2000).

## 2.5.4 Immobilization of biorecognition element

Enzyme immobilization appears as a key factor to develop efficient biosensors with appropriate performances such as good operational and storage stability, high sensitivity, high selectivity, short response time and high reproducibility. Immobilized biomolecules have to maintain their structure, their function, to retain their biological activity after immobilization, to remain tightly bound to the surface and not to be desorbed during the use of the biosensor. Moreover, an ideal biosensor has to be stable for long-term application. The type of immobilization method affects activity and stability of enzymatic biosensors. The methods used include the traditional procedures of physical wrapping and chemical cross-linking or entrapment, and others methods such as immobilization by affinity (**Figure 2.5.4.1**).

1) **Adsorption** is a **rapid** and **simple procedure**, especially for disposable biosensors. Physical adsorption based on **van der Waals attraction** between biomolecule and solid support surface is the **method most often used**. The most important drawbacks of this technique are that bonding forces between biomolecule and support are weak and cannot easily be controlled. As a consequence, the biological component can be leached during the assay, depending on experimental conditions such as pH, ionic strength, temperature, and solvent (Albareda-Sirvent *et al.*, 2000). Disposable electrochemical DNA sensors have been prepared by adsorption of synthetic single-stranded oligonucleotides on graphite SPEs (Marrazza *et al.*, 1999; Marrazza *et al.*, 2000; Del Giallo *et al.*, 2005).

2) Immobilization by **intra or intermolecular cross-linking** has also been used to coat electrode surfaces with specific biotransducer molecules. The method is based on the formation of three-dimensional (3D) links between the biological material and bi or multifunctional reagents. The resulting modified biological material is completely insoluble in water and can be adsorbed on a solid surface. Configurations based on multiple-layer deposition using **glutaraldehyde as a cross-linking agent for enzyme immobilization** are among the methods most often reported for immobilization of enzymes in planar biosensor devices. Because the resulting 3D network sometimes affects the kinetic and diffusion properties of the enzyme, optimization of the immobilization step is needed to preserve enzyme activity and appropriate diffusion characteristics (Albareda-Sirvent *et al.*, 2000). This strategy has been used to develop biosensors for uric acid (Gilmartin and Hart, 1992, carbohydrates (Bilitewski *et al.*, 1993), catechol (Kirgoz *et al.*, 2005), and pesticides (Solna *et al.*, 2005).

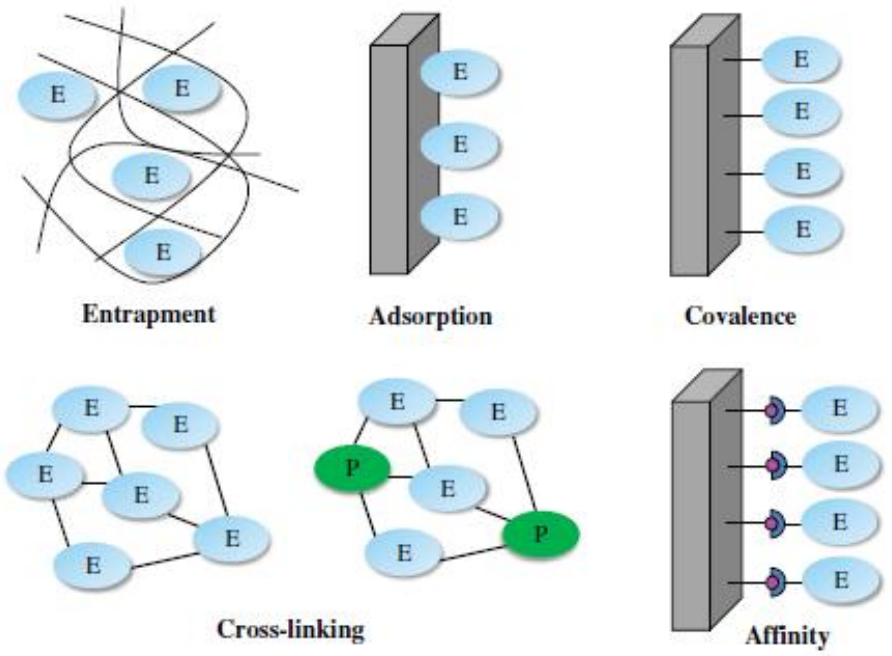



**Figure 2.5.4.1** – Schematic representation of the main diferente method of enzyme immobilization. E: enzyme, P: inert protein.

3) **Covalent immobilization** of the biorecognition component on the SPEs surface can also be achieved by use of the self assembled monolayer (SAM) procedure. In this process the electrode surface should be coated by a gold layer. Active groups (–COOH, –NH2, –OH) on the surface of the monolayer are then reacted with the biomolecule leading to **covalent bonding**. The important characteristics of SAM, for example the stability, reproducibility, and uniformity of the monolayer, have led to the use of this type of immobilization in several applications, for example detection of contamination of food by pathogenic bacteria, and assessment of environmental pollution (Farabullini *et al.*, 2007).

4) Biocomponent immobilization by **entrapment** within a suitable matrix which is then deposited on the screen-printed support can improve the stability of the biorecognition component. Immobilization in matrices such as gels, polymers, pastes, or inks can also be as simple as **physical adsorption**. The biological material is usually mixed and well homogenized with the supporting material and then applied over the electrode as an additional membrane that must be dried or polymerized. The principal advantage of this technique is its compatibility with **mass fabrication techniques**. Kalab and Skladal (Kalab and Skladal, 1997) reported the preparation of a biosensor for 2,4-dichlorophenoxyacetic acid herbicides based on a screen-printed amperometric transducer and a monoclonal AB against 2,4-dichlorophenoxyacetic acid entrapped within a thin Nafion film which was then directly immobilized on the electrode surface. **Electropolymerization** is a special example of the entrapment technique. The enzyme, or any biological molecule, is homogenized in a monomer matrix and this is then deposited by **electropolymerization**. Conducting polymers include polypyrrol, polythionine, polyaniline, and polyindole (Albareda-Sirvant *et al.*, 2000; Gao *et al.*, 2003; Grenann *et al.*, 2005).

5) Immobilization by **affinity** is a strategy to create (bio)affinity bonds between an **activated support** (e.g. with lectin, avidin, metal chelates) and a **specific group** (a tag) of the protein sequence (e.g. carbohydrate residue, biotin, histidine). This method allows **controlling the biomolecule orientation** in order to avoid **enzyme deactivation** and/or **active site blocking**. Several affinity methods have been described to immobilize enzymes through (strept)avidin-biotin (Esseghaier *et al.*, 2008), lectin-carbohydrate (Bucur *et al.*, 2005) and metal cation-chelator interactions (Halliwell *et al.*, 2002). An enzyme can contain affinity tags in its sequence (e.g. a sugar moiety) but, in some cases, the affinity tag (e.g. biotin) needs to be attached to the protein sequence by genetic engineering methods such as site-directed mutagenesis, protein fusion technology and post-transcriptional modification (Andreescu and Marty, 2006).

In conclusion, there are different types of biomolecules that we can immobilize and several methods to perform its immobilization on SPEs previously (or not) modified with nanoparticles. Below it is presented some biosensors which are applied on food analysis showing in each case the limit of detection and the range calibration (**Table 2.5.4.1**).

**Table 2.5.3.1 -** Biorecognition compontent of biosensors based on SPEs. Adapted from Tudorache and Bala, 2007.

| Biocomponent of biosensor | | Application | Sensitivity (μmol $L^{-1}$) | Ref. |
|---|---|---|---|---|
| Enzyme | L-Ascorbic acid oxidase | Uric acid | 2.5 | [34] |
| | Glucose oxidase with the mediator tetrathiafulvalene beta-galactosidase, and glucose oxidase and invertase mutarotase and glucose oxidase | Glucose<br>Lactose<br>Sucrose | – | [35] |
| | Tyrosinase modified with methylphenazonium-zeolite | Phenols | $2.5\times10^{-4}$ | [36] |
| | Organophosphorus hydrolase | Hydrolyzed paraoxon<br>Methyl parathion | $9.0\times10^{-2}$<br>$7.0\times10^{-2}$ | [37] |
| | Parathion hydrolase from Pseudomonas sp. isolated from contaminated soil | Parathion | 1.0 | [38] |
| | Nitrate reductase derived from yeast methyl viologen mediator | Nitrates | 4.1 | [39] |
| | Organophosphorus hydrolase | V-type nerve agents<br>VX (O-ethyl-S-2-diisopropylaminoethyl methylphosphonothioate)<br>RNX (O-isobutyl-S-2-diethylaminoethyl methylphosphonothioate) | 2.0<br>$8.0\times10^{-1}$ | [40] |
| Genetically modified enzyme | Glucose oxidase (GOD) genetically modified by adding a poly-lysine chain at the C-terminal with a peptide linker inserted between the enzyme and poly-lysine chain | Glucose | – | [41] |
| | Genetically engineered acetylcholinesterases from *Drosophila melanogaster* | Carbaryl<br>Carbofuran<br>Pirimicard | $1.0\times10^{-2}$<br>$8.0\times10^{-4}$<br>$2.0\times10^{-2}$ | [42] |
| | Genetically modified acetylcholinesterase from *Drosophila melanogaster* | Methamidophos pesticide | 1.0 | [43] |
| Antibody | Anti-tyrotropin antibody | Tyrotropin | | [44] |
| | Anti-progesterone monoclonal antibody | Progesterone | 2.0–5.0 | [45] |
| | Monoclonal sheep anti-progesterone antibody | Progesterone | $1.0\times10^{-3}$ | [46] |
| | Polyclonal anti-2,4-dichlorophenoxyacetic acid antibody | 2,4-Dichlorophenoxyacetic acid herbicide | $1.0\times10^{-2}$ | [47] |
| | Monoclonal anti-2,4-dichlorophenoxyacetic acid antibody | 2,4-Dichlorophenoxyacetic acid herbicide | $1.0\times10^{-1}$ | [48] |
| | Anti-rabbit IgG | Rabbit IgG | $7.0\times10^{-3}$ | [49] |
| | Anti-aflatoxin M1 | Aflatoxin M1 | $2.5\times10^{-2}$ | [50] |
| | Monoclonal mouse anti-phenanthrene | Phenanthrene | $8.0\times10^{-1}$ | [51] |
| Nucleic acids (DNA/RNA) | Single-stranded nucleic acid-modified strips | trna<br>ssDNA<br>dsDNA | 3.0<br>$2.5\times10^{1}$<br>$3.0\times10^{1}$ | [52] |
| | Single-stranded oligonucleotides | Daunomycin<br>Polychlororinated biphenyls<br>Aflatoxin B1 | $3.0\times10^{2}$<br>$2.0\times10^{2}$<br>$3.0\times10^{4}$ | [64] |
| | Single-stranded oligonucleotides | Human apolipoprotein E | – | [65] |
| | 25-mer Oligonucleotide, from the E. coli lacZ gene | Short DNA sequences from the *E. coli* pathogen | – | [55] |
| | DNA | Human DNA antibodies of systemic lupus erythematosus and bronchial asthma | – | [57] |
| Bacteria/ yeast/tissue culture cell | Pseudomonas cell | Phenol | 1.0 | [60] |
| | *Pseudomonas putida* ML2 | Benzene | $1.0\times10$ | [61] |
| | Secreted placental alkaline phosphatase | Herpes simplex virus | – | [62] |

**Table 2.5.4.1 -** Enzyme-biosensors based on SPEs dedicated to food analysis. Adapted from Tudorache and Bala, 2007.

| Analyte(s) | Biosensor configuration | Biosensor characteristic(s) | | Ref. |
|---|---|---|---|---|
| | | LOD (μmol $L^{-1}$) | LR (μmol $L^{-1}$) | |
| Lactic acid | Enzyme (lactate and malate dehydrogenase) immobilized by sol–gel network based on orthosilicates | – | 0–2 | [110] |
| Malic acid | | | 0–15 | |
| Acetaldehyde | Entrapment of aldehyde dehydrogenase and nicotinamide adenine dinucleotide ($NAD^+$) in a sol–gel matrix on electrodes modified with the mediator MBRS | – | – | [111] |
| Acetaldehyde | *Disposable sensor* – $NAD^+$ and the enzyme (D-lactate/aldehyde dehydrogenase) adsorbed on an MB-modified electrode | 1.0<br>30 | 5–500<br>50–1000 | [76] |
| D-Lactic acid | *Reusable sensor* – enzyme (D-lactate/aldehyde dehydrogenase) immobilization by sol–gel procedure on an MBRS-modified electrode | 6.0<br>50 | 10–250<br>75–1000 | |
| D-Lactic acid | *Disposable sensor* – deposition of the enzyme (D-lactate/L-lactate/aldehyde dehydrogenase) on an MB-modified electrode | 50<br>30 | 75–1000<br>50–1000 | [103] |
| Acetaldehyde<br>L-Lactic acid | *Reusable sensor* – entrapment (photopolymerization/metal chelate affinity) of the enzyme (D-lactate/L-lactate/aldehyde dehydrogenase) on an MBRS-modified electrode | 6.0<br>1.0<br>10 | 10–250<br>5–500<br>20–200 | |
| Acetaldehyde | Sol–gel entrapment of aldehyde dehydrogenase on an MBRS-modified electrode | – | 10–260 | [112] |
| L-Lactate | Immobilization of lactate dehydrogenase on electrochemically polymerized MB film accomplished by electrodeposition from monomer solution containing enzyme | 100 | 1000–1200 | [113] |
| Ethanol | Adsorbed alcohol oxidase on the CoPC-modified electrode; coated the electrode with nitrocellulose acetate/polycarbonate membrane | – | 120–2000 | [115] |
| Ethanol | Immobilization of alcohol oxidase with PCS hydrogel | – | 10–3000 | [116] |
| Ethanol | Cross-linked QH-ADH to an Os-complex-modified poly(vinylimidazole) redox polymer using poly(ethylene glycol) diglycidyl ether | 1.0 | 1–250 | [117] |
| Paraoxon<br>Carbaryl | Wild-type enzyme combined with different engineered variants of NbAChE | 1.0<br>20 | – | [122] |
| Carbaryl<br>Parathion methyl | AChE immobilized on the electrode | – | 2–90 (ppb)<br>1–10 (ppb) | [123] |
| Pirimiphos methyl | AChE immobilized on the electrode | $3.5\times10^{-6}$ | – | [124] |

MB - Meldola blue
MBRS - Reinecke salt of Meldola blue
CoPC - Cobalt phthalocyanine
PCS - Poly(carbamoyl)sulfonate
QH-ADH - Quinoprotein alcohol dehydrogenase
NbAChE - *Nippostrongylus brasiliensis* acetylcholinesterase

# Chapter III – Materials, apparatus and methods

## 3.1 Reagents

### 3.1.1 Fabrication of SPE

Several **inks** were used in the fabrication of **screen-printed electrodes (SPEs)**, namely Electrodag PF-407 A (carbon ink), Electrodag 6037 SS (silver/silver chloride ink) and Electrodag 452 SS (dielectric ink) supplied by Achenson Colloiden (Scheemda, Netherlands).

### 3.1.2 Stock solutions

Spermidine (Spd) ($C_7H_{19}N_3$, Acros Organics, Fisher Scientific, Loures, Portugal), monoamine oxidase A (MAO) (Sigma-Aldrich, Steinheim, Germany), diamine oxidase (DAO) (Sigma-Aldrich, Steinheim, Germany) and bovine serum albumin (BSA) (Sigma-Aldrich, Steinheim, Germany) were prepared in phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$, Panreac, Barcelona, Spain). The pH was adjusted by NaOH (J.T. Baker, Deventer, Netherlands) and HCl (Sigma-Aldrich, Steinheim, Germany).

Aliquots and main solutions of Spd and DAO were stored in a -20ºC refrigerator, while MAO solutions were stored in -80º C refrigerator. BSA and Glutaraldehyde solutions (Sigma-Aldrich, Steinheim, Germany) were stored at 4º C.

## 3.2 Apparatus and software

SPEs were produced on on a **DEK 248 printing machine** (DEK, Weymouth, UK) using polyester screens with appropriate stencil designs mounted at 45º to the printer stroke (**Figure 3.2.1**).

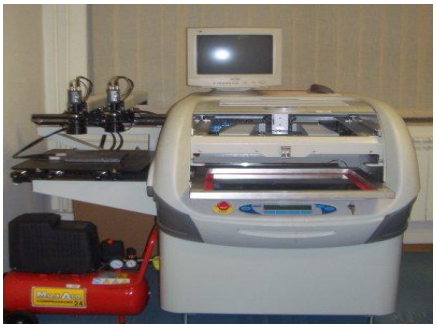


**Figure 3.2.1 -** DEK 248 printing machine

Electrochemical measurements were performed by Autolab PGSTAT128N electrochemical system (**Figure 3.2.2**) with General Purpose Electrochemical System (GPES) software version 4.9 (Eco Chemie B.V., Utrecht, Netherlands).

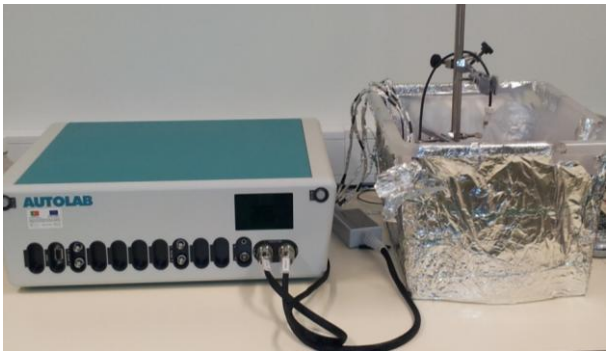



**Figure 3.2.2 -** Autolab PGSTAT128N electrochemical system

# 3.3 Methods

## 3.3.1 Preparation of SPEs

1) Electrodes are fabricated by using a DEK 248 screen-printing system (**Figure 3.2.1**), a screen polyester mesh and polyurethane squeegees. Perform a sequential layer deposition on a polyester film (0.5 mm thickness) to obtain 44 screen-printed configurations of three electrodes each one (working, reference and counter electrode) per film. 4 $mm^2$ carbon working electrodes are design (**Figure 3.3.1.1**).

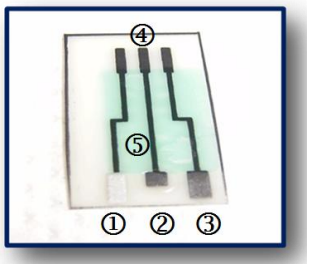



**Figure 3.3.1.1 -** Design of a home-maid SPCE (1 - Reference electrode: Ag/AgCl; 2- Working electrode; 3- Counter-electrode; 4- Electrical connections; 5- Dielectric protection layer)

2) Mechanical clean of the counter and working electrodes surfaces by a thin grain sandpaper.

3) Using the cyclic voltammetry method record 20 cycle voltammograms between -2 V and 2 V with a 100 $mVs^{-1}$ scan rate in a 0.1 M KCl solution for electrode activation.

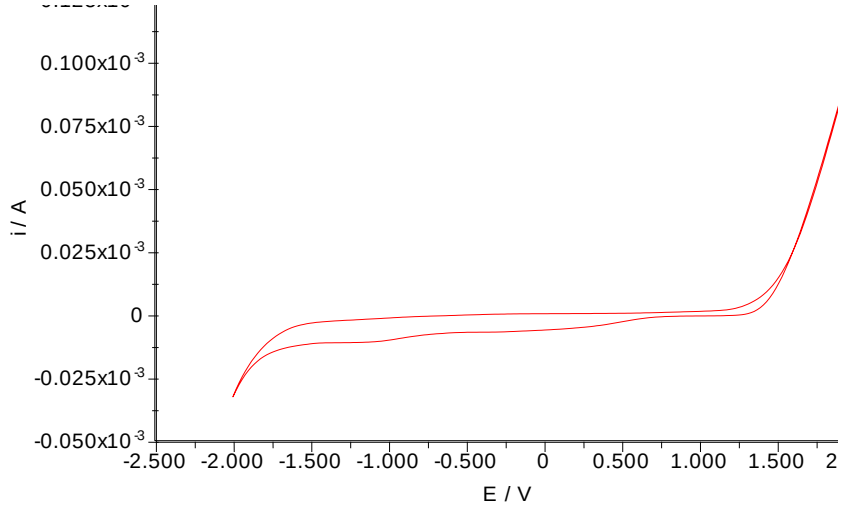



**Figure 3.3.1.2 -** Working electrode surface activation

New screen printed electrodes do not need this treatment.

## 3.3.2 Metallic nanoparticles deposition on SPE

There are two methods to deposit two different metallic nanoparticles:

**a) Gold deposition**

1) Prepare a 0.1 mM solution of $HAuCl_4$ in 0.5 M $H_2SO_4$.
2) Submerge the electrode in a 4 mL $HAuCl_4$ solution.
3) Apply an accumulation potential of 0.18V during 15s.

**b) Silver deposition**

1) Prepare 0.04 M acidic (pH = 2) Britton-Robinson buffer
2) Prepare a 5mM $AgClO_4$ solution
3) Submerge the electrode in a 4 mL Britton-Robinson buffer and add 81.6 µL of $AgClO_4$.
4) Apply an accumulation potential of -0.8 V during 120s.

## 3.2.3 Enzyme immobilization

There are two methods to immobilize two different enzymes:

**a) MAO immobilization**

1) Prepare phosphate buffer pH 7.5 (0.05 M);
2) Prepare MAO (120 mg/mL), BSA (5 mg/mL) (1) and Glutaraldehyde (27 mg/mL) (2) solutions in phosphate buffer pH 7.5;
3) Add 5 µL of MAO solution, 6.8 µL of solution 1, 3.2 µL of phosphate buffer pH 7.5 in a eppendorf (3);

4) Take 5 µL of solution 3 and solution 2 to make a 10 µL mixture on other eppendorf (4).
5) Lastly, mix 5 µL of solution 4 with 5 µL of solution 2, and take 5 µL of this solution (5) and deposit in the working electrode surface.
6) Set the electrode in the 4ºC refrigerator and wait for 1h30.

**b) DAO immobilization**

1) Prepare DAO solution (120 mg/mL) in phosphate buffer pH 7.5 (0.05 M).
2) Add 10 µL of DAO solution, 6.8 µL of solution 1, 3.2 µL of phosphate buffer pH 7.5 in a eppendorf (3);
3) Take 5 µL of solution 3 and solution 2 to make a 10 µL mixture on other eppendorf (4).
4) Lastly, mix 5 µL of solution 4 with 5 µL of solution 2, and take 5 µL of this solution (5) and deposit in the working electrode surface.
5) Set the electrode in the 4ºC refrigerator and wait for 1h30.

# Chapter IV – Results and discussion

## 4.1. Sensitive diaminoxidase biosensor based on carbon SPE for Spd determination

### 4.1.1 First results and signal optimization

In this particular biosensor, the basic underlying chemistry is the action of diamine oxidase (DAO) that catalyzes the oxidative deamination of spermidine to 3-aminopropionaldehyde, hydrogen peroxide ($H_2O_2$) and ammonia ($NH_3$) (Duhazé et al., 2002). The involved reaction is shown in **Figure 4.1.1.1**.

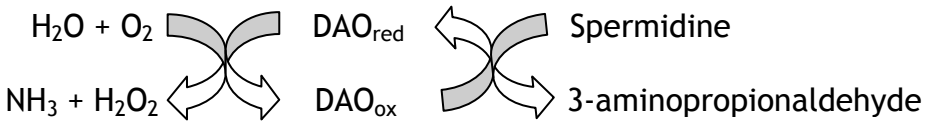



**Figure 4.1.1.1 -** Oxidative deamination of spermidine to 3-aminopropionaldehyde, hydrogen peroxide and ammonia by DAO

Catalysis proceeds through a ping-pong mechanism divided into two half-reactions:

$$DAO_{OX} + RCH_2NH_2 \Leftrightarrow DAO\text{-}RCH_2NH_2 \rightarrow DAO_{RED} + RCHO$$

$$DAO_{RED} + O_2 \rightarrow DAO_{OX} + H_2O_2 + NH_3$$

The overall reaction will became rate limited if either Spd or oxygen concentration are too low. Spd concentration can be measured by chronoamperometry, by monitoring current intensity related with the oxidative deamination of Spd.

The electrochemical characterization of this biosensor included experiments carried out in absence of DAO (**Figure 4.1.1.2**).

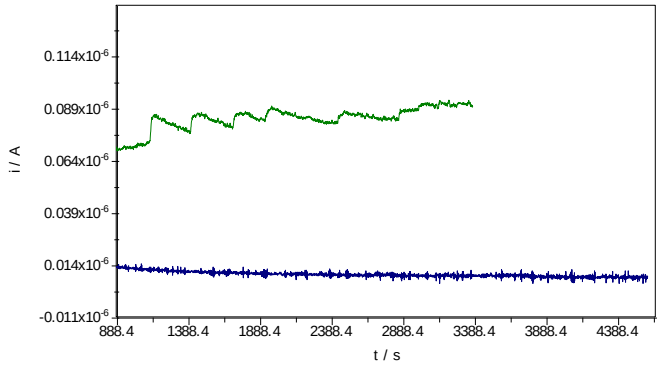



**Figure 4.1.1.2 -** Several Spd additions ($1x10^{-4}$ M) to a DAO immobilize (green line) and a without DAO immobilize (blue line) sensor. The phosphate buffer pH and the applied potential used were respectively 7.5 and + 0.7 V (theoretical value for optimal enzyme operation and oxygen reduction to $H_2O_2$ potential).

No chronoamperometric response was observed by adding several aliquots of Spd $1x10^{-4}$ M solution to the non-modified electrode. However, a clear response can be observed with the biosensor immobilized with DAO, thus providing the essential role of DAO on this type of response.

Because reduction of $H_2O$ (l) to $OH^-$ (aq) in presence of oxygen has a standard potential of 0.82 at pH near 7, the chronoamperometric response was also evaluated for the addition of pure water aliquots (**Figure 4.1.1.3 a** and **b**). After several additions of 50 µl of Spd ($1x10^{-5}$ M) to a DAO immobilized biosensor two additions of 50 µl of pure water were made without any effect (**Figure 4.1.1.3 a**). The experiment was repeated with the additions of 100 µl of Spd ($1x10^{-5}$ M) and two additions of 100 µl of pure water. However a weak signal was observed (**Figure 4.1.1.3 b**) it cannot be quantified because it returns to baseline, proving that reduction of water does not interfere with Spd chronoamperometric signal.

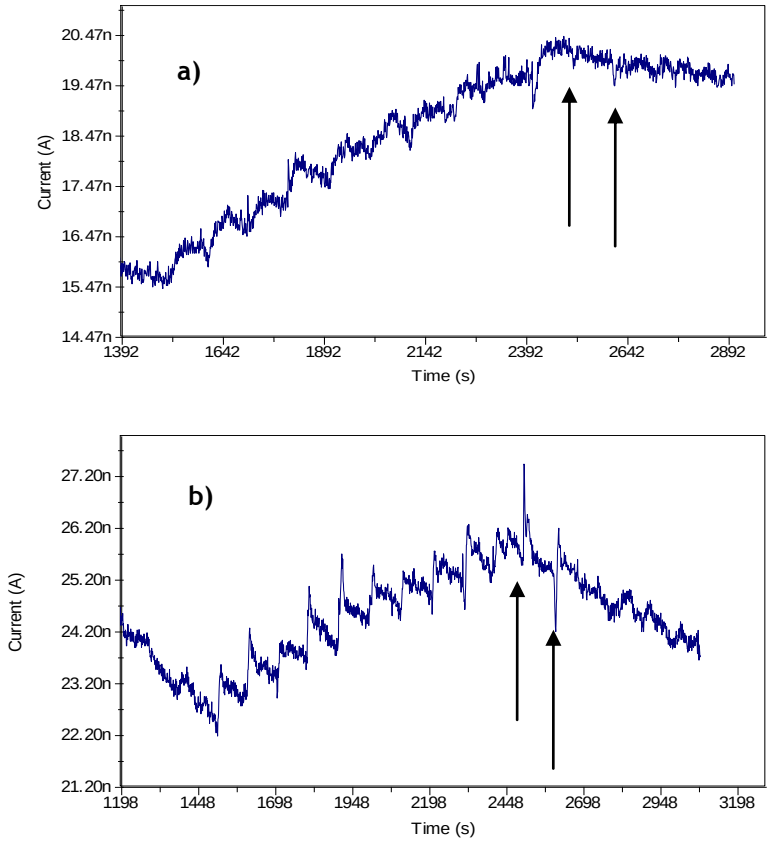


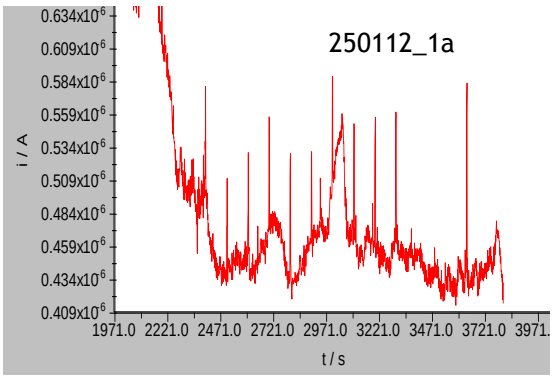

**Figure 4.1.1.3 -** Chronoamperometric response to several Spd additions ($1x10^{-5}$ M) and two additions of pure water (indicated by an arrow) to DAO immobilized biosensor. **a)** 50 µl additions. **b)** 100 µl additions. The phosphate buffer pH and applied potential used were respectively 8.3 and +0.6 V.

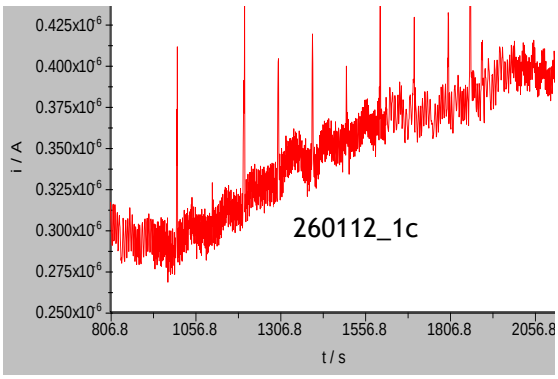
A specific biosensor design is largely dependent upon the transducer operating principle, the involved analytes and the working environment. So, after sensor architecture optimization, experimental chronoamperometric variables must be optimized. Chronoamperometric current depends directly on experimental variables such as applied potential and solution pH (Crouch et al., 2005). Their effect on current response was estimated one-at-time.

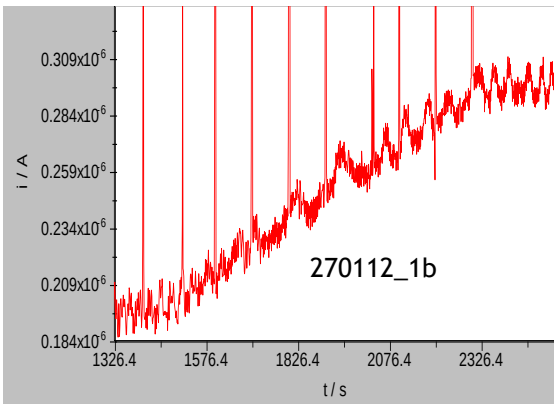
The effect of applied potential has been evaluated by considering the following potentials: +0.7 V, + 0.8 V and + 0.9 V. The results are shown in **Tables 4.1.1.1**, **4.1.1.2** and **4.1.1.3** with the respective chronoamperograms.

**Tables 4.1.1.1** - Experimental data resume for DAO by applying + 0.7 V. Solutions prepare in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M. NQ = Not quantified.

| File | Valid Additions | Position | Equation | $R^2$ |
|---|---|---|---|---|
| **250112_1a** | | 1st | NQ | NQ |
| **260112_1c** | 10 | 3rd | y = 1.19E$^6$x + 4.3045 | 0.9885 |
| **270112_1b** | 10 | 2nd | y = 1.21E$^6$x - 5.1782 | 0.9981 |
| **010212_1b** | | 2nd | NQ | NQ |



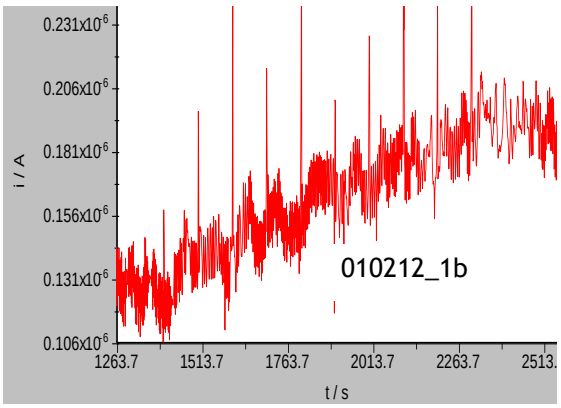
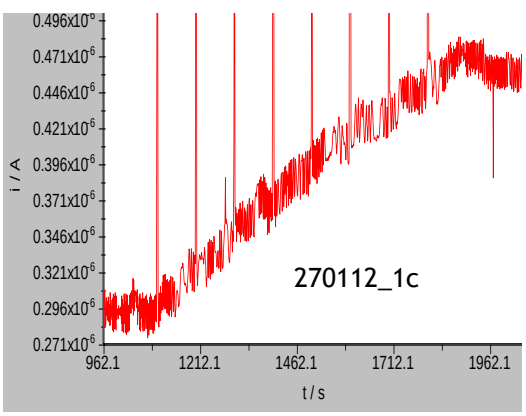
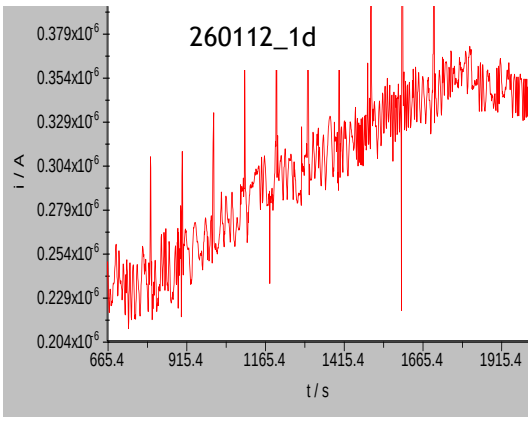
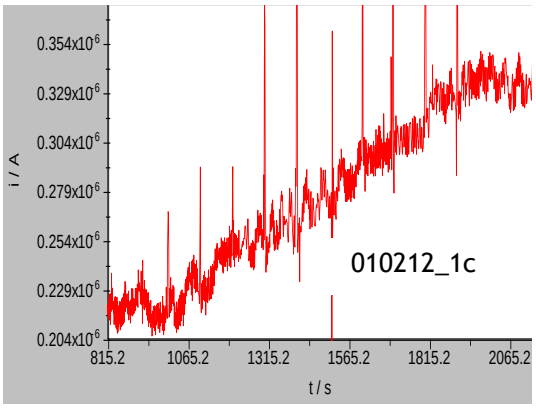



**Table 4.1.1.2** - Experimental data resume for DAO by applying +0.8 V. Solutions prepared in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M.

| File | Valid Additions | Position | Equation | $R^2$ |
|---|---|---|---|---|
| **260112_1d** | 10 | $4^{th}$ | $y = 1.33E^{6}x + 3.2837$ | 0.9936 |
| **270112_1c** | 8 | $2^{nd}$ | $y = 2.34E^{6}x + 1.3605$ | 0.9991 |
| **010212_1c** | 10 | $3^{rd}$ | $y = 1.28E^{6}x - 0.4899$ | 0.9929 |
| **010212_2d** | 9 | $4^{th}$ | $y = 1.34E^{6}x - 1.7590$ | 0.9871 |



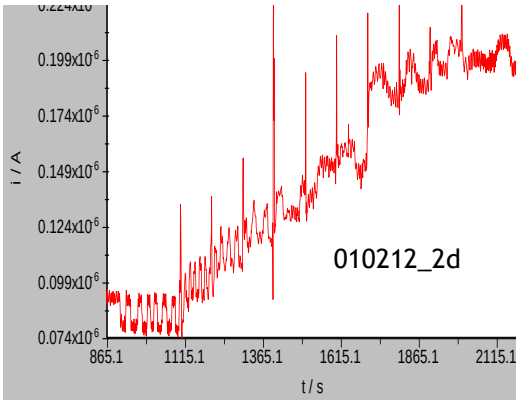
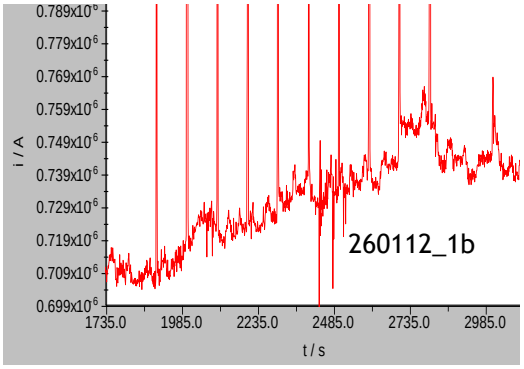
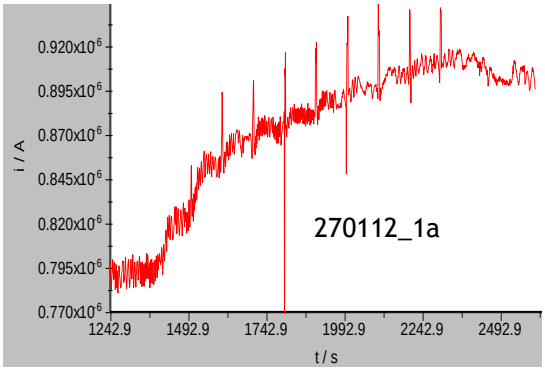
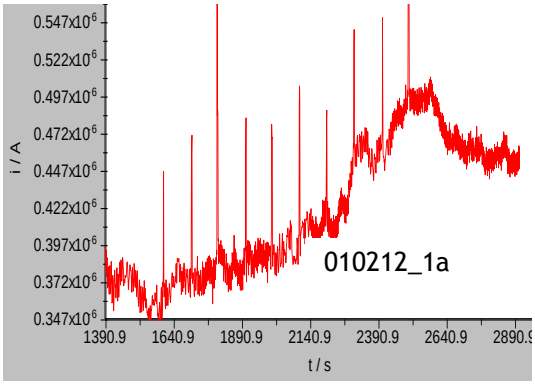



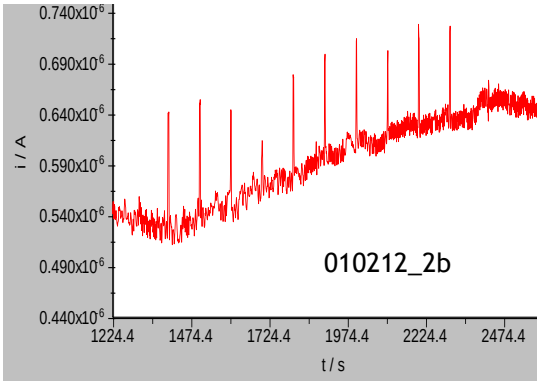
**Table 4.1.1.3** - Experimental data resume for DAO by applying +0.9 V. Solutions prepared in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M

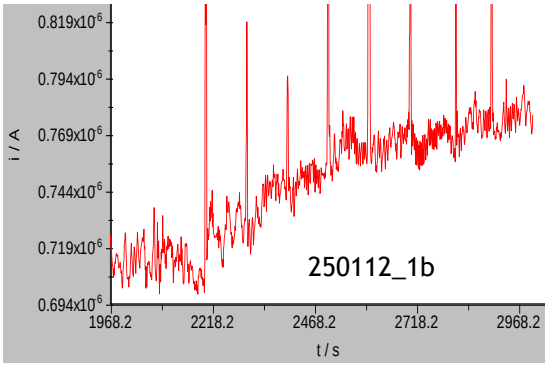
| File | Valid Additions | Positior | Equation | $R^2$ |
|---|---|---|---|---|
| **250112_1b** | 6 | 2nd | $y = 8.95E^{5}x - 4.6876$ | 0.9534 |
| **250112_1c** | 9 | 3rd | $y = 1.69E^{6}x + 1.8450$ | 0.9905 |
| **260112_1b** | 9 | 2nd | $y = 8.75E^{5}x + 33.277$ | 0.9878 |
| **270112_1a** | 8 | 1st | $y = 4.88E^{5}x + 1.9636$ | 0.9798 |
| **010212_1a** | 7 | 1st | $y = 6.52E^{5} + 24.185$ | 0.9145 |
| **010212_2b** | 7 | 2nd | $y = 3.39E^{6}x - 10.8462$ | 0.9926 |

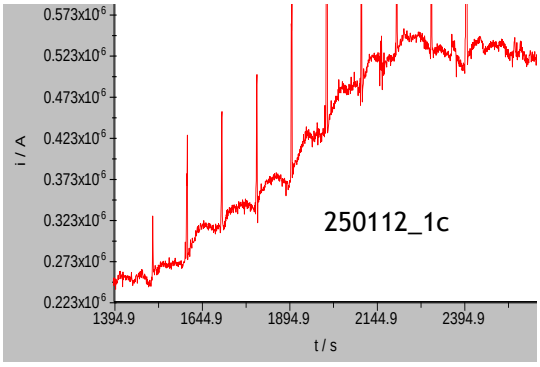


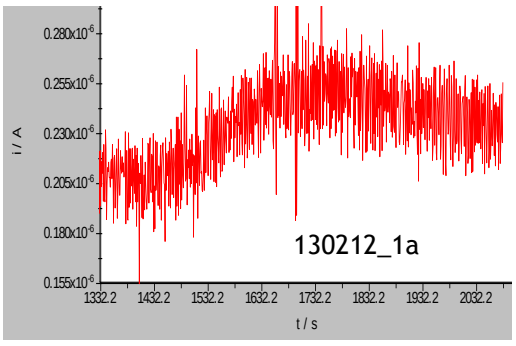
Normally DAO biosensor can be used to run between five to six experiments with good signals. The experiment order using the same biosensor (defined as "Position") is an important factor to be considered because in the first carried out experiment, enzyme may not be yet adapted to the solution environment. This will be less significant along the biosensor utilization until it reaches a point that the overuse is the most important feature ($5^{th}$ or $6^{th}$ utilization).

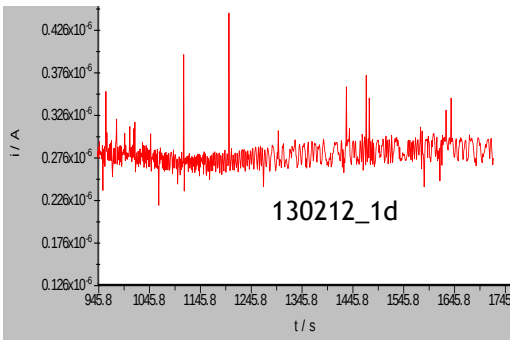
Comparing the noises at different potentials, the signals obtained at +0.8 V seems to be better than the ones obtained at + 0.7 V and +0.9 V. If we define the number of valid additions as a measure of assay consistent, it's easy to see that + 0.8 V give us a high number comparing to the other potentials. The $R^2$ value, determination coefficient, shows the linearity of the assay. The best potential that fits in this description (higher $R^2$ values) is also + 0.8 V. So, the more effective potential under the study conditions seems to be +0.8 V.

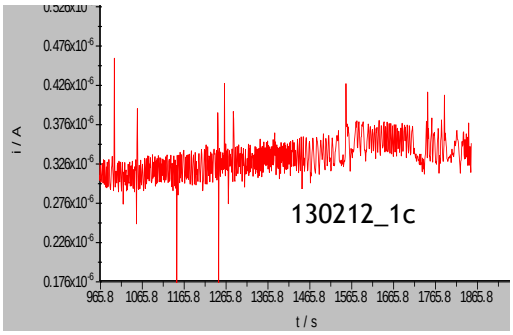
The phosphate buffer pH influence study has been done using pHs 6, 7, 8 and 9. The pH selection was made considering the optimal operation pH for DAO, which are around 7.5.
The results are shown in **Tables 4.1.1.4**, **4.1.1.5** and **4.1.1.6** with the respective chronoamperograms.

**Table 4.1.1.4** - Experimental data resume for DAO comparing phosphate buffer pH 6 to pH 8. Applied potential +0.8 V. Additions of 50 µl Spd $10^{-3}$ M. NQ = Not Quantified

| File | Valid Additions | pH | Position | Equation | $R^2$ |
|---|---|---|---|---|---|
| **130212_1a** | | 6 | $1^{st}$ | NQ | NQ |
| **130212_1b** | | 6 | $2^{nd}$ | NQ | NQ |
| **130212_1c** | | 6 | $3^{rd}$ | NQ | NQ |
| **130212_1d** | 10 | 8 | $4^{th}$ | y = 3.67E$^{6}$x + 21.2! | 0.9936 |



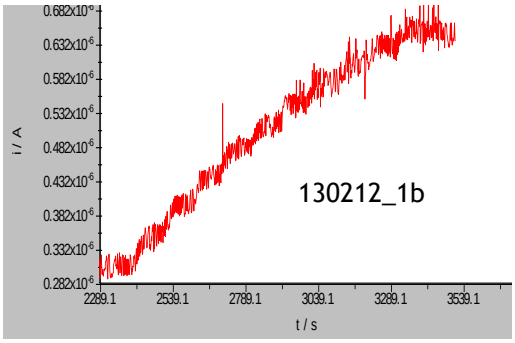
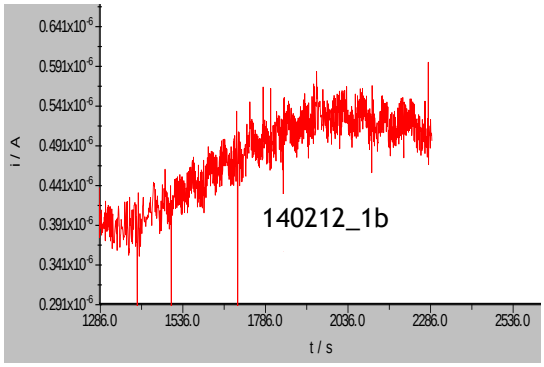
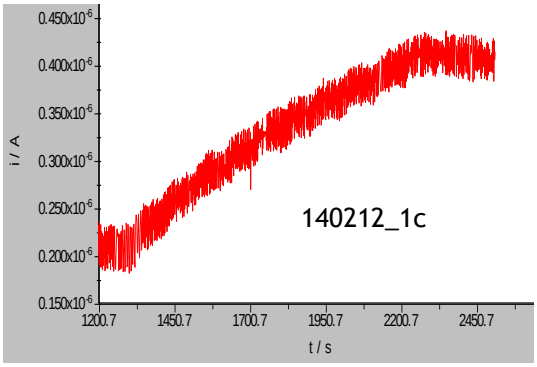
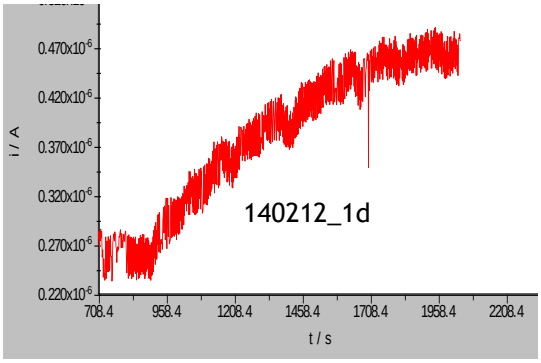



**Table 4.1.1.5** - Experimental data resume for DAO comparing phosphate buffer pH 7 to pH 8. Applied potential +0.8 V. Additions of 50 µl Spd $10^{-3}$ M. NQ = Not quantified

| File | Valid Addition | pH | Position | Equation | $R^2$ |
|---|---|---|---|---|---|
| **140212_1b** | | 7 | 1st | NQ | NQ |
| **140212_1c** | | 7 | 2nd | NQ | NQ |
| **140212_1d** | | 7 | 3rd | NQ | NQ |
| **140212_1e** | 8 | 8 | 4th | $y = 5.15E^{6}x + 116.8$ | 0.9929 |



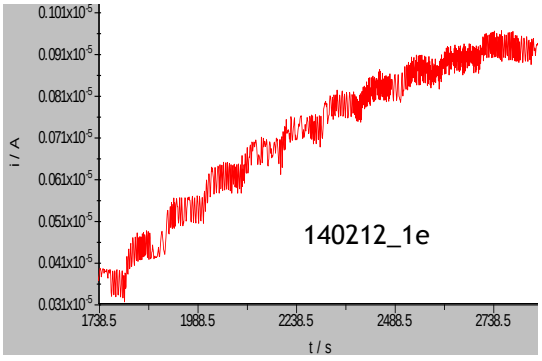
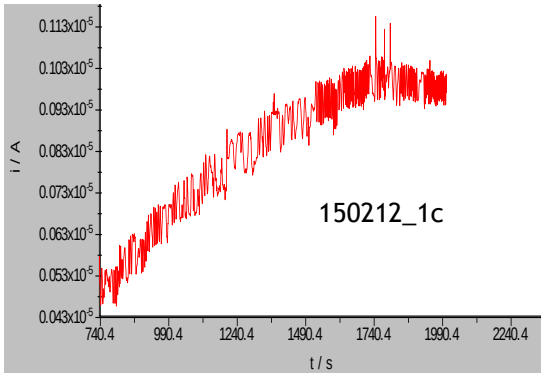
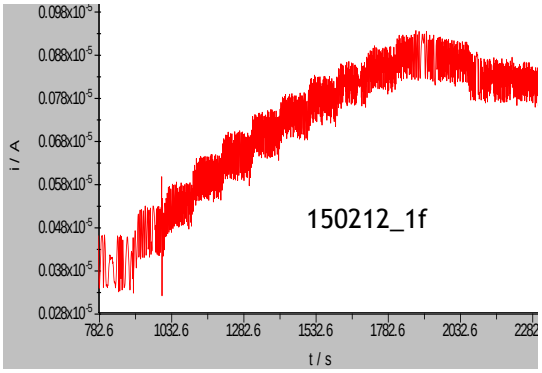
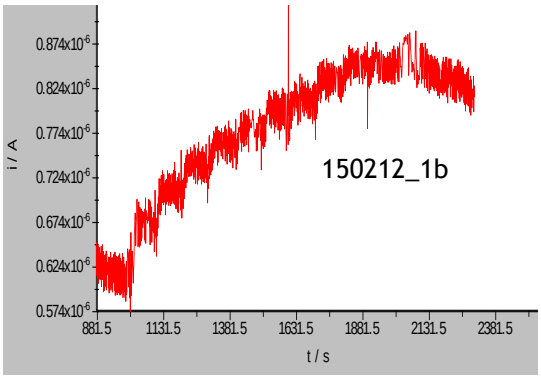



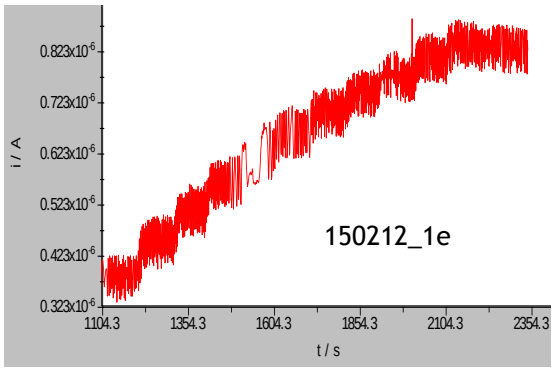
**Table 4.1.1.6** - Experimental data resume for DAO comparing phosphate buffer pH 9 to pH 8. Applied potential +0.8 V. Additions of 50 µl Spd $10^{-3}$ M. NQ = Not quantified

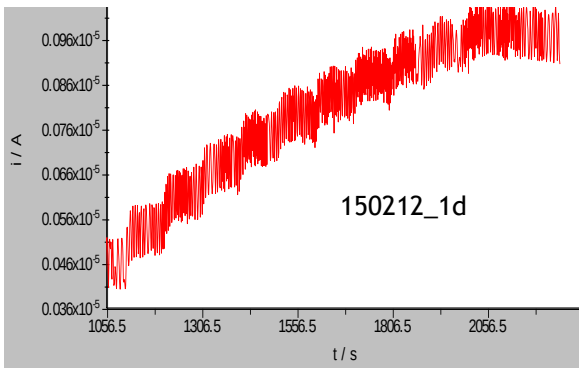
| File | Valid Additior | pH | Position | Equation | $R^2$ |
|---|---|---|---|---|---|
| 150212_1b | 8 | 9 | 1st | y = 2.29E$^6$x + 50.676 | 0.9921 |
| 150212_1c | | 9 | 2nd | NQ | NQ |
| 150212_1d | 9 | 9 | 3rd | y = 4.76E$^6$x + 82.109 | 0.9915 |
| 150212_1e | 10 | 8 | 4th | y = 4.62E$^6$x + 39.027 | 0.9948 |
| 150212_1f | 8 | 8 | 5th | y = 4.85E$^6$x + 49.125 | 0.9906 |



As we can see this study has been done by adding Spd to a biosensor under different phosphate solutions pHs. The same biosensor was used for comparison of pH 8 behavior, with selected pH, thus removing the error of preparing different biosensors. By analyzing these results we can say that acidic (pH 6) and neutral pHs (pH 7) do not favors the activity of immobilize enzyme. On the other hand a high value of pH increases its activity, pH 8 and 9 have the best results. To choose which of them will be used as optimal pH, others experiments were made and resumed below in **Tables 4.1.1.7** and **4.1.1.8**. It is also shown two examples of chronoamperograms for each pH (**Figure 4.1.1.4** and **4.1.1.5**).

**Table 4.1.1.7 -** Resume of all chronoamperometric experiments with the following characteristics: + 0.8 V as applied potential; phosphate buffer pH 8; and DAO as immobilized enzyme.

| File | Valid Additions | Additions | Position | Equation | $R^2$ |
|---|---|---|---|---|---|
| 130212_1d | 10 | 50 µL Spd $10^{-3}$M | $4^{th}$ | $y = 3.67E^6x + 21.257$ | 0.9936 |
| 140212_1e | 8 | 50 µL Spd $10^{-3}$M | $4^{th}$ | $y = 5.15E^6x + 116.83$ | 0.9929 |
| 150212_1e | 10 | 50 µL Spd $10^{-3}$M | $4^{th}$ | $y = 4.62E^6x + 39.027$ | 0.9948 |
| 150212_1f | 8 | 50 µL Spd $10^{-3}$M | $5^{th}$ | $y = 4.85E^6x + 49.125$ | 0.9906 |
| 160212_1c | 8 | 25 µL Spd $10^{-3}$M | $3^{th}$ | $y = 4.23E^6x + 53.158$ | 0.9917 |
| 160212_1d | 9 | 50 µL Spd $10^{-3}$M | $4^{th}$ | $y = 6.37E^6x + 34.575$ | 0.9977 |
| 220212_1b | 8 | 50 µL Spd $10^{-3}$M | $2^{th}$ | $y = 5.13E^6x + 176.38$ | 0.9905 |

**Table 4.1.1.8 -** Resume of all chronoamperometric experiments with the following characteristics: + 0.8 V as applied potential; phosphate buffer pH 9; and DAO as immobilized enzyme.

| File | Valid Addition | Additions | Positio | Equation | $R^2$ |
|---|---|---|---|---|---|
| 150212_1b | 8 | 50 µL Spd $10^{-3}$M | $1^{st}$ | $y = 2.29E^6x + 50.676$ | 0.9921 |
| 150212_1d | 9 | 50 µL Spd $10^{-3}$M | $3^{rc}$ | $y = 4.76E^6x + 82.109$ | 0.9915 |
| 210212_1b | 8 | 50 µL Spd $10^{-3}$M | $2^{nc}$ | $y = 5.13E^6x + 176.38$ | 0.9905 |
| 210212_1c | 8 | 50 µL Spd $10^{-3}$M | $3^{rc}$ | $y = 6.74E^6x + 217.64$ | 0.9903 |
| 210212_1f | 6 | 50 µL Spd $10^{-3}$M | $6^{th}$ | $y = 6.00E^6x + 219.35$ | 0.9914 |

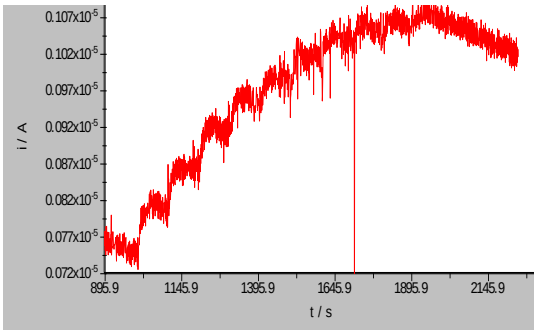



**Figure 4.1.1.4 -** Chronoamperogram relative to 220212_1b with phosphate buffer pH 8

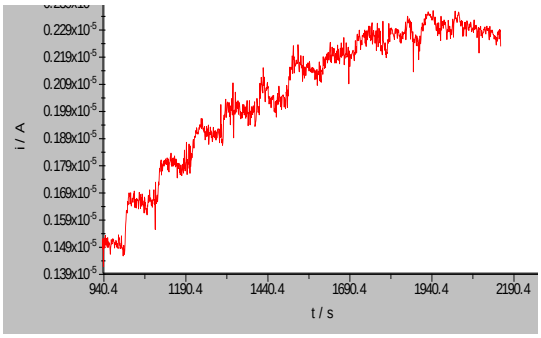



**Figure 4.1.1.5 -** Chronoamperogram relative to 210212_1c with phosphate buffer pH 9

Using the information showed in previous tables, the correct phosphate buffer pH to be used was defined as 8, because at this pH the $R^2$ value is higher for the highest number of valid additions and the noise was lower.

To conclude, the selected optimal conditions are respectively +0.8 V and 8 for applied potential and phosphate buffer pH.

As known chronoamperometric current depends directly on Spd concentration and this parameter influence should also be analyzed. Because we did not have the opportunity to do these experiments additions were fixed to 50 µl Spd $10^{-3}$ M.

## 4.1.2 Figures of merit

The figures of merit of a method are as a rule, reproducibility, repeatability and limit of detection. Reproducibility is a measure of the scatter or the drift in a series of results performed over a period of time. It is generally determined for the analyte concentrations within the usable range. Precision (determined in terms of reproducibility) is usually discussed in terms of standard deviation (SD) and relative standard deviation (RSD).

**Detection Limit**

The evaluation of the detection limit was carried out based on variability of a blank. The first step to determinate the detection limit is to get a linear relation between Spd concentration and the corresponding current signal.

To construct the calibration curves (**Figure 4.1.2.1**) a chronoamperogram (**Figure 4.1.2.2**) using Spd solution with concentrations ranging between $9.901E^{-6}$ and $9.091E^{-5}$ M (**Table 4.1.2.1)** was record.

**Table 4.1.2.1 -** Experimental data to construct the calibration curve

| Spd concentration (M) | Intensity (nA) |
|---|---|
| $9.901E^{-6}$ | 45 |
| $1.961E^{-5}$ | 92 |
| $2.913E^{-5}$ | 126 |
| $3.846E^{-5}$ | 173 |
| $4.762E^{-5}$ | 205 |
| $5.660E^{-5}$ | 233 |
| $6.542E^{-5}$ | 265 |
| $7.407E^{-5}$ | 297 |
| $8.257E^{-5}$ | 321 |
| $9.091E^{-5}$ | 342 |

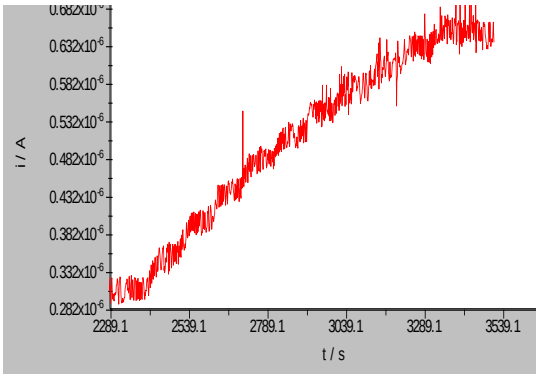



**Figure 4.1.2.1 -** Chronoamperogram for construction of calibration curve. + 0.8 V as applied potential; 8 as phosphate buffer pH; and additions of 50 µl Spd $10^{-3}$ M.

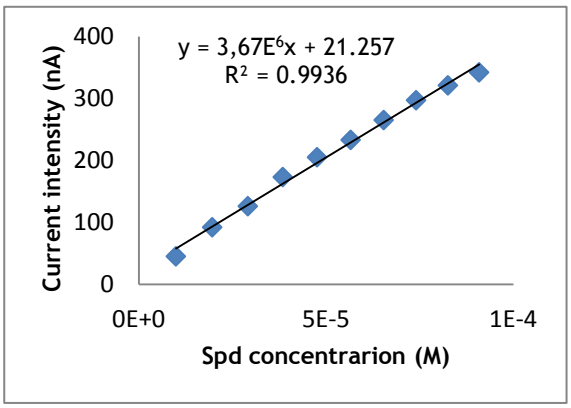



**Figure 4.1.2.2 -** Calibration curve and $R^2$ value using results of **Table 4.1.2.1.**

After invalid point's removal, the calibration curve as the following equation:

$$\mathbf{y = 3.67E^{6}x + 21.257}$$

$$\mathbf{R^2 = 0.9936}$$

**With a sensibility of $3.67x10^{6}$ nA $mol^{-1}$ $dm^3$**

Knowing that a blank do not have Spd, therefore do not have any signal, the variability of a sample which contains a small amount of Spd ($9.901E^{-6}$ M) was used to determine the detection limit. By adding this amount of Spd respective current signals were recorded (**Table 4.1.2.2**).

**Table 4.1.2.2** – Data to calculate the average and standard deviation of a sample with small amount of Spd. + 0.8 V as applied potential; 8 as phosphate buffer pH; and additions of 50 µl Spd 10-3 M.

| Spd concentration (M) | Intensity (nA) |
|---|---|
| $9.901E^{-6}$ | 45 |
| $9.901E^{-6}$ | 90 |
| $9.901E^{-6}$ | 68 |
| $9.901E^{-6}$ | 60 |
| $9.901E^{-6}$ | 43 |
| $9.901E^{-6}$ | 50 |

$\bar{x}$ = 59.33; SD = 17.75

By a probability level of 5% (α=ß=0.05), the *t* unilateral value for 5 degrees of freedom is 2.015. Therefore, the detection limit is,

$$D_l = 2.015\sqrt{1+\frac{1}{6}} \times 17.75 = 38.63$$

where 6 it is the number of replicates and 17.75 corresponds to its SD value.

The detection limit expressed in terms of concentration is,

$$X_d = 2 \times \frac{38.63}{3.67E^6} = 2.11E^{-5}M$$

where $3.67E^6$ is the calibration curve slope.

These values determined by Massart et al., 1997 method has a high associate error because of the small amount of data (increases the *t* unilateral value) and large discrepancy between data values (e.g. 45 to 90) which contributes for the SD and, consecutively, the $D_L$ increment.

***Repeatability and reproducibility***

Repeatability and reproducibility are extremely important characteristics to control in an analytical method. In this specific case there are few results to reach a conclusion about the repeatability value. It is necessary to do more experiments with the same biosensor at the same conditions to have a minimum number of results to calculate this feature. On the other hand, there are enough results to obtain the reproducibility of this analytical method.

To evaluate the method reproducibility three experiments were carried out with three different biosensors operating at the same conditions. The results are shown in **Table 4.1.2.3**

and respective chronoamperograms and calibration curves presented between **Figure 4.1.2.3 and 4.1.2.8**.

**Table 4.1.2.3 -** Experimental data for the determination of method reproducibility.

| Spd (M) | Intensity (nA) | | |
|---|---|---|---|
| | 140212_1e | 150212_1f | 220212_1b |
| $9.901E^{-6}$ | 90 | 60 | 150 |
| $1.961E^{-5}$ | 180 | 130 | 260 |
| $2.913E^{-5}$ | 260 | 190 | 320 |
| $3.846E^{-5}$ | 310 | 240 | 390 |
| $4.762E^{-5}$ | 360 | 290 | 430 |
| $5.660E^{-5}$ | 420 | 340 | 500 |
| $6.542E^{-5}$ | 460 | 370 | 530 |
| $7.407E^{-5}$ | 510 | 400 | 550 |
| $8.257E^{-5}$ | 540 | 440 | 600 |
| $9.091E^{-5}$ | 570 | 460 | 630 |

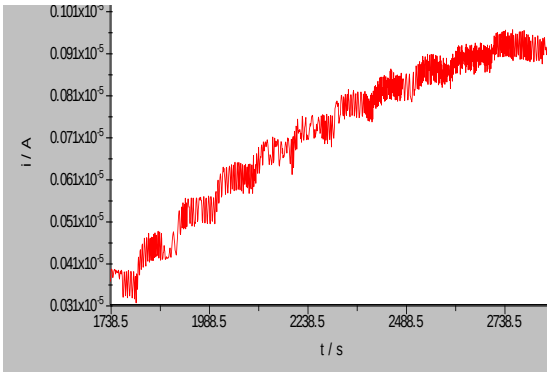



**Figure 4.1.2.3 -** Chronoamperogram of 140212_1e for the evaluation of method reproducibility.

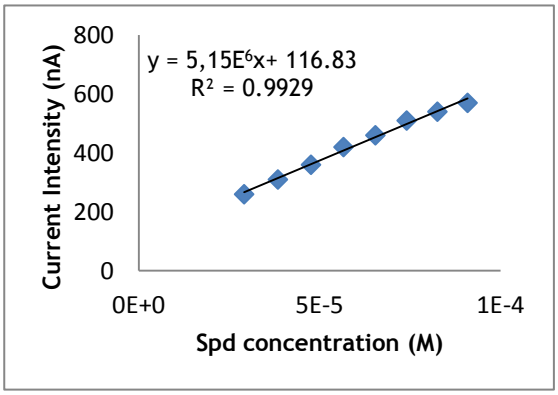



**Figure 4.1.2.4 -** Calibration curve and $R^2$ value of 140212_1e for the evaluation of method reproducibility.

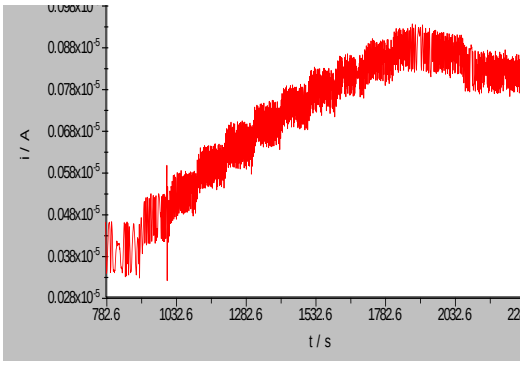



**Figure 4.1.2.5 -** Chronoamperogram of 150212_1f for the evaluation of method reproducibility.

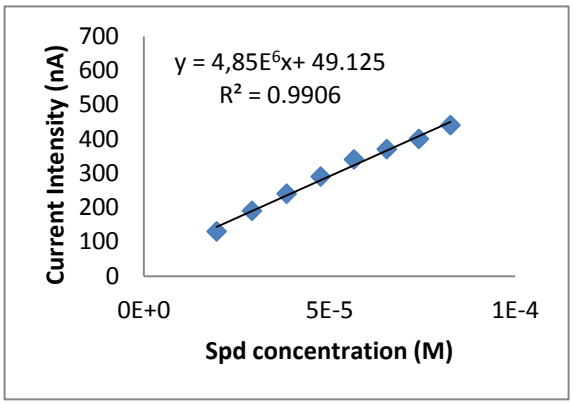



**Figure 4.1.2.6 -** Calibration curve and $R^2$ value of 150212_1f for the evaluation of method reproducibility.

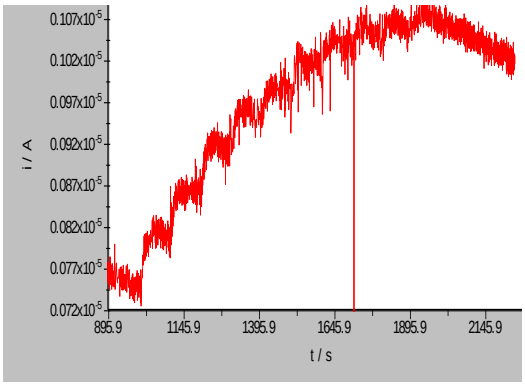



**Figure 4.1.2.7 -** Chronoamperogram of 220212_1b for the evaluation of method reproducibility.

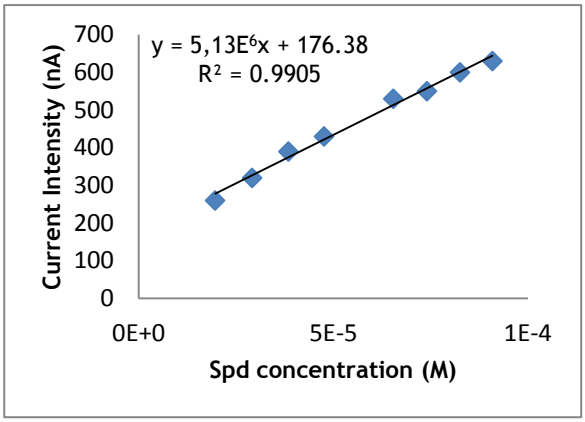



**Figure 4.1.2.8 -** Calibration curve and $R^2$ value of 220212_1b for the evaluation of method reproducibility.

To calculate RSD it is necessary the different curves slope (**Table 4.1.2.4**). The RSD value is calculated by the ratio between the slope standard deviation and the slope average. In this case a reproducibility of 3.33% was obtained.

**Table 4.1.2.4 -** Respective file slope for evaluation of method reproducibility

| File | Slope ($E^6$) |
|---|---|
| 140212_ | 5.15 |
| 150212_ | 4.85 |
| 220212_ | 5.13 |

## 4.1.3 Comparison with previous work

Some experiments were also performed at University of Burgos. Below we can see two groups of images corresponding to an experiment made at University of Beira Interior (**Figure 4.1.2.1** and **4.1.2.2**) and other made at University of Burgos (**Figure 4.1.3.1** and **4.1.3.2**). These experiments were made in two different non modified DAO biosensors. It is interesting to see that even with initials conditions totally different, we have a close slope similarity, showing the same sensibility level. However, the equation b values are pretty different. In absence of Spd we are expected to get a current signal near zero, but as we can see in **Figure 4.1.2.2** we have 21.257 nA of current intensity. On the other hand, **Figure 4.1.3.2** shows us a basal current signal of 0.3739 nA, when it does not have any Spd in solution. These values discrepancy (21.257 nA and 0.3739 nA) suggests a much higher background noise for the experiments made at Beira Interior when compared with the ones completed at Burgos University. Consequently, it seems incorrect to compare Burgos and Beira Interior

experiments. In order to low background noise of Beira Interior equipment several adapters (**Figure 4.1.3.5**) were built and experimented, with no signification improvement. So we purpose, in a future work, connect only the autolab system to a specific land that is separated of others investigation systems, providing an isolated autolab system.

**Table 4.1.3.1** resumes the most important characteristics of DAO not modified with nanoparticles biosensor constructed in University of Beira Interior.

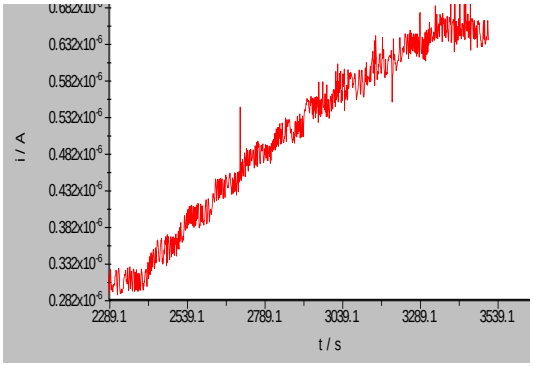



**Figure 4.1.3.1** - Chronoamperogram for construction of calibration curve. +0.8 V as applied potential; 8 as phosphate buffer pH; and additions of 50 µl Spd $10^{-3}$ M. University of Beira Interior

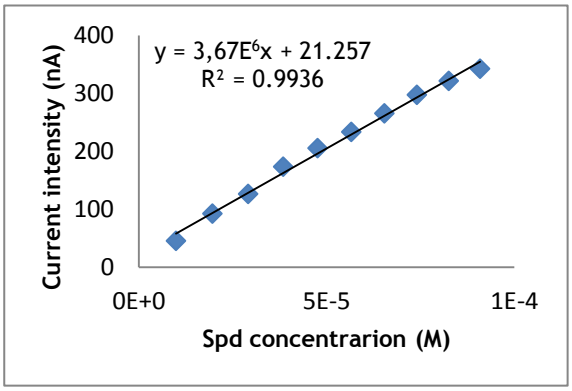



**Figure 4.1.3.2** - Calibration curve and $R^2$ value corresponding of **Figure 4.1.3.1** chronoamperogram.

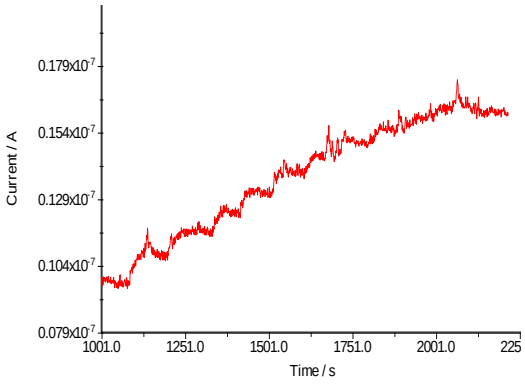



**Figure 4.1.3.3** - Chronoamperogram for construction of calibration curve. +0.6 V as applied potential; 8.3 as phosphate buffer pH; and additions of 100 µl Spd $10^{-5}$ M. University of Burgos

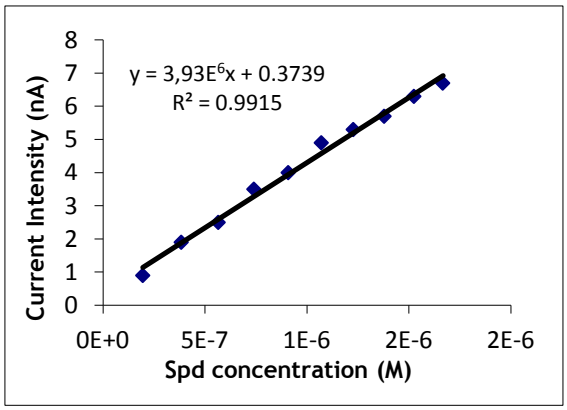



**Figure 4.1.3.4** - Calibration curve and $R^2$ value corresponding of **Figure 4.1.3.3** chronoamperogram.

**Figure 4.1.3.5 -** Constructed adapters for try to resolve the background noise.

**Table 4.1.3.1** – Resume of the most important characteristics obtained on construction of DAO non modified Biosensor at Beira Interior University. NQ = Not quantified

| Characteristic | Value |
|---|---|
| Applied potential (V) | + 0.8 |
| Phosphate buffer pH | 8 |
| Sensibility (nA $mol^{-1}dm^3$) | $3.67E^6$ |
| Detection Limit (M) | $2.11E^{-5}$ |
| RSD (%) Repeatability | NQ |
| RSD (%) Reproducibility | 3.33 |

# 4.2. Sensitive monoaminoxidase biosensor based on carbon SPE for Spd determination

## 4.2.1 First results and signal optimization

The mechanism of monoamine oxidase (MAO) action is the same that was established in **subsection 4.1.1** for DAO. As well as presented before, water interference is not expected for this particular biosensor. As said before, the theoretical mechanism is divided into two half-reactions:

$$MAO_{OX} + RCH_2NH_2 \Leftrightarrow MAO\text{-}RCH_2NH_2 \rightarrow MAO_{RED} + RCHO$$

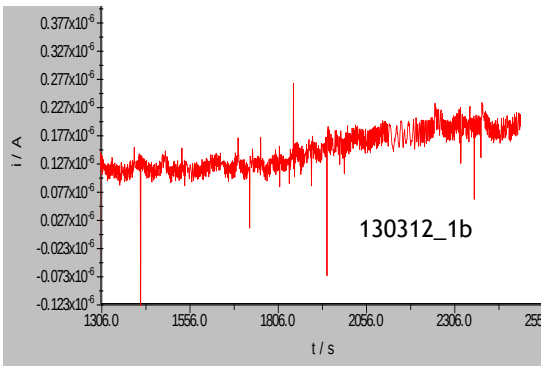
$$MAO_{RED} + O_2 \rightarrow MAO_{OX} + H_2O_2 + NH_3$$

and overall reaction will became rate limited if either Spd or oxygen concentration are too low. By monitoring current intensity Spd concentration can be measured.

As showed before, for biosensor optimization is essential to find the optimal operation potential. So, experiments have been carried out where phosphate buffer pH has been fixed at 8 (the optimal pH in DAO biosensor case) and applied potential changed between +0.7 V and + 0.9 V. The results are shown in **Tables 4.2.1.1**, **4.2.1.2** and **4.2.1.3** with the respective chronoamperograms.

**Table 4.2.1.1 -** Experimental data resume for MAO by applying +0.5 V. Solutions prepared in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M. NQ = Not quantified.

| File | Valid Additions | Position | Equation | $R^2$ |
|---|---|---|---|---|
| 130312_1b | - | 2nd | NQ | NQ |
| 140312_1f | - | 6th | NQ | NQ |



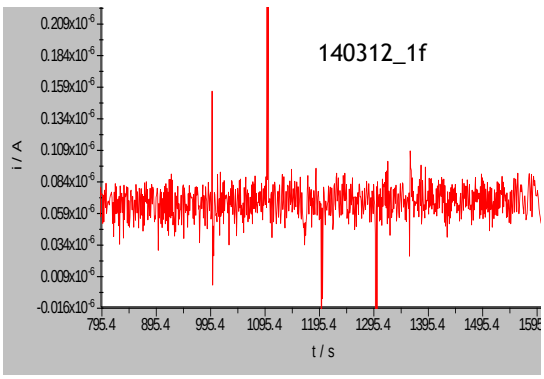
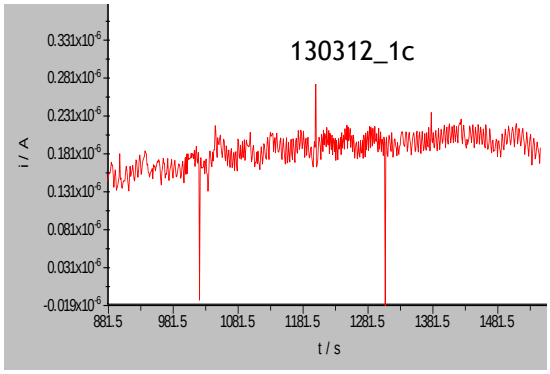



**Table 4.2.1.2 -** Experimental data resume for MAO by applying +0.6 V. Solutions prepared in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M. NQ = Not quantified

| File | Valid Additions | Position | Equation | $R^2$ |
|---|---|---|---|---|
| 130312_1c | - | 3rd | NQ | NQ |
| 140312_1e | - | 5th | NQ | NQ |



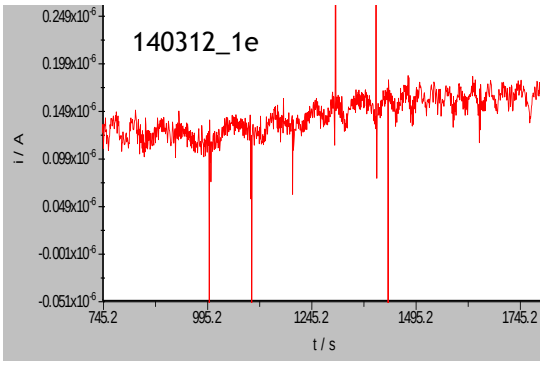
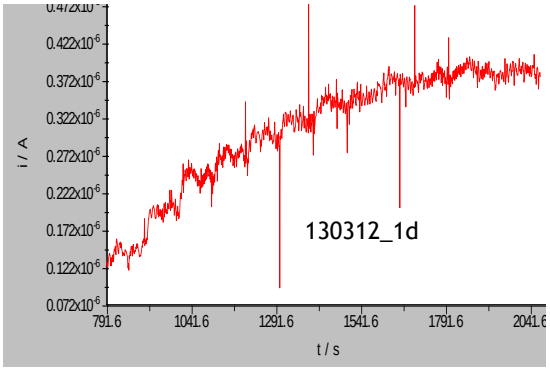
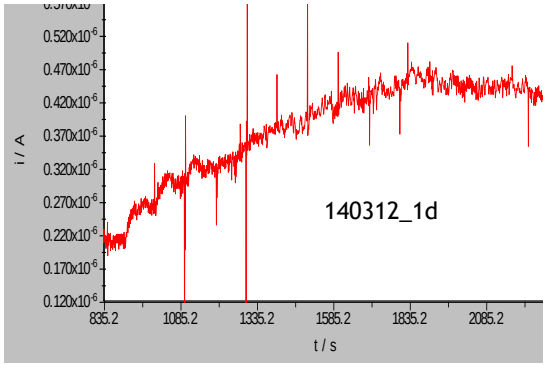




**Table 4.2.1.3 -** Experimental data resume for MAO by applying +0.7 V. Solutions prepared in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M. NQ = Not quantified

| File | Valid Additions | Position | Equation | $R^2$ |
|---|---|---|---|---|
| 130312_1d | | 4th | NQ | NQ |
| 140312_1d | | 4th | NQ | NQ |
| 150312_1b | 8 | 2nd | y = 2.22E$^6$x + 7.41( | 0.9949 |



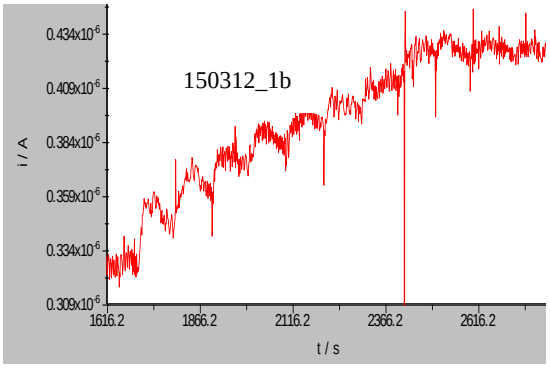
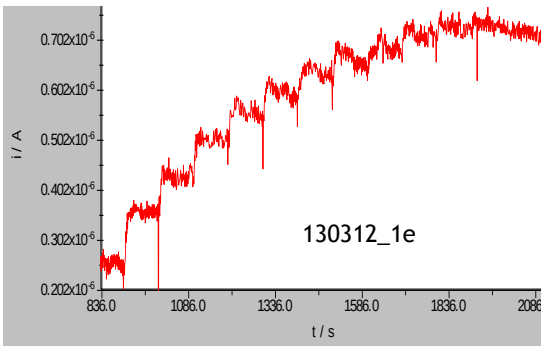
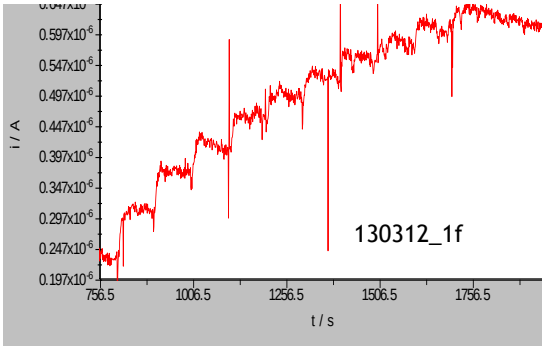
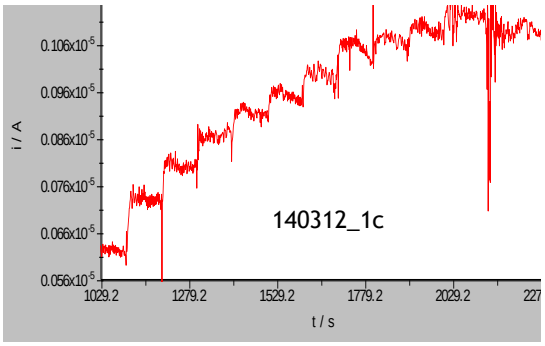



**Table 4.2.1.4 -** Experimental data resume for MAO by applying +0.8 V. Solutions prepared in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M. NQ = Not quantified

| File | Positio | Valid Additior | Equation | $R^2$ |
|---|---|---|---|---|
| 130312_1e | 5th | 8 | y = 4.89E6x + 83.911 | 0.9722 |
| 130312_1f | 6th | 8 | y = 3.59E6x + 79.082 | 0.9909 |
| 140312_1c | 3rd | 8 | y = 4.17E6x+ 121.01 | 0.9985 |
| 150312_1c | 3rd | 9 | y = 3.22E6x + 32.164 | 0.9915 |



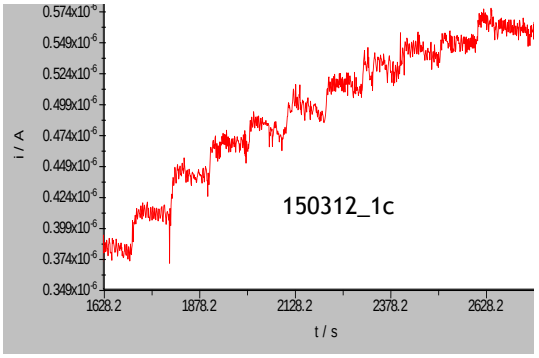
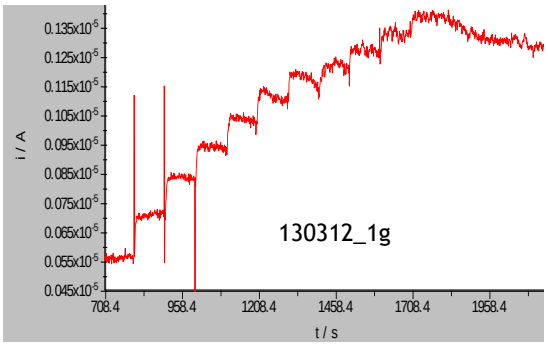
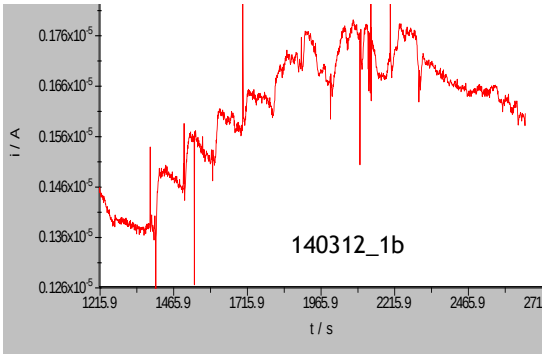
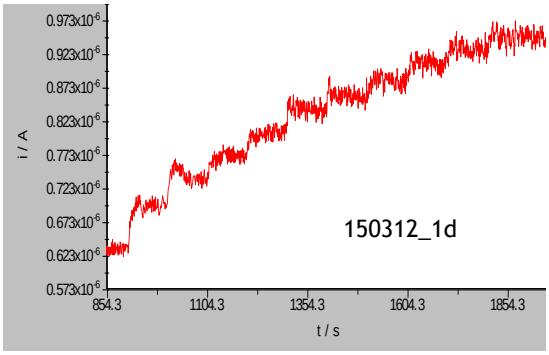




**Table 4.2.1.5 -** Experimental data resume for MAO by applying +0.9 V. Solutions prepared in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M excepted in 150312_1d was 25µl Spd $10^{-3}$ M. NQ = Not quantified

| File | Position | Valid Additions | Equation | $R^2$ |
|---|---|---|---|---|
| 130312_1g | 7th | 8 | y = 7.69E6x + 124.4 | 0.9934 |
| 140312_1b | 2nd | - | NQ | NQ |
| 150312_1d | 4th | 8 | y = 5.89E6x + 41.70 | 0.9947 |






As we expected, applied potentials lower than +0.7 V do not have the correct response, because it is necessary a potential bigger or equal to +0.7 V to induce Spd oxidation by MAO. The 140312_1c and 140312_1b files show consecutive experiments made at the same biosensor, but applying different potentials: +0.8 and +0.9 V, respectively. It is interesting to find that with the same conditions but just applying a different potential we have totally different response, with much more quality by applying +0.8 V. So we define it as the optimal potential.

In the biosensor optimization process, pH determination is also as important as the potential applied, however we did not perform this type of experiments because the pH activity range of MAO is similar to DAO. Thus, pH 8 has been selected for phosphate buffer preparation.

Enzyme immobilization is an extreme careful process involving important molecules such as: Bovine Serum Albumin (BSA), Glutaraldehyde (GA) as well as enzyme. GA is the cross-linker agent and BSA is used to promote its correct immobilization. Due the close proximity, it is important to know how these molecules interfere on the enzyme environment, damaging or benefiting its activity. Considering this, we try to find how the biosensor response was by manipulating BSA and GA concentrations.

### 4.2.2 Study of BSA/MAO concentration ratio effect on biosensor response

One of the solutions used in the immobilization procedure is made using BSA, MAO and phosphate buffer pH 7.5 (solution 3). The following group of experiments (**Figure 4.2.2.1 to 4.2.2.8**) was made in order to evaluate the BSA concentration influence in MAO response to several Spd additions. This study was made by preparing two solutions 3 with two different concentrations of BSA: 2.5 mg/ml (half the standard concentration) and 10 mg/ml (double the standard concentration). Five additions of 50 µl Spm $10^{-3}$ M were made in each experiment.

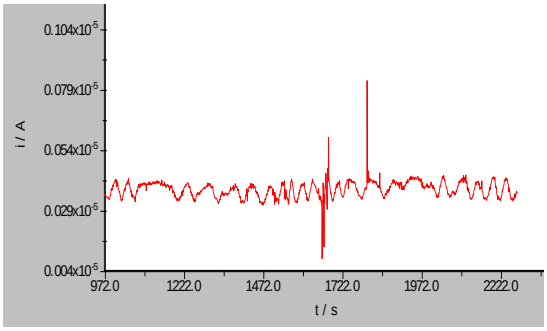



**Figure 4.2.2.1** - Experiment 040512_1b corresponding to a solution 3 with the following characteristics 5 µl MAO; 6.8 µl BSA (2.5 mg/ml) and 3.2 µl phosphate buffer pH 7.5.

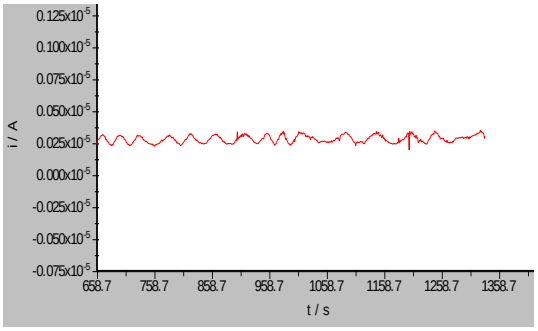



**Figure 4.2.2.2** - Experiment 040512_1c corresponding to a solution 3 with the following characteristics 5 µl MAO; 6.8 µl BSA (2.5 mg/ml) and 3.2 µl phosphate buffer pH 7.5.

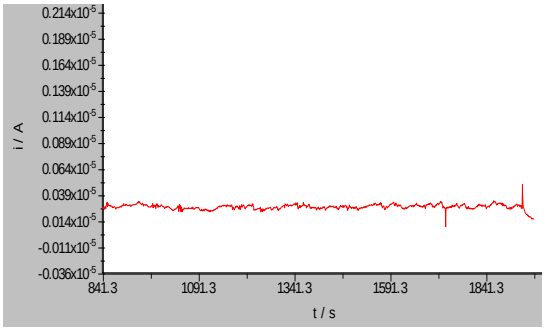



**Figure 4.2.2.3** - Experiment 040512_2b corresponding to a solution 3 with the following characteristics 5 µl MAO; 6.8 µl BSA (10 mg/ml) and 3.2 µl phosphate buffer pH 7.5.

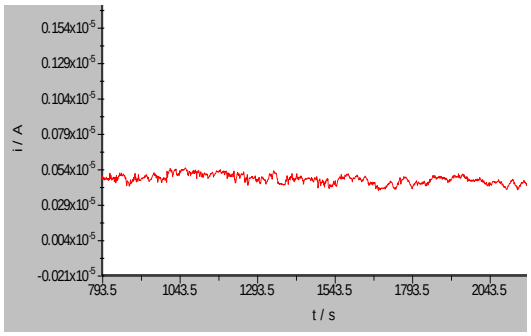



**Figure 4.2.2.4** - Experiment 040512_3b corresponding to a solution 3 with the following characteristics 5 µl MAO; 6.8 µl BSA (2.5 mg/ml) and 3.2 µl phosphate buffer pH 7.5.

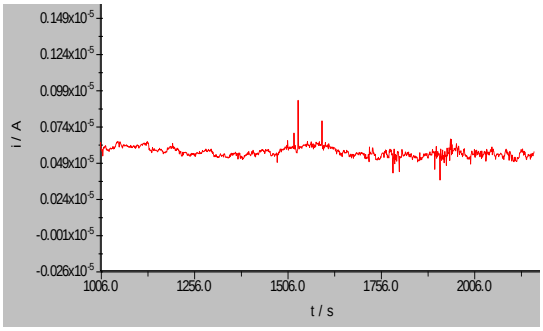



**Figure 4.2.2.5** - Experiment 080512_1b corresponding to a solution 3 with the following characteristics 5 µl MAO; 6.8 µl BSA (2.5 mg/ml) and 3.2 µl phosphate buffer pH 7.5.

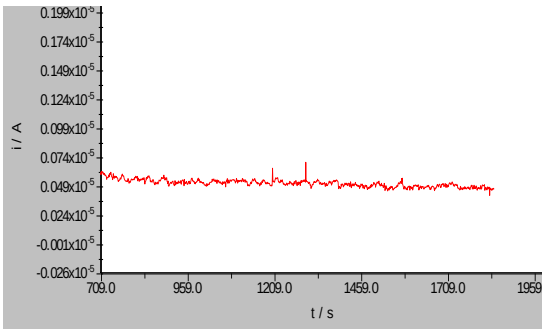



**Figure 4.2.2.6** - Experiment 080512_1c corresponding to a solution 3 with the following characteristics 5 µl MAO; 6.8 µl BSA (2.5 mg/ml) and 3.2 µl phosphate buffer pH 7.5.

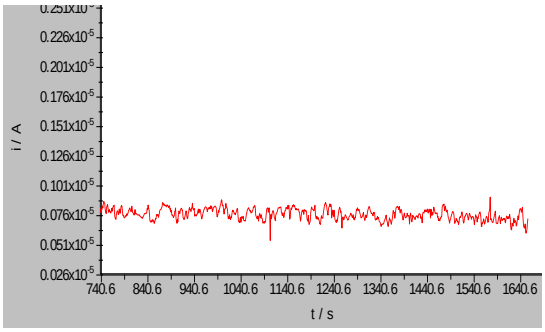



**Figure 4.2.2.7** - Experiment 080512_2b corresponding to a solution 3 with the following characteristics 5 µl MAO; 6.8 µl BSA (10 mg/ml) and 3.2 µl phosphate buffer pH 7.5.

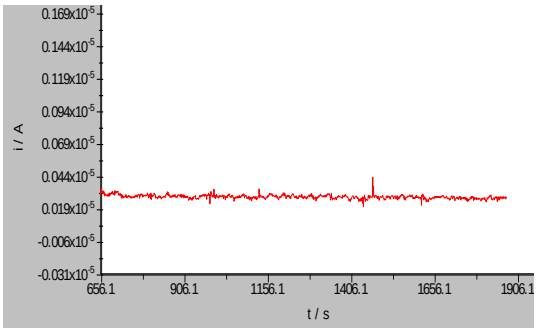



**Figure 4.2.2.8** - Experiment 080512_3b corresponding to a solution 3 with the following characteristics 5 µl MAO; 6.8 µl BSA (10 mg/ml) and 3.2 µl phosphate buffer pH 7.5.

As we can be seen, the manipulation (decrease or increase) of BSA concentration results in a decrease of the signal quality, which demonstrates the essential role of this biomolecule in stabilization of the immobilized enzyme. Thus, we can conclude that the optimal BSA concentration is 5 mg/ml.

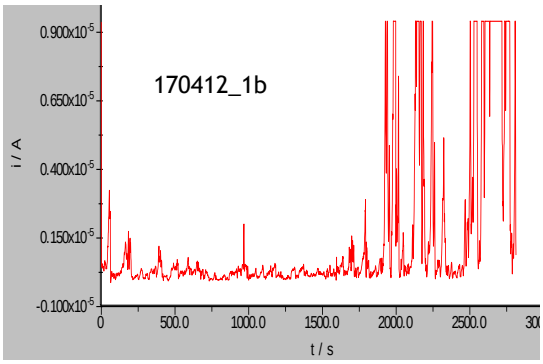
## 4.2.3 Study of GA/MAO concentration ratio effect on biosensor response

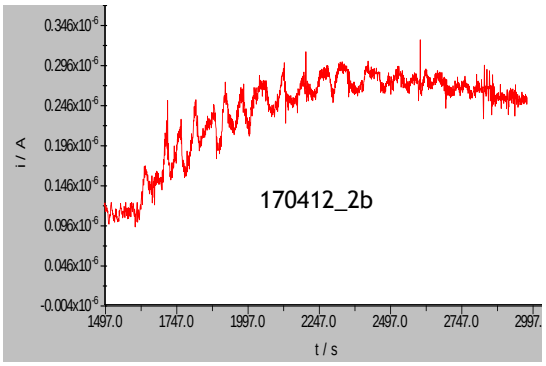
GA is a cross-linking agent used as an interface between the electrode surface and the enzyme. Is, therefore, essential the analysis of its concentration influence, lows values may imply loss of enzyme and high values may affect enzyme kinetic and diffusion properties, resulting on a loss of activity.

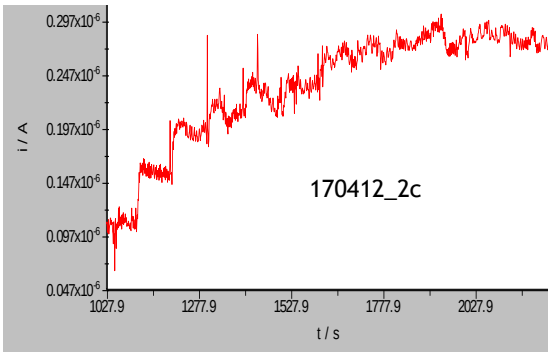
The behavior of used electrodes was first checked by cyclic voltammetry, before enzyme immobilization. Then, MAO was immobilized separately with two GA solutions with different concentrations: one four times less concentrated (6.75 mg/ml) and other four times more concentrate (108 mg/ml) than the standard GA solution (27mg/ml). Below, are shown in **Tables 4.2.3.1** and **4.2.3.2** all experiments and the respective graphics.

**Table 4.2.3.1** - Experimental data resume for GA 6.75 mg/ml. Solutions prepared in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M. +0.8 V as applied potential. NQ = Not quantified

| File | Valid Additions | Equation | $R^2$ |
|---|---|---|---|
| 170412_1b | | NQ | NQ |
| 170412_2b | | NQ | NQ |
| 170412_2c | 7 | $y = 1.62E^6x + 52.240$ | 0.9883 |
| 170412_2d | 6 | $y = 1.65E^6x + 40.178$ | 0.9832 |



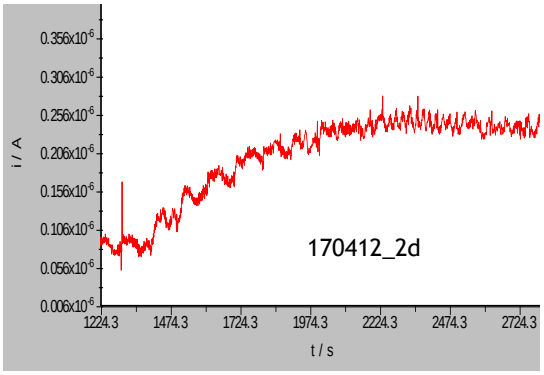
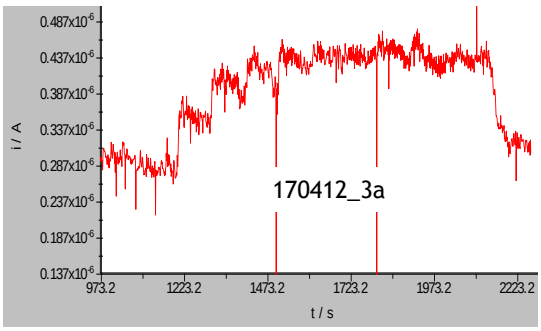
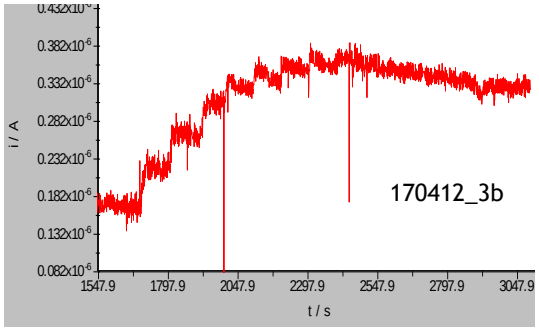
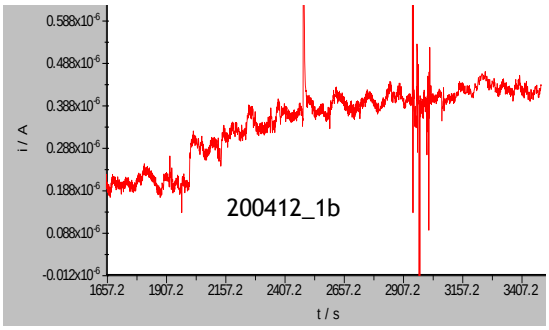



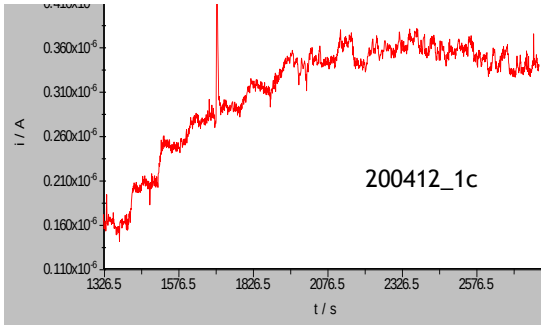
**Table 4.2.3.2 -** Experimental data resume for GA 108 mg/ml. Solutions prepared in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M. +0.8 V as applied potential. NQ = Not quantified

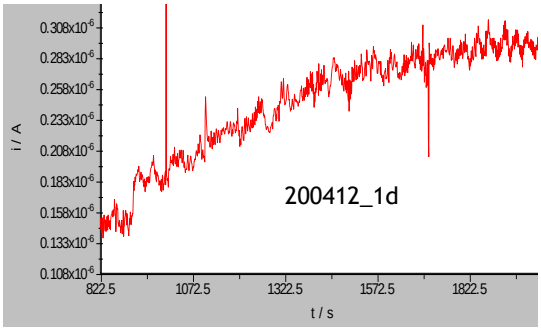
| File | Valid Additions | Equation | $R^2$ |
|---|---|---|---|
| 170412_3a | 5 | $y = 2.33E^{6}x + 47.280$ | 0.9673 |
| 170412_3b | 7 | $y = 2.36E^{6}x + 52.108$ | 0.9112 |
| 200412_1b | | NQ | NQ |
| 200412_1c | 7 | $y = 2.45E^{6}x + 34.493$ | 0.9680 |
| 200412_1d | | NQ | NQ |
| 200412_2a | 7 | $y = 1.58E^{6}x + 62.967$ | 0.9736 |

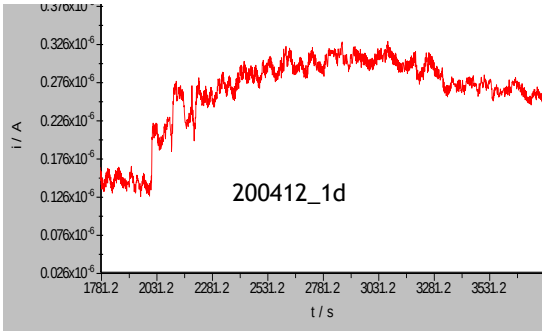


As showed above, it seems that the variation of GA concentration does not favor the signal quality. It is interesting to mention that the number of valid additions decreases when we decrease or increase the GA concentration. In addition, there are a significant number of experiments that could not be quantified because of the background noise.

MAO was immobilized in two different sensors (**Figure 4.2.3.1**) with the purpose to see if the background noise decreased. The conditions previously optimized were used in the same way on both biosensors: + 0.8 V as applied potential, phosphate buffer ph 8 and BSA and GA standard concentrations (5 mg/ml and 27 mg/ml, respectively). Results are showed in **Tables 4.2.3.3** and **4.2.3.4** with the respective graphics.

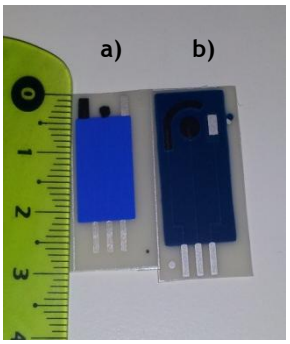
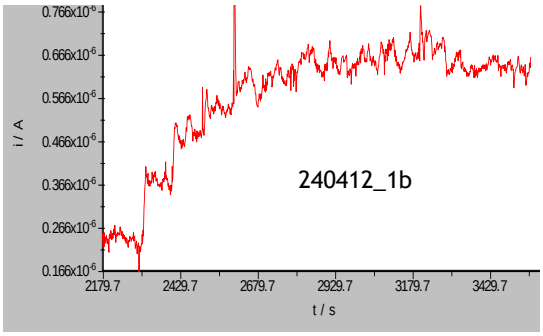
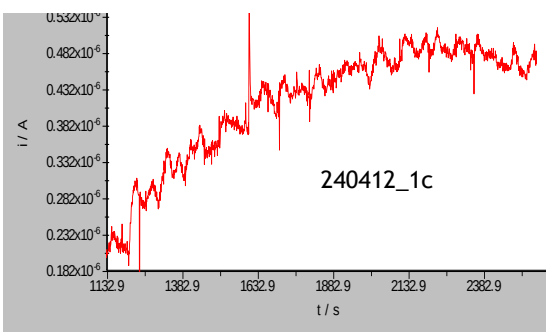


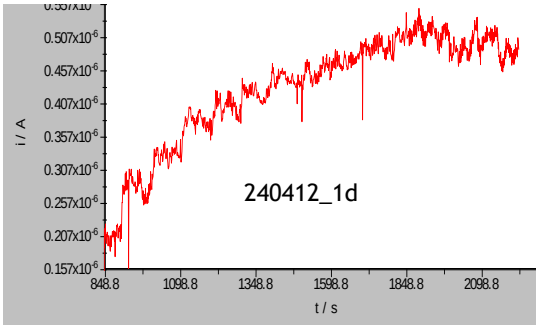

**Figure 4.2.3.1** - Two SPEs examples. **a)** Type of SPE used in all work realized in University of Beira Interior **b)** SPE to be compared.

**Table 4.2.3.3** – Experimental data resume for Sensor a). Solutions prepared in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M. +0.8 V as applied potential.

| File | Valid Additions | Equation | $R^2$ |
|---|---|---|---|
| 240412_1b | 7 | $y = 3.24E^{6}x + 213.83$ | 0.9389 |
| 240412_1c | 7 | $y = 3.03E^{6}x + 46.379$ | 0.9900 |
| 240412_1d | 7 | $y = 3.15E^{6}x + 54.235$ | 0.9737 |
| 240412_1e | 5 | $y = 4.00E^{6}x + 33.353$ | 0.9588 |



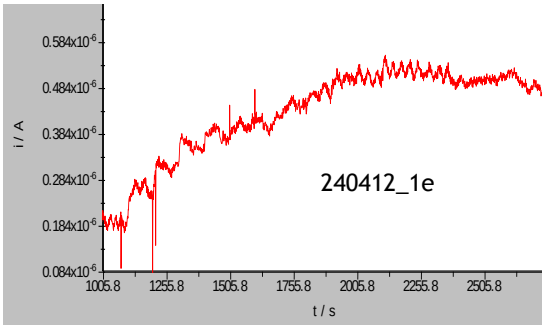
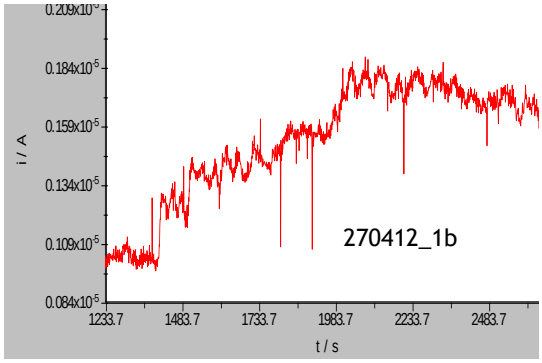
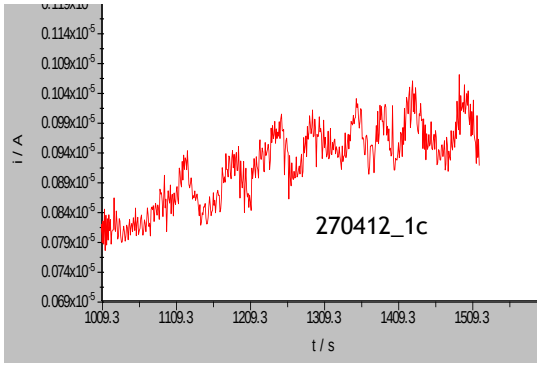
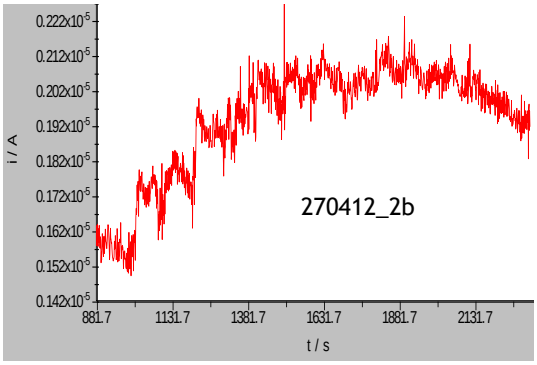



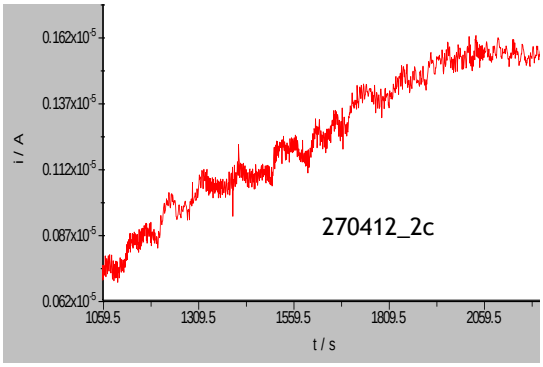

**Table 4.2.3.4** - Experimental data resume for Sensor b). Solutions prepared in phosphate buffer pH 8. Additions of 50 µl Spd $10^{-3}$ M. +0.8 V as applied potential. NQ = Not quantified

| File | Valid Additions | Equation | $R^2$ |
|---|---|---|---|
| 270412_1b | 7 | $y = 9.34E^{6}x + 108.26$ | 0.9957 |
| 270412_1c | | NQ | NQ |
| 270412_2b | 6 | $y = 7.29E^{6}x + 88.864$ | 0.9726 |
| 270412_2c | 9 | $y = 9.24E^{6}x + 27.414$ | 0.9926 |






Comparing the two types of sensors when using the same glutaraldehyde concentration (27 mg/ml), seems that the Sensors b) could have the best response because showed better $R^2$ values in a highest number of valid additions. Although no more experiments with these sensors were made.

## 4.2.4 Figures of merit

With the available results we can determinate the MAO biosensor sensibility and reproducibility. Data for sensibility determination are shown in **Table 4.2.4.1**. **Figure 4.2.4.1** and **4.2.4.2** represents the chronoamperogram and calibration curve.

***Sensibility***

**Table 4.2.4.1 -** Experimental data to construct the calibration curve. For analysis purpose was rejected the first point

| Spd Concentration (M) | Current Intensity (nA) |
|---|---|
| $9.901E^{-6}$ | 26 |
| $1.961E^{-5}$ | 58 |
| $2.913E^{-5}$ | 84 |
| $3.846E^{-5}$ | 95 |
| $4.762E^{-5}$ | 109 |
| $5.660E^{-5}$ | 129 |
| $6.542E^{-5}$ | 146 |
| $7.407E^{-5}$ | 158 |
| $8.257E^{-5}$ | 169 |
| $9.091E^{-5}$ | 181 |

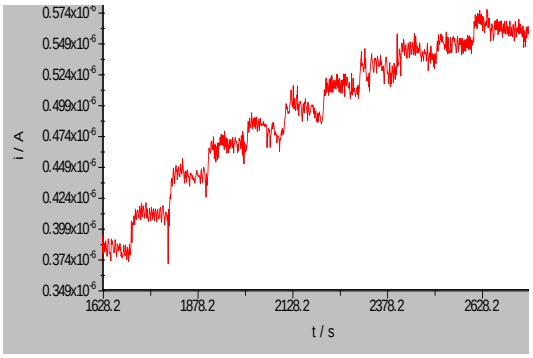



**Figure 4.2.4.1** - Chronoamperogram for construction of calibration curve. + 0.8 V as applied potential; 8 as phosphate buffer pH; and additions of 50 µl Spd $10^{-3}$ M.

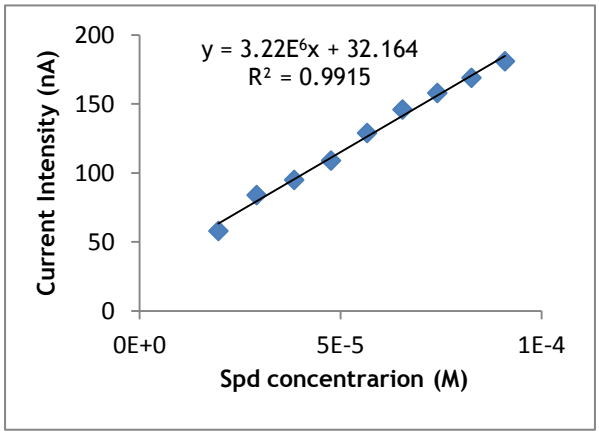



**Figure 4.2.4.2** - Calibration curve and $R^2$ value using results of Table **4.2.4.1.**

After invalid point's removal, the calibration curve as the following equation:

$$\mathbf{y = 3.22E^{6}x + 32.164}$$

$$\mathbf{R^2 = 0.9915}$$

**With a sensibility of $3.22x10^6$ nA $mol^{-1}$ $dm^3$**

***Reproducibility***

Repeatability and reproducibility are extremely important characteristics to control in an analytical method. In this specific case there are few results to reach a conclusion about the repeatability value. It is necessary to do more experiments with the same biosensor at the same conditions to have a minimum number of results to calculate this feature. On the other hand, there are enough results to obtain the reproducibility of this analytical method.

To evaluate the method reproducibility three experiments were carried out with three different biosensors operating at the same conditions. The results are shown in **Table 4.2.4.2**. and respective chronoamperograms and calibration curves presented between **Figure 4.2.4.3 and 4.2.4.8**.

**Table 4.2.4.2 -** Experimental data to construct the calibration curves to evaluate the MAO biosensor reproducibility

| Spd (M) | Intensity (nA) | | |
|---|---|---|---|
| | 130312_1f | 140312_1c | 240412_1c |
| $9.901E^{-6}$ | 75 | 60 | 69 |
| $1.961E^{-5}$ | 138 | 130 | 104 |
| $2.913E^{-5}$ | 177 | 190 | 145 |
| $3.846E^{-5}$ | 230 | 240 | 168 |
| $4.762E^{-5}$ | 262 | 290 | 192 |
| $5.660E^{-5}$ | 296 | 340 | 213 |
| $6.542E^{-5}$ | 317 | 370 | 242 |
| $7.407E^{-5}$ | 346 | 400 | 246 |
| $8.257E^{-5}$ | 368 | 440 | 258 |
| $9.091E^{-5}$ | 402 | 460 | 277 |

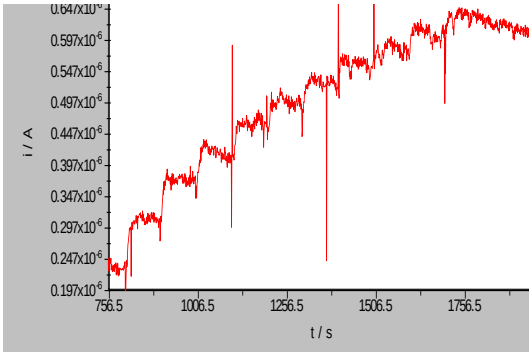



**Figure 4.2.4.3 -** Chronoamperogram of 130312_1f for the evaluation of method reproducibility.

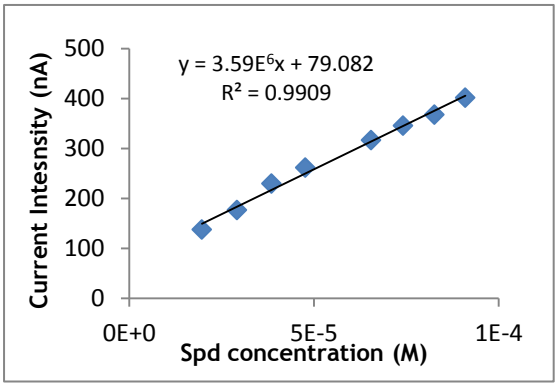



**Figure 4.2.4.4 -** Calibration curve and $R^2$ value of 130312_1f for the evaluation of method reproducibility.

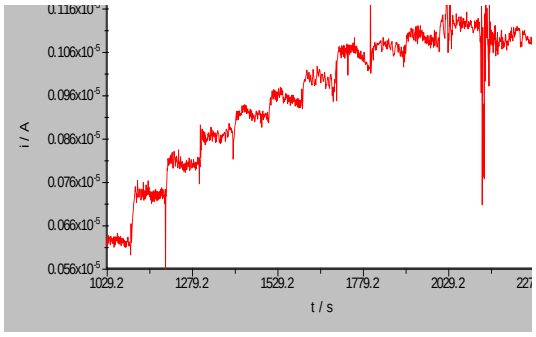



**Figure 4.2.4.5 -** Chronoamperogram of 140312_1c for the evaluation of method reproducibility.

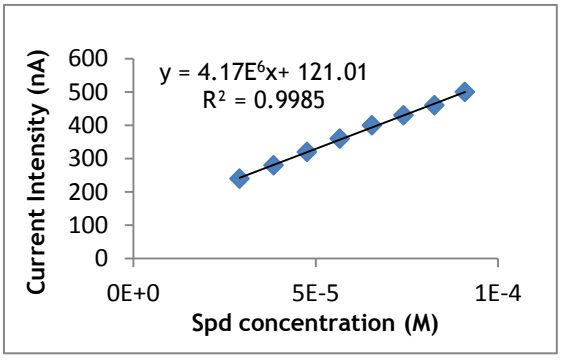



**Figure 4.2.4.6 -** Calibration curve and $R^2$ value of 140312_1c for the evaluation of method reproducibility.

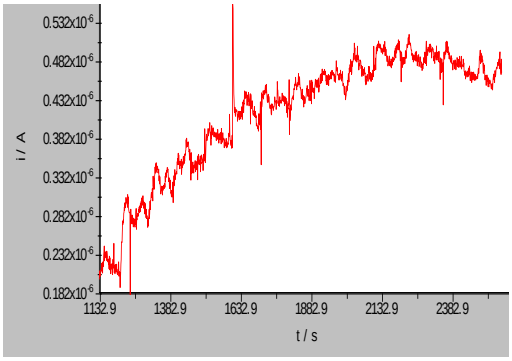



**Figure 4.2.4.7** - Chronoamperogram of 240412_1c for the evaluation of method reproducibility.

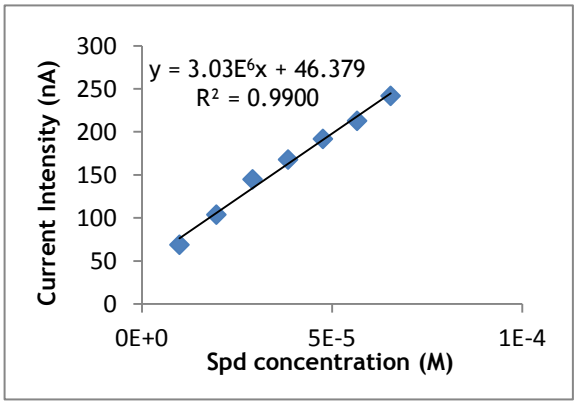



**Figure 4.2.4.8** - Calibration curve and $R^2$ value of 240412_1c for the evaluation of method reproducibility.

To calculate RSD it is necessary the different curves slope (**Table 4.2.4.3**). The RSD value is calculated by the ratio between the slope standard deviation and the slope average. In this case a reproducibility of 15.85% was obtained.

**Table 4.2.4.3** - Respective file slope for evaluation of method reproducibility

| File | Slope ($E^6$) |
|---|---|
| 130312_1f | 3.59 |
| 140312_1c | 4.17 |
| 240412_1c | 3.03 |

Below are showed a resume Table which compares the two enzyme biosensors constructed in University of Beira Interior (**Table 4.2.4.4**).

**Table 4.2.4.4** - Results of DAO and MAO biosensor constructed in University of Beira Interior. NQ = Not quantified

| | DAO Biosensor | MAO Biosensor |
|---|---|---|
| Sensibility (nA $mol^{-1}dm^3$) | $3.67E^6$ | $3.22E^6$ |
| Detection limit (M) | $2.11E^{-5}$ | NQ |
| RSD (%) reproducibility | 3.33% | 15.85% |

Based on **Table 4.2.4.4**, DAO biosensor has a sensibility and RSD for reproducibility better than MAO. In addition, it is essential to refer that the DAO solution has around an 80 times less activity than MAO, which reinforces the DAO biosensor choice.

# 4.3. Sensitive modified with Gold nanoparticles diaminoxidase biosensor based on carbon SPE for Spd determination

As we said before, one form of SPEs modification consists of the incorporation of **metallic nanoparticles** on the working electrode surface. Due to their reduced size, **metallic nanoparticles** exhibit important physical and electrical properties which make them very useful for the construction of more sensitive electrochemical sensors and biosensors (Ren *et al.*, 2005; Starowicz *et al.*, 2006; Welch and Compton, 2006). **Silver** and gold **nanoparticle-modified carbon SPEs** show important advantages when they are used as working electrodes in electrochemical techniques (Domínguez-Renedo *et al.*, 2008). Thus, different experiments were performed using a modified gold nanoparticles DAO biosensor, to determinate features such as: detection limit, sensibility, repeatability and reproducibility. These results were made at University of Burgos, therefore, as we explained before, we could not compare them with non-modified DAO biosensor performed at University of Beira Interior.

## 4.3.1 Figure of Merits

***Detection Limit***

The first step for LOD calculation is the obtainment of a linear relation between spermidine concentration and electrochemical signal.

Several calibrations were done at biosensor selected optimal conditions, that after invalid points removal were used for the construction of detection curves using the DETARCHI program (Sarabia et al., 1994) (**Table 4.3.1.1**).

**Table 4.3.1.1** - All valid experiments resume of SPEs immobilized with DAO and with gold nanoparticles. Spermdine prepared in phosphate buffer pH 8.9. + 0.6 V as applied potential.

| File | $R^2$ | Equation | Scale Estimate | Detection Limit | Signal Detection |
|---|---|---|---|---|---|
| 110909_A2 | 0.9955 | $y = 2{,}093E^{6}x + 1.8462$ | 0.05917 | $1.791E^{-7}$ | $-2.910E^{-7}$ |
| 110909_A3 | 0.9993 | $y = 2.77E^{6}x + 1.130$ | 0.20416 | $3.262E^{-7}$ | $-6.656E^{-8}$ |
| 150909_A1 | 0.9905 | $y = 2.69E^{6}x + 2.276$ | 0.86984 | $1.492E^{-6}$ | $3.971E^{-7}$ |

| | | | | | |
|---|---|---|---|---|---|
| 150909_A2 | 0.9994 | y = 3.04E$^{6}$x 0.685 | 0.15105 | 2.189E10$^{-7}$ | -3.43E$^{-8}$ |
| 150909_A3 | 0.9999 | y = 3.61E$^{6}$x 0.377 | 0.04400 | 5.458E10$^{-8}$ | -2.772E$^{-8}$ |
| 150909_A4 | 0.9997 | y = 3.67E$^{6}$x 0.434 | 0.10321 | 1.357E10$^{-7}$ | -2.936E$^{-8}$ |
| 150909_A5 | 0.9998 | y = 3.68E$^{6}$x 0.180 | 0.05506 | 7.090E10$^{-8}$ | -1.343E$^{-8}$ |
| 160909_2_A1 | 0.9996 | y = 3.67E$^{6}$x 0.247 | 0.12758 | 1.702E10$^{-7}$ | 2.035E$^{-8}$ |
| 160909_2_A2 | 0.9965 | y = 3.52E$^{6}$x 0.827 | 0.20406 | 2.981E10$^{-7}$ | 1.591E$^{-7}$ |

Almost all signal detections were negative which does not have any chemical meaning. Thus, the evaluation of the detection limit in a DAO biosensor modified by electrodeposition with gold nanoparticles was also carried out based on the variability of a blank.

To construct the calibration curve (**Figure 4.3.1.2**) a chronoamperogram (**Figure 4.3.1.1**) using Spd solution with concentrations ranging between 6.951E$^{-7}$ and 6.542E$^{-6}$ M (**Table 4.3.1.2)** was record.

**Table 4.3.1.2 -** Experimental data to construct the calibration curve.

| Spd concentration (M) | Intensity (nA) |
|---|---|
| 6.951E$^{-7}$ | 2.3 |
| 1.381E$^{-6}$ | 4.7 |
| 2.057E$^{-6}$ | 7 |
| 2.724E$^{-6}$ | 9.5 |
| 3.382E$^{-6}$ | 11.8 |
| 4.031E$^{-6}$ | 14.3 |
| 4.671E$^{-6}$ | 16.7 |
| 5.303E$^{-6}$ | 19.2 |
| 5.927E$^{-6}$ | 21.4 |
| 6.542E$^{-6}$ | 23.6 |

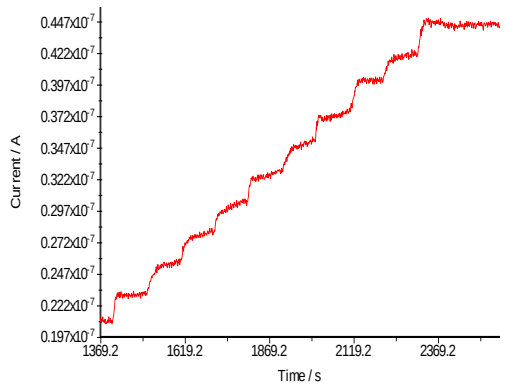



**Figure 4.3.1.1** - Chronoamperogram for construction of calibration curve. +0.6 V as applied potential; 8.9 as phosphate buffer pH; additions of 35 µl Spd $10^{-3}$ M; and deposition of gold nanoparticles

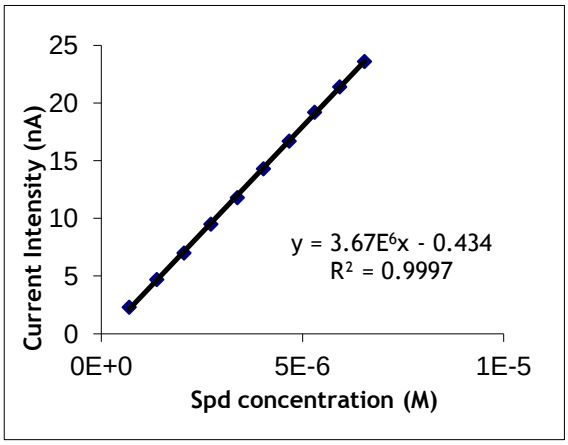



**Figure 4.3.1.2** - Calibration curve and $R^2$ value using results of **Table 4.3.1.2.**

After invalid point's removal, the calibration curve as the following equation:

**$y = 3.67E^{6}x - 0.434$**

**$R^2 = 0.9997$**

**With a sensibility of $3.67x10^{6}$ nA $mol^{-1}$ $dm^3$**

The variability of a sample which contains a small amount of Spd ($6.951E^{-7}$M) was used to determine the detection limit. By adding this amount of Spd respective current signals were recorded (**Table 4.3.1.3**).

**Table 4.3.1.3** - Data to calculate the average and standard deviation of a sample wit small amount of Spd

| Spd concentration (M) | Intensity (nA) |
|---|---|
| $6.951E^{-7}$ | 1.9 |
| $6.951E^{-7}$ | 3.5 |
| $6.951E^{-7}$ | 1.6 |
| $6.951E^{-7}$ | 2.1 |
| $6.951E^{-7}$ | 2.3 |
| $6.951E^{-7}$ | 2.3 |
| $6.951E^{-7}$ | 2.1 |
| $6.951E^{-7}$ | 2.6 |

$\bar{x}$ = 2.3; SD = 0.5682

By a probability level of 5% (α=ß=0.05), the *t* unilateral value for 7 degrees of freedom is 1.895. Therefore, the detection limit is,

$$D_l = 1.895\sqrt{1+\frac{1}{8}} \times 0.5682 = 1.142$$

where 8 it is the number of replicates and 1.142 corresponds to its SD value.

The detection limit expressed in terms of concentration is on the same range of the ones presented on **Table 4.3.1.1**,

$$X_d = 2 \times \frac{1.142}{3.67E^6} = 6.22^{-7}M$$

where $3.67E^6$ is the calibration curve slope.

***Repeatability and reproducibility***

As said before, repeatability and reproducibility are extremely important characteristics to determinate in an analytical method. To evaluate the method repeatability of a DAO biosensor modified by electrodeposition with gold nanoparticles were carried out three experiments with same biosensor. The results are shown in **Table 4.3.1.4**. and between **Figure 4.3.1.3 and 4.3.1.8**.

**Table 4.3.1.4 -** Experimental data for the determination of method repeatability

| **Spd (M)** | **Intensity (nA)** | | |
|---|---|---|---|
| | 150909_A3 | 150909_A4 | 150909_A5 |
| $6.951E^{-7}$ | 2.1 | 2.3 | 2.3 |
| $1.381E^{-6}$ | 4.6 | 4.7 | 4.8 |
| $2.057E^{-6}$ | 7 | 7 | 7.4 |
| $2.724E^{-6}$ | 9.5 | 9.5 | 9.9 |
| $3.382E^{-6}$ | 11.9 | 11.8 | 12.4 |
| $4.031E^{-6}$ | 14.2 | 14.3 | 14.7 |
| $4.671E^{-6}$ | 16.3 | 16.7 | 17.1 |
| $5.303E^{-6}$ | 18.8 | 19.2 | 19.4 |
| $5.927E^{-6}$ | 21 | 21.4 | 21.5 |
| $6.542E^{-6}$ | 23.2 | 23.6 | 23.8 |

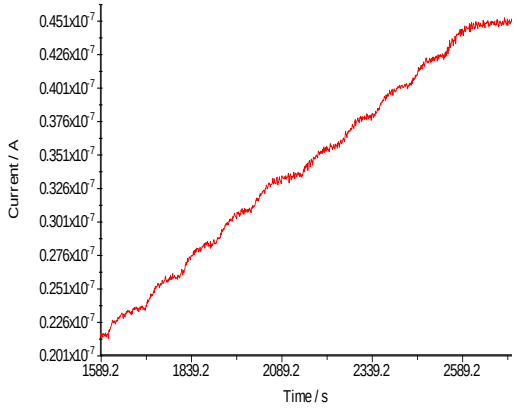



**Figure 4.3.1.3 -** Chronoamperogram of 150909_A3 for the evaluation of method repeatability.

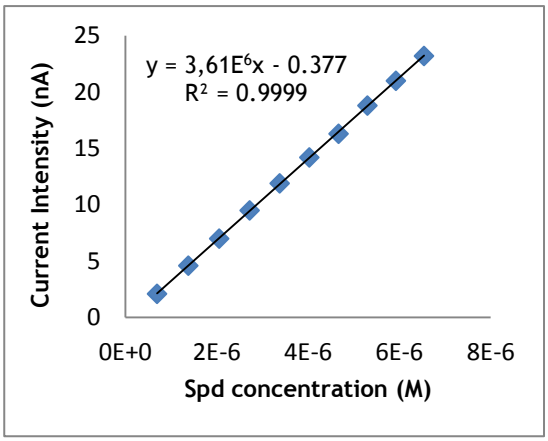



**Figure 4.3.1.4 -** Calibration curve and $R^2$ value of 150909_A3 for the evaluation of method repeatability.

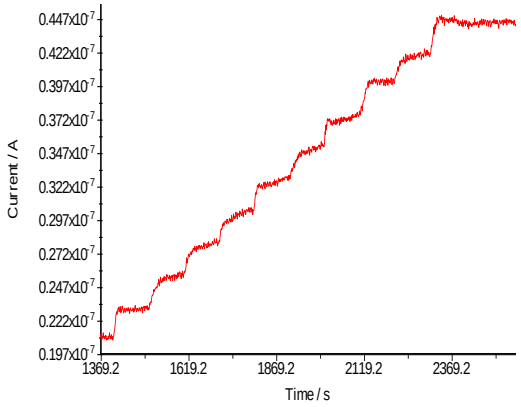



**Figure 4.3.1.5 -** Chronoamperogram of 150909_A4 for the evaluation of method repeatability.

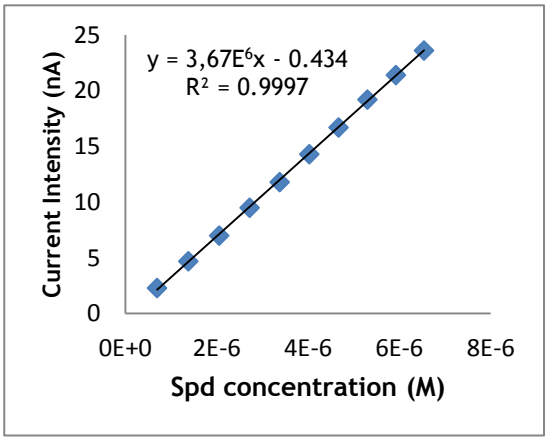



**Figure 4.3.1.6 -** Calibration curve and $R^2$ value of 150909_A4 for the evaluation of method repeatability.

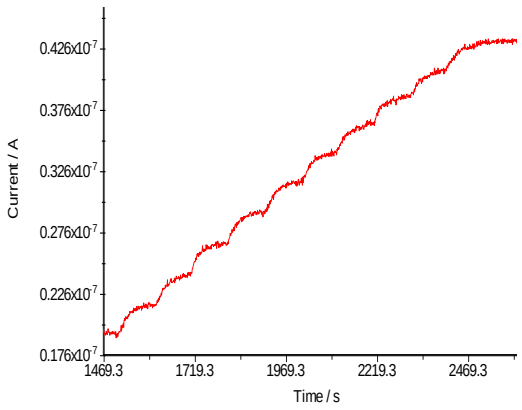



**Figure 4.3.1.7 -** Chronoamperogram of 150909_A5 for the evaluation of method repeatability.

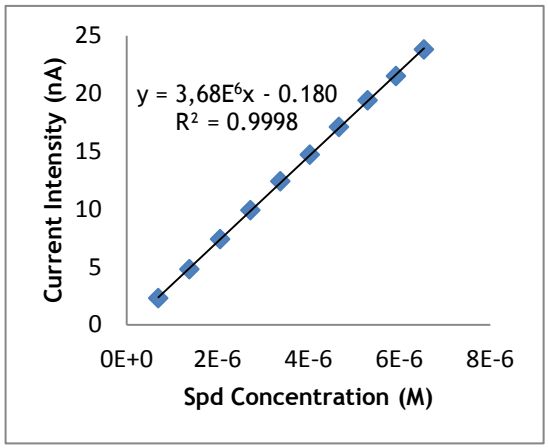



**Figure 4.3.1.8 -** Calibration curve and $R^2$ value of 150909_A5 for the evaluation of method repeatability.

To calculate RSD it is necessary the different curves slope (**Table 4.3.1.5**). In this case a repeatability of 1.04% was obtained.

**Table 4.3.1.5 -** The respective file slope for the evaluation of method repeatability.

| File | Slope ($E^6$) |
|---|---|
| 150909_A3 | 3.61 |
| 150909_A4 | 3.67 |
| 150909_A5 | 3.68 |

To evaluate the method reproducibility of a DAO biosensor modified by electrodeposition with gold nanoparticles three experiments with three different biosensors were carried out. The results are shown in **Table 4.3.1.6**. and between **Figure 4.3.1.9 and 4.3.1.14**.

**Table 4.3.1.6 -** Experimental data for the determination of method reproducibility.

| Spd (M) | Intensity (nA) | | |
|---|---|---|---|
| | 110909_A3 | 150909_A2 | 160909_2_A2 |
| $6.951E^{-7}$ | 2.1 | 2.3 | 2.3 |
| $1.381E^{-6}$ | 4.6 | 4.7 | 4.8 |
| $2.057E^{-6}$ | 7 | 7 | 7.4 |
| $2.724E^{-6}$ | 9.5 | 9.5 | 9.9 |
| $3.382E^{-6}$ | 11.9 | 11.8 | 12.4 |
| $4.031E^{-6}$ | 14.2 | 14.3 | 14.7 |
| $4.671E^{-6}$ | 17.3 | 16.7 | 17.1 |
| $5.303E^{-6}$ | 18.8 | 19.2 | 19.4 |
| $5.927E^{-6}$ | 21 | 21.4 | 21.5 |
| $6.542E^{-6}$ | 23.2 | 23.6 | 23.8 |

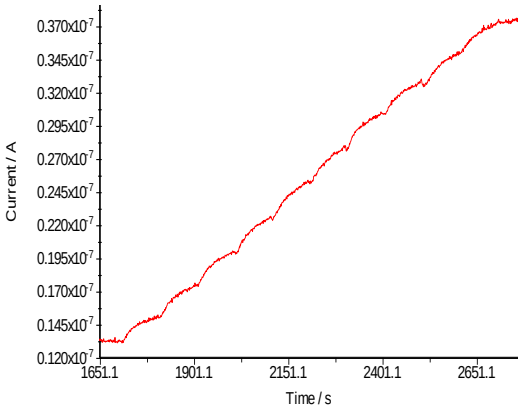



**Figure 4.3.1.9** - Chronoamperogram of 110909_A3 for the evaluation of method reproducibility.

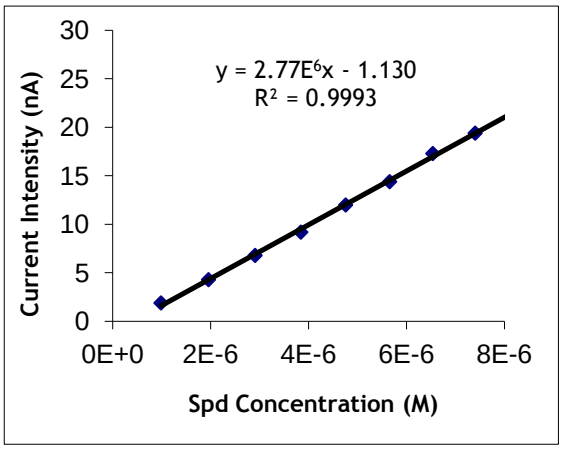



**Figure 4.3.1.10** - Calibration curve and $R^2$ value of 110909_A3 for the evaluation of method reproducibility.

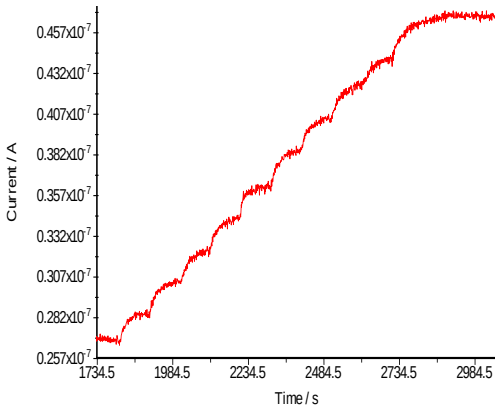



**Figure 4.3.1.11** - Chronoamperogram of 150909_A2 for the evaluation of method reproducibility.

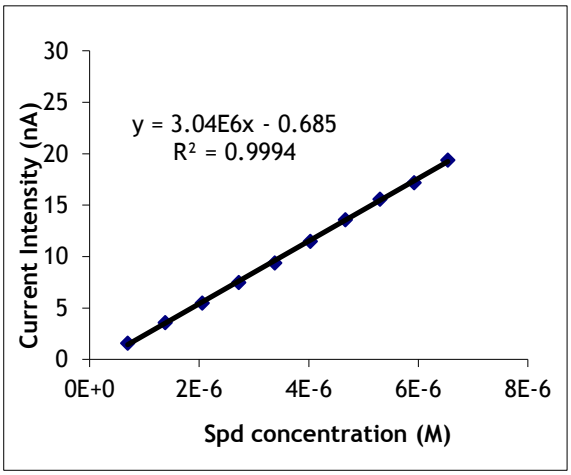



**Figure 4.3.1.12** - Calibration curve and $R^2$ value of 150909_A2 for the evaluation of method reproducibility.

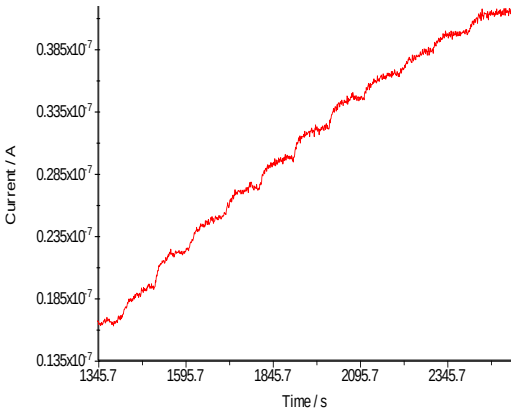



**Figure 4.3.1.13** - Chronoamperogram of 160909_2_A2 for the evaluation of method reproducibility.

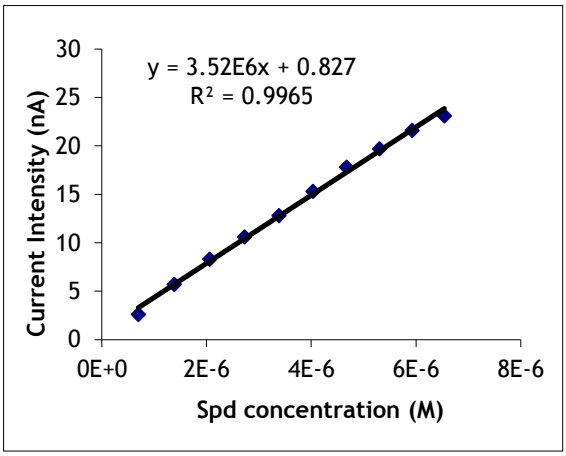



**Figure 4.3.1.14** - Calibration curve and $R^2$ value of 160909_2_A2 for the evaluation of method reproducibility.

By using the different curves slope (**Table 4.3.1.7**) a reproducibility of 12.21% was obtained.

**Table 4.3.1.7 -** The respective file slope for the evaluation of method reproducibility.

| File | Slope ($E^6$) |
|---|---|
| 110909_A3 | 2.77 |
| 150909_A2 | 3.04 |
| 160909_2_A2 | 3.52 |

# 4.4. Sensitive modified with Gold nanoparticles monoaminoxidase biosensor based on carbon SPE for Spd determination

## 4.4.1 Figures of merit

**Table 4.4.1.1** shows several calibrations that were done at biosensor selected optimal conditions. After invalid points removal for the construction of detection curves, DETARCHI program (Sarabia et al., 1994) was used. These experiments were made a MAO biosensors previously modified with Gold nanoparticles.

**Table 4.4.1.1 - V**alid experiments resume of SPEs immobilized with MAO and with gold nanoparticles. Spd prepared in phosphate buffer pH 8.9; +0.7 V as applied potential.

| File | $R^2$ | Equation | Scale Stimate | Detection Limit | Signal Limit |
|---|---|---|---|---|---|
| 230909_A2 | 0.9995 | y = 7,39E$^{6}$x + 0.4797 | 0.04960 | 3.645E$^{-8}$ | 1.357E$^{-8}$ |
| 230909_A3 | 0.9910 | y = 1.49E$^{7}$x + 6.7116 | 1.59142 | 6.489E$^{-7}$ | 1.760E$^{-6}$ |
| 230909_A4 | 0.9997 | y = 1.33E$^{6}$x + 1.880 | 0.25827 | 9.495E$^{-8}$ | 3.049E$^{-7}$ |
| 240909_A1 | 0.9931 | y = 1.36E$^{7}$x + 5.0138 | 1.08956 | 4.796E$^{-7}$ | 1.240E$^{-6}$ |
| 240909_A2 | 0.9964 | y = 1.68E$^{7}$x + 4.5246 | 0.62090 | 1.674E$^{-7}$ | 4.206E$^{-7}$ |
| 240909_2_A2 | 0.9930 | y = 1.27E$^{7}$x + 3.9226 | 1.02888 | 3.746E$^{-7}$ | 4.727E$^{-7}$ |
| 240909_2_A3 | 0.9994 | y = 1.53E$^{7}$x + 0.7495 | 0.24378 | 7.849E$^{-8}$ | 1.591E$^{-7}$ |
| 250909_A2 | 0.9928 | y = 1.26E$^{7}$x + 4.2907 | 0.78653 | 3.262E$^{-7}$ | 7.293E$^{-7}$ |
| 280909_2_A1 | 0.9973 | y = 8.96E$^{6}$x + 2.2241 | 0.48075 | 2.648E$^{-7}$ | 3.919$^{-7}$ |
| 290909_A1 | 0.9998 | y = 1.96E$^{7}$x + 0.6771 | 0.36686 | 8.218E$^{-8}$ | 1.505E$^{-7}$ |
| 290909_A2 | 0.9997 | y = 2.35E$^{7}$x + 0.4744 | 0.39873 | 8.269E$^{-8}$ | 1.989E$^{-7}$ |
| 290909_A3 | 0.9976 | y = 2.43E$^{7}$x + 1.6963 | 0.71294 | 1.345E$^{-7}$ | 2.066E$^{-7}$ |
| 290909_A4 | 0.9956 | y = 2.34E$^{7}$x + 4.5178 | 1.15417 | 2.892E$^{-7}$ | 1.161E$^{-6}$ |

### *Detection Limit*

Consider the previous Table it is easy to see how detection limit varies. The lowest achieved LOD was $3.645E^{-8}$ M and the highest was $6.489E^{-7}$ M. The LOD average is $2.354E^{-7}$ M and its SD is $1.843E^{-7}$.

### *Sensibility*

Relatively to this feature we could say that most of the slope magnitude order it is found at 1x10^7 where the highest is $2.43E^{7}$ nA $mol^{-1}dm^{3}$.

### *Repeatability and reproducibility*

There are enough data to determine how biosensor behaves when submitted to several experiments. As said before, to evaluate the repeatability three experiments with the same biosensor were carried out. Calibrates are shown in **Table 4.4.1.2** as well as the chornoamperograms between **Figure 4.4.1.1** and **4.4.1.6**.

**Table 4.4.1.2 -** Experimental data for method repeatability determination.

| Spd (M) | Intensity (nA) | | |
|---|---|---|---|
| | **290909_A2** | **290909_A3** | **290909_A4** |
| $3.984E^{-7}$ | 9 | 10 | 11 |
| $7.937E^{-7}$ | 19 | 20 | 22 |
| $1.186E^{-6}$ | 29 | 30 | 33 |
| $1.575E^{-6}$ | 38 | 41 | 43 |
| $1.961E^{-6}$ | 47 | 51 | 52 |
| $2.344E^{-6}$ | 56 | 60 | 62 |
| $2.724E^{-6}$ | 64 | 69 | 69 |
| $3.100E^{-6}$ | 73 | 78 | 77 |
| $3.475E^{-6}$ | 82 | 85 | 85 |
| $3.846E^{-6}$ | 91 | 93 | 92 |

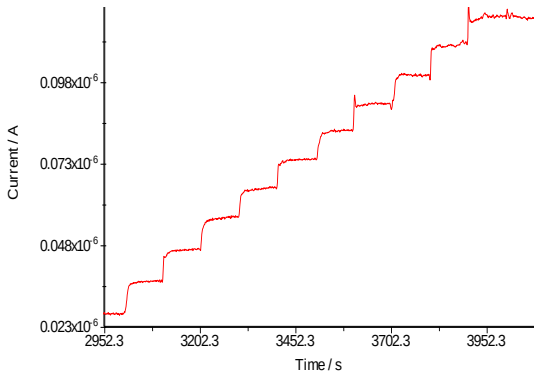



**Figure 4.4.1.1 -** Chronoamperogram of 290909_A2 for the evaluation of method repeatability.

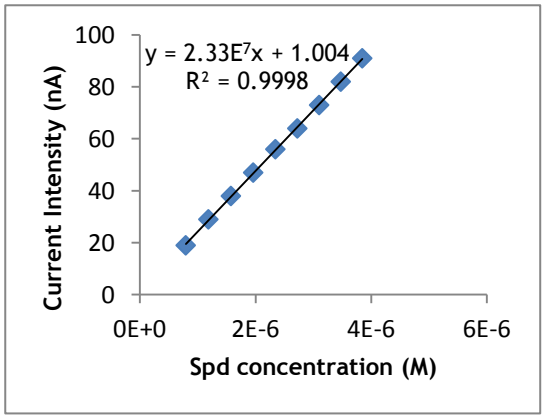



**Figure 4.4.1.2 -** Calibration curve and $R^2$ value of 290909_A2 for the evaluation of method repeatability.

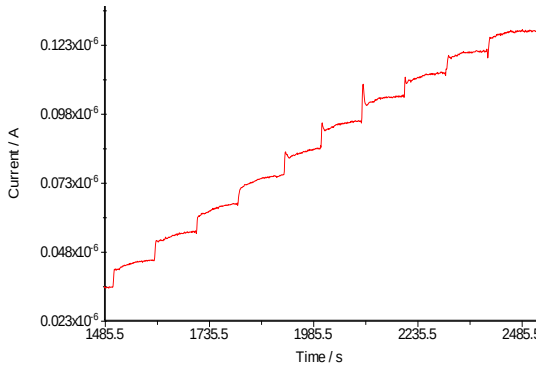



**Figure 4.4.1.3 -** Chronoamperogram of 290909_A3 for the evaluation of method repeatability.

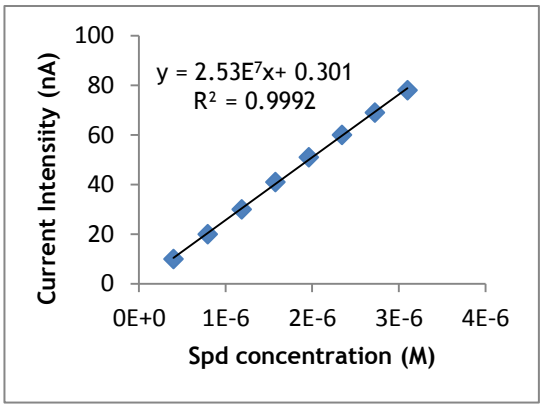



**Figure 4.4.1.4 -** Calibration curve and $R^2$ value of 290909_A3 for the evaluation of method repeatability.

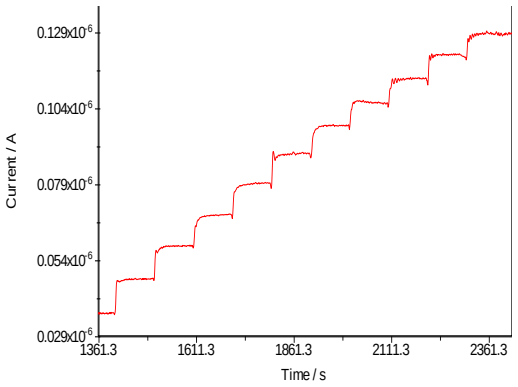



**Figure 4.4.1.5 -** Chronoamperogram of 290909_A4 for the evaluation of method repeatability.

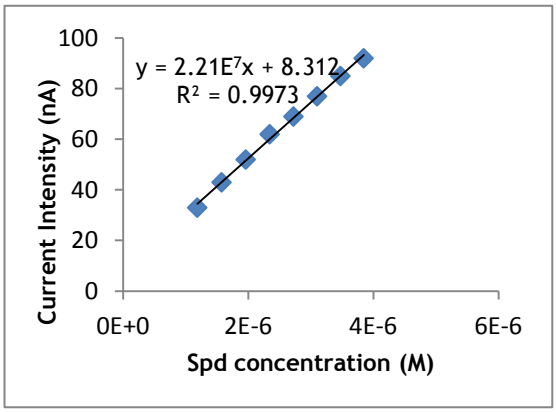



**Figure 4.4.1.6 -** Calibration curve and $R^2$ value of 290909_A4 for the evaluation of method repeatability.

By using the different curves slope (**Table 4.4.1.3**) a repeatability of 6.90% was found.

**Table 4.4.1.3 -** The respective file slope for the evaluation of method repeatability.

| File | Slope (E′) |
|---|---|
| 290909_A2 | 2.23 |
| 290909_A3 | 2.53 |
| 290909_A4 | 2.21 |

We can also determine biosensor reproducibility from data presented in **Table 4.4.1.1.** For this we selected a group of three experiments made at three different biosensors prepared in the same conditions. Calibrates are shown in **Table 4.4.1.4** and chornoamperograms between **Figure 4.4.1.7** and **4.3.1.12**.

**Table 4.4.1.4 -** Experimental data for the determination of method reproducibility

| Spd (M) | Intensity (nA) | | |
|---|---|---|---|
| | 230909_A4 | 240909_A1 | 240909_2_A2 |
| $5.964E^{-7}$ | 9 | 10 | 10 |
| $1.186E^{-6}$ | 18 | 20 | 17 |
| $1.768E^{-6}$ | 26 | 30 | 25 |
| $2.344E^{-6}$ | 33 | 39 | 33 |
| $2.913E^{-6}$ | 41 | 47 | 42 |
| $3.475E^{-6}$ | 48 | 54 | 49 |
| $4.031E^{-6}$ | 56 | 61 | 55 |
| $4.580E^{-6}$ | 63 | 68 | 61 |
| $5.123E^{-6}$ | 70 | 73 | 65 |
| $5.660E^{-6}$ | 77 | 80 | 71 |

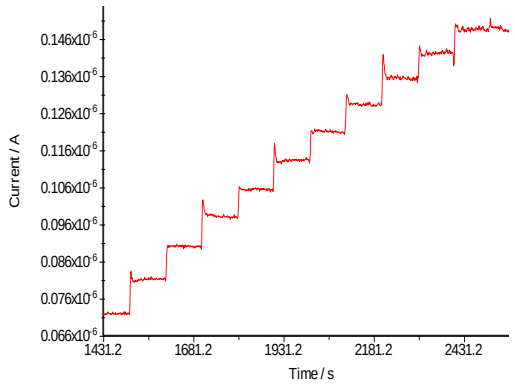



**Figure 4.4.1.7 -** Chronoamperogram of 230909_A4 for the evaluation of method reproducibility.

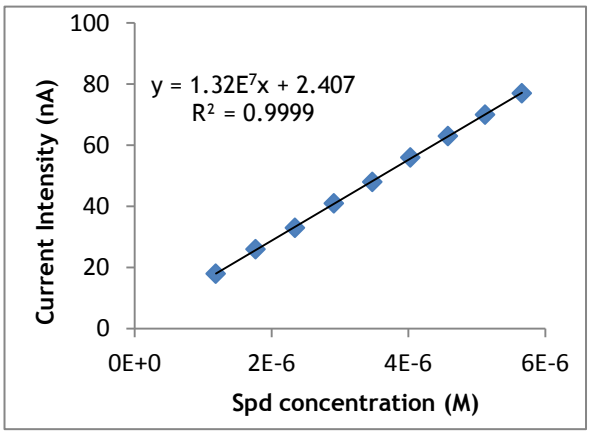



**Figure 4.4.1.8 -** Calibration curve and $R^2$ value of 230909_A4 for the evaluation of method reproducibility.

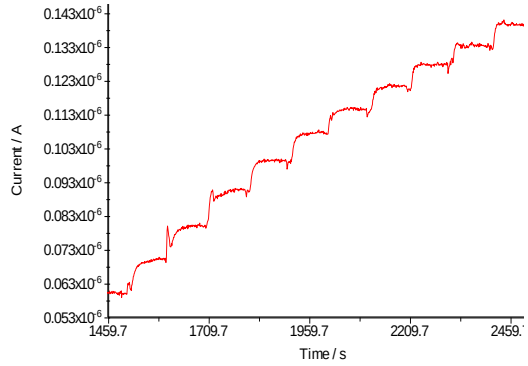



**Figure 4.4.1.9 -** Chronoamperogram of 240909_A1 for the evaluation of method reproducibility.

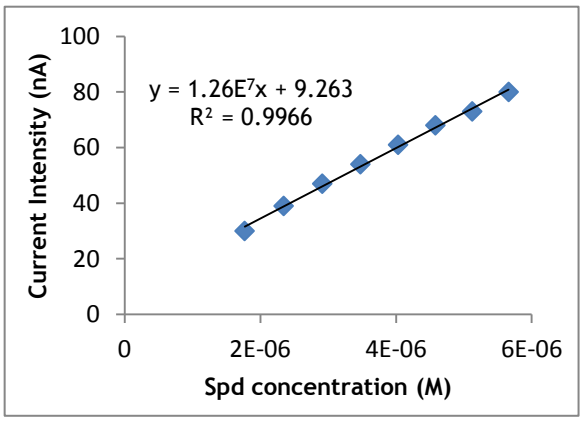



**Figure 4.4.1.10 -** Calibration curve and $R^2$ value of 240909_A1 for the evaluation of method reproducibility.

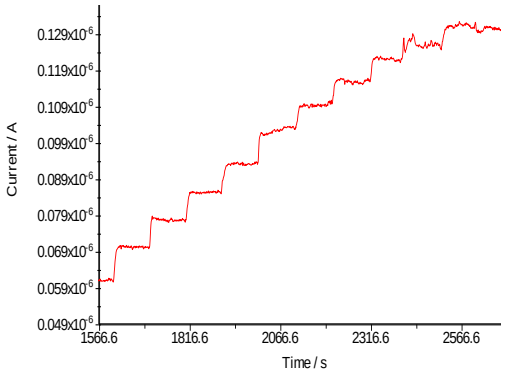



**Figure 4.4.1.11** - Chronoamperogram of 240909_2_A2 for the evaluation of method reproducibility.

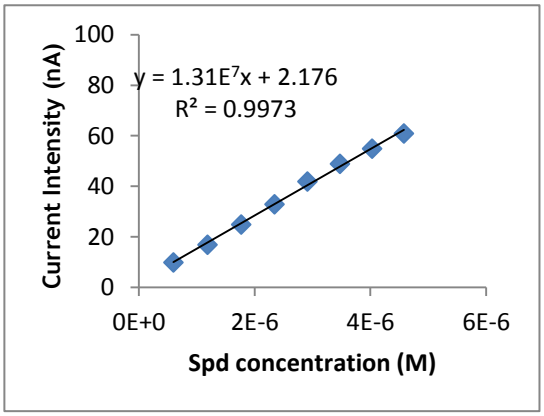



**Figure 4.4.1.12** - Calibration curve and $R^2$ value of 240909_2_A2 for the evaluation of method reproducibility.

Based on the different curves slope (**Table 4.4.1.5**) a reproducibility of 2.37% was obtained.

**Table 4.4.1.5** - The respective file slope for the evaluation of method reproducibility

| File | Slope ($E^7$) |
|---|---|
| 230909_A4 | 1.37 |
| 240909_A1 | 1.26 |
| 240909_2_A2 | 1.31 |

# 4.5. Sensitive modified with Silver nanoparticles monoaminoxidase biosensor based on carbon SPE for Spd determination

## 4.5.1 Figures of merit

These experiments were made in MAO biosensors previously modified with silver nanoparticles. **Table 4.5.1.1** shows several calibrations that were done at biosensor selected optimal conditions. After invalid points removal for the construction of detection curves the DETARCHI program (Sarabia et al., 1994) was used.

**Table 4.5.1.1** – Valid experiments resume of SPEs immobilized with MAO and with silver nanoparticles. Spd prepared in phosphate buffer pH 8.9 and +0.7 V as applied potential.

| File | $R^2$ | Equation | Scale stimate | Detection Limit | Signal Limit |
|---|---|---|---|---|---|
| 090909_A2 | 0.9996 | y = 2,84E$^6$x + 0.459 | 0.16743 | 2.609E$^{-7}$ | 8.397E$^{-8}$ |
| 090909_A3 | 0.9994 | y = 4,06E$^6$x - 0.165 | 0.28152 | 3.068E$^{-7}$ | 4.754E$^{-8}$ |
| 100909_A2 | 0.9932 | y = 2,18E$^6$x - 2.075 | 0.43907 | 9.551E$^{-7}$ | -1.440E$^{-7}$ |
| 100909_A3 | 0.9983 | y = 2,91E$^6$x - 1.724 | 0.21052 | 3.464E$^{-7}$ | -1.615E$^{-7}$ |
| 100909_A4 | 0.9990 | y = 2,97E$^6$x - 0.400 | 0.16082 | 3.177E$^{-7}$ | 1.403E$^{-8}$ |
| 100909_2_A1 | 0.9962 | y = 7,19E$^6$x + 4.415 | 0.24975 | 1.769E$^{-7}$ | 2.980E$^{-7}$ |
| 100909_2_A2 | 0.9921 | y = 6,36E$^6$x + 3.271 | 1.02713 | 1.091E$^{-6}$ | 1.190E$^{-6}$ |
| 100909_2_A4 | 0.9932 | y = 5,88E$^6$x + 9.367 | 1.27477 | 1.059E$^{-6}$ | 1.258E$^{-6}$ |

***Detection Limit***

Although, some results have a negative signal limit, the majority is positive. Thus we have reliable information about how detection limit fluctuate. The lowest achieved LOD was 1.769E$^{-7}$ M and the highest was 1.091E$^{-6}$M. The LOD average is 5.642$^{-7}$M and its standard deviation is 3.949E$^{-7}$.

### *Sensibility*

Relatively to this feature we could say that most of the slope magnitude order it is found at 1x10^6 where the highest is 7.19E$^{6}$ nA mol$^{-1}$dm$^{3}$.

### *Repeatability and reproducibility*

To evaluate the repeatability were carried out three experiments with the same biosensor in the same conditions. The results are shown in **Table 4.5.1.2** and between **Figure 4.5.1.1** and **4.5.1.6**.

**Table 4.5.1.2 -** Experimental data for method repeatability determination

| Spd (M) | Intensity (nA) | | |
|---|---|---|---|
| | 100909_A2 | 100909_A3 | 100909_A4 |
| 9.901E$^{-7}$ | 0.8 | 1.8 | 2.6 |
| 1.961E$^{-6}$ | 2.5 | 4 | 5.3 |
| 2.913E$^{-6}$ | 3.8 | 6.4 | 8.4 |
| 3.846E$^{-6}$ | 6 | 9.1 | 11.2 |
| 4.762E$^{-6}$ | 7.7 | 11.7 | 13.8 |
| 5.660E$^{-6}$ | 9.8 | 14.6 | 16.2 |
| 6.542E$^{-6}$ | 12 | 17.5 | 18.8 |
| 7.407E$^{-6}$ | 14.2 | 20 | 21.2 |
| 8.257E$^{-6}$ | 16.7 | 22.4 | 24 |
| 9.091E$^{-6}$ | 17.7 | 24.8 | 27.1 |

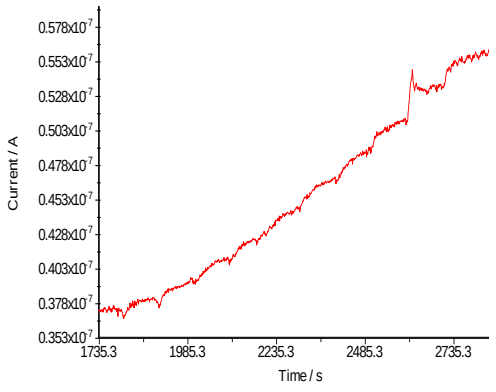



**Figure 4.5.1.1 -** Calibration curve and $R^2$ value of 100909_A2 for the evaluation of method repeatability.

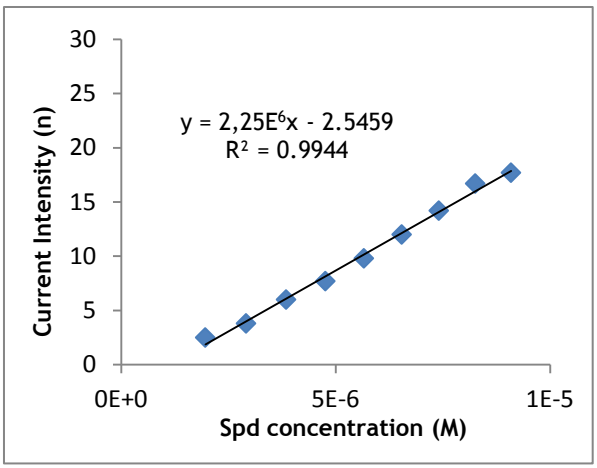



**Figure 4.5.1.2 -** Calibration curve and $R^2$ value of 100909_A2 for the evaluation of method repeatability.

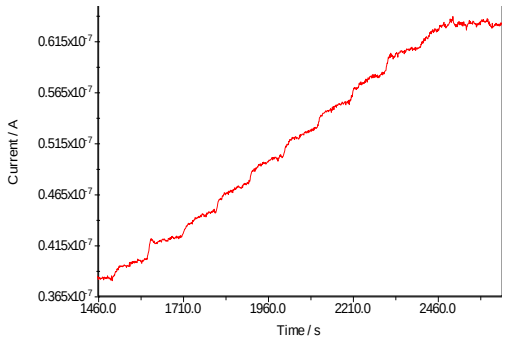



**Figure 4.5.1.3** - Chronoamperogram of 100909_A3 for the evaluation of method repeatability

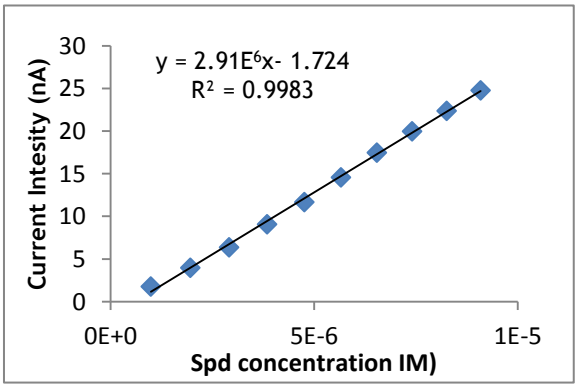



**Figure 4.5.1.4 -** Calibration curve and $R^2$ value of 100909_A3 for the evaluation of method repeatability.

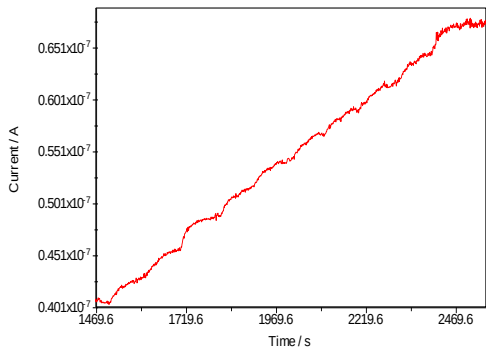



**Figure 4.5.1.5** - Chronoamperogram of 100909_A4 for the evaluation of method repeatability

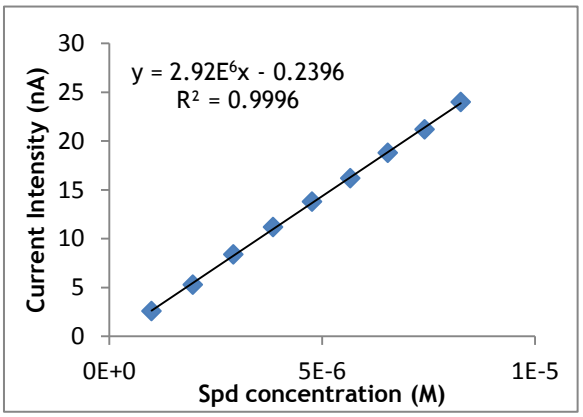



**Figure 4.5.1.6 -** Calibration curve and $R^2$ value of 100909_A4 for the evaluation of method repeatability.

Based on the different curves slope (**Table 4.5.1.3**) a repeatability of 14.31% was achieved.

**Table 4.5.1.3 -** The respective file slope for the evaluation of method repeatability.

| File | Slope ($E^6$) |
|---|---|
| 100909_A2 | 2.25 |
| 100909_A3 | 2.91 |
| 100909_A4 | 2.96 |

Three different biosensors prepared in the same conditions were used to check its reproducibility (**Table 4.5.1.4.**, **Figure 4.5.1.7** to **4.5.1.12**).

**Table 4.5.1.4 -** Experimental data for method reproducibility determination

| Spd (M) | Intensity (nA) | | |
|---|---|---|---|
| | 090909_A3 | 100909_A3 | 100909_2_A2 |
| $9.901E^{-7}$ | 3.9 | 1.8 | 8 |
| $1.961E^{-6}$ | 7.5 | 4 | 15 |
| $2.913E^{-6}$ | 11.6 | 6.4 | 23 |
| $3.846E^{-6}$ | 15.5 | 9.1 | 29 |
| $4.762E^{-6}$ | 19.1 | 11.7 | 35 |
| $5.660E^{-6}$ | 23 | 14.6 | 40 |
| $6.542E^{-6}$ | 26.8 | 17.5 | 44 |
| $7.407E^{-6}$ | 30.1 | 20 | 49 |
| $8.257E^{-6}$ | 33.4 | 22.4 | 52 |

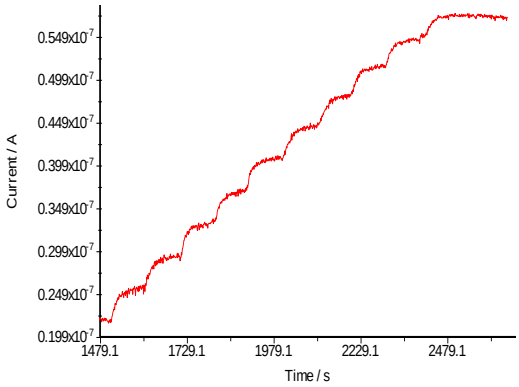



**Figure 4.5.1.7 -** Chronoamperogram of 090909_A3 for the evaluation of method reproducibility.

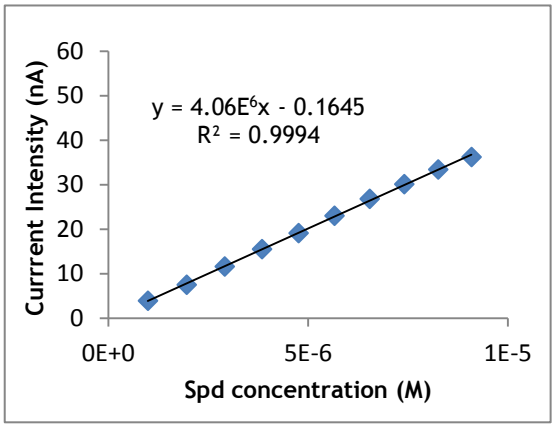



**Figure 4.5.1.8 -** Calibration curve and $R^2$ value of 090909_A3 for the evaluation of method reproducibility.

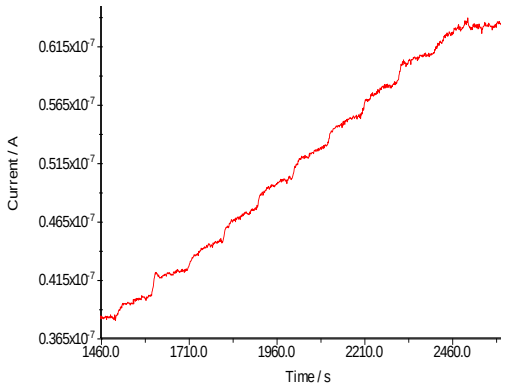



**Figure 4.5.1.9 -** Chronoamperogram of 100909_A3 for the evaluation of method reproducibility.

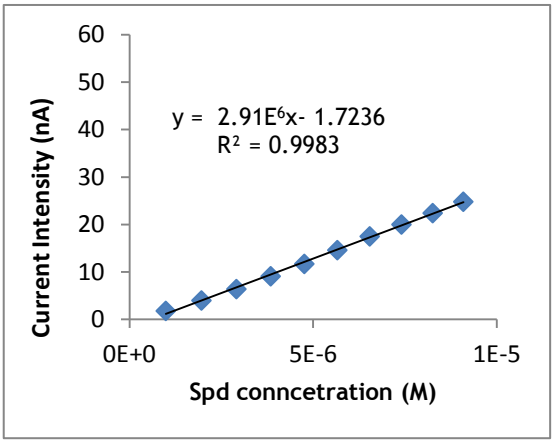



**Figure 4.5.1.10 -** Calibration curve and $R^2$ value of 100909_A3 for the evaluation of method reproducibility.

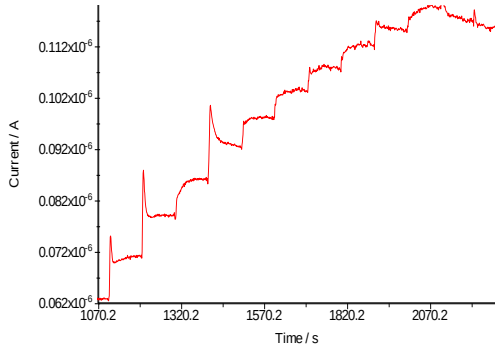



**Figure 4.5.1.11** - Chronoamperogram of 100909_2_A2 for the evaluation of method reproducibility.

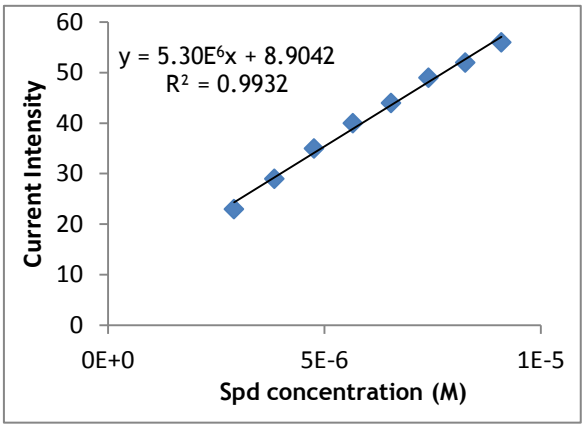



**Figure 4.5.1.12** - Calibration curve and $R^2$ value of 100909_2_A2 for the evaluation of method reproducibility.

Considering the different curves slope (**Table 4.5.1.5**) a reproducibility of 29.22% was found.

**Table 4.5.1.5** - The respective file slope for the evaluation of method reproducibility.

| File | Slope ($E^6$) |
|---|---|
| 090909_A3 | 4.06 |
| 100909_A3 | 2.91 |
| 100909_2_A2 | 5.30 |

Finally a comparison between the different nanoparticles modified biosensors is presented. **Table 4.5.1.6** shows the most useful and relevant information.

**Table 4.5.1.6** - Figures of merits for the studied nanoparticles modified biosensors. Biosensors constructed in University of Burgos. NQ = Not quantified

| | DAO | | MAO | |
|---|---|---|---|---|
| | GOLD | SILVER | GOLD | SILVER |
| Sensibility (nA $mol^{-1}dm^3$) | $3.67E^6$ | NQ | $2.43E^7$ | $7.19E^6$ |
| Detection Limit (M) | $6.22E^{-7}$ | NQ | $2.35E^{-7}$ | $5.64E^{-7}$ |
| RSD (%) Repeatability | 1.04 | NQ | 6.90 | 14.31 |
| RSD (%) Reproducibility | 12.21 | NQ | 2.37 | 29.22 |

This resume table clarify the diferences between the two used nanoparticles. In MAO case it is evident that electrodeposicion of Gold nanoparticles makes a much more powerful biosensor than the electrodeposicion of Silver nanoparticles, since all the compared parameters are better for the gold modified biosensor. The analyzis is more difficult when we compare the two enzymes, althoug MAO biosensor seems to have the higher sensibility and the lower limit of detection, the enzymatic activity difference between MAO and DAO (80 times higher for MAO) can not be forgotten. So, at the end, both enzymes are potentially useful for Spd biosensors construction.

As mention in the introduction section one of most common analytical techniques used for determination of biogenic amines in biological samples is HPLC. By comparing limit of detection reached using this technique with the ones obtained with the studied biosensors, we realize that the values for HPLC can be in the range of pmol for polyamines (Lozanov *et al.*, 2007), much lower than the values of LOD obtained by us . However HPLC, is highly sensitive (Lozanov *et al.*, 2007) it is not cheap technique and straightforward to perform, making the biosensors prepared by us a good option to quantify biogenic amines.

# 4.6 Biosensor enzymatic activity determination by isothermal titration microcalorimetry

## 4.6.1 Introduction

Almost every significant life process is dependent on enzyme activity. Therefore the assay and pharmacological regulation of enzymes are key elements in clinical diagnosis and therapeutics. Enzymes are proteins that function as biological catalysts and they play a crucial role in nearly all processes that take place in living organisms. Therefore studies of these molecules and the reactions they catalyze have been a core activity of biochemists for at least the past fifty years.

Isothermal titration microcalorimetry (ITC) may be used as a tool for obtaining enzyme kinetic constants and the overall apparent molar enthalpy for catalytic reactions. ITC is useful, in this regard, since it directly measures the heat change as catalysis proceeds and this is proportional to the rate of the reaction. ITC is a well established, powerful, versatile and high-sensitivity technique that is widely used for measuring the thermodynamics of equilibrium association reactions (Wiseman et al., 1989; Haq et al., 2000). In addition, well designed experiments can yield an approximate value for the equilibrium association constant ($K_A$) for the enzyme-substrate complex when product formation is the limiting step, as well as the stoichiometry (n) of the reaction. Therefore ITC can yield an almost complete thermodynamic profile for any biomolecular complex formation.

Traditionally, enzymologists have relied upon spectrophotometric assays where the presence of a chromophere/fluorophore on either the substrate or product allows quantification of turnover rate. When the substrate/product is spectroscopically invisible and they cannot be derivatized, biochemists usually have to carry out meticulous and very careful time course experiments. Here the catalysis is allowed to proceed for different time periods after which the amount of formed product is determined using chromatographic, electrophoretic or mass spectrometric means. Clearly these methods can be difficult, requiring a high level of expertise, and can be expensive and slow (Wiseman *et al.*, 1989; Haq *et al.*, 2000). ITC resolves this problem, most physiologically relevant or chemically interesting compounds can be studied without any consideration of whether or not the compound is spectroscopically active. ITC also offers a general methodology that can be used to obtain Michaelis-Menten rate information on enzyme function. ITC is already a proven technology for gaining detailed thermodynamic insights into equilibrium binding phenomena, and therefore factors such as baseline stability, reproducibility and overall sensitivity are well established. Monitoring the rate of a reaction using changes in thermal power is nondestructive, direct and very sensitive (Wiseman et al., 1989; Haq et al., 2000). Therefore it is possible to use ITC to obtain enzyme activity data since rates are determined directly. In addition numerous injections of substrate

can be made into one reaction cell, thus eliminating experimental error associated with the preparations of different samples. ITC also allows experimental conditions such as temperature, pH, and ionic strength to be easily modulated.

## 4.6.2 Results Analysis

**Pseudo-First-Order Assays**

A thermodynamically favorable chemical reaction is driven by a decrease in free energy, which is the sum of an enthalpic and an entropic term ($\Delta G = \Delta H + T\Delta S$); the enthalpic component is observed as heat. Thus an assay method based on measuring the rate of heat generation accompanying conversion of substrate to product should give a measure of the enzyme reaction rate. Heat ($Q$) measured as a function of time ($dt$) is defined as the thermal power (Todd *et al.*, 2001):

$$Power = \frac{dQ}{dt}$$

The amount of heat associated with converting $n$ moles of substrate to product is given by

$$Q = n \times \Delta H_{app} = [P]_{Total} \times V \times \Delta H_{app}$$

where $V$ is the volume of the solution in the reaction cell, $P$ is the molar concentration of product generated, and $\Delta H_{app}$ is an experimentally determined molar enthalpy for the reaction. Thus a measure of termal power generated by an enzyme immediately gives knowledge of the reaction rate (Todd *et al.*, 2001):

$$Power = \frac{dQ}{dt} = \frac{d[P]}{dt} \times V \times \Delta H_{app}$$

**Enzyme kinetics**

When an enzyme (E) interacts with its substrate (S), an enzyme-substrate complex (ES) is formed. This complex is converted to its transition state (ES), then enzyme-product complex (EP). Finally, the enzyme-product complex dissociates to give free enzyme and product. In biological systems, where substrate concentration is much higher than enzyme concentration, the reaction is simplified to:


where $k_1$ is rate constant for formation of ES and $k_{-1}$ is rate constant for dissociation of ES. [E] is the total enzyme concentration in the reaction. The catalytic rate constant is kcat. And reaction rate $R_f$ is determined by the Michaelis-Menten equation:

$$R_f = \frac{V_{max} \times [S]_t}{K_M + [S]_t}$$

where $V_{max}$ is maximum observable velocity, $[S]_t$ is instantaneous substrate concentration, and $K_M$ is Michaelis constant. The lower the $K_M$, the greater the affinity between enzyme and substrate. Since $V_{max}$ is equal to $k_{cat} \bullet [E]$, Equation below is rewritten as:

$$R_f = \frac{K_{cat} \times [E] \times [S]_t}{K_M + [S]_t}$$

A hyperbolic rate plot is generated in a graph of $R_f$ vs. $[S]_t$ (**Figure 4.6.2.1**). $V_{max}$ is the rate when the curve plateaus, and $K_M$ is equal to $[S]_t$ at 1/2 $V_{max}$, where half of the enzyme molecules are in ES form.

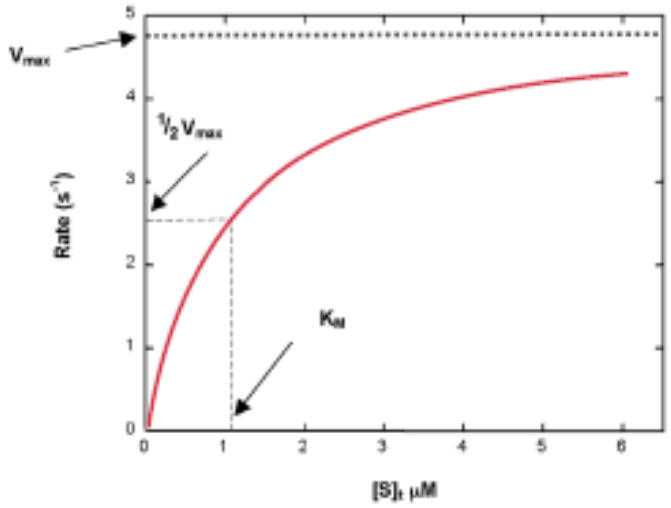



**Figure 4.6.2.1** - Hyperbolic curve of a $R_f$ vs. $[S]_t$ graphic with kinetic parameters indication.

### 4.6.3 Experimental data

In this section, we plan to evaluate MAO activity immobilized in an SPE by using ITC. For this purpose is imperative to reproduce optimal MAO biosensor operation conditions during these experiments.

To actually perform the ITC assay it is necessary to determined enzymatic reaction molar enthalpy by having relatively high concentrations of enzyme in the cell, relatively low amounts of substrate in the injection syringe, and by leaving sufficient gaps between injections to ensure that all of the substrate is converted to product. The resultant peaks can be used to obtain $\Delta H_{app}$ in the normal way. Considerer this, a MAO biosensor prepared under optimal conditions described in **section 4.2** was used for enthalpy reaction determination (**Experiment 1** - **Figure 4.6.3.1**).

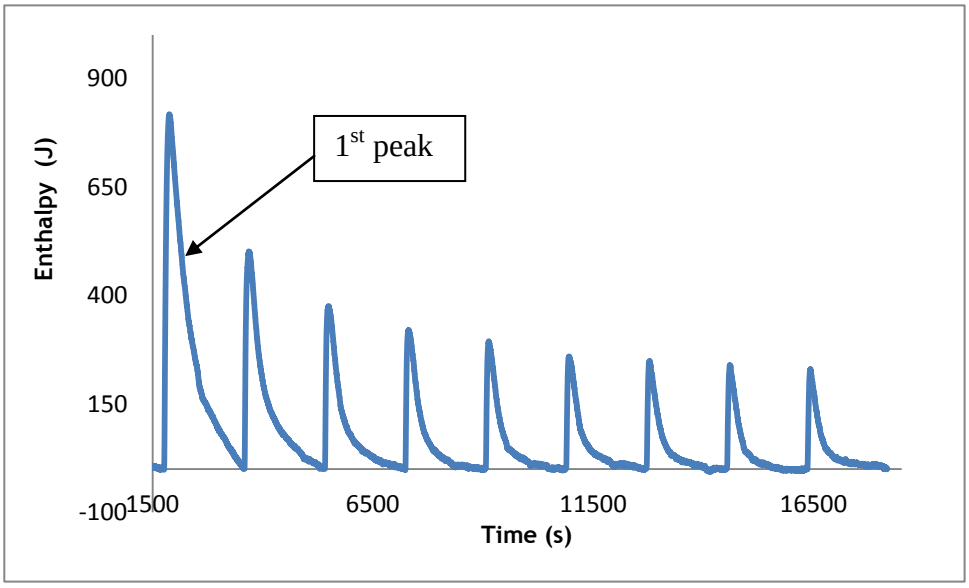



**Figure 4.6.3.1** - Exothermic data for evaluation of MAO activity. Nine injections of 5µl, $10^{-1}$ M Spd were added to a 100 µl phosphate buffer pH 8 solution containing the electrode immobilized with MAO.

For the purpose of data analysis, we ignored the first injection. A key feature of this data is that the magnitude of ΔH decreases after each injection, until the last four, where it stabilizes. This effect could be due to product inhibition. Thus, to evaluate the reason for this behavior, other experiments were conducted. All the previously procedure was repeated, but at this time using an electrode without MAO immobilized (**Experiment 2**- **Figure 4.6.3.2**).

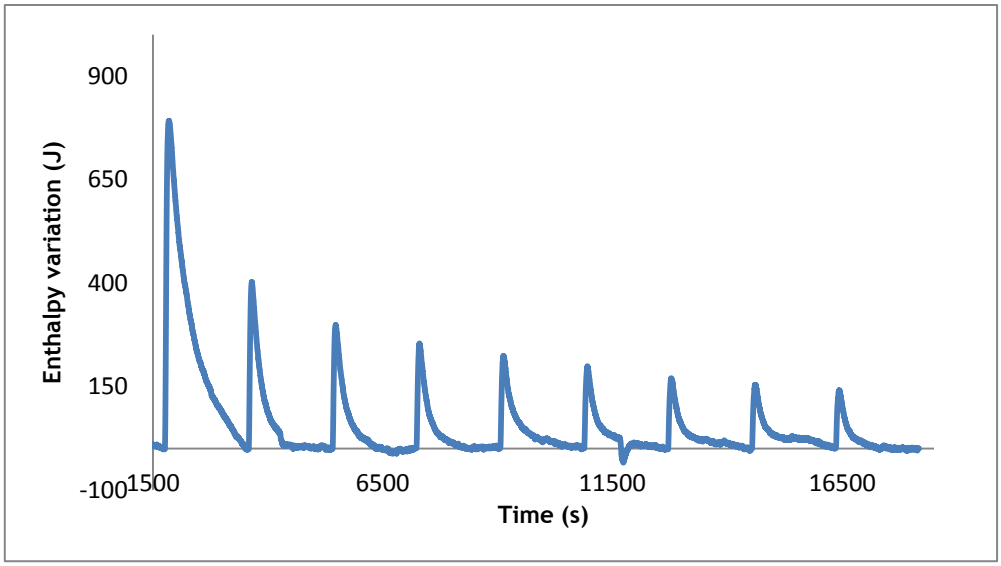



**Figure 4.6.3.2** - Exothermic data for evaluation of MAO activity. Nine injections of 5µl, $10^{-1}$ M Spd were added to a 100 µl phosphate buffer pH 8 solution containing the electrode without MAO immobilized.

As can be seen, a similar response was obtained when an electrode without MAO immobilized is used. Thus, the reason why the magnitude of $\Delta H_{app}$ decreases after each injection (except for the last four) is not due to product inhibition. This is probably due to a dilution phenomenon occurring when Spd is added to the phosphate solution. Hence, we conducted another experiment (**Experiment 3)** without electrode, which perfectly shows how heat of dilution influenced the previous results (**Figure 4.6.3.3**).

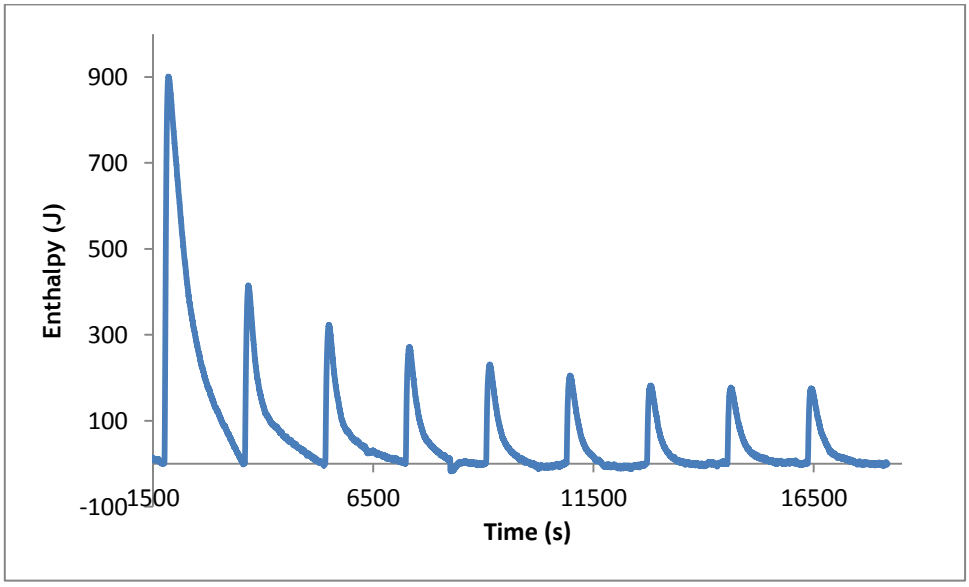



**Figure 4.6.3.3** - Exothermic data for evaluation of MAO activity. Nine injections of 5µl, $10^{-1}$ M Spd were added to a 100 µl phosphate buffer pH 8 solution.

These high exothermic values of heat of dilution may be due to spermidine protonation. As previously discussed in Chapter I, Spd has its amine group's protonated in acidic and soft alkaline pHs (Gucliucci, 2004). Due to this dilution phenomenon is not wrong to call this enthalpy as dilution enthalpy. So, factor dilution, instead of Spd concentration, will be directly related with enthalpy. Considering this, a representation of **dilution factor vs dilution enthalpy** is presented on **Figure 4.6.3.4** considering the data on **Table 4.6.3.1.**

**Table 4.6.3.1 -** Experimental data resume for experiments 2 (electrode without MAO immobilized) and 3 (buffer solution), of six injections of 5µl, $10^{-1}$ M Spd and respective measured enthalpies (a thirty-minute gap was left between each injection).

| Dilution Factor | Dilution Enthalpy (mJ) | |
|---|---|---|
| | Experiment 2 | Experiment 3 |
| 0.04762 | -2.1189 | -2.5961 |
| 0.04545 | -1.5157 | -1.8612 |
| 0.04348 | -1.2687 | -1.2755 |
| 0.04167 | -1.1512 | -0.8336 |
| 0.04000 | -1.0927 | -0.7542 |
| 0.03846 | -0.6922 | -0.8526 |

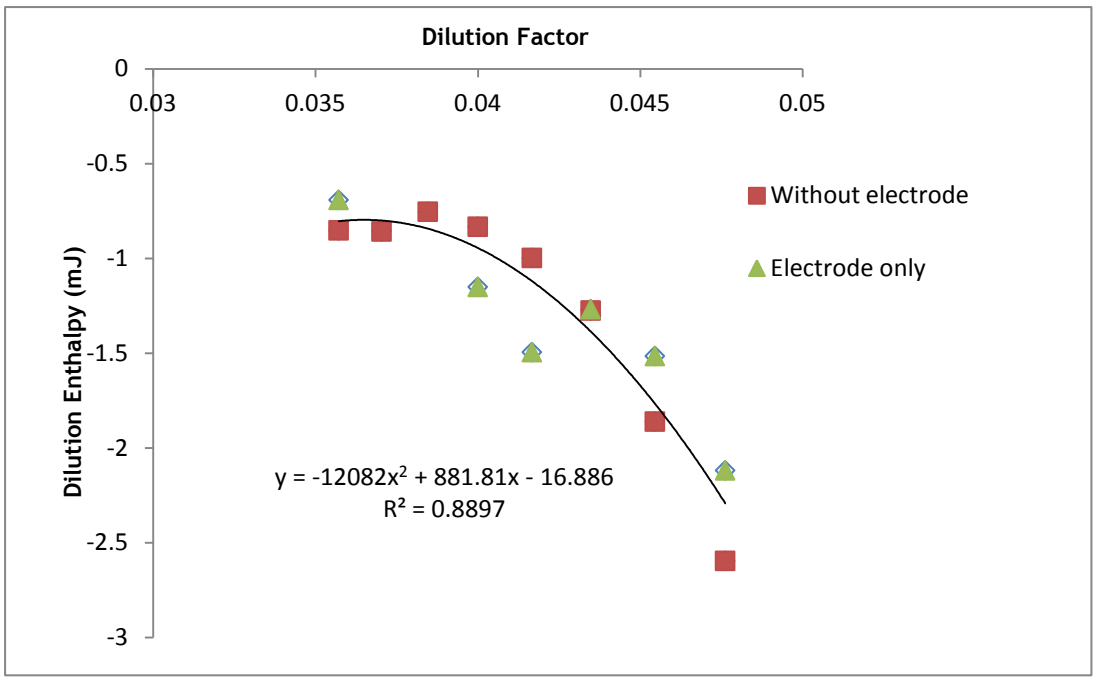



**Figure 4.6.3.4** - Dilution Factor vs. Enthalpy for two different experiments: without electrode (square) and electrode only (triangle).

As showed on **Figure 4.6.3.4**, both experiments have similar behavior. This fact contributes to emphasize the importance of heat dilution on these experiments, since the expected electrode surface adsorption enthalpy was not the major heat. So we can look down the adsorption input to enthalpy. We can also state that during Spd injections the influence of heat dilution decreases and stabilizes after the fifth addition, which indicates us a major dilution enthalpy influence for the initial injections.

Knowing that total heat results from enzyme reaction enthalpy, dilution enthalpy and adsorption enthalpy:

$$\Delta H_{total} = \Delta H_{enzyme\ reaction} + \Delta H_{dilution} + \Delta H_{adsorption}$$

enzyme reaction enthalpy can be obtained by subtracting dilution plus adsorption enthalpy (relation with dilution factor represented by y = -12082x2 + 881.81x - 16.886) from total enthalpy The table for graphic construction (**Table 4.6.3.2**) is showed below as well the respective graphic (**Figure 4.6.3.5**).

**Table 4.6.3.2 -** Experimental data resume for experiment 1 (electrode MAO immobilized) and enthalpy of reaction calculation (six injections of 5µl, $10^{-1}$ M Spd and respective measured enthalpies, a thirty-minute gap was left between each injection).

| Dilution Factor | $\Delta H_{Total}$ (mJ) | $\Delta H_{Dilution+ADSORPTION}$ (mJ) | $\Delta H_{enzyme\ reaction}$ (mJ) |
|---|---|---|---|
| 0.04546 | -2.2286 | -1.7665 | -0.4621 |
| 0.04348 | -1.6609 | -1.3858 | -0.2752 |
| 0.04167 | -1.4433 | -1.1196 | -0.3237 |
| 0.04000 | -1.3452 | -0.9448 | -0.4004 |
| 0.03846 | -1.1674 | -0.8430 | -0.3244 |
| 0.03571 | -1.0979 | -0.8035 | -0.2944 |

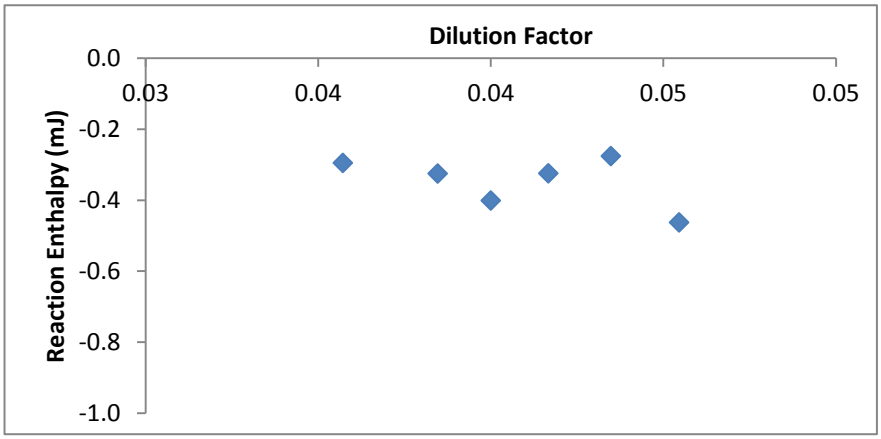



**Figure 4.6.3.5 -** Dilution factor vs enzyme reaction enthalpy. Average $\Delta H_{enzyme\ reaction}$ equal to -0.347 mJ.

From above experiments $\Delta H_{enzymatic\ reaction}$ has the value of -0.347 mJ. To actually perform the ITC assay, in order to derive the Michaelis-Menten defined enzyme parameters ($K_M$, $k_{cat}$ and $V_{max}$) a second type of experiment needs to be carried out. Rate data can be obtained by having relatively low amounts of enzyme in the cell, relatively high amounts of substrate in the injection syringe and by leaving shorter gaps between injections. The aim here is to ensure that subsequent to each injection, steady-state conditions are maintained and no more than 5% of the substrate is depleted prior to the next injection. These experiments were not performed because microcalorimeter calibration stops working, making impossible the quantification of heat. This problem has been already solved, and we intent to soon collect Michaelis-Menten defined enzyme parameters necessary to evaluate enzyme activity.

# Chapter V – Conclusion

The different results can be summarized in the following conclusions for the respective section:

**a) DAO immobilized biosensor without nanoparticles**

1. No chronoamperometric response was observed by adding several aliquots of Spd to the non-modified electrode. However, a clear response can be observed with the biosensor immobilized with DAO, thus providing the essential role of DAO on this type of response.
2. When adding several water aliquots no interference was detected on the chronoamperometric signal.
3. The selected optimal conditions are respectively +0.8 V and 8 for applied potential and phosphate buffer pH.
4. Concerning the figure of merit values this method presents a sensibility of $3.67E^{6}$ nA $mol^{-1}dm^{3}$, a detection limit of $2.11E^{-5}$ M and a reproducibility of 3.33%. Repeatability was not quantified.

**b) MAO immobilized biosensor without nanoparticles**

1. Immobilization process was optimized to evaluate the GLU and BSA concentration influence. The previously standard concentration was established as the optimal concentration, in other words, 27 mg/ml and 5 mg/ml for GLU and BSA respectively.
2. The selected optimal conditions are, as well as for DAO, respectively +0.8 V and 8 for applied potential and phosphate buffer pH.
3. This method has a sensibility of $3.22E^{6}$ nA $mol^{-1}dm^{3}$, however the detection limit as well as, repeatability were not quantified due the limited number of calibrates. Method reproducibility has been evaluated as 15.85%.

**e) Gold modified DAO biosensor**

1. The selected optimal conditions are respectively +0.6 V and 8.9 for applied potential and phosphate buffer pH.
2. Regarding to the figure of merit values this method has a sensibility of $3.67E^{6}$ nA $mol^{-1}$ $dm^{3}$ and the detection limit of $6.22E^{-7}$ M. The RSD (%) values for repeatability and reproducibility were respectively 1.04 and 12.21.

**c) Gold modified MAO biosensor**

1. The selected optimal conditions are respectively +0.7 V and 8.9 for applied potential and phosphate buffer pH.

Concerning figure of merit values a sensibility of $2.43E^{7}$ nA $mol^{-1}dm^{3}$ and a detection limit of $2.35E^{-7}$ M was reached with this biosensor. The RSD (%) values for repeatability and reproducibility were 6.90 and 2.37, respectively.

**d) Silver modified MAO biosensor**

1. This type of biosensor has an optimal operation at an applied potential of +0.7 V and a pH of 8.9 (phosphate buffer).
2. In terms of achieved results, this method has a sensibility of $7.19E^{6}$ nA $mol^{-1}dm^{3}$ and a detection limit of $5.64E^{-7}$ M. The repeatability and reproducibility were calculated as 14.31% and 29.22%, respectively.

**f) ITC**

1. Adsorption input to enthalpy can be looked down since major contribution to enthalpy is due to dilution phenomenon and enzymatic reaction.
2. The value for $\Delta H_{enzymatic\ reaction}$ was calculated as -0.347 mJ, the same magnitude order for other enzymes.

## Future Works

To complete the biosensor construction it is necessary to check which Spd similar compounds interfere with biosensor signal. Also, we intent to apply the developed biosensors to real samples like food or biological fluids. Finally for method validation, it will be necessary to compare results with results obtained by another analytical method (e.g. HPLC).

Concerning ITC, Michaelis-Menten enzyme parameters need to be defined in order to evaluate MAO activity. Finally DAO activity has to be also evaluated.

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