Master degree in Nanostructured Materials for Nanotechnology Applications

Enzymatic optical (nano)biosensors based on "in situ"synthesized nanoclusters

Final Master Project

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ABBREVIATIONS

DAO	Diamine Oxidase
Put	Putrescine
Cad	Cadaverine
His	Histamine
AuNPs	Gold Nanoparticles
NPs	Nanoparticles
NCs	Nanoclusters
SPR	Surface Plasmon Resonance
ES	Enzymatic Substrate
BA	Biogenic Amines
HRP	Horseradish Peroxidase
OAB	o-aminobenzaldehyde
RT	Room temperature
RPM	Revolutions per minute
STEM	Scanning and tunnelling electron microscope
Au	Gold
Ag	Silver

SUMMARY

Nowadays food quality controls are very strict to ensure human health. There are many of them, to detect ripening, based on the determination of histamine (a biogenic amine), but there is not anyone based on putrescine and/or cadaverine. The aim of this project is to detect and quantify, if it is possible, those biogenic amines taking advantage of the enzymatic reaction between them and diamine oxidase, DAO. The product of this reaction turns into its cyclic form and forms a complex with a gold precursor. This is the beginning of the gold nanoparticles (AuNPs) synthesis, which can be measured by absorbance due to its optical properties based on the surface plasmon resonance (SPR) phenomenon. Therefore, absorbance due to nanoparticles can be related to biogenic amine's concentration.

In addition, a characterization is done to improve the conditions and the throughput of the reaction taking advantage of the reaction kinetics. Some experiments are proposed to understand the mechanism of the gold nanoparticles formation and, real and synthetic samples are analysed.

The final purpose of this investigation line is to fabricate a biosensor to be implemented in food packages; therefore, the characterization is also done at room temperature to mimic real conditions.

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1.-INTRODUCTION

1.1.- NATURAL RIPENING INDICATORS AND QUALITY INDEXES

Biogenic amines, such as cadaverine, putrescine and histamine, are ubiquitous in biological matrices, not only in animal and plant cells, they are also produced by microbial decarboxylation of amino acids. The amount and type of amine formed is strongly influenced by the food composition, microbial flora and by several parameters (temperature[1,2], food additives, moisture, ripening, etc) which allow bacterial growth during food storage. The estimation or even quantification of the biogenic amine's levels is very important due to its impact on human health and food quality[3].

These amines concentration can change during food processing and storage, because of that their formation has been proposed as an index of chemical quality.

Histamine has been frequently reported as the main agent of chemical quality[4]. At present, the legal maximum level of biogenic amines in fish products has been established only for histamine at 50 mg/kg by the Food and Drug administration (FDA) of the United States of America. The European Union established 100 mg of histamine per kg in 1991, and 300 mg of total biogenic amines per kilogram. However, histamine is not a good indicator for decomposition because its accumulation during storage is lower than other biogenic amines such as putrescine and cadaverine. An example of a sardine ripened can be observed in figure 1[5]; although the maximum is reached by histamine, then it decreases until a level lower than cadaverine. Therefore, a biosensor is required to determine the other both biogenic amines, especially cadaverine.

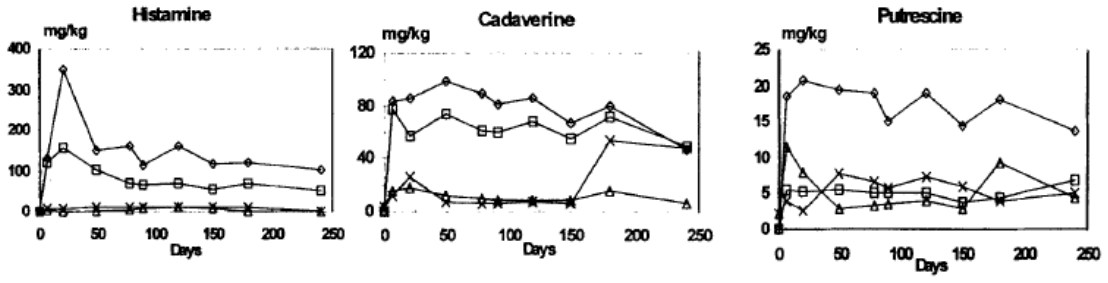

Figure 1.Change in biogenic amines during ripening of sardine. Squares represent the frozen sardine[5].

1.2.- OPTICAL (NANO) BIOSENSORS

A chemical biosensor, it is a device that transforms (bio)chemical information into analytical useful information[6]. They are composed by:

- Receptor: With biological nature, is immobilized to interact with a molecule and to provide the chemical signal. Sensibility depends on that step. If the receptor is not able to produce the chemical signal, an indicator is used for that purpose.
- Transductor: Transforms the chemical signal into an analytical signal usually an electric signal. In the case of optical biosensor the transductor is the detector.
- Signal conversion and amplification stage: Property that has been measured and its variations are registered to be able to observe and quantify them.

Taking advantage of the specific properties of nanoparticles and nanomaterials, there are new types of optical biosensors based on their optical properties. These nanomaterials have been used as:

- Receptors, by covalent interactions such as hydrogen bonds, Van der Waals forces. Not very selective.
- Biorreceptor's support, increasing its stability and allowing miniaturization being very simple to manage.
- Indicators in colorimetric and fluorimetric systems increasing sensibility, stability and biocompatibility. For that purpose the nanomaterials must by bonded to the receptor. The most common use of nanoparticles.

1.3.- NANOMATERIALS FOR BIOSENSORS. DETERMINATION OF BIOGENIC AMINES

Metallic (Au and Ag) nanoparticles are widely used because if they are excited at a certain wavelength, absorption is observed due to the dispersion of the radiation. That phenomenon, surface plasmon resonance (SPR), is caused by the coupling between the surface NPs electrons and the radiation.

In the case of gold nanoparticles, they have intrinsic optical properties due to its size and present a wide band of absorption at a certain wavelength (λ=520 nm) and turns the

solution from colourless to violet. These can be used as optical biosensor if absorbance is measured, or even as a colorimetric method due to the visual colour change.

An example of metallic nanoparticles used as biosensor for the determination of biogenic amines is developed by Shih-Yu Tseng et al.[7], whose have been working in a new method based on the embedding of metal nanoparticles onto flexible papers using reversal nanoimprint lithography. The NP-embedded papers can serve as gas sensors for the detection of volatile biogenic amines (BAs) released from spoiled food.

Focussing on the use of optical biosensors, the determination of the biogenic amines that this project is interested in, cadaverine (cad), putrescine (put) and especially histamine (his), can be easily done following its enzymatic reaction with diamine oxidase, DAO.

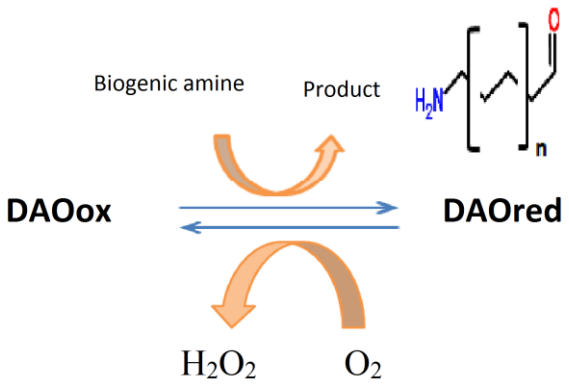

Figure 2. Scheme of the enzymatic reaction between DAO and several enzymatic substrates (put, cad).

For example, K.M.A. El-Nour at al.[8] have developed a new optical sensor for rapid screening with high sensitivity for the existence of biogenic amines in poultry meat samples. Gold nanoparticles (d=11-19 nm) function as a fast and sensitive biosensor for detection of histamine resulting from bacterial decarboxylation of histidine as a spoilage marker for stored poultry meat.

2.-HYPHOTESIS AND OBJECTIVES

Taking advantage of the redox process that is involved in an enzymatic reaction with oxidase-type enzymes, it is supposed that if this reaction takes place in the presence of a gold precursor, gold could be reduced forming gold nanoparticles (figure 3) and the concentration of the formed nanoparticles should be proportional to the concentration of analyte in the sample.

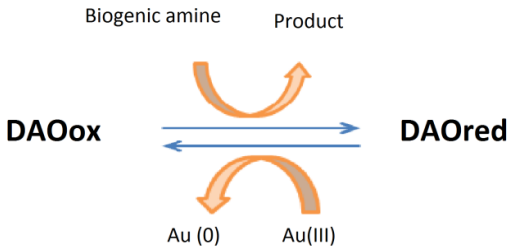

Figure 3. Enzymatic redox process where gold is involved.

Based on this hypothesis, this project is focussed on an "in situ" gold nanoparticles synthesis that takes advantage of the enzymatic reaction between DAO and an enzymatic substrate which can be putrescine and/or cadaverine (a biogenic amine, BA). This reaction can be followed by the spectroscopic properties of the metallic nanoparticles formed during the enzymatic reaction.

The reaction has to be understood, also the mechanism and the role of each compound that takes part in the synthesis. Conditions might be improved to obtain the maximum throughput, sensibility and selectivity.

Taking into account the final goal of this investigation line, synthesis has to be also studied, at least, at room temperature.

Real samples of decomposed food are very complex. Because of that, optimum synthesis conditions might be applied to the identification, detection, and if it is possible quantification, of the enzymatic substrates that are present in the samples.

The final purpose of this investigation line is an enzymatic optical biosensor based on this determination where gold nanoparticles are the indicators and the receptor is the enzyme to promote the enzymatic reaction.

3.-INSTRUMENTATION AND REAGENTS

3.1.-REAGENTS

- Di-Sodium Hydrogen Phosphate anhydrous (Na_2HPO_4, 141.96 g/mol) from Panreac.
- Gold Chloride hydrate (III) acid ($HAuCl_4$, 339.79 g/mol) from Sigma-Aldrich (27988-77-8).
- Diamine oxidase 89,5 IU/ml from Bioresearch (DAO-B14G02)
- Putrescine dihydrochloride (161.07 g/mol) from Sigma Aldrich (333-93-7).
- Cadaverine dihydrochloride (179.10 g/mol) from Sigma Aldrich (1476-39-7).
- Histamine dihydrochloride (184.07 g/mol) from Sigma Aldrich (56-92-8).
- 2-aminobenzaldehyde (121.14 g/mol) from Sigma-Aldrich (592-23-7).
- Real samples of fish in unhealthy conditions (obtained from the Aragon Public Health Service). They are composed by:
    - Putrescine: 5mg/l = $5.7 \cdot 10^{-5}$M
    - Cadaverine: 19 mg/l = $1.86 \cdot 10^{-4}$M
    - Histamine: 45 mg/l = $4.05 \cdot 10^{-4}$M
    - Tyramine: 15mg/l = $1.08 \cdot 10^{-4}$M
    - Phenylethylamine: 2.5mg/l = $2.07 \cdot 10^{-5}$M
    - Tryptamine: 1mg/l = $6.25 \cdot 10^{-6}$M

3.2.-INSTRUMENTATION

- UV-Vis spectrophotometer Agilent 9453A.
- UV-Vis spectrophotometer Analytikjena Specord 210.
- Transmission Electron Microscope, TEM, FEI Tecnai F30.
- Analytical scale balance A&D-GR-202.
- pH-Meter micropH from Crison.

3.3.-APPARATUS

- Incubator: Eppendorf thermomixer compact.
- Magnetic stirrer A-01 SBS.

- Micropipette of variable volume: Pipetman Gilson (2-20 µl, 50-200µl and 200-1000µl), Socorex (5-50µl, 10-100µl and 100-1000 µl) and Eppendorf (10-100 µl, 30-300µl and 100-1000µl)
- Centrifuge from Thermo Scientific MultifugeX1R.
- Centrifugal filters (50000 and 10000 Da) from Merck Millipore.

3.4.- METHODS

- Enzyme separation: DAO has pyridoxal on its natural structure. The solution that is used has extra-pyridoxal that disturbs measurements because its absorption is a wide peak that includes AuNPs wavelength (λ=520 nm). To separate the solution and obtain only the enzyme it is centrifuged (10 minutes, 4000 rpm) using centrifugal filters of 50000 Da: pyridoxal passes through meanwhile the enzyme remains in the upper part. After that, it is diluted with buffer (pH≈7) using the same volume as the initial to keep constant its concentration.

- UV-Vis spectrophotometer: The formation of gold nanoparticles is followed by absorption because they exhibit SPR phenomenon. Two different instruments are used: Spectrophotometer Agilent 9453A has only one path length of 1 cm where blank should be done before measuring samples, and UV-Vis spectrophotometer Analytikjena Specord 210 that has 8 path lengths of 1 cm to measure samples and another path length of the same size to measure the blank.

- STEM samples preparation: A drop of the sample with nanomaterials is dispersed in water onto a carbon-coated copper grid.

4.-RESULTS AND DISCUSSION

4.1.- PREVIOUS STUDIES

In previous studies, the enzymatic reaction between DAO and Put or Cad has been followed by the changes on the absorbance of DAO wavelength at λ=405 nm or λ=420 nm (figure 4a); however, the sensibility is not very high and the enzyme is very hard to be regenerated. The last problem can be solved using another enzyme, catalase, that allows its total regeneration (figure 4b); nevertheless, sensibility continue being a trouble.

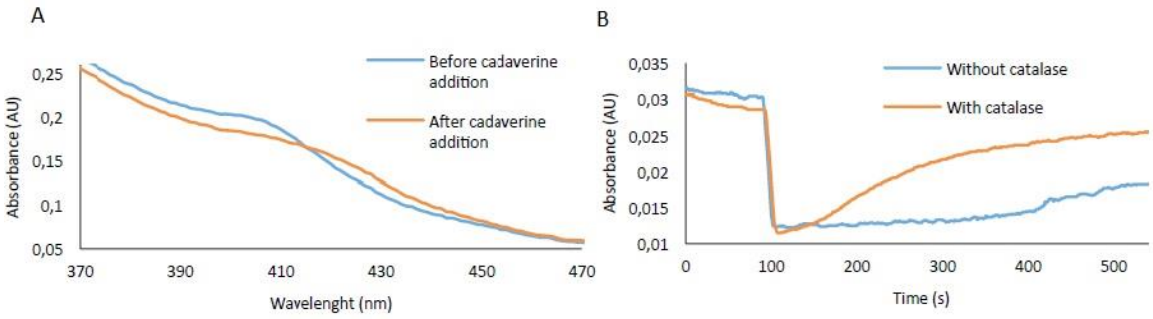

Figure 4. (A) DAO spectrum before and after adding Cad and (B) DAO regeneration with and without catalase[9].

In order to increase the sensibility, it has been coupled two a second enzymatic reaction in which H_2O_2 reacts in the presence of HRP (figure 5):

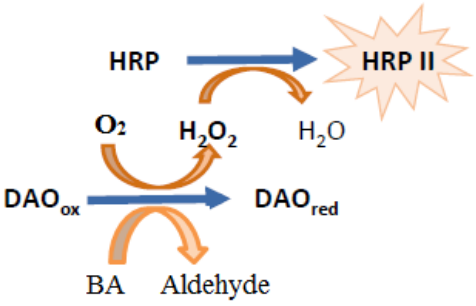

Figure 5. H_2O_2 is measured by a connected second reaction[10].

The reaction between H_2O_2 and the enzyme, HRP can be followed through the absorbance changes in the molecular absorption properties of HRP due to its different

oxidation states[11]. The problem in this case is that HRP is not very specific or selective; therefore signals can be altered by other compounds present in the sample.

A method to study this reaction is required to obtain results with more sensibility than in the case of using the molecular absorption properties of DAO and also with higher selectivity than in the case of using the optical properties of HRP. The proposal of this project is to study the enzymatic determination of BA based on the optical properties of the AuNPs generated during the DAO-BA enzymatic reaction.

This proposal is based on the previous results obtained in the case of the GOx-AuNps studies[12].

4.2.- STUDY OF THE REACTION AND MECHANISM

It is observed that the enzymatic reaction between BA and DAO provides the appropriate conditions to obtain AuNPs. Starting in the same conditions that were obtained in the GOx-AuNPs studies[12], the initial concentration of $HAuCl_4$ was $5.25 \cdot 10^{-4}$ M. The enzymatic substrate concentration might be higher than the concentration of dissolved oxygen ($2.2.10^{-}4$M) in solution, therefore $1 \cdot 10^{-3}$M is prepared. Taking into account the enzymatic reaction and the concentration of ES, enough concentration of the enzyme is required: 4 IU/ml.

Using those initial conditions the reaction is studied with putrescine and cadaverine as enzymatic substrates. The concentration of NPs can be related to the absorbance at λ=520 nm and depends on the enzymatic substrate's concentration.

- DAO: 4IU/ml
- $HAuCl_4$: $5.25 \cdot 10^{-4}$ M
- Cad: $1 \cdot 10^{-3}$ M
- Put: $1 \cdot 10^{-3}$M

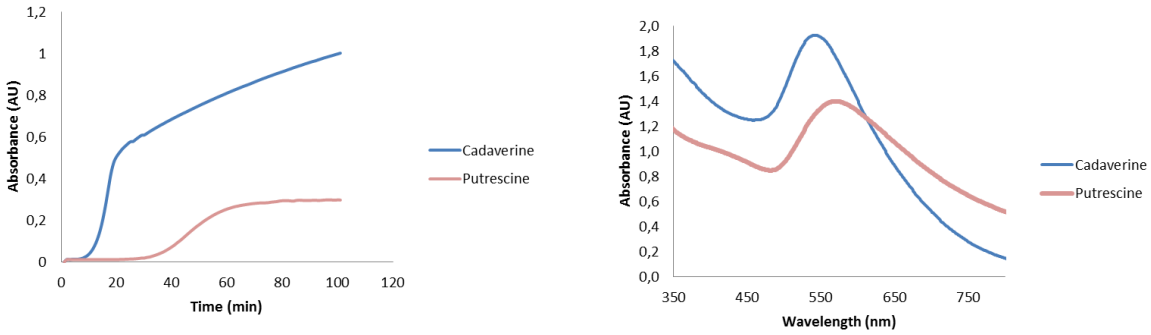

Figure 6. Formation of AuNPs using cadaverine $1 \cdot 10^{-3}$ M and putrescine $1 \cdot 10^{-3}$ M as enzymatic substrates (λ=520nm).

It can be observed in figure 6 that AuNPs are formed, and the reaction can be followed at gold nanoparticles wavelength, where the maximum absorbance appears. In addition both substrates provide different spectra:

- Cadaverine provides a higher final absorbance, therefore forms a higher amount of AuNPs.
- The molecular absorption spectrum of putrescine is broader than that of cadaverine one so, putrescine provides a higher polydispersity.
- AuNPs formation is quicker with cadaverine than with putrescine.

Until now, there was not possible the differentiation of cadaverine from putrescine with DAO, because both are lineal diamines that react in a very similar way. Yet, there are several differences in the formation of AuNPs depending on the biogenic amine that is used.

The key might be in the mechanism of the reaction. Taking into account that both products of the enzymatic reaction have the same functional groups and that they only differ in a carbon of the lineal chain, it can be due to the fact that the product turns into its cyclic form once it is formed (figure 7)[13].

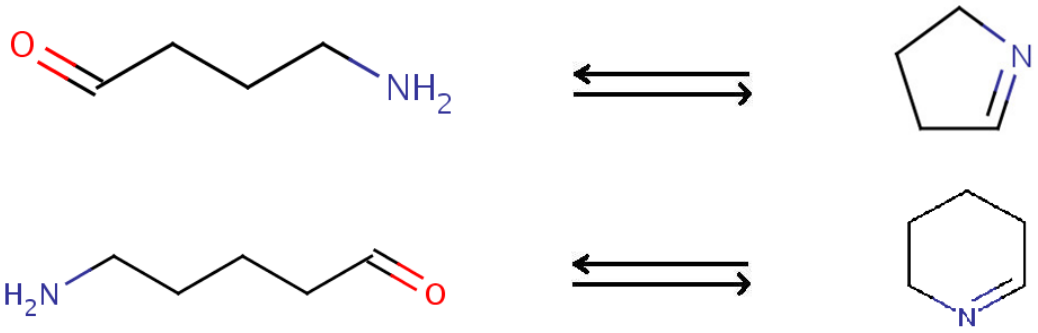

Figure 7. Cyclic form of the products of the enzymatic reaction.

There are some studies that verify the spontaneous cyclization of the aldehyde that comes from the enzymatic reaction, obtaining Δ-pyrroline from putrescine and Δ-piperideine from cadaverine. The formation of those compounds can be demonstrated using o-aminobenzaldehyde, which condenses with the cyclic azomethine to form yellow or orange, respectively, 1,2-dihydroquinazolinium derivatives[13].

Using this compound to verify the formation of the cycle, the following experiments are prepared. Final concentrations are enclosed in table 2:

Table 1. Final concentrations in cuvette.

DAO	OAB	Put	Cad	$HAuCl_4$
4 IU/ml	$2.5 \cdot 10^{-4}$M	-	$1 \cdot 10^{-3}$M	-
4 IU/ml	$2.5 \cdot 10^{-4}$M	-	$1 \cdot 10^{-3}$M	$5.25 \cdot 10^{-4}$M
4 IU/ml	$2.5 \cdot 10^{-4}$M	$1 \cdot 10^{-3}$M	-	-
4 IU/ml	$2.5 \cdot 10^{-4}$M	$1 \cdot 10^{-3}$M	-	$5.25 \cdot 10^{-4}$M

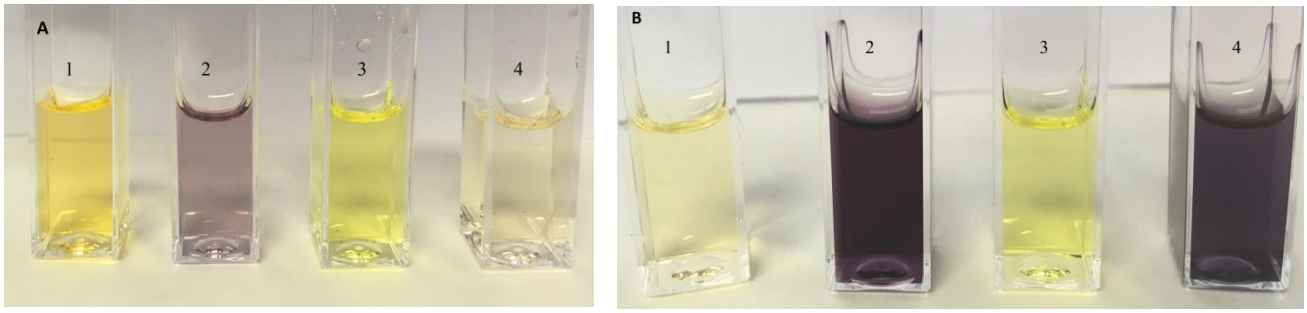

Figure 8. Experimets 1-4 (from the left to the right) (A) after 20 minutes and (B) after 24 hours.

To check if the cyclic compound is formed 1 and 3 are used, while 2 and 4 are used to verify if the cyclic compound is the reactant that allows the formation of AuNPs. First of all cuvettes are prepared without the gold precursor. Once yellow and orange are observed, $HAuCl_4$ is added in 2 and 4. In figure 8 cyclic forms are corroborated (orange(1) and yellow (3) colours are observed) and they are the precursors required for AuNPs formation (2 and 4). Cadaverine provides a cycle of 6 members while the one for putrescine has 5 members; it can be an explanation of the difference in reaction time, because ring tension is higher in the last one, taking more time for the formation of the cyclic form.

Also the role of the DAO obtaining AuNPs has to be studied. For that purpose the enzymatic reaction with cadaverine is separated using a centrifugal filter of 10000 Da: the upper part contains the enzyme, and the part that passes through the centrifugal filter contains only the product of the enzymatic reaction. After that, $HAuCl_4$ is added to the fraction that passes through to check if nanoparticles can be synthesized with the cyclic product and without the enzyme. At the same time the synthesis of AuNPs is prepared with the enzymatic reaction (DAO and cadaverine) to be able to compare the same reaction with and without the enzyme, and understand its role in the reaction.

It is observed that the reaction takes place in both fractions, even without the enzyme, so it is not necessary for the formation of AuNPs. However, if it is not present, AuNPs tend to aggregate and they precipitate (figure 9, annex 1); Therefore DAO is a stabilizer of the nanoparticles.

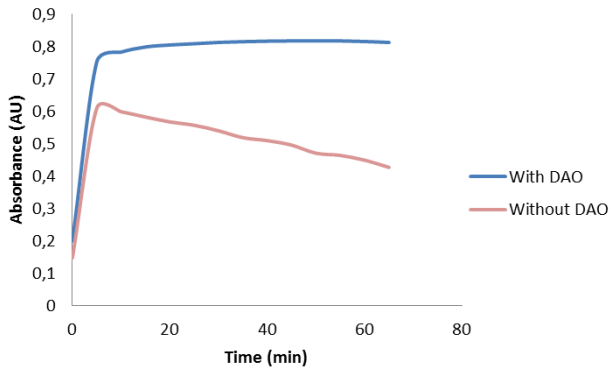

Figure 9. Formation of AuNPs with and without DAO.

In order to obtain the best results, parameters of the synthesis reaction have to be optimized. First of all the concentration of the reactants is studied at room temperature. After that, temperature of the reaction is also modified to decrease the reaction time by increasing the kinetics.

-ES (Cad, Put): Depending on the concentration of ES, the final amount of AuNPs variates. Both are directly proportional, therefore the higher is the first one, the higher amount of nanoparticles is obtained.

- Enzyme, DAO: The role of the enzyme is to produce the aldehyde that promotes the formation of AuNPs by the enzymatic reaction; and it works also as nanoparticle's stabilizer. Some experiments are prepared with different DAO concentrations, keeping constant the concentration of the other compounds that are involved in the synthesis: cadaverine concentration is $1 \cdot 10^{-4}$ M and gold precursor concentration is $5.25 \cdot 10^{-4}$ M.

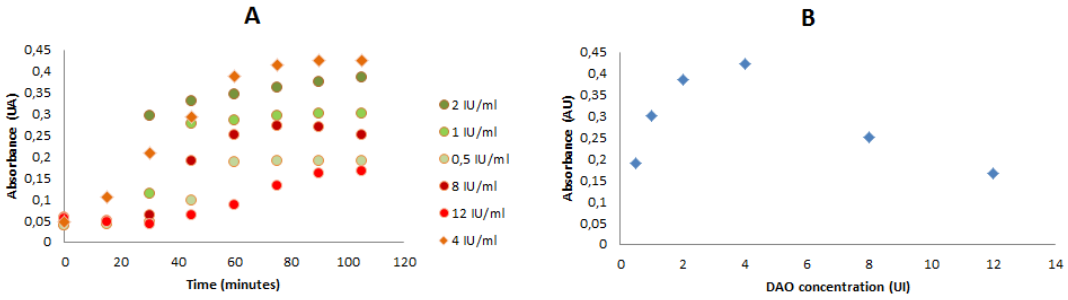

Figure 10. (A) Comparison of several DAO concentrations with time and (B) comparison of the absorbance after 110 minutes of reaction time (λ=520nm).

In figure 10A is represented the reaction evolution of the same experiment with different concentrations of enzyme where: in the red-scale circles are represented those concentrations higher than the one that was used until now (orange diamonds), and those that are lower are represented with the green-scale circles. It is observed that the highest absorbance is obtained with a DAO concentration of 4 UI/ml. This result is corroborated in figure 10B where the absorbance of all experiments at 110 minutes of reaction time is represented and, again, the best result is obtained for that enzyme concentration.

-Tetracholoauric acid, $HAuCl_4$: Some cuvettes are prepared with a cadaverine concentration of $1 \cdot 10^{-4}$M, DAO concentration of 4 UI/ml (optimized) and different concentrations of gold.

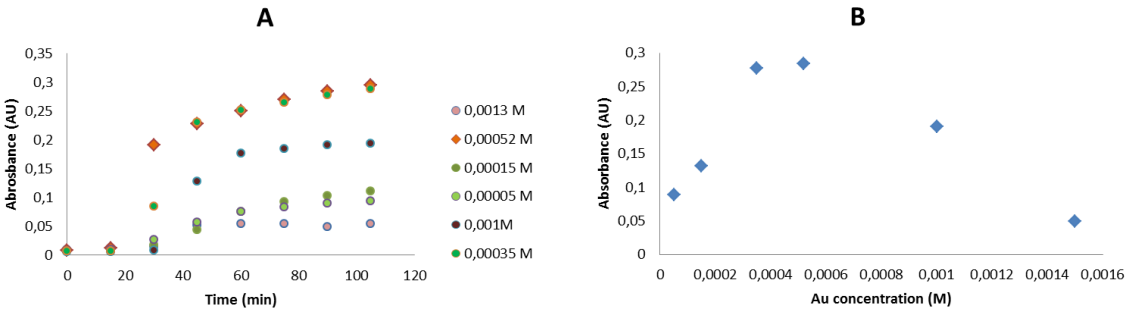

Figure 11. (A) Time representation of some experiments with different $HAuCl_4$ concentrations and (B) the absorbance representation of these experiments at 110 minutes.

Gold precursor results are represented in figure 11: A it is expressed in time where orange diamonds are the concentration of gold precursor that has been used until now; red-scale circles are higher concentrations while lowers are green-scale circles. B represents the absorbance of all experiment at a certain time (110 minutes). It is observed that the maximum is very similar for the two concentrations, but the one that achieves the higher absorbance quicker is $5.25 \cdot 10^{-4}$M.

-Temperature: Kinetic factor is very important because reaction time can hardly decrease by increasing the temperature, although the enzymatic denaturation has be considered. Therefore a compromise should be achieved in order to reach the best results without denaturizing the enzyme.

For that purpose the same experiment is repeated at different temperatures: 30ºC, 40ºC, 50ºC, 60ºC, 70ºC. Final concentrations are into brackets:

1870µl buffer+45µl DAO (4IU/ml) + 64µl Cad ($1 \cdot 10^{-4}$M) + 21µl $HAuCl_4$ ($5.25 \cdot 10^{-4}$M)

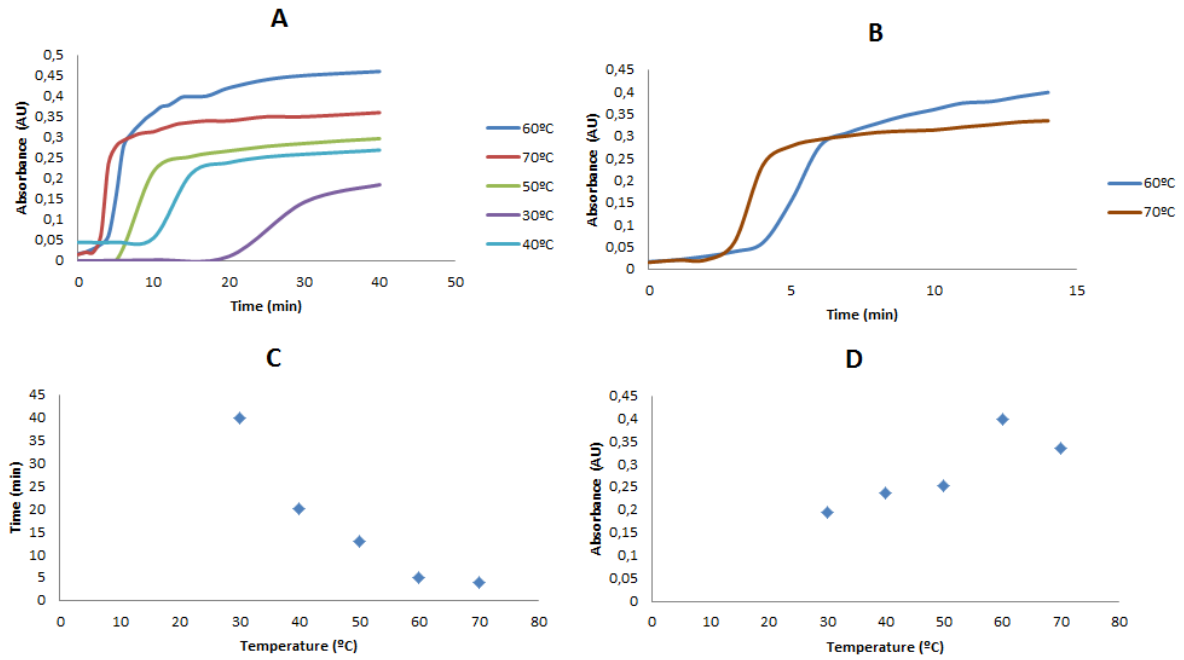

Figure 12. (A) Comparison of the same experiment at different temperatures, (B) comparison between the two highest temperatures, (C) comparison of reaction time and (D) comparison of the maximum absorbance reached.

Temperature study shows that, in general, increasing this parameter reaction time decreases while throughput increases (figure 12A). Reaction time depends exponentially on the temperature (figure 12C) meanwhile absorbance presents a maximum at 60 ºC (figure 12D) and at higher temperatures this parameter decreases. The two highest temperatures present a very similar reaction time and final absorbance because of that they are compared in figure 12B: it is observed that the difference on time is only 2 minutes, what can be assumed, while the difference on the other parameter is higher. Taking results of both parameters into account it is observed that throughput presents a maximum and then decreases; it can be due to the fact that enzymes have an optimum range of temperature to work, and out of that range it denatures and starts to decrease its activity. Therefore, 60 ºC can be considered as the optimum temperature for the method of AuNPs synthesis.

Once optimum conditions have been reached, a calibration is required in order to obtain the minimum enzymatic substrate concentration that the method is able to detect and quantify, and to obtain the linear range.

Some experiments are prepared covering a wide range of cadaverine concentrations, from $1 \cdot 10^{-3}$M to $3 \cdot 10^{-6}$M (figure 13). Blanks are also corroborated (annex 2).

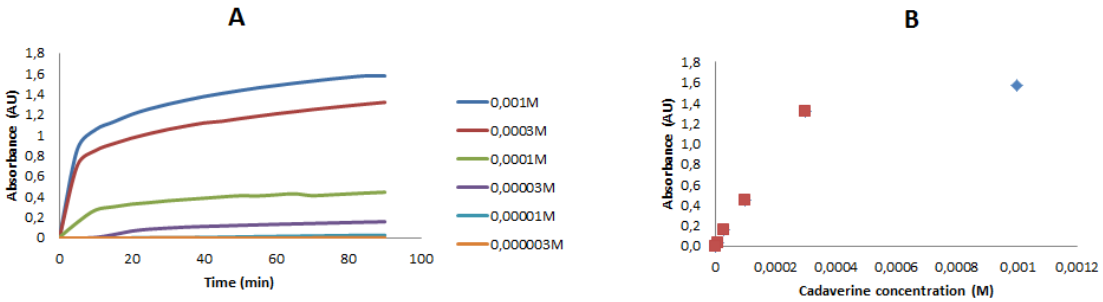

Figure 13. Cadaverine calibration curve at 60ºC (A) evolution of absorbance during an hour and (B) absorbance of each concentration when reaction time is one hour (λ=520nm).

The lowest concentration of cadaverine is not detected, and the highest is out of the linear range, therefore the calibration curve covers from $3 \cdot 10^{-4}$M to $1 \cdot 10^{-5}$M (red squares) with a determination coefficient of 0.9991.

Now the same cuvettes are prepared with putrescine however, due to the fact that kinetic for that enzymatic substrate is slower, the lowest concentration is not prepared (figure 14). Blanks are also corroborated (annex 2).

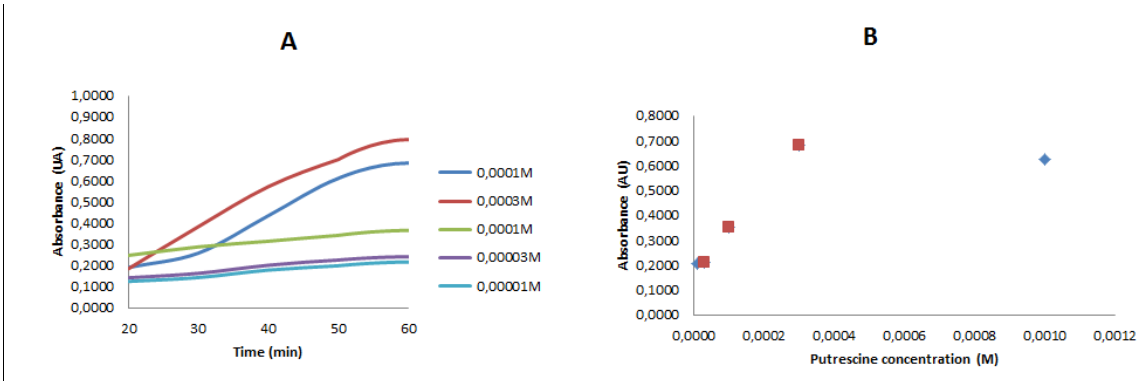

Figure 14. Putrescine calibration curve at 60ºC (A) evolution of absorbance during an hour and (B) absorbance of each concentration when reaction time is one hour (λ=520nm).

The lowest concentration is not detected, and the higher is out of the linear range, therefore the calibration curve covers from $3 \cdot 10^{-4}$M to $3 \cdot 10^{-5}$M (red squares) with a determination coefficient of 0.9974. In this case linear range and final absorbance are smaller than in the case of cadaverine, while reaction time is much higher.

At this point it is important to point out that it is observed a difference both in reaction time and in the final absorbance between concentrations that are above oxygen concentration ($2.2 \cdot 10^{-4}$M) and those that are under this concentration. It can be due to the fact that oxygen plays an important role in the enzymatic reaction, because when it is consumed DAO on its reduced form predominates until oxygen is trapped from the aire and regeneration starts. In putrescine calibration curve the difference is obviously because only concentrations above oxygen provide nanoparticles.

The final purpose of this study line is the fabrication of a biosensor based on gold nanoparticles for the determination of biogenic amines in decomposed food, which is stored in the fridge or even at room temperature, but never at 60ºC. Because of that the same calibration curves are obtained at room temperature.

At 25ºC putrescine is the first, to check if decreasing temperature, although kinetic factor is not available and reaction time increases, it allows the detection of lower concentrations (figure 15):

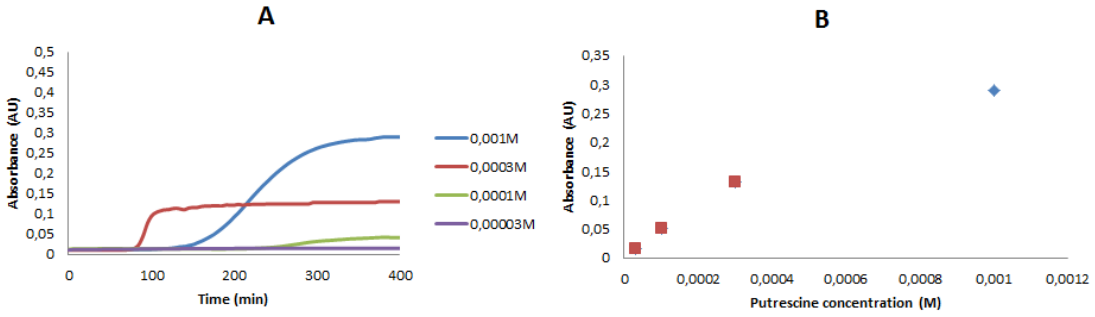

Figure 15. Putrescine calibration curve at room temperature (A) evolution of absorbance during an hour and (B) absorbance of each concentration when reaction time is two hours (λ=520nm).

Putrescine is again only detected at concentrations above $2.2 \cdot 10^{-4}$M. The biosensor could not work only detecting that BA because a very high concentration is required to be able to detect that amine.

Cadaverine experiments are prepared until a concentration of $3 \cdot 10^{-5}$M (figure 16):

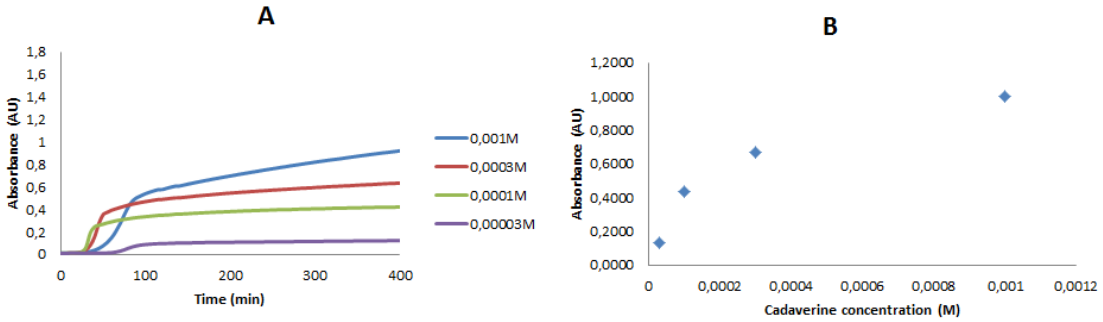

Figure 16. Cadaverine calibration curve at room temperature (A) evolution of absorbance during an hour and (B) absorbance of each concentration when reaction time is two hours (λ=520nm).

For that biogenic amine, sensibility does not differ with temperature meanwhile for putrescine this parameter decreases with temperature. It can be due to ring tension, which is very high for the last one, and this barrier can only be jumped with an energy contribution, heat in this case. These conclusions again corroborate the cyclic form of enzymatic reaction's product as promoter of AuNPs synthesis.

Taking into account that oxygen concentration affects too much to the reaction, spectra that are obtained from a concentration above and under $2.2 \cdot 10^{-4}$M are analysed for both amines:

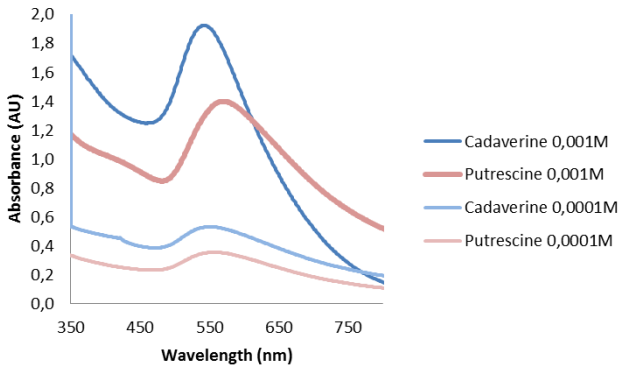

Figure 17. Spectro of concentrations above and under $2.2 \cdot 10^{-4}$M of putrescine and cadaverine.

It is observed in figure 17 that oxygen affects not only at kinetic of the reaction, but also to the dispersity, amount and size of the nanoparticles. For those concentrations that are above $2.2 \cdot 10^{-4}$M (DAOred predominates) there is a very well defined peak and it can be observed that the final absorbance in much higher than those samples with a concentration of $1 \cdot 10^{-4}$M. To check size and dispersity of nanoparticles STEM images are shown in figure 18 and table 2 summarizes it:

Table 2. Gold nanoparticles size depending on enzymatic substrate's nature and its concentration.

Enzymatic substrate	Concentration	AuNPs size (nm)	Dispersion of the sample	Size of aggregations	Size of NPs aggregated
Cadaverine	$1 \cdot 10^{-3}$M	11-20	Polydispersed	70 nm	13 nm
Cadaverine	$1 \cdot 10^{-4}$M	3 (NCs)	Monodispersed	-	-
Putrescine	$1 \cdot 10^{-3}$M	30-40	Polydispersed	80 nm	10 nm
Putrescine	$1 \cdot 10^{-4}$M	<1 (NCs)	Monodispersed	20 nm	1 nm

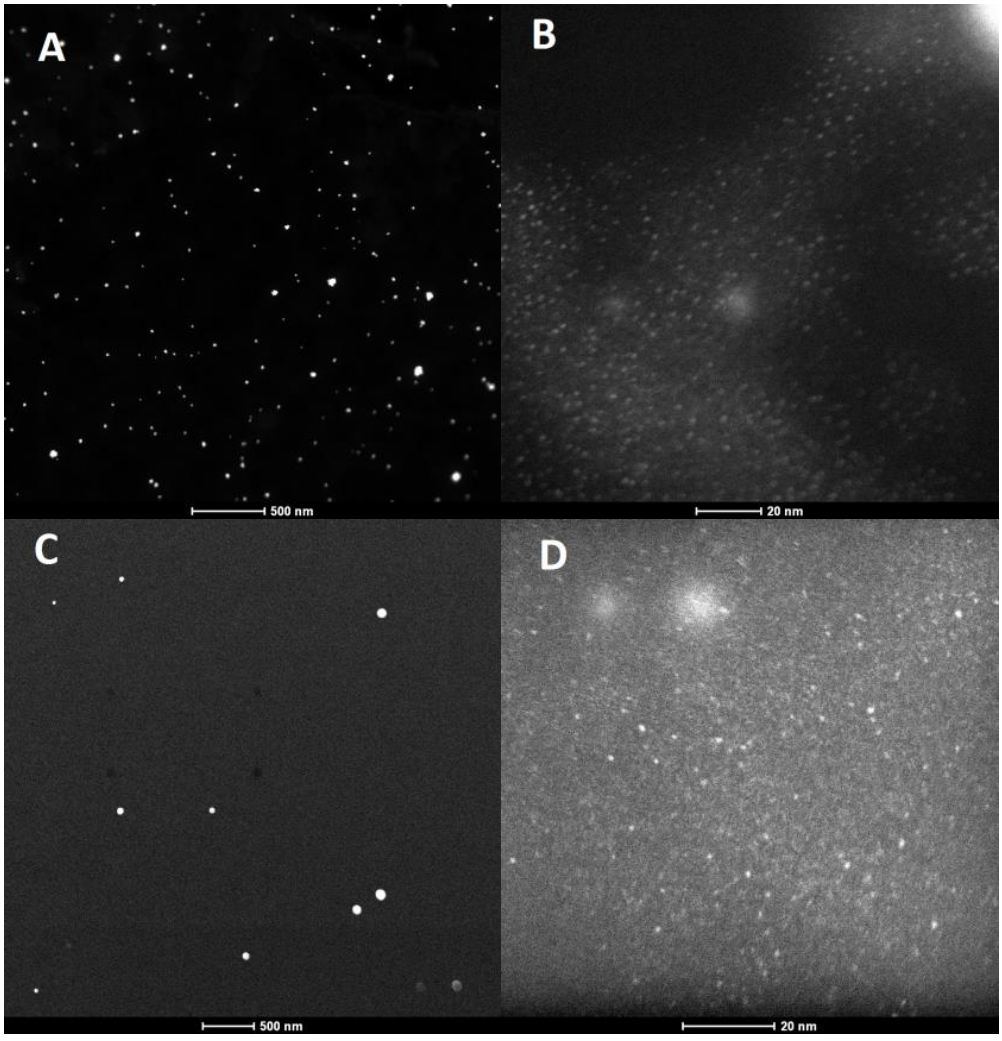

Figure 18.STEM images using as enzymatic substrates: (A) cadaverine $1 \cdot 10^{-3}$ M (500 nm), (B) cadaverine $1 \cdot 10^{-4}$ M (20nm), (C) putrescine $1 \cdot 10^{-3}$ M (500nm) and (D) putrescine $1 \cdot 10^{-4}$ M (20nm).

STEM images in figure 18 demonstrate the importance of oxygen and the role of DAOred as nanoparticle's stabilizer. Samples with an ES concentration lower than $2.2 \cdot 10^{-4}$M present monodispersity and nanoclusters, NCs (particle's size lower than 3 nm); it justifies the absence of absorbance, because NCs are supposed to be characterized by its intrinsic fluorescence. On the other hand those samples with a higher concentration than oxygen are polydispersed and NPs are bigger. It is also corroborated that cadaverine provides a higher concentration of nanoparticles than putrescine; it can be due to the nanoparticle's synthesis kinetics, because the last one is the slowest.

To try to increase the throughput, same experiments are studied using OAB. Theoretically this compound forms a complex with the cyclic form of the aldehyde, which is the product of the enzymatic reaction. Therefore, if the whole aldehyde is on that form, throughput of nanoparticles synthesis might increase. To verify if this hypothesis is correct, the following cuvettes (table 4) are prepared at 60ºC:

Table 3.Composition of cuvettes to corroborate if OAB increases throughput and decreases the detection limit.

Cuvette	DAO	OAB	Cad	$HAuCl_4$
1	4IU/ml	$2{,}5 \cdot 10^{-4}$M	$1 \cdot 10^{-4}$M	$5.25 \cdot 10^{-4}$ M
2	4IU/ml	-	$1 \cdot 10^{-4}$M	$5.25 \cdot 10^{-4}$ M
3	4IU/ml	$2{,}5 \cdot 10^{-4}$M	$1 \cdot 10^{-5}$M	$5.25 \cdot 10^{-4}$ M
4	4IU/ml	-	$1 \cdot 10^{-5}$M	$5.25 \cdot 10^{-4}$ M

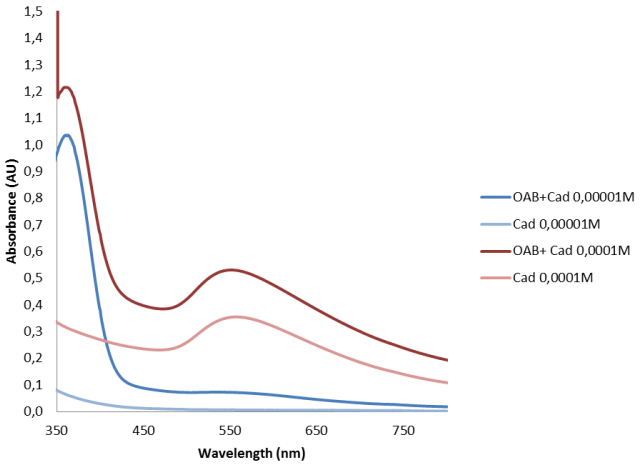

Figure 19. Comparison of results obtained with and without OAB.

It is corroborated in figure 19 that OAB increases the throughput of the synthesis reaction because increases the amount of aldehyde on its cyclic form; therefore the hypothesis of this form as precursor of AuNPs, grows stronger again. In addition, the lower concentration that could be detected with o-aminobenzaldehyde decreases ($1 \cdot 10^{-5}$ M) although the detection limit is not lower.

4.5.- ANALYSIS OF SYNTHETIC AND REAL SAMPLES

Real decomposed samples are not characterized by the formation of only cadaverine and putrescine. There are more compounds that are formed when food rots, an example of a fish contains (table 5):

Table 4. Composition in biogenic amines of a decomposed fish.

Compound	Concentration (M)
Putrescine	$5.7 \cdot 10^{-5}$
Cadaverine	$1.86 \cdot 10^{-4}$
Histamine	$4.05 \cdot 10^{-4}$
Tyramine	$1.08 \cdot 10^{-4}$
Phenylethylamine	$2.07 \cdot 10^{-5}$
Tryptamine	$6.25 \cdot 10^{-6}$

Before trying to analyse a real sample, some synthetic mixtures are prepared in order to predict results when more than one enzymatic substrate is involved in the reaction. First of all putrescine and cadaverine, that has been characterized and quantified along this project, are mixed. To achieve this goal the same cuvettes are prepared with a final volume of 2 ml using optimum concentrations of DAO and $HAuCl_4$, a constant concentration of cadaverine, $1 \cdot 10^{-3}$M, $1 \cdot 10^{-4}$M and $3 \cdot 10^{-5}$M, and different concentrations of putrescine that are enclosed in table 6:

Table 5. Cuvette concentrations mixing putrescine and cadaverine.

Cad concentration (M)	Put concentration (M)	Cad concentration (M)	Put concentration (M)	Cad concentration (M)	Put concentration (M)
$1 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	$1 \cdot 10^{-4}$	$1 \cdot 10^{-3}$	$3 \cdot 10^{-5}$	$1 \cdot 10^{-3}$
$1 \cdot 10^{-3}$	$3 \cdot 10^{-4}$	$1 \cdot 10^{-4}$	$3 \cdot 10^{-4}$	$3 \cdot 10^{-5}$	$3 \cdot 10^{-4}$
$1 \cdot 10^{-3}$	$1 \cdot 10^{-4}$	$1 \cdot 10^{-4}$	$1 \cdot 10^{-4}$	$3 \cdot 10^{-5}$	$1 \cdot 10^{-4}$
$1 \cdot 10^{-3}$	$3 \cdot 10^{-5}$	$1 \cdot 10^{-4}$	$3 \cdot 10^{-5}$	$3 \cdot 10^{-5}$	$3 \cdot 10^{-5}$

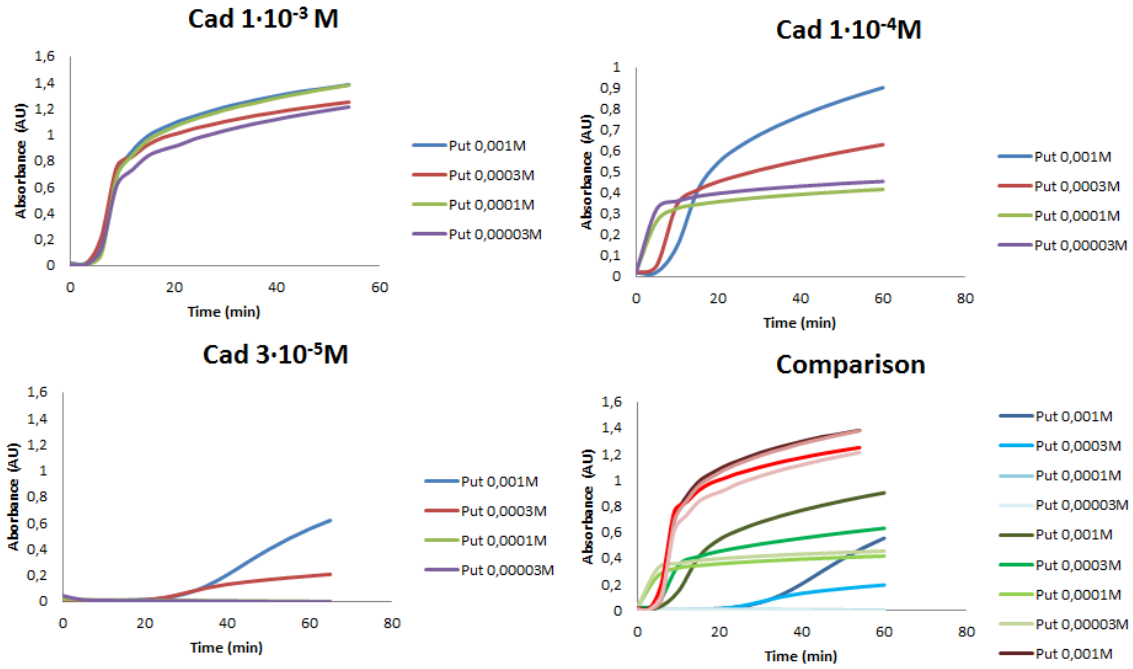

Figure 20. Experimental results of table 6 (λ=520nm).

Different concentrations of cadaverine provide different reaction time and initial ratio of change of absorbance (figure 20). When the concentration of cadaverine is constant, depending on the concentration of putrescine, differences are also observed. To try to be able to quantify those changes, the variation of absorbance in a certain time is calculated in both cases (figure 21), to try to fit it with a concentration of each enzymatic substrate.

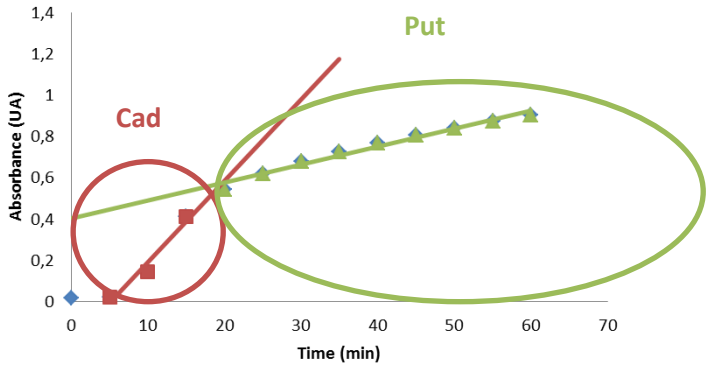

Figure 211. Variation of absorbance in a certain time for both biogenic amines (λ=520nm).

Cadaverine's absorbance change at different concentrations is studied from the beginning to 15 minutes (table 7):

Table 6. Study of cadaverine's absorbance change in mixtures with putrescine.

	Cad: 0.001M	Cad 0.0001M	Cad 0.00003M
[Put]	Δabs/Δt (0-15 min)	Δabs/Δt (0-15 min)	Δabs/Δt (0-15 min)
0 M	0.0766	0.0246	0.00033
0.001M	0.0647	0.0263	-0.00026
0.0003M	0.0606	0.0263	-0.00026
0.0001M	0.0634	0.0215	-0.00026
0.00003M	0.0553	0.0244	-0.00026
Average	**0.0610**	**0.0246**	**-0.00026**
Deviation	0.0042	0.0023	0
DSR(%)	**6.8127**	**9.1456**	**0**

The maximum deviation (DSR) is around 10%. The determination of cadaverine in presence of putrescine can only be done in a ratio 1:1 to 1:10 between both biogenic amines.

The same study is done for putrescine, but in this case the range of time that is used goes from 15 to 60 minutes (table 8):

Table 7. Study of putrescine's absorbance change in mixtures with cadaverine.

	Put: 0.001M	Put: 0.0003M	Put: 0.0001M	Put: 0.00003M
[Cad]	Δabs/Δt (15-60 min)	Δabs/Δt (15-60 min)	Δabs/Δt (15-60 min)	Δabs/Δt (15-60 min)
0.001M	0.009808	0.008185	0.010518	0.009365
0.0001M	0.010882	0.004822	0.001591	0.001587
0.00003M	0.012116	0.194909	-0.00023	-0.00024
Average	**0.010935**	**0.069305**	**0.003958**	**0.003572**
Deviation	0.001155	0.108789	0.005753	0.005099
DSR(%)	**10.56174**	**156.9703**	**145.3382**	**142.7453**

Putrescine study give not as successful as cadaverine results. Deviation is very high, it cannot be assumed; therefore putrescine concentration cannot be predicted in a mixture of several biogenic amines, may be due to the very low rate of the reaction at room temperature.

The other simple biogenic amine that can be found in real samples is histamine. It is formed by a cycle of 5 members (two of them nitrogen) and a lineal chain that ends with an amine group as it can be observed in figure 22.

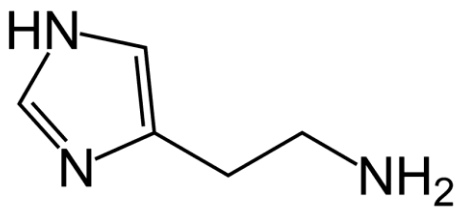

Figure 22. Histamine.

Synthetic samples with histamine, cadaverine and putrescine are prepared and turbidity is observed when gold is added. After 24 hours a precipitate is observed.

Due to the fact that the only one that has not been studied for the formation of gold nanoparticles is histamine, this reaction might be due to that amine. The enzymatic reaction between DAO and histamine has also been hardly studied, and turbidity has never been observed, therefore it might be due to the interaction between gold and the amine.

To confirm this interaction, there are proposed the following approaches:

– Due to the interaction between gold and histamine, there should not be enough "free" gold to be able to interact with the other amine, therefore throughput of the formation of gold nanoparticles decreases.
  It could be solved adding more amount of the gold precursor.

To check this possible solution, some experiments are prepared with the same amount of biogenic amines trying to simulate real sample concentrations and different concentrations of gold precursor (table 9 and figure 23):

Table 8. Cuvette concentrations.

Cuvette	DAO	Cad	Put	Hist	$HAuCl_4$
1	4IU/ml	$1\cdot10^{-4}$M	$3\cdot10^{-5}$M	$4.05\cdot10^{-4}$M	$1.05\cdot10^{-3}$M
2	4IU/ml	$1\cdot10^{-4}$M	$3\cdot10^{-5}$M	$4.05\cdot10^{-4}$M	$1.58\cdot10^{-3}$M
3	4IU/ml	$1\cdot10^{-4}$M	$3\cdot10^{-5}$M	$4.05\cdot10^{-4}$M	$2.1\cdot10^{-3}$M

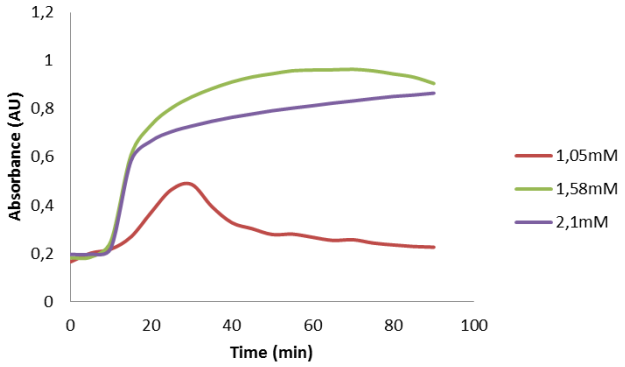

Figure 23. Comparison of gold concentrations (λ=520nm).

In a study at the beginning of this project it was observed that there was a maximum of absorbance at a certain gold precursor concentration. Now there are more biogenic amines present in the sample, therefore $HAuCl_4$ concentration required is different but its behaviour should be similar, a maximum might be achieved. In figure 23 it is verified and the highest absorbance is reached at a concentration of $1.58 \cdot 10^{-3}$M.

– Formation of gold nanoparticles is followed by absorbance and turbidity is observed immediately so, the initial signal is not zero because it provides an interference. If the signal due to the other amines is not very high, it cannot be observed.

  It could be solved by a centrifugation step that separates the precipitated gold-histamine, and then an extra concentration of gold precursor is added to react with the other amines.

To corroborate this approach, it is tested the formation of AuNPs in real samples with and without the centrifugation step and it is observed that this pre-treatment of the sample avoids the interference of this precipitated.

After that, to be able to compare results from both approaches, a real sample is separated in two fractions (table 10) in order to compare the efficiency of both methods in exactly the same sample.

Table 9. Cuvettes composition to check both possibilities.

Cuvette	DAO	$HAuCl_4$	Centrifuge
1 (1st possibility)	4 IU/ml	$1.58 \cdot 10^{-3}$M	No
2 (2nd possibility)	4 IU/ml	$1.05 \cdot 10^{-3}$M	Yes

Cuvette that is centrifuged requires an extra addition of gold precursor after this treatment. The quantity is the same as it is added at the beginning. Therefore, the total amount of $HAuCl_4$ added to the sample is twice the optimum gold precursor concentration obtained in 4.3.: the first addition promotes the precipitation of the complex gold-histamine, and the second one promotes the formation of AuNPs. Results are represented in figure 24.

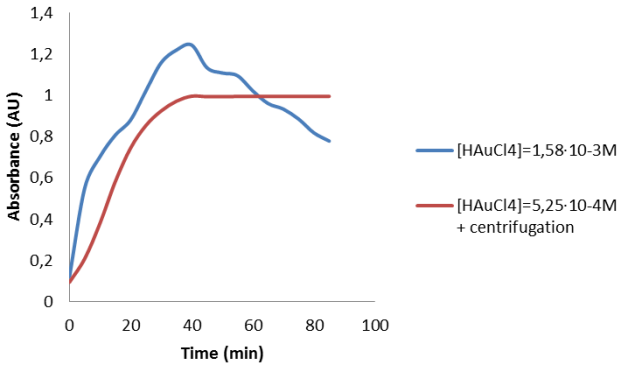

Figure 24. Real samples with two possible treatments to avoid histamine perturbations (λ=520nm).

The first situation, adding more gold precursor, provides a quicker signal but it is not very stable. It can be due to the fact that histamine is still being present; therefore it can interact with gold once absorbance has started to increase.

The second situation proposes a pre-treatment of the sample, a centrifugation to eliminate all the complex histamine-gold. If it can be applied it provides very stable signals yet, samples have to be able to suffer that pre-treatment. If the final biosensor is implemented in a food contained, it cannot be applied or a previous step might be implemented to eliminate the histamine.

Putrescine and cadaverine provide different AuNPs depending on its concentration during the enzymatic reaction. If it is lower than $2.2 \cdot 10^{-4}$M, nanoclusters are obtained instead of nanoparticles due to its size (≤ 3nm). NCs are characterized by its intrinsic fluorescence but it has not been corroborated and those nanoclusters have not been characterized in this study and can be done in a future investigation.

The determination of both amines at the same time has not been totally successful because only cadaverine can be quantified. A possibility is to measure areas instead of slopes. The total area can be measured and the concentration of putrescine can be obtained by the difference between the total are and that due to cadaverine (which can be obtained by the area during the first 15 minutes of reaction).

Taking into account that the final purpose of this investigation line it is to fabricate a biosensor implemented in food package, the gold precursor and the enzyme have to be immobilized in a solid support and have to be able to work at low temperature (4-6ºC at least).

The enzymatic reaction between the enzyme (DAO) and the enzymatic substrate (putrescine and/or cadaverine) forms an amine-aldehyde that suffers a cyclization in a spontaneous manner. That product is the precursor for the formation of gold nanoparticles. The enzyme promotes the enzymatic reaction and also plays a role as stabilizer of the nanoparticles.

Optimum conditions for the synthesis of gold nanoparticles are enclosed in table 11:

Table 10. Optimum parameters for AuNPs synthesis.

Factor to optimize	Value
DAO	4 UI/ml
$HAuCl_4$	$5.2 \cdot 10^{-4}$M
Temperature	60ºC

Calibration curves (table 12) are obtained for both enzymatic substrates at the optimum temperature, but also at room temperature because the final purpose of this study line is to fabricate a biosensor that works at low temperature.

Table 11. Parameters of cadaverine and putrescine calibration curves.

Enzymatic substrate	Temperature	Linear range (M)	R^2
Cadaverine	60ºC	$3 \cdot 10^{-4}$-$1 \cdot 10^{-5}$	0.9991
Cadaverine	RT	$1 \cdot 10^{-4}$-$3 \cdot 10^{-5}$	-
Putrescine	60ºC	$3 \cdot 10^{-4}$-$3 \cdot 10^{-5}$	0.9974
Putrescine	RT	$< 2 \cdot 10^{-4}$	-

Enzymatic substrate concentrations that are above oxygen concentration provide polydispersed nanoparticles meanwhile those that are under this concentrations are monodispersed nanoclusters. Focusing on nanoparticles concentration, cadaverine samples are more concentrated (table 13).

Table 12. Characterization of AuNPs.

Enzymatic substrate	Concentration	AuNPs size (nm)	Dispersion of the sample
Cadaverine	$1 \cdot 10^{-3}$M	11-20	Polydispersed
Cadaverine	$1 \cdot 10^{-4}$M	3	Monodispersed

Putrescine	$1 \cdot 10^{-3}$M	30-40	Polydispersed
Putrescine	$1 \cdot 10^{-4}$M	<1	Monodispersed

O-aminobenzaldehyde increases the synthesis of the nanoparticles because it keeps the product of the enzymatic reaction on its cyclic form. This compound cannot decrease the detection limit.

Real samples have more than one enzymatic substrate: cadaverine, putrescine, histamine, tryptamine, tyramine and phenylethylamine; therefore signals cannot be predicted with individual calibration curves.

In synthetic mixtures with cadaverine and putrescine, the increase in absorbance during the first minutes can be attributed to cadaverine and can be predicted. After that, the slope is due to the other biogenic amine, but it cannot be quantified.

When histamine is added to the mixture, turbidity is observed due to its interaction with $HAuCl_4$. To solve this problem there are two possibilities:

-To increase the gold precursor concentration: provides not stable signals, but it can be the solution for a biosensor where everything except the enzymatic substrate is immobilized.

-To centrifuge: provides stable signals, this is a pre-treatment that can only be applied if the sample can be manipulated before interacting in the biosensor.

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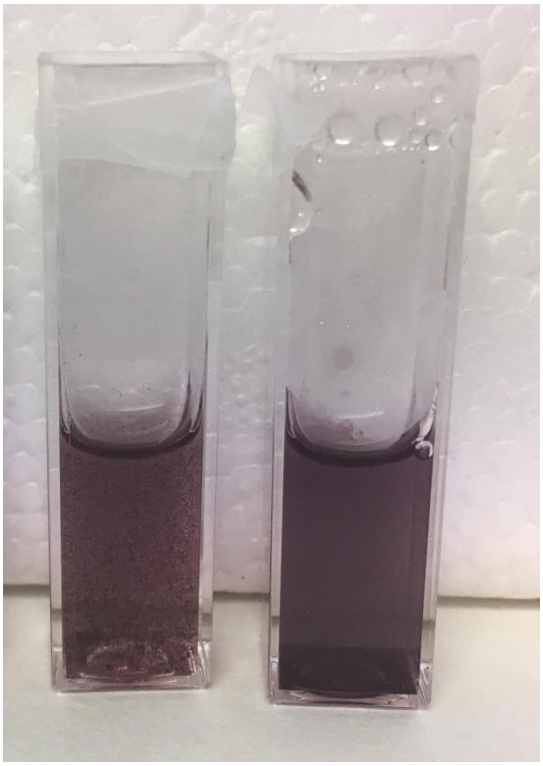
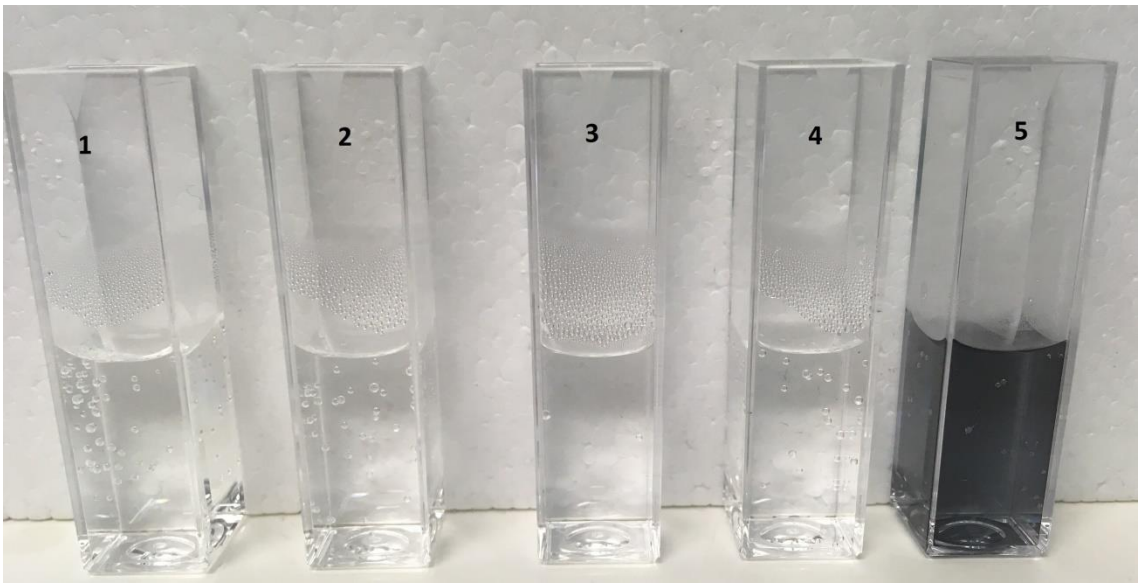
8.-ANNEXS

Annex 1: On the left side the formation of AuNPs without DAO, there can be seen aggregations and turbidity meanwhile on the right side, in presence of DAO, there are stabilized NPs because there is no turbidity.



Annex 2: Due to the fact that optimum conditions are at 60ºC, blanks have to be corroborated to check that the kinetic factor does not promote secondary reactions.



Cuvettes 1 (without DAO) and 2 (without ES) are blanks of cadaverine, 3 (without DAO) and 4 (without ES) are blanks of putrescine, and 5 is the reaction to be able to compare.