Scientific report

regarding the project implementation in the period January 2012 - December 2013

During **2012** were carried out the activities included the work plan for the achievement of general objective of the project, an electronic system based on chemical sensors and biosensors for the analysis of biogenic amines. In the following pages will be described the activities carried out, objectives achieved and dissemination activities carried out in this first year of the project.

1. Modeling and simulating studies of interaction between biogenic amines and receptors of sensors and biosensors were carried out by using HyperChem and Matlab software, respectively. From the modeling studies was determined the mechanism of interaction between biogenic amines and sensitive compounds, in the case of sensors and enzymes from the receptor element, in the case of biosensors.

Amine oxidases interact with biogenic amines by means of metal ions located in the active center of the enzyme cleaving amino groups. From the enzymatic reaction results a carbonyl compound, ammonia and H_2O_2. Monoamine oxidases and diamine oxidases have a high selectivity, this fact being related to the nature and chemical structure of the active center and to conformation of the protein chain.

When the enzyme is putrescine oxidase, the biocatalytic reaction takes place by the interaction of the amino group of putrescine and the active center of the enzyme results an aldehyde-amine compound, ammonia and H_2O_2. H_2O_2 is detected by electrochemical oxidation on the surface of the biosensor by applying a suitable potential, which depends on the nature of the electrode and the presence of electron mediators.

In the case of horseradish peroxidase, an enzyme which have the active center outside of the protein molecule, the interaction does not occur directly with the amine molecule but with H2O2 generated from the action of an amine oxidase (e.g. diamine oxidase) by means of the Fe (III) ion located in the active center of the enzyme. Therefore, the peroxidase can be used for the construction of bi-enzyme biosensors containing an amine oxidase and peroxidase.

Tyrosinase can be used as a biocatalyst for the detection of biogenic amines containing phenolic groups in the molecule. The mechanism of interaction is different for monophenols (e.g. tyramine) and diphenols (e.g. dopamine). Interaction occurs between phenolic group from the molecule and the active center of the enzyme that contains two Cu ions. The catalytic reaction occurs in two steps; in the first stage take place the hydroxylation the ortho position (for monophenols) followed by oxidation of diphenols to o-quinone. Ortho-quinone is reduced electrochemically at a potential that depends on the nature of the electrode and of electron transfer mediators.

In all cases studied by modeling, the biosensor detection mechanism is governed by the transfer of electrons (the slowest step) and applying a potential leads to an acceleration of the reaction due to electrochemical transformation of reaction products resulting from the enzymatic reaction.

The simulations using Matlab software were performed considering that the electrochemical biosensor has a flat geometry, the enzyme layer is deposited on the surface and then covered with a semi permeable membrane to ions, reagents and reaction products. It was determined the effect of pH and temperature on the biosensor response. On the other hand, the influence of limiting factor on reaction rate, diffusion and transfer of electrons, on the range of linearity (linear dependence of biosensor response and analyte concentration) and response time of the biosensor. It has been shown that diffusion has a huge influence on the range of linearity (variations of the order of magnitude) and a lower influence on the biosensor response. If the rate limiting step is the transfer of electrons influence the linearity range is reduced while response times vary significantly.

From the results presented here it can be concluded that in these studies was determined the mechanism of interaction between biogenic amines and receiver element of sensor or biosensor by modeling and simulation. In addition, the influence of the factor limiting of reaction rate, pH and temperature on

linearity and time domain response of the biosensor during amperometric or potentiometric measurements.

2. Selection of sensors and biosensors design

It was carried out a market study in order to choose among commercially available electrodes that are optimal for the purpose of this project. Thus, for small amounts of sample was choose the screen-printed sensors based on different materials from Dropsens. For larger amounts of sample were acquired carbon electrodes in the form of wire. Also, it were designed and constructed novel sensor designs. Furthermore, it were constructed electrodes in the form of platinum disk, carbon paste electrodes, ITO electrodes, screen-printed electrodes from Au in the form of sensor arrays, all of them with adequate size and low cost. In the case of carbon paste electrodes was changed the chemical composition of carbon paste by using different materials based on carbon (graphite, carbon nanoparticles, carbon nanotubes, carbon nanofibres) and different electroactive materials (Lu, Gd and Dy bisphthalocyanines, Co-phthalocyanine, Fe-phthalocyanine, di-Litium phthalocyanine, ferrocene) which are sensitive to biogenic amines and can be electron transfer mediators in the case of biosensors. From exploratory measurements using potentiometric and voltammetric techniques it was established that voltammetric methods are more appropriate because these present higher sensitivities. Equilibrium potential of sensors and biosensors in the analyzed sample will be used to establish correlations with other physico-chemical parameters of sample. It was established that the study of the electrochemical behavior of sensors and biosensors will be carried out using cyclic voltammetry. For increasing of sensitivity and in peaks resolution will be used the square wave voltammetry. For routine measurements will be used chronoamperometry applying optimum potential for oxidation or reduction of target compound or a product obtained from the enzymatic reaction.

In conclusion, it was chose and build appropriate designs of sensors and biosensors, the system can be adapted depending on the amount of sample available and on the physico-chemical properties of these.

3. Improvement of sensors qualities and design of novel sensors and biosensors

The activities carried out were aimed to develop the design of novel sensors and biosensors by chemical and biochemical modification of commercial electrodes or electrodes build in the laboratory. Modifier materials were purchased after a rigorous market study. Some of these commercial materials (Co-phthalocyanine, pyrrole, aniline, etc.) were purified by recrystallization or distillation. For electrochemical or chemical synthesis of other sensitive materials were purchased reagents and solvents required. As in the case of commercial materials it was necessary an advanced purification because the presence of impurities in the sensitive material can decisively influence the sensors or biosensors characteristics.

3.1. Synthesis and characterization of molecular materials

For the construction of sensors and biosensors are required sensitive material with adequate properties that are able to provide a measurable response when these interacting with the analyte. Based on the experience in this field, it were synthesized a series of coordinative compounds. Bisphthalocyanines of some lanthanide ions (Lu, Gd, Dy) were synthesized using a method that does not use solvents. Thus, appropriate amounts of lanthanide acetate and phthalonitrile are mixed in the solid state and then heated and maintained at a temperature of 250°C for 3 hours. As a result of the reaction it was obtained a blue-green solid (a mixture of the neutral form and the reduced form of the bisphthalocyanine). The solid was cooled, solved in chloroform and passed through a neutral Al_2O_3 chromatographic column using $CHCl_3$ in order to separate the neutral form of bisphthalocyanine ("green" form). Separation was monitored by using thin layer chromatography and UV-Vis spectroscopy. The raw product was purified by recrystallization from heptane obtaining a green solid (reaction yield is near to 25% for all three bis-phthalocyanines synthetized). Bisphthalocyanines obtained were characterized by UV-Vis, NIR and FTIR demonstrating their purity and presence of characteristic peaks in the UV-Vis, NIR and FTIR spectra. Therefore, it were obtained, purified and physicochemical characterized several compounds that will be tested as sensitive materials for detection of biogenic amines.

3.2. Synthesis and characterization of conducting polymers

For the preparation of sensors based on conducting polymers were used the following monomers: pyrrole, aniline and 3-methylthiophene. As doping agents were used several compounds that allow obtaining polymer films with morphologies, sensitivities and different redox properties. Thus, for the electrochemical synthesis of polyaniline were used: HCl, HNO_3, H_3PO_4, CH_3COOH, $HClO_4$ and $H_2C_2O_4$. For the synthesis of polypyrrole were used $K_4[Fe(CN)_6]$, $Na_2[Fe(CN)_5NO]$, $H_3PW_{12}O_{40}$, H_2SO_4, Na_2MoO_4, sodium salt of 9,10-anthraquinon-2-sulfonic acid acid, sodium dodecansulfonat, sodium decansulfonat, p-toluenesulfonic acid acid and phosphate buffer of pH 7. In the case of poly-3-methylthiophene doping agents used were: $LiClO_4$, $LiCF_3SO_3$, tetrabutylammonium perchlorate and tetrabutylammonium tetrafluoroborate.

From the solution containing monomer and doping agent were synthesized polymer films with different properties using different electrochemical techniques such as chronoamperometry, chronopotentiometry, cyclic voltammetry and square wave voltammetry. It was shown that by using chronoamperometry the polymeric films obtained are optimal for use in the construction of sensors and biosensors. This technique allows a strict control of the deposited layer thickness and of over-oxidation degree.

Synthesized polymers were characterized by IR spectroscopy in order to determine the degree of over-oxidation. From the analysis of IR spectra were determined the optimal conditions of polymerization so that the polymer is not over-oxidated. A maximum potential of 0.8V was used for electrochemical synthesis ensures a very low degree of polymer over-oxidation.

The morphology of polymer films was determined by scanning electron microscopy (SEM) and atomic force microscopy (AFM). These studies were conducted during the research stage at the University of Valladolid (Spain). It were shown that the parameters having a major influence on the morphology of the polymeric films are chemical nature of doping agent and electrochemical technique used for synthesis.

In conclusion, conducting polymers were synthesized and characterized spectroscopically and microscopically. From the spectroscopic, microscopic and electrochemical studies were demonstrated that polypyrole is most suitable for the construction of sensors and biosensors, due to the compatibility with biogenic amines and to the double role of electron mediator and immobilization matrix, in the case of biosensors. Poly-3-methylthiophene has the disadvantage that the electrochemical synthesis is carried out only from acetonitrile solution, increasing manufacturing costs. Polyaniline was synthesized and presents suitable electrochemical properties only in strongly acidic solutions, solutions that cannot be used for enzymes.

3.3. Fabrication of novel sensors and biosensors

For fabrication of sensors and biosensors were used different methods that have as objective deposition of sensitive material on a solid support, with a particular design, suitable for electrochemical measurements.

a) The choice of substrates was made based on nature of sensitive material and the optimal method of deposition. For this purpose Pt wire was purchased from which it were fabricated electrodes in the form of disc. Other substrates that were used for fabrication of electrodes were ITO (indium tin oxide) coated glass, useful for electrochromism measurements. Also, it were used screen-printed gold electrodes in a sensor array configuration. It were purchased and used different screen printed electrodes containing the same device the working electrode, the counter and the reference electrode. The materials used in construction of these screen printed electrodes are carbon (C), carbon nanotubes, carbon nanofibers, graphene, C-cobalt phthalocyanine, C-Prussian blue, platinum, C-platinum nanoparticles and Au. For electrochromism measurements were acquired optical-transparent screen printed electrodes based on ITO. Another type of electrodes used were C wire electrodes. Electrode area was between 0.785mm^2 and 12.56mm^2 for disc electrodes, 1cm^2 of ITO electrodes and electrodes 52,5mm^2 for wire electrodes.

The electrochemical characteristics of all electrodes were studied. It were shown that some of them can be used as voltammetric sensors without any changes. In other cases modifying of them with other sensitive materials, commercial or synthesized in the laboratory, was absolutely necessary. For the construction of biosensors was necessary the modification with enzyme of receptor element.

b) Depending on the size of the substrate and the amount of material available were used several methods for deposition of sensitive layer.

b.1. In the case of sensors based on conducting polymers was used electrodeposition technique (electrosynthesis) from a solution containing the monomer and doping agent. The solvent was water in the case of polyaniline and polypyrrole and acetonitrile in the case of poly-3-methylthiophene. The optimal electrochemical technique for electrosynthesis was chronoamperometry, which ensure an uniform deposition, a short deposition time (30-120s) and by controlling the potential, over-oxidation process of polymer. Furthermore, this technique controls very precise of electric charge used on electrosynthesis and it can be accurately calculate the thickness of the deposited polymer. It were electrosynthetized polymeric films with thicknesses between 200 nm and 50μm. Optimal thickness of polymer for the fabrication of sensors is between 2 and 10μm, thickness that provides mechanical stability and good sensitivity. Also, this thickness is ideal for immobilizing the enzyme in the case of biosensors.

b.2. In the case of other sensitive materials were used other deposition methods described in the following paragraphs. The most performant technique used is Langmuir-Blodgett technique (employed during the research stage at the University of Valladolid), technique providing the control of sensitive layer of sensor or biosensor at molecular level. ITO substrate was used as substrate and sensitive materials were bis-phthalocyanines of Lu, Gd and Dy. In order to facilitate the deposition of nanostructured monolayers arachidic acid was used, also. The quality of monolayers was studied by BAM microscopy. When biosensors were fabricated, the enzyme was introduced in aqueous sub-phase (0.01M phosphate buffer and 0.1 M NaCl) and the mediator on the surface of the subphase. In the first step were recorded surface pressure isotherms determining the surface pressure where monomolecular layer has a high degree of order and can be transferred to the solid support. At the optimal surface pressure it were transferred by immersion-emersion cycles, a variable number of monolayers, between 10 and 30, depending on sensitive properties of materials.

For disk screen-printed electrodes cast or drop-and-dry deposition technique was used both for deposition of sensitive material and enzyme, in the case of biosensors.

ITO substrate was used also in the case of layer-by-layer techniques (LBL) and spin-coating.

For the fabrication of other sensors was used carbon paste electrodes technique in which carbonaceous material (graphite, carbon nanotubes, C nanopowder) was mixed with mineral oil in a ratio that can ensure a good electrical conductivity and mechanical strength (the weight ratio is 1: 1.3). For increased sensitivity were used sensitive substances or enzymes, which were deposited on the surface of carbon paste electrodes. In some cases, sensitive materials were placed within the carbon paste (for example, bis-phthalocyanines). The percentage of the phthalocyanine in relation to the carbon material is 15%.

b.3. Immobilization of enzymes on electrodes was achieved by several methods, namely: physical adsorption, by retaining in solid matrix (carbon paste), by electropolymerization and by Langmuir Blodgett technique. In order to increase the stability of the enzyme layer, regardless of the method of deposition , it was used cross-linking process with glutaraldehyde. The enzymes employed were tyrosinase, peroxidase, mono- and diamine oxidase. Enzyme layer comprises between 100 and 300 units per biosensor.

In the case of electronmediators , these were deposited on the sensitive element following two strategies. The mediator and enzyme layers were deposited separately by the same technique or using different techniques. For example, over the film of polypyrrole obtained by electrochemical synthesis the enzyme was adsorbed and then cross-linking reaction is performed. Also, in this category is included the modification of carbon paste electrodes, metalic or screen-printed by enzyme adsorption followed by cross-linking.

When there was a physical and chemical compatibility between the enzyme and the mediator, mixed layers were deposited by an appropriate method. This is the case of bis-phthalocyanines which were deposited together with enzyme by Langmuir-Blodgett technique, of polypyrrole electrosyntetised from a solution containing monomer, doping agent and enzyme or of carbon pastes made from carbonaceous material, mediator, enzyme, and conglomerate agent.

3.4. Sensors and biosensors prepared were characterized by spectroscopic (UV-Vis, NIR and IR) and microscopic (SEM, and AFM BAM) techniques.

From the analysis of UV-Vis, NIR and IR spectra was determined the ordering degree of molecules, molecular orientation relative to the solid substrate (perpendicular, parallel or under other angle), the formation of new covalent bonds, the existence of enzyme on biosensor receptor element, etc. BAM (Brewster angle microscopy) allowed determining the morphology of monolayer before transferring on solid substrate. Morphology of receptor element was determined by SEM and AFM.

For the sensors and biosensors prepared by using Langmuir-Blodgett technique was determined that bis-phthalocyanine molecules were oriented almost perpendicular to the substrate surface of ITO, the arachidic acid molecules were perpendicular to the surface and form a bi-layer and the enzyme molecules are retained in two-layer structures similar to cellular membranes. In addition, this biomimetism promotes the enzymatic activity due to changing quaternary structure and accessibility of the active center for analyte molecules, as shown in the measurements carried out with this type of biosensors.

In the case of enzyme immobilization by using cross-linking reaction it were identified novel covalent bonds between enzyme molecules and between enzyme and immobilization matrix, e.g. polypyrrole. From measurements carried out with these biosensors was found that cross-linking leads to a decrease in enzymatic activity but also is an increasing of biosensors durability. This is related to the change in conformation of the enzyme. Therefore, there must be an equilibrium between sensitivity of biosensors and their durability.

Microscopic techniques were shown that mixed layers deposited by Langmuir-Blodgett technique have a very low roughness due to the homogeneity of monolayers transferred onto solid substrates. In the case of polypyrrole, morphology depends on the nature doping agent and the electrochemical technique used.

In addition to the originally proposed work plan it were carried out a series of studies on the determination of electroactive compounds in emulsions with the purposes of determining the capacity of sensors and biosensors to function in this type of environment. Results obtained with polypyrrole sensors were excellent and were published. This study was necessary because the sensors and biosensors will be used for the analysis of biogenic amines in foods with a minimal processing of samples, thus in complex heterogeneous environments. It was also studied the encapsulating of enzymes before immobilization in order to increase the sensitivity of biosensors.

Therefore, in this year were carried out all the activities fixed in the working plan obtaining novel sensors and biosensors, with new designs, from different sensitive materials deposited by means of nanotechnologies characterized by spectroscopic and microscopic methods.

Dissemination of results

Dissemination of research results was carried out by publishing ISI papers, publication of a chapter in a monograph and participation to international and national conferences.

Publicarea de articole ISI

1. C. Apetrei, *Novel method based on polypyrrole-modified sensors and emulsions for the evaluation of bitterness in extra virgin olive oils*, **Food Research International** 48 (2012) 673–680; doi:10.1016/j.foodres.2012.06.010, journal impact factor 3.15; relative influence score 2.47386.

2. I.M. Apetrei, C. Apetrei, *Amperometric biosensor based on polypyrrole and tyrosinase for the detection of tyramine in food samples*, **Sensors & Actuators: B. Chemical**, 178 (2013) 40-46; http://dx.doi.org/10.1016/j.snb.2012.12.064, journal impact factor 3.898; relative influence score 1.85283.

Chapters in international monographs

1. C. Apetrei, M. Ghasemi-Varnamkhasti, *Biosensors in food PDO authentication*, Chapter 11, in **Comprehensive Analytical Chemistry**, Volume 60 , 2013, Pages 279-297, **Food Protected Designation of Origin - Methodologies and Applications**, Ed. A. Gonzalvez and M. de la Guardia, Elsevier, ISBN: 9780444595621, http://dx.doi.org/10.1016/B978-0-444-59562-1.00011-6

Participation in international conferences and papers published in the proceedings of conferences

1. I.M. Apetrei, C.V. Popa (Ungureanu), D. Tutunaru, C. Apetrei, *Biosensors based on different carbonaceous materials for the analysis of biogenic amines*, **The Frontiers of Microscopy Virtual**

Conference, Elsevier, 21 March 2012, Poster, http://www.materialstoday.com/virtualconference/the-frontiers-of-microscopy

2. I.M. Apetrei, D. Tutunaru, C.V. Popa (Ungureanu), C. Apetrei, *Electrochemical study of biogenic amines with conducting polymer sensors,* **International Conference of Applied Sciences, Chemistry and Chemical Engineering (CISA), Sixth Edition**, Bacau, April 24-27, 2012, Poster, http://cisaconf.ub.ro

Article published: pages 16-20, ISSN 2066-7817

3. I.M. Apetrei, D. Tutunaru, C.V. Popa (Ungureanu), C. Apetrei, *Development of amperometric biosensor based on tyrosinase immobilized in phosphate-doped polypyrrole film for detection of biogenic amines,* **14th International Meeting on Chemical Sensors - IMCS 2012**, May 20-23, 2012, Nuremberg, Germany, Poster, http://www.ama-science.org/home/details/1068

Article published:pages 855-858, ISBN 978-3-9813484-1-5, DOI 10.5162/IMCS2012/P1.1.16

4. C.V. Popa (Ungureanu), I.M. Apetrei, D. Tutunaru, C. Apetrei, *Biosensing properties of novel biosensors towards biogenic amines,* **1st International Conference on Analytical Chemistry RO - ICAC'2012**, 18 – 21 Septembrie 2012, Targoviste, Romania, Poster, awarded ***Best Poster Award,*** http://www.icstm.ro/ICAC2012

5. I.M. Apetrei, D. Tutunaru, C.V. Popa (Ungureanu), C. Apetrei, *Fish freshness monitoring using chemical modified voltammetric electrodes*, **Centenary Of Education in Chemical Engineering**, November 28-30, 2012, Iasi, Romania, oral presentation, http://www.ch.tuiasi.ro/CNIC2012/index.html

6. C. Apetrei, *Biosensors based on nanotechnologies*, **Materials Today Virtual Conference: Nanotechnology**, Elsevier, December 11-13, 2012, Poster, http://www.materialstoday.com/virtual conference/materials-today-virtual-conference-nanotechnology

Participation in national conferences

1. D. Tutunaru, I. M. Apetrei, *Applications of biosensors in medicine*, **Zilele Medicale Galatene**, 6-7 November 2012, Galati, Romania, oral presentation

During **2013**, the second year of the project, were carried out the activities included in the additional contract signed at the beginning of 2013. In the following pages will be described the activities carried out, objectives achieved and dissemination activities carried out in the second year from project implementation.

Objective 1. Fundamental studies on the interactions between the sensitive layer and the analyzed samples

Activities carried out for achievement of this objective were presented below.

1.1 The study of interactions between biogenic amines and active layer of sensors

It was determined that the physico-chemical interaction between the active layer of sensors and biogenic amines depends on the nature of the sensitive material, on surface morphology and on the particularities of the analyte. In the case of sensors based on polypyrrole doped with different electroinactive anions was determined that a very important factor is the electrosynthesis of the polymer procedure. The surface morphology is different in the case of polypyrrole doped with the same doping agent. It was determined that the best sensitivity presents the sensors prepared using chronopotentiometry. In general, polypyrrole shows spherical arrangements with many active centers that allow an effective interaction between the active layer and the analytes. The figure presented below shows the SEM image obtained for the polypyrrole doped with anthraquinon-sulfonic anion, electrosynthetized by chronoamperometry (applied potential 0.8V and deposition time of 720 s).

Detection of biogenic amines is allowed in two ways. On the one hand, polypyrrole participate in oxidation-reduction reactions that are influenced by the physicochemical properties of the sample. Scheme of these processes is :

$$\left[P^+ / A^-\right]_f + K_s^+ \overset{e^-}{\longleftrightarrow} \left[P / A^- / K^+\right]_f$$

$$\left[P^+ / A^-\right]_f + Cl_s^- \overset{e^-}{\longleftrightarrow} \left[P^{++} / A^- / Cl^-\right]_f$$

where:
- P - polypyrrole;
- A^- - doping anion;
- K^+_s - cation from the sample;
- Cl^-_s - anion from the sample;
- s - solution;
- f - polymeric film (active layer).

Thus, concentration, pH, ionic strength, etc. influences the redox processes of polypyrrole by changing peak currents, changing the shape of the peaks and shifting of peak potentials to higher or lower values. All these changes are quantified and are used for identification, discrimination, classification and quantification of biogenic amines in samples. Polypyrrole doped with electroactive anions (ferrocyanide ions, nitroprusside ion, etc.) shows characteristic peaks related to polypyrrole and to the doping ion.

On the other hand, in the case of amines (e.g., dopamine, epinephrine, histamine or trimethylamine) was observed that these compounds present own redox peaks in the potential range studied. The biogenic amines can be detected directly or indirectly by means of sensors based on polypyrrole doped with different doping agents.

In the case of sensors based on phthalocyanines, the experimental observations are similar. In this case, surface morphology, which is correlated with the phthalocyanine deposition method, influence significantly the sensor properties. The figure shows the AFM image of a thin film of lutetium bisphthalocyanine deposited by Langmuir - Blodgett technique.

Phthalocyanines shows characteristic oxidation-reduction processes related to chemical structure and to nature of the central ion. The cobalt phthalocyanine shows the characteristic peaks due to oxidation-reduction of Co ions, and in the case of bisphthalocyanines the characteristic peaks are related to redox processes of the phthalocyanine rings.

$$[Co^{I}Pc]^- \leftrightarrow [Co^{II}Pc]^0 \leftrightarrow [Co^{III}Pc]^+$$

$$[LuPc_2]^- \leftrightarrow [LuPc_2]^0 \leftrightarrow [LuPc_2]^+$$

Redox processes of phthalocyanines are influenced by the physicochemical properties of the sample analysed. It was also determined that phthalocyanines shows electrocatalytic effect favoring the redox processes of different chemical species present in the sample. The biogenic amines are oxidized at potentials much lower due to the action of phthalocyanine catalyst present in the sensitive layer of the sensor. The most marked electrocatalytic effect was observed in the case of lutetium bisphthalocyanine. Thus, biogenic amines can be detected directly or indirectly by the sensors based on phthalocyanines.

1.2 The study of interactions between biogenic amines and active layer of biosensors

Biosensors developed during this project were enzymatic biosensors based on different materials as immobilizing matrix (screen-printed electrodes based on carbon modified with different nanomaterials, e.g. carbon nanotubes, nanoparticles of Pt or Au, screen-printed electrodes modified with a layer of polypyrrole, metalic electrodes coated with polypyrrole, unmodified carbon paste electrodes modified with phthalocyanines, etc.) and enzymes able to detect biogenic amines.

It was determined that immobilizing matrix plays a crucial role in terms of analytical performances and characteristics of biosensors developed.

It was determined that the method of immobilization plays an essential role in maintaining catalytic activity of the enzyme immobilized in the solid substrate of biosensor. Among the methods of immobilization the best method is cross-linking, if a strict control of reaction time is achieved. This is due to

particularity of immobilizing matrix, which has a certain porosity and allows a good adsorption of the enzyme and of functional groups that can participate in cross-linking reactions with glutaraldehyde. Identification of functional groups, their modification as a result of participating in the chemical reactions were performed by IR spectroscopy.
The detection mechanism of biosensors depends on the nature of the immobilized enzyme in the active layer. Tyrosinase catalyze biochemical reactions of a particular category of biogenic amines, namely catecholamines. General mechanism for detection of catecholamines is:

If the analyzed catecholamine has in the chemical structure one -OH group, in the first stage of occurs enzymatic hydroxylation of catecholamine. The next step take place the oxidation of hydroxylated derivative to corresponding quinone derivative. Quinone formed in the enzymatic reaction is electrochemically reduced at biosensor surface. The response of biosensor consist in the development of a cathodic current that can be measured by voltammetric or amperometric methods.
In the case of amine oxidases, the reaction mechanism is similar if the sensitive layer of the biosensor is immobilized diamine oxidase or monoamine oxidase. The differences consist in different sensitivity of biosensors from the same analyte when the enzyme is different. The histamine detection scheme is presented in the case of a biosensor based on diamine oxidase (DAO).
Enzymatic reaction leads to the de-amination of histamine and aldehyde derivative is electrochemically oxidized at the biosensor surface. The electrochemical process can be monitored by voltammetric or amperometric methods.

1.3 Study of the enzymatic kinetics of biosensors

For the study of enzymatic kinetics were recorded calibration curves of different types of biosensors immersed on solutions of biogenic amines in optimal conditions.
In all cases were obtained characteristic dependences of an enzymatic Michaelis-Menten type kinetics. Thus, for small concentrations a linear increase of biosensor response with increasing concentration was observed, then a plateau was obtained (stationary state) which corresponds to a saturation state of the biosensor. In this stage, all active sites on the surface of the biosensor participate to the enzymatic reaction, so if there an increase in the concentration of substrate cannot be detected by the biosensor.
From the linearity range detection limits were determined using $3\sigma/m$ criteria, where σ is the standard deviation of the signal in the blank solution and m is the slope of the calibration curve, and it correspond to biosensor sensitivity (increasing of biosensor signal when the concentration increases with one unit).
Limits of detection and quantification of biosensors developed in this project are in the 10^{-7} - 10^{-6} M range. These results indicate that the biosensor performances were optimal to be used in practical applications on real samples. These biosensors will be used for analysis of foods, drugs or biological samples.
From data obtained from the calibration curve Hill coefficient was calculated, the Hill coefficient giving information about the mechanism of enzymatic reactions. In electrochemical measurement the current is the kinetic parameter dependent on the concentration of the analyte in solution. From the plot of Hill equation the coefficient h was determined. The equation for calculation of Hill coefficient is:

$$\lg \frac{I_{\max}}{I_{\max} - I} = -\lg K_M^{app} + h \cdot \lg[S]$$

where:
- I_{max} - maximum reaction rate;
- I - reaction rate;
- K_M^{app} - apparent Michaelis-Menten constant;
- h - Hill coefficient;
- [S] - concentration of analyte.

In all cases was obtained values of h around 1 (ideal value), which demonstrates that the reaction kinetics at the biosensor surface is fitted to a Michaelis-Menten type kinetics. When the values of h are slightly larger than one there is a positive cooperative effect between active sites occupied by analyte molecule. When values are slightly lower than one there is a negative cooperative effect.
From the calibration data, by plotting 1/I versus 1/c were calculated the characteristic parameters of enzymatic reaction, the maximum reaction rate (I_{max}) and the apparent Michaelis-Menten constant (K_M^{app}) using Lineweaver-Burk equation:

$$\frac{1}{I} = \frac{1}{I_{\max}} + \frac{K_M^{app}}{I_{\max}[S]}$$

K_M^{app} values are lower or comparable to those obtained when the enzyme is in solution. This demonstrates that the enzyme immobilization of the biosensor sensitive element does not diminish the biocatalytic activity. For this reason biosensors developed in this project shows features and superior analytical performance compared to other biosensors reported in the literature.

1.4 Comparison of experimental data with those obtained through modeling and simulation

Experimental data obtained with sensors and biosensors developed in this research project were compared with data obtained through modeling and simulation. Good correlations were obtained for the sensors based on polypyrrole doped with ions without electroanalytical activity and for the unmodified carbon-based electrodes. In more complex systems involving multiple chemical and electrochemical reactions, correlations continue to be good, but the differences between modeled and experimental data are higher. This is due to the construction of models that were developed using some approximations.
In the case of biosensors good correlation with experimental data were obtained when amperometry was used as method for the recording of biosensors responses. Modeling of enzymatic and electrochemical processes occurring at the biosensors surface were used to explain the functioning mechanism of biosensors.
In conclusion, the results obtained were useful for understanding the mechanism of interaction between the analyte and the sensing element of the sensor or biosensor. This thing allowed the development of sensors and biosensors with good analytical and performance characteristics for identification and quantification of biogenic amines.

Objective 2. Data processing and interpretation of results

The activities carried out for achieving this objective is presented below.

2.1 Defining of methods for data analysis. Exploratory analysis

For statistical analysis of the experimental data were considered the specificity of data resulting from the electrochemical measurements. In the case of voltammetric methods (cyclic voltammetry, square wave voltammetry) data are current-potential value pairs. The number of pairs of data is large, hundreds of values and therefore for the correct interpretation of the experimental data should be used multivariate data analysis. In the case of amperometric determinations, interpretation of experimental results is carried out using basic statistical methods in Excel or Origin.

2.2 Pre-processing of experimental data

The importance of this stage is very high for a correct analysis of experimental data. This step has as objective the increasing the quality and representativeness of the data. At this stage was extracted useful information from the global information recorded for a system (bio)sensor-sample. For example, from a voltammetric curve was selected a number of significant parameters for the samples, such as potential or

peak intensities. This reduction voltammetric data is useful for explaining the thermodynamic characteristics (e.g. potential of a redox couple) or kinetics (scanning rate influences the responses of (bio)sensors). However, using this method is lose a large amount of useful information such as dynamic characteristics of voltammetric curves. For increasing the quality and quantity of useful information several strategies were used. The first method is the reduction of variables using kernel functions, bell-shapped functions of unitary area. By multiplying 10 kernel functions with voltammetric curve results 10 coefficients related with the dynamic characteristics of voltammetric curve. By this method, voltammetric curve is divided into 10 intervals and the area under the curve was calculated for every interval. Consequently, 10 representative values for each voltammetric curve are obtained. It was developed a application in Matlab, which calculated the kernel coefficients, that will be used as input parameters for Principal Component Analysis. In the case of curves obtained by square wave voltammetry kernel technique is applied to complete curve as is saved from the software of potentiostat. The application calculates the total range of the potential axis values, it is divides into ten equal parts and calculates the area under the curve corresponding to each interval.

Curves are obtained using cyclic voltametry are bivaluated, to each potential values correspond two current values. Pre-processing software dedicated to the analysis of cyclic voltammograms separate the anodic and cathodic waves and these curves are analyzed in parallel. In some cases it is used only the anodic curve because cathodic and anodic curves are complementary. By the analysis only of anodic curve the computation time was reduced to half.

Other methods which were implemented and used was the use of genetic algorithms to select the representative data from the voltametric curves. Selection of parameters was performed using Genetic Algorithm in Matlab software. This method is good when the selected data will be used to classify the samples analyzed.

Another method that was implemented is Discrete Wavelet Transform for compression of data from voltammetric curves. The software is part of the tools used in Matlab Wavelet Toolbox.

Pre-processing method to be used depends on the number of samples, the number of sensors or biosensors and multivariate analysis purposes, discrimination or classification. The methods will be used in the third stage of the project related to analyses of foods, drugs or biological samples.

2.3 Application of Principal Component Analysis method for processing of experimental data

Voltammograms obtained from the analysis of different samples show a wide variety of both peaks related to activity of the sensor and to the sample analyzed. In the case of biosensors, the peaks correspond to chemical, electrochemical or enzymatic processes. In the first attempts to analyze the signals the potential peak were evaluated and these data were used as input principal component analysis (PCA). This method led to good results, considering that potential from occurring peaks are reproducible. One of the problems of using peak potential as input for PCA were the need that the number of parameters representatives must be equal for all the curves analyzed. However, the number of peaks depends on the sample analyzed and the sensor or biosensor used. Using this method loses a large amount of information contained in voltammetric curves. In addition, the differences between the curves obtained for different samples are not only the difference between the peak potentials but also the shape of the curve, shape and relative intensity of peaks, etc.

Processing of all data that forms voltammograms is slow and expensive, because it is not possible to use a program to give the results of multivariate analysis in a reasonable time. Another drawback is evident in the case of mixed sensor networks (based on polypyrrole sensors, sensors based on phthalocyanine, biosensors) where the potential range used is different, so voltammograms contain a different number of pairs of current-potential data. All these inconveniences were solved by development of computer software that extract useful information from the experimental curves. Pre-processed data will be organized as a matrix input for PCA.

The data matrix is imported in Matlab or The Unscrambler and is scaled in order to minimize the differences in magnitude between the experimental data. Scaling method used employed the normal distribution, the *zscore* function in Matlab, or normalization routine in The Unscrambler software.

2.4 Application of classification methods (PLS-DA and SIMCA) for the processing of experimental data

The Unscrambler and Matlab software and after importing the matrix with the experimental data permit the analysis of data by means of different statistical models for exploratory analysis, discrimination and classification. PCA was the starting point of multivariate analysis and is the method most used. It is an unsupervised method, which allowed analyzing and exploring data structure, finding correlations or categories of samples, study the weight of sensors as well as the outlier detection and removal (data with excessive variance in the context of data analysis).

PLS-DA and SIMCA were applied with the aim of assessing the classification and recognition of sensor and biosensors arrays, in those cases where there is a representative number of samples in order to establish well-defined and representative classes of samples.

PLS-DA is a deterministic method of classification based on Linear Discriminant Analysis (LDA), method of pattern recognition similar with PCA but supervised, based on PLS algorithm for assigning samples to one or other of the known classes. In PLS-DA, these category variables known represent a category, in other words, have qualitative character. When PLS-DA is implemented to qualitative variables are assigned numerical values and regression models are constructed. Unknown samples are assigned to these categories or not, depending on the signals recorded by sensors and biosensors array. Results are presented in different ways, for example belonging to a class or category and error sensitivity and selectivity of the model.

SIMCA was used as supervised classification method. It is a probabilistic method that relies on the construction of independent models for each existing classes using PCA. Since the PCA determine latent variables and class structure, if they are indeed different, mathematical models that define each of them will be mandatory different. Unknown samples were assigned to classes, in relation with theirs data characteristics.

Classification methods were defined, implemented and verified in the analysis of experimental data and will be used to analyze the data obtained from monitoring and classification of samples.

2.5 Establishing of correlations between sensor or biosensors signals and the results of physico-chemical and sensory analysis

PLS1 and PLS2 regression methods were used to determine correlations between the signals of sensors or biosensors obtained from the analysis of samples (data matrix X, calibration) and other type of values associated with the same samples obtained by other methods of analysis (data matrix Y, predicted) as physico-chemical analyzes and sensory analyzes. Thus, in the case of sensors based on polypyrrole and biosensors based on carbon-DAO were established very good correlations with the pH or storage time of the fish in refrigerator.

Therefore, the activities carried out have led to the definition of software able to interpret experimental data by means of multivariate analysis according to the characteristics of sensors and biosensors responses, if voltammetric or amperometric methods were used.

In conclusion, during this year all research activities included in the work plan were carried out and the objectives were fully achieved. The practical results obtained were processed, interpreted, compiled and published in scientific journals or presented at scientific meetings.

Dissemination of results in 2013

When was signed the additional contract in 2013 it were decided that in this stage of the project will be published minimum 2 ISI papers and the participation in at least 3 scientific meetings.

Dissemination of research results include the publication 4 ISI papers, one sent for publication, publishing a chapter in a international monograph and participation in 9 international or national conferences. Therefore, the objectives of dissemination activity were fully accomplished.

Publishing of ISI papers

1. I. M. Apetrei, C. Apetrei, *Amperometric tyrosinase based biosensors for serotonin detection*, **Romanian Biotechnological Letters** 18(3) (2013) 8253-8262; http://www.rombio.eu/vol18nr3/Content.html, journal impact factor 0.349; relative influence score 0.115.

2. I. M. Apetrei, M. L. Rodriguez-Mendez, C. Apetrei, J. A. de Saja, *Fish Freshness Monitoring Using an E-tongue Based on Polypyrrole Modified Screen-Printed Electrodes*, **IEEE Sensors Journal** 13 (2013) 2548 - 2554; http://dx.doi.org/10.1109/JSEN.2013.2253317, journal impact factor 1.475; relative influence score 1.247.

3. I. M. Apetrei, C. Apetrei, *Voltammetric e-tongue for the quantification of total polyphenol content in olive oils*, **Food Research International** xxx (2013) xxx–xxx; http://dx.doi.org/10.1016/j.foodres.2013.04.032, journal impact factor 3.15; relative influence score 2.44.

4. I. M. Apetrei, C. Apetrei, *Biosensor based on tyrosinase immobilized in single-walled carbon nanotubes modified glassy carbon electrode for epinephrine detection*, **International Journal of Nanomedicine** 8 (2013) 4391-4398; http://dx.doi.org/10.2147/IJN.S5, journal impact factor 3.463; relative influence score 1.355.

5. I. M. Apetrei, D. Tutunaru, C. V. Popa (Ungureanu), C. Apetrei,*Electrochemical biosensors for catecholamines*, **Revue Roumaine de Chimie**, 2013, sent 13.09.2013, manuscript 56/2013.

Publication of BDI papers

1. I. M. Apetrei, D. Tutunaru, A. Nechita, C. Georgescu, Disposable amperometric biosensor for adrenaline detection, Analele Universităţii "Dunărea De Jos" din Galaţi, Fascicula XVII, Medicină, 2013, vol. 1/2013, 11-15.

Chapters in international monograph

1. I. M. Apetrei, C. Apetrei, Capitol *Characterization of Red Wines Polyphenolics Employing Sensors and Biosensors*, Carte, ***Wine: Phenolic compounds, Classification and Health benefits***,2014,Nova Science Publishers, *accepted October 2013*.

Participation in international conferences and papers published in the proceedings of conferences

1. I.M. Apetrei, C.V. Popa (Ungureanu), C. Apetrei, *Amperometric biosensor for the detection of histamine in food products*, **International Conference of Applied Sciences, Chemistry and Chemical Engineering (CISA), Seventh Edition**, Bacau, May15-18, 2013, Oral presentation, http://cisaconf.ub.ro

Published paper: pages 180-183, Alma Mater Publishing House, Bacau, ISSN 2066-7817.

2. I.M. Apetrei, C.V. Popa (Ungureanu), C. Apetrei, *Disposable Biosensors Based on Carbonaceous Screen-Printed Electrodes and Diamine Oxidase*, **European Biotechnology Congress**, Bratislava, Slovakia, May 16-18, 2013, Poster, http://www.eurobiotech2013.eu/.

Published abstract: S65, Current Opinion in Biotechnology, ISSN 0958-1669, DOI: http://dx.doi.org/10.1016/j.copbio.2013.05.174

3. I.M. Apetrei, C. Apetrei, Biosensors based on nanostructured layers for the detection of histamine, EuroNanoForum 2013, Dublin, Ireland, June 18-20, 2013, Poster.

Published abstract: http://www.euronanoforum2013.eu/poster-participation/

4. I.M. Apetrei, C. Apetrei, Biosensor based on tyrosinase immobilized in single-walled carbon nanotubes screen-printed electrode for tyramine detection, 18th Romanian International Conference on Chemistry and Chemical Engineering, Sinaia, September 4-7,2013, oral prezentation.

Published abstract: RICCCE18, Papers and Abstracts, pag. S2-21, Politehnica Press, Bucuresti, ISSN 2344-1895.

5. I. M. Apetrei, D. Tutunaru, C. V. Popa (Ungureanu), C. Apetrei, *Electrochemical Biosensors for Catecholamines*, **International Conference of Physical Chemistry - ROMPHYSCHEM 15**, Bucuresti, September 11-13, 2013, Keynote oral presentation

Published abstract: Abstracts, ROMPHYSCHEM 15, pag. 78, ISSN 2286-1327.

6. I. M. Apetrei, D. Tutunaru, C. V. Popa (Ungureanu), C. Apetrei, *Biosensor array for the determination of biogenic amines in food samples*, **The 6th International Symposium Euroaliment - around food**, Galati, October 3-5, 2013, Poster

Published abstract: Papers of the International Symposium EuroAliment, pag. 28, Galati University Press, ISSN 1843-5114.

7. C. Apetrei, Expert sensory system with applicability in food industry**, The 6th International Symposium Euroaliment - around food**, Galati, October 3-5, 2013, oral prezentation.

Published abstract: Papers of the International Symposium EuroAliment, pag. 29, Galati University Press, ISSN 1843-5114.

8. C. Apetrei, Biosensors based on based on nanostructured biomaterials, **Materials Today Virtual Conference: Biomaterials**, Elsevier, 19-21 November 2013.

Participation in national conferences

1. C. Popa (Ungureanu), C. Apetrei, *Biosensors based on carbonaceous screen-printed electrodes and diamine oxidase*, **Conferinta Stiintifica a Scolilor Doctorale din Universitatea „Dunarea de Jos" din Galati (CSSD-UDJG)**, 16-17 Mai, 2013, Poster, awarded *2nd Poster Award*, http://www.ugal.ro/stiri/conferin%C8%9Ba_%C8%98tiin%C8%9Bifica_a_%C8%98colilor_doctorale

Published abstract: Book of Abstracts, Scientific Conference of Doctoral Schools from UDJ Galati, CSSD-UDJG 2013, First Edition, pag. 84, Galati University Press.

The results obtained in this research project so far are promising and the most interesting part in the terms of application are the researches will be carried out in the third year of the project. The importance of biogenic amines in foods, drugs or biological samples and the development of novel sensors and biosensors for identifying, quantifying and monitoring of these compounds is of great scientific and practical interest. The results obtained so far give us the hope that the project will be fully implemented based on financial resources required in the initial plan of the project, purchased equipment and specialized personnel in this field member of project.

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