Scientific report for the entire period of project implementation
"Development of an electronic system based on electrochemical sensors and biosensors for the control of biogenic amines"
1 January 2012 – 30 June 2016

The report is structured for each stage of the project. The implementation of the project regarding the activities and the results was structured in five stages corresponding to each year (from 2012 to 2016). The dissemination activities developed in the project is presented for the entire period of the project implementation. Finally, there are described the conclusions and the final remarks.
Implementation plan of the project established by the research contract is presented in the next table:

Year	Objectives	Activities	Results to be delivered
2012	**1. Modeling and simulating studies of interaction between biogenic amine compounds and receptor element of sensors and biosensors.**	**1.1. Modeling of interaction mechanism between biogenic amines and receptor element of sensors in absence and applying a potential**	**Publication of minimum 2 ISI papers** **Participation to at least 3 scientific conferences**
		1.2. Modeling of interaction mechanism between biogenic amines and receptor element of biosensors in absence and applying a potential	
		1.3. Simulation of chemical species dynamics at the surface of sensors and biosensors	
		1.4. Theoretical study about the influence of some parameters (pH, temperature) on the biosensor response	
	2. Selection of sensors and biosensors designs	2.1. Study of commercial sensors designs	
		2.2. Design of novel sensors	
		2.3. Use of voltammetry and potentiometry in measurements of sensors and biosensors responses	
	3. Improvement of sensors qualities and design of novel sensors and biosensors	3.1. Synthesis and characterization of molecular materials	
		3.2. Synthesis and characterization of conducting polymers	
		3.3. Fabrication of novel sensors and biosensors: selection of the substrates, selection and application of deposition methods, immobilization of enzymes onto electrodes	
		3.4. Characterization of sensors and biosensors by spectroscopic (UV-Vis, NIR and IR) and microscopic (SEM, BAM and AFM) techniques	
2013	**1. Fundamental studies of the interaction between sensitive layer and samples under study**	1.1. Study of interactions between biogenic amines and active layer of sensors	Publication of minimum 2 ISI papers Participation to at least 3 scientific conferences
		1.2. Study of interactions between biogenic amines and active layer of biosensors	
		1.3. Study of enzyme kinetics of biosensors	
		1.4. Comparison of experimental data with those obtained in modeling and simulation studies	
	2. Data treatment and interpretation of the results	2.1 Establishment of methods for data analysis. Exploratory analysis	
		2.2 Pre-processing of experimental data	
		2.3 Application of Principal Component Analysis method for processing of	

		DA and SIMCA) for processing of experimental data	
		2.5 Establishment of correlations between sensors and biosensors signals and the results of physico-chemical analysis or sensory	
2014	**1. Test of the sensing capabilities of the sensors and biosensors**	1.1. Study of the selectivity of sensors and biosensors	Publication of minimum 2 ISI papers Participation to at least 2scientific conferences
		1.2. Study of the stability of sensors and biosensors	
		1.3. Study of the reproducibility of sensors and biosensors	
		1.4. Study of the lifetime of sensors and biosensors	
		1.5. Determination of the detection limits of sensors and biosensors	
		1.6. Determination of the response time of sensors and biosensors	
		1.7. Determination of the reversibility and the recovery of sensors and biosensors	
2015	**1. Test of sensors and biosensors in real samples. Applicative studies**	1.1. Selecting of sensors and biosensors arrays for applications in real samples	Publication of minimum 2 ISI papers Participation to at least 2scientific conferences
		1.2. Selection of the methods for data treatment	
		1.3. Analysis of biogenic amines in meat products, cheeses and fermented beverages	
2016	**1. Test of sensors and biosensors in real samples. Applicative studies**	1.4. Monitoring of food freshness	Publication of minimum 2 ISI papers Participation to at least 2scientific conferences
		1.5. Analysis of biogenic amines in fruits	
		1.6. Analysis of biogenic amines in clinical samples	
		1.7. Validation of the systems by the establishment of correlations between the results obtained with sensors and biosensors and the results of physico-chemical, sensory, biochemical and medical analyses	

During 2012 it 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.

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 outand the objectives achieved 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:

- 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.

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:

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:

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.

During 2014, the third year from the project, were carried out the activities included in the additional contract signed in 2014 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 performed, objectives achieved and dissemination activities carried out in the period January - December 2014.

The objective this year was **Testing of sensitive performance of sensors and biosensors;** for this purpose were performed the activities necessary for a better characterization of sensors and biosensors developed in previous years of the project.

Some of the most important characteristics for use in practice of sensors and biosensors are related to their life time (reproducibility, stability and durability), to the selectivity for the target compound and to the limit of detection. Thus, all types of sensors and biosensors developed in previous activities of the project were tested in order to select the best ones in the terms of sensitive performances for applications on real samples.

1.1 Study of sensors and biosensors selectivities

In studies regarding selectivity were used sensors and biosensors fabricated and characterized in previous stages of the project. The results will be presented below.

Sensors based on polypyrrole doped with different doping agents and **the sensors based on phthalocyanine** detect biogenic amines in samples on the basis of their influence on the characteristic peaks of modifying materials or present their own peaks due redox reactions at the electrode surface. Thus, in the case of dopamine, epinephrine, histamine and trimethylamine characteristic peaks are obtained when cyclic voltammetry or square wave voltammetry were used as detection methods.

For determination of the selectivity of responses provided by **sensors based on carbon paste modified with phthalocyanines** voltammetric curves were recorded by square wave voltammetry in aqueous solutions of KCl, phosphate buffer solutions of pH 6, 7 and 8, $KClO_4$ and others. It was observed that when sensors are immersed in different electrolyte solutions, recorded voltammograms are stable, depending on the nature and concentration of the electrolyte. These peaks are influenced by the presence of interfering substances. Therefore, if the solutions of electrolytes are added electroactive substances can be observed changes of peak potentials and their currents (related to the redox processes of phthalocyanines, of polypyrrole, etc.) but also the appearance of peaks due electroactive substances present in solution. Voltammograms obtained become more complex with the increasing of number of active compounds present in the sample. Therefore, the sensors are useful in studies of monitoring the freshness of food; voltammetric signals are processed using multivariate data analysis methods in order to select useful information for this type of analysis.

In the case of solutions containing two or more electroactive amines, to increase the resolution of the peaks, square wave voltammetry was used. In these conditions are obtained voltammograms with well separated peaks that are useful in quantitative determinations based on regression curves. For example, the sensor based on carbon paste (CPE) modified with $LuPc_2$ can detect and quantify dopamine and histamine in a complex sample containing besides the two amines and other interfering compounds such as tyramine, tyrosine, cysteine and various ions (Na^+, Ca^{2+}, Cl^-).

It were studied the interferences from successive exposure of **sensors based on Langmuir-Blodgett films (LB) of phthalocyanine** at different electrolyte solutions. For this purpose were prepared ITO based electrodes coated with LB films of $LuPc_2$, $GdPc_2$ and $DyPc_2$. Each sensor was immersed in potassium chloride solution. After recording of five consecutive cyclic voltammograms, the electrodes were removed from the solution, washed with bidistilled water and let to dry. Subsequently, the sensors were immersed

in phosphate buffer solution pH 7 or biogenic amines and cyclic voltammograms were recorded. Finally, the electrodes were immersed in a solution of KCl and cycled six times in order to eliminate potential interference species adsorbed on LB film. After this measurement protocol it was observed that oxidation and reduction peaks corresponding phthalocyanine are modified and new peaks were observed that do not exist in voltammograms of sensor recorded in 0.1 M KCl solution. Therefore, the original signal of the sensor is significantly altered and cannot be recovered the initial response. The results showed that some chemical species present in the solution are adsorbed on LB film during cycling of and remain in LB film causing a "memory" effect, in other words there is an irreversible contamination of the electrode.

Similar studies were performed for **sensors based on carbon paste modified with phthalocyanine**. In this case, the differences observed between voltammograms recorded in 0.1 M KCl solution with a new sensor and voltammograms same sensor in 0.1 M KCl solution used in the analysis of biogenic amines are much lower than in the case of LB sensors, the sensors can be recovered and reused by polishing or cycling in KCl solution.

In the case of **sensors based on polypyrrole** doped with different doping agents, modifying the sensor signal after measurement of solutions of biogenic amines is reduced, and the voltammetric signal is recovered through cycling (5-6 cycles) in a solution of KCl or phosphate buffer solution of pH 7.

Study of interference in the case of biosensors were performed according to the enzyme used and the potential interference that may be present in real samples analysis. Detection method used was amperommetry in the optimal operating conditions of biosensors (applied potential, pH, ionic strength, temperature, continuous stirring).

In the case of biosensor based screen printing electrodes (SPE) modified with carbon nanotubes functionalized with carboxyl groups and tyrosinase (**Ty/SWCNT-COOH/SPE**) was determined the interferences of different compounds from the sample on analytical signal corresponding to the analyte. The result was expressed as the limit of tolerance, which is the maximum concentration of foreign substances that cause a relative error of about ± 5% in the determination of tyramine. Concentrations of interfering substances were 0.01 M Ca^{2+} and Cl^-; 0.001 M putrescine, histamine, tyrosine and glutathione. Tolerance ratios to interfering substances in determination of 100 μM concentration of tyramine were 2500 for Ca^{2+} and Cl^-; 300 for putrescine, histamine and glutathione; 20 for tyrosine. Therefore, this biosensor is useful for the selective determination of tyramine in complex samples containing biogenic amines. The absence of peak currents modifications in the presence of interfering chemical species demonstrated that Ty/SWCNT-COOH/SPE biosensor is a very good biosensor for the detection and quantification of tyramine in complex samples. Use of this biosensor in detecting of tyramine in fish products has the advantage of high selectivity because other biogenic amines do not interfere. Detection of tyramine is related to presence of phenolic group in the chemical structure of tyramine.

In the case of biosensors based on electrodes of Pt modified with polypyrrole doped with phosphate ions and containing enzyme tyrosinase immobilized (Ty/PO_4-PPy/Pt), the studies on the selectivity were conducted in this way. It was studied the influence of different compounds that could interfere in the determination of 50 μM tyramine in optimal experimental conditions. The result was expressed as the limit of tolerance. The concentration of interfering substances were 0.1 M Mg^{2+}, Ca^{2+} and Cl^-; 0.01 M histamine, putrescine, tryptophan, spermine, phenylalanine and glutathione. The tolerance ratios to a interfering compounds in the detection of 50 μM tyramine were 2000 for Mg^{2+}, Ca^{2+} and Cl^-; 200 for histamine, putrescine, spermine and glutathione; 100 for tryptophan; 20 for phenylalanine. Selectivity in case of interference caused by some phenolic compounds was studied by cyclic voltammetry. Cyclic voltammograms of catechol solution containing 50 μM or 50 μM phenol added to a solution of tyramine 50 μM present two cathodic peaks, one at -0.05 V (related with phenol or catechol) and one at -0.250 V (related with tyramine). In the case of amperometric detection of tyramine at -0.250 V was determined that phenol and catechol cause an additive error in determining tyramine, but less than 2%. However, this biosensor is useful for the selective determination of tyramine in complex samples containing biogenic

amines. The absence of significant changes in peak currents recorded in the presence of interfering species demonstrates that the biosensor Ty/PO_4-PPy/Pt can be considered an optimal biosensor for the detection and quantification of tyramine in complex samples.

The use of this biosensor for the detection and quantification of tyramine in fermented foods has the advantage of a high selectivity for other amino compounds that do not interfere. This is due to the specific action of the tyrosinase with tyramine, which is a double functional compound which contains an amino group and a phenolic group.

Also, for the biosensor based on vitreous carbon (GCE) modified with single wall carbon nanotubes (SWCNT) and diamine oxidase (DAO) (**DAO/SWCNT-GCE**) was studied the influence of interfering substances on detection of 50 μM epinephrine for the biosensor based on glassy carbon modified with single wall carbon nanotubes and diamine oxidase. Concentrations of interfering substances were 0.01 M Na^+ and $S_2O_5^{2-}$; 0.001 M urea, uric acid, and citric acid. Tolerance ratios to interfering substances on detection of 50 μM epinephrine were 2400 for Na^+ and $S_2O_5^{2-}$; 800 for urea, 180 for uric acid and 180 for citric acid. Glucose and glycine in concentrations of 0.001 M have no influence on the biosensor response on the detection of 50 μM epinephrine. These results demonstrate that the biosensor DAO/SWCNT-GCE is a biosensor with optimal characteristics for the determination and quantification of epinephrine.

Similar values of tolerance ratios were obtained for **biosensors based on diamine oxidase** (DAO) immobilized on **different carbonaceous materials** (graphite, carbon nanotubes, graphene and carbon nanofibres - the support is carbon paste or vitreous carbon electrodes) **polypyrrole doped with phosphate ions** by the casting method followed by cross-linking with glutaraldehyde on determination of 50 μM putrescine. High selectivity is related to specific biocatalytic action of the enzyme.

Biosensors fabricated by immobilizing **monoamine oxidase (MAO) in mixed LB film of arachidic acid (AA) and dysprosium (III) bisphthalocyanine ($DyPc_2$)** present tolerance ratios of 240-280 to interfering substances (glucose, ascorbic acid, uric acid, cysteine) on detection of histamine50 μM. High selectivity is due to specific biocatalytic action of the enzyme and mediator effect of phthalocyanine on electrons transfer.

1.2 Study of the sensors and biosensors stability

To assess the stability of **sensors based on Langmuir-Blodgett thin films (LB) of phthalocyanine**, there were recorded several consecutive cycles in 0.1 M KCl solution. The first cyclic voltammograms is slightly different from the second one. Then successive voltammograms are highly reproducible. For example, an LB film of $GdPc_2$ immersed in 0.1 M KCl aqueous solution shows a very high reproducibility, so after 100 successive scans what is seen is only a slightly decreases the peak intensity below 3.5% for all sensors studied. When the measurements were stopped and the sensor based on the LB film is removed from the solution (even for a few minutes), significant changes in cyclic voltammograms recorded were observed. Irreproducibility caused by removing the electrode from solution and storage were assessed following the procedure described below: after the registration of three cycles in 0.1 M KCl solution the electrodes are removed from the solution and washed with ultrapure water. Then the electrodes are kept using three methods. One of the electrodes is kept in bidistilled water, the second in a solution of 0.1 M KCl, and the third is held in the air. Periodically (from 15 to 15 minutes), the sensors are re-immersed in KCl solution and three cyclic voltammograms were recorded. Cyclic voltammograms recorded after storage differ significantly from the first voltammograms recorded (when the electrode is new), highlighting the loss of signal repeatability regardless the condition of electrode storage. The changes observed differ from one sensitive material to another and from an electrolyte to another, and are primarily related to lowering of peak intensity, peak shifting to higher potential values and the appearance of new peaks or "shoulders" of the initial peaks. This effect can be attributed to the changing of LB films structure (the degree of order) after immersion in solutions of electrolytes and performing of voltammetric measurements. Although, LB technique allows to obtain very high ratio electrode surface/volume, which facilitates the electrochemical

processes at the sensors surfaces, and the lack of stability and reproducibility of the measurement are the main disadvantages of electrodes based on LB films However, the LB technique is very reproducible, allowing the fabrication of practically identical sensors. Differences between sensors prepared in the same conditions are less than 0.5% in terms of response obtained by cyclic voltammetry. This type of sensors were used as disposable sensors.

In order to test the viability of sensors based on **carbon paste (CPE) modified with phthalocyanines ($LnPc_2$)** were conducted a series of systematic experiments to investigate the electrochemical behavior in time and utilization capacity. It should be emphasized that in all cases, the sensors need a stabilization phase, which consists in recording of five cyclic voltammograms in order to establish a equilibrium between the sensor and the solution analyzed. To determine the stability of responses provided by sensors based on carbon paste modified with phthalocyanines, voltammetric curves were recorded by square wave voltammetry in aqueous solutions of KCl and biogenic amines (in phosphate buffer pH 7), removing the sensor from the analyzed solution after each measurement. It is noticed that the peak potentials are almost constant (% RSD <1%) and the variations of peaks intensities with increasing number of repetitions are very low (% RSD <3%) for 100 repetitions. Variation of entire curve measured as % RSD of the 10 kernel coefficients is <5% for all this type of sensors ($GdPc_2$, $LuPc_2$, $DyPc_2$). From these data it can be stated that the voltammetric sensors based CPE modified with phthalocyanines shows good stability and can be used in routine measurements. In addition, the sensor surface can be recovered by polishing the electrode surface with a filter paper and cycling in 0.1 M KCl solution.

Regarding the repeatability of sensors based on **polypyrrole doped with different doping agents** should be mentioned that the first five cycles recorded are slightly different from those recorded later, but after this this stabilization step the voltammetric cycles are highly reproducible. After 100 cycles was observed only a slight and progressive decrease in peak intensities (there is an overall decrease in signal intensity about 3%). On the other hand, when cycling is stopped and the sensors are removed from the solution and stored in air or in ultrapure water for several minutes, and then reintroduced into solution, no changes are observed in electrochemical signals obtained (variations are less than 1% for all studied sensors). These results show the good short-term stability and repeatability of the response of sensor based on polypyrrole.

In the case of biosensors, at this stage was studied the biosensor based on **polypyrrole and tyrosinase** in order to determine the stability of the biosensor response when it is used intensively. Thus, there was recorded a total of 50 cycles in 50 μM dopamine solution (electrolyte solution concentration is 0.01 M phosphate buffer, pH 7.0) finding that there is a decrease in cathodic current of 2%. Biosensors are stable and can be used to analyze a large number of samples without a significant loss of sensitivity. In all quantitative determinations, quantification must be carried out comparing with a reference sample.

Under optimal conditions of pH, ionic strength and temperature the biosensor based on screen-printed electrodes (SPE) modified with single wall carbon nanotubes (SWCNT) and tyrosinase **(Ty-SWCNT/SPE**) has a good stability, at least two hours of continuous use. Note that cyclic voltammograms recorded in 100 μM tyramine solution (electrolyte solution is phosphate buffer 0.01 M, pH 7.0) slightly decrease in intensity after two hours of continuous use. Cathodic peak current shows a decrease of 3.75% after two hours of continuous use. Therefore, the biosensor has a very good stability.

The stability of the Ty-SWCNT/SPE biosensor signal was confirmed by recording a large number of cyclic voltammograms (100 cycles) in 50 μM serotonin solution (electrolyte solution is phosphate buffer 0.01 M, pH 7.0) . Cathodic peak current decreases with 3.5% confirming the stability of Ty-SWCNT/SPE biosensor to intensive use.

Biosensor **DAO/SWCNT-GCE** shows high stability for continuous use in 50 μM epinephrine solution. The recording of 50 cycles in the potential range 0.5 - 0.5V causes a decrease of voltammetric signal of 2.5%. Similar decreases of voltammetric signal were obtained and for biosensors based diamine oxidase (DAO)

immobilized on different carbonaceous materials (graphite, carbon nanotubes, graphene and carbon nanofibers) to cycling in 50 μM putrescine, showing a good stability of biosensors.

Biosensors based on monoamine oxidase (MAO) immobilized on mixed LB film of AA and $DyPc_2$ shows a very goog stability during cycling, voltammetric signal decreasing 8.5% after 30 cycles. This type of biosensors will be used as disposable biosensors. LB method allows obtaining biosensors with a good reproducibility.

Sensors and biosensors based on SPE shows good stability when were used in the analysis of biogenic amines. Registering a large number of cycles do not lead to significant drops of voltammetric signal. After removing of biosensors and sensors based on SPE are retired from the sample solution and keep for a few hours and re-immersed in the solution to be analyzed, a modification of the signal is observed. It was found that this change is related to degradation of the reference electrode (Ag screen-printed). To ensure accurate measurements, sensors and biosensors based SPE should be used as disposable sensors and biosensors.

1.3 Study of sensors and biosensors reproducibility

Reproducibility of fabrication of **sensors and biosensors based on LB films** is very good, the differences between responses obtained by voltammetry is less than 1%. The differences between the responses obtained by amperometric are less than 0.8%. This is the main advantage of using the LB technique in the deposition of sensitive layers for applications in sensors and biosensors. This technique is optimal for the fabrication of disposable sensors and biosensors.

Reproducibility of fabrication of sensors and biosensors based of CPE modified with phthalocyanines and enzymes is good, the differences between the signals is less than 4% for sensors and biosensors prepared in the same conditions. Despite the fact that the preparation of these sensors and biosensors is handling, the strict compliance of protocol manufacturing provide sensors and biosensors of good quality.

Reproducibility of fabrication of **sensors and biosensors based on polypyrrole** depends on the reproducibility of electrosynthesis, the stage when the polypyrrole is deposited on the electrode by using chronoamperometry (CA). In order to test the reproducibility of sensors fabrication by chronamperometry was calculated the charges consumed on the deposition of polypyrrole in the presence of different doping agents. Five sensors were fabricated for each doping agent. Relative standard deviation (% RSD) is lower than 6% for all doping agents used in electrosynthesis (Table 1).

Table 1. Reproducibility data using CA techniques for preparation of PPy doped with different doping agents

These data confirm the high reproducibility of the method used for the preparation of sensors and biosensors based on polypyrrole.

Also, it was studied the reproducibility of fabrication of **biosensors based on enzyme-modified SPE**. For example, the reproducibility of fabrication of Ty-SWCNT-COOH/SPE biosensors (SWCNT-COOH - carbon nanotubes functionalized with carboxyl groups) was studied by using cyclic voltammetry. It was fabricated three biosensors, it were recorded responses and the voltamperometric signals were compared. The results obtained(cathodic peak current) when Ty-SWCNT-COOH/SPE biosensor was immersed in the solution of tyramine 100 μM (in 0.01 M phosphate buffer, pH 7.0), expressed as % RSD were less than 2%, which demonstrates that the method of fabrication the biosensor has a good quality. This test confirmed the reproducibility of the procedure for immobilizing the enzyme on a solid matrix based on carbon nanotubes functionalized with carboxyl groups.
Similar results were obtained for other enzymes (MAO and DAO) immobilized on nanostructured carbon materials (graphene, SWCNT, SWCNT-COOH, SWCNT-NH_2). The rigorous control of the protocol of fabrication and storage conditions of sensitive compounds are essential for reproducible fabrication of sensors and biosensors.

1.4 Study of the lifetime of sensors and biosensors.

As shown in studies on the stability, **sensors based on LB films of phthalocyanine** are disposable sensors and the possibility of their use in practice is fully justified because the LB technique allows the preparation of sensors with identical characteristics. The same consideration is correct for all types of **biosensors manufactured by means of LB techniques**. These sensors and biosensors shows very good analytical properties, reversible behavior and very low detection limits. Therefore they will be used as disposable sensors and biosensors for the analysis of biological samples of interest in medicine.
Durability (long term stability) of sensors based on **carbon paste electrodes modified with phthalocyanines** was tested by recording voltammetric signals for a period of one month. The sensors were stored in tightly closed boxes in a refrigerator at 4°C. Voltammetric curves were recorded for CPE-based sensors and phthalocyanine in aqueous solutions of electrolytes (KCl solution, phosphate buffer solution pH 6, 7, 8, etc.) and biogenic amines. Variation of voltammetric curves were monitored over time.
By quantifying of changes observed in this time were analyzed regarding potentials peaks changes, peak current and whole variation curves (represented by 10 kernel coefficients). For example, phthalocyanines peak potentials after a month of use (five cyclic voltammograms recorded daily) shows a relative standard deviation less than 2.5% for all sensors and electrolytes analyzed. The same study carried out in the case of biogenic amines showed that peak potential of sensor shows a relative standard deviation less than 5% for all sensors and amines analyzed.
Another study were carried out to test the durability of these sensors in terms of peaks potential, peaks current and a voltammetric curves include the study of four solutions biogenic amines (dimethylamine, trimethylamine, histamine, putrescine) with the same electrode ($LuPc_2$-CPE) for one month. After immersion in each solution of amine several voltammetric curves were recorded (five cycles). Between measurements carried out for each amine, the sensors were washed with ultrapure water and were kept in a hermetically sealed box in the fridge.
In Table 2 are presented the values of relative standard deviation of peak potentials and peak currents of $GdPc_2$ in dimethylamine solution (electrolyte was 0.1M KCl).

Table 2. The values of relative standard deviation of peaks potential for Gd bisphthalocyanine in dimethylamine solution (electrolyte is 0.1 M KCl) after one month

As can be observed in Table 2, the relative standard deviation values for peak potentials and peak currents of phthalocyanine are lower than 5%.

% RSD values for total voltammetric curve were calculated based on the 10 kernel coefficients. The values calculated were below 7% for all the solutions analyzed. This result demonstrates that the sensors based CPE modified with phthalocyanine shows a good long-term stability. This type of sensor can be used for long periods of time and, in addition, if the sensor response is modified this can be recovered.

One of the most important advantages of these sensors is that it is possible the recovering (cleaning) of active surface by removing the first layer by polishing with a filter paper. Another method is the regeneration of sensor signal using cyclic voltammetry by recording 10 cycles in 0.1 M KCl aqueous solution.

In conclusion, the stability of sensors based CPE depends on the number of measurements and the type of samples in which they are immersed. An electrode stored under appropriate conditions, can be used for at least one month after that the regeneration of the active area is required.

For testing the durability of **polypyrrole-based sensors** cyclic voltammograms were recorded in 0.1 M KCl solution in the successive days, for 5 days. The changes observed corresponding to voltammograms recorded were a slight decrease in peak currents. Values relative standard deviation (% RSD) were calculated from the average values of sensor responses (measured as peak intensities). In Table 3 were presented the values of % RSD calculated for cyclic voltammograms recorded in five consecutive days with a sensor based on Ppy/DSA immersed in 0.1 M KCl solution.

Table 3. Values of % RSD calculated for peak currents of cyclic voltammetry of sensor Ppy/DSA registered in 0.1 M KCl during five consecutive days

These data indicate a good stability for a period of five days to all sensors, % RSD values are lower than 20%.

In case of using integral curve (10 kernel coefficients for a curve) % RSD values are with 2-3% higher than values calculated for peak current.

Instead,% RSD values are less than 7% when are take into account the peaks potentials.

It must be emphasized that sensors can be replaced easily because fabrication of sensors based on polypyrrole is very reproducible.

In another study were analyzed the biosensors based on polypyrrole and tyrosinase with the purpose of determining the sustainability and preservation of enzyme activity at room temperature and in the refrigerator. The stability of the enzyme was monitored biosensors for measuring the signal in a solution of known concentration of dopamine (50 μM). At room temperature (20°C) enzymatic activity decreases rapidly, observing a decrease of cca. 70% of the initial enzyme activity in 10 days. A much smaller decrease of biosensor response was observed when the electrode is kept at 4°C in refrigerator. It was calculate that the biosensor preserve 85% from the initial activity of the enzyme after a month. After this period, a accelerated decrease of enzymatic activity was observed. The conclusion of this study was that in order to

maintain the sensitivity of biosensors for a relatively long period of time, they must be kept in the refrigerator.
Similar results were also obtained when the enzyme immobilized in the carbon matrix was DAO, 77% from the enzyme activity is maintained after one month of storage in refrigerator.
The stability of the Ty-SWCNT/GCE biosensor during storage in a refrigerator (at 4°C) was confirmed using the biosensor every 24 hours for a month. Evolution of biosensor response during time is as follows. After 15 days, the biosensor response began to decrease. However, the biosensor was maintained 89% of the response (cathodic current) after one month of storage. The stability of Ty-SWCNT/GCE biosensor is good, the biosensor being able to use for at least a month with a proper drift correction.
Durability study of biosensors based on SPE and LB was not necessary because their stability is low (few hours).

1.5 Determination of detection limits of sensors and biosensors

For determination of the limit of detection (LOD) were constructed calibration curves of sensors and biosensors. Measurements were carried out in optimal conditions of pH, temperature, electrolyte etc. LOD was calculated using the relationship LOD = $3 \times \sigma / m$ (σ-relative standard deviation of the biosensor signal corresponding to the lowest concentration from the calibration plot, m- slope of calibration curve). Values of detection limits are between 10^{-5}-10^{-6}M in the case of sensors and 10^{-6}-10^{-8}M for biosensors. In the following tables are presented some results obtained for a series of sensors and biosensors developed and characterized in this research project.

Table 4. LOD values for sensors based on CPE modified with different phthalocyanines for different amine compounds

Tabelul 5. LOD values for sensors based on polypyrrole doped with different doping agents on detection trimethylamine

Tabelul 6. LOD values for sensors based on polypyrrole doped with different doping agents on detection of ammonia and putrescine

Table 7. LOD values of SPE sensors based on carbon nanofibers, carbon nanotubes, and graphene

Table 8. LOD values for different biosensors developed in this project

These good results are related to the nature and structure of the sensing element containing nanostructured materials, compatible with biogenic amines and using of electron mediators and enzymes that facilitate the transfer of electrons, increasing sensitivity and selectivity.

1.6 Determination of the response time of the sensors and biosensors

The response time of the sensors and biosensors depends on the technique used for measurement. Thus, if the technique used is cyclic voltammetry, the time for recording of the response can vary among 30 seconds and 5 minutes, depending on the scanning speed and potential range used. For the sensors based on carbon modified with phthalocyanines, cyclic voltammograms were recorded with a scan rate of 100 $mV \times s^{-1}$ and a range of potential from -1V to 1.3V. The same conditions were used in the case of LB sensors-based on phthalocyanines. For sensors based on polypyrrole, cyclic voltammograms were recorded with a scan rate of 50 $mV \times s^{-1}$ and a potential range from -1V to 0.5V.

In the case of biosensors, cyclic voltammograms were recorded with a scanning rate of 50 mV × s^{-1} and a potential range from -0.5V to 0.5V. For amperometric measurements, biosensors response was quantified as the time to the equilibrium current from the initial state to the new steady state equilibrium which is reached by adding a known amount of analyte. Response time values ranging among 3 and 10s in the optimal operating conditions such as applied potential, pH, ionic strength and temperature.
In conclusion, the time required for analysis is much reduced. Therefore, sensors and biosensors developed in this project are able for on-line in-line and real-time measurements of food and biological samples.

1.7 Determination of reversibility and recovery of sensors and biosensors

Recovery of sensors and biosensors after the use in the analysis of samples depended on the type of sample and number of measurements. The sensors based on carbon paste were recovered after using in electrochemical measurements, the modifications are reversible, by using two methods: polishing of surface with a paper filter and by cycling in 0.1M KCl solution. Both methods provide a good recovery of sensors.
Method of cycling in 0.1M KCl solution is the best method for recovery of sensors based on polypyrrole. By this method the contaminants were eliminates from the surface of the sensors, the modification of polypyrrole is one reversible.
In the case of biosensors, the recovery of voltammetric and amperometric signal was not possible after an intensive use. Contamination of biosensor are related to irreversible modification of enzyme conformation and was not possible to recover the initial state. Storage in inappropriate conditions led to the protein denaturation and losing of biocatalytic activity of enzyme.
In conclusion, the research carried during 2014 were dedicated to the development of sensors and biosensors completely characterized in the terms of analytical performances, life time and condition of use. The knowledge of sensors and biosensors behavior will be used for selection of sensors and biosensors for applications on real samples.

In the period January-December **2015 were** carried out activities included in the project implementation plan, activities needed to achieve the overall objective of the project, an electronic system based on chemical sensors and biosensors for analysis of biogenic amines.

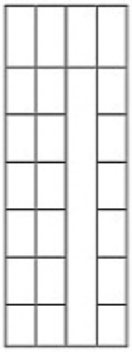
Testing of sensors and biosensors

1. Selection of sensors and biosensors arrays for applications on real samples

For the analysis of real samples were selected sensors and biosensors with appropriate properties for the aim of this research and those that are able to detect biogenic amines with adequate sensitivity.

The first category of sensors that were included in sensors array of electronic system are sensors based on polypyrrole doped with different anions. Doping anions and electrosynthesis conditions are presented in Table 1.

Table 1. The conditions of electropolymerization used in the development of polypyrrole sensors



The second category of sensors included in the sensors array are ***screen-printed electrodes*** based on carbon nanomaterials, employed as received or modified in the laboratory with phthalocyanines. Screen-printed carbon electrodes (4 mm in diameter) purchased from Dropsens (www.dropsens.com, models 110 CNT, 110 GPH, 110 CNF) were used. These screen-printed electrodes are designed for the development of sensors and biosensors with an enhanced electrochemical active area. Carbon nanomaterials (Carbon Nanotubes-CNT, Carbon Nanofibres-CNF and Graphene-GPH) have excellent mechanical, electrical, and thermal properties thus become excellent modifier to the carbon matrix for enhancing the sensitivity and selectivity.

From the category of biosensors were included a variety of biosensors in the array; the most important ones are summarized below.

Carbon paste based biosensor for serotonin. Carbon paste electrodes were prepared by mixing carbon nanopowder (<50 nm particle size (TEM), ≥99% trace metals basis, Sigma-Aldrich) and the cobalt(II) phthalocyanine (15%, w/w). Nujol was used as the binder of the multi-component composite mixture. Paste was packed into the body of a 1mL PVC (polyvinylchloride) syringe and compacted. A metallic copper wire was used as an electrical contact. The tyrosinase (Ty) was immobilized on the above CoPc-CPE (CPE modified with CoPc) by drop-and-dry technique followed by cross-linking with glutaraldehyde.

Glassy carbon electrode based biosensor for catecholamines. The glassy carbon electrode (GCE) surface was polished with alumina paste, washed with ultrapure water before and rinsed in methanol. The electrode active part was a 4 mm diameter disk. The other parts of carbon electrode were covered with isolating epoxy resin. After cleaning process, the GCE surface was coated with 10 μL of the SWCNTS suspension (1.0 mg×mL^{-1} in methanol). The solvent was evaporated in air at room temperature. The

enzyme, Ty, was immobilized on the above GCE modified with SWCNTs (SWCNT-GCE) by drop-and-dry technique followed by cross-linking.

Polypyrrole doped with anions used in the case of sensors were used for immobilization of DAO by cross-linking. Different types of ***biosensors based on polypyrrole and aminooxidases*** (monoamine oxidase A, monoamine oxidase B, and diamine oxidase, respectively) were also included in the array. They have different sensitivities and selectivities towards biogenic amines. For example the biosensor based on diamino oxidase (DAO) from Porcine kidney, E.C. 1.4.3.6), cross-linked by glutaraldehyde on electrosynthesized polypyrrole films is high sensitive for histamine detection.

A biosensor for the determination of dopamine and epinephrine by using ***tyrosinase*** (from mooshroom, E.C. 1.14.18.1), cross-linked by glutaraldehyde on ***graphene modified screen-printed carbon electrodes*** was developed and included in the array.

For the determination of catecholamines a biosensor based on ***tyrosinase***, cross-linked by glutaraldehyde on *amide functionalized single-walled carbon nanotubes* modified screen-printed carbon electrodes was included in the array.

Ty-SWCNT-COOH/SPE *biosensor* for tyramine. 5.0 $mg \times mL^{-1}$ of tyrosinase solution was prepared with 0.01 M phosphate buffer solution at pH 7.0. 50 µL of 0.01 M phosphate buffer (pH 7.0) containing 5 $mg \times mL^{-1}$ of Ty was drop coated onto 12.56 mm^2 area of SWCNT-COOH (*carboxyl functionalized single-walled carbon nanotubes*) thick film, and dried at 4°C for 10 min. Then the electrode was treated with glutaraldehyde vapour to immobilize Ty onto SWCNT-COOH/SPE surface resulting in a Ty-SWCNT-COOH/SPE biosensor. After that, the biosensor was fully washed with ultrapure water to remove all chemicals physically adsorbed.

A biosensor based on a ***carbon screen-printed electrode modified with Prussian Blue and diamine oxidase*** was included in the array.

The biosensor ***based on Langmuir – Blodgett film of tyrosinase, arachidic acid and dysprosium bis-phthalocyanine*** for the electrochemical detection of tyramine and dopamine was tacken into acount for the array.

In conclusion, in this task were selected the sensors and biosensors optimal for detection and/or quantification of biogenic amines. The novel sensors and biosensors are based on different electrode designs, different materials for immobilization, electron mediators and enzymes. Multi(bio)sensor system developed is able to detect all categories of biogenic amines.

2. Selecting methods for data processing

The objective of this activity is to establish the methods of data analysis in order to discrimination and classification of samples analyzed and establishing correlations between different types of measurements.

For processing the data obtained with sensors and biosensors developed in this project they were optimized and used several methods of multivariate data analysis: principal component analysis (PCA), partial least squares - discriminant analysis (PLS-DA), analysis of variance (ANOVA), Soft independent modelling of class analogies (SIMCA), t test, multiple regression by partial least squares method for a parameter or several parameters (PLS1 and PLS2).

Sensors and biosensors provide complex voltammograms (variety of peaks at different potentials and with different currents). The intrinsic complexity and cross-selectivity of the signals generated by the array of sensors and biosensors are advantages because the data set contains large amount of information about the sample. But, the fact that the whole data set contains meaningful information can difficult the data processing. In consequence, a pre-treatment step to reduce of the number of variables (without loss of information) is required.

For the analysis of the data obtained with sensors or biosensors developed in this project it is required a preprocessing step. Cyclic voltammograms (CV) and the voltammograms obtained by square wave voltammetry (SWV) are differently analyzed.

Using this method, SWV curve is multiplied by a number of 10 kernel functions, and further integrated with respect to potential. Ten parameters for each SWV are obtained. An example of applying the kernel method to data obtained by square wave voltammetry is presented in Figure 1.

Figure 1. Applying of the kernel method for the voltamograms registered by SWV

Cyclic voltammograms were mathematically pre-processed and used as data source for multivariate data analysis. Using kernel method, the cyclic voltammogram curve (i vs. E) is divided in anodic and cathodic part. Then, the anodic curve is multiplied by a number of 10 smooth, bell-shaped windowing functions, and integrated with respect to potential. By this pre-processing technique the information throughout the global response is reduced to 10 representative parameters per each curve. An example of applying the kernel method to data obtained by cyclic voltammetry is presented in Figure 2.

Figura 2. Aplicarea metodei kernel pentru voltamogramele inregistrate prin CV

Once the voltammograms have been pre-processed and the number of variables reduced, such variables are used as the input for multivariate data analysis using for instance Principal Component Analysis or Partial Least Squares Discriminant Analysis as discrimination and classification methods. Other methods were also employed as described below.
In the following paragraphs are discussed some of the results obtained in the research work carried out and applying different methods of data analysis.

Array of sensors based on Ppy. Discrimination of biogenic amines

The responses obtained when using SPE modified with polypyrrole showed a high degree of complexity, since transient responses observed in the voltammograms are related to the electrode material and to the nature and concentration of the amine molecules present in the solutions (and to the interactions electrode-solution).
This makes possible to use the sensors in an array configuration. The pattern of responses generated by the array is a fingerprint of the sample studied. This pattern can be related with certain features or characteristics of the samples by means of chemometrics.
In order to evaluate the discrimination capabilities of the array of voltammetric sensors, Principal Component Analysis was conducted using the information obtained from the array formed by Ppy modified SPE sensors. Figure 3 shows the PCA results as a three-dimensional scores plot of principal

components that allow obtaining well-defined and separated clusters.

Figure 3. PCA score plot of the cyclic voltammograms of the amine solutions with Ppy –based sensor array

PCA has been validated by full cross validation method and an optimal number of 5 principal components have been used. The first three principal components explain the 97% of the information (PC1= 57%; PC2=24%; PC3=16%).

The separated clusters indicate that the five solutions could be clearly discriminated from each other. In addition, the positions of the clusters are related to the electrochemical properties of the tested solutions. It has to be noticed that the cluster corresponding to the ammonia, appears in the left side of the diagram, far apart from the rest of the amines. Aliphatic amines appear in the right side of the diagram. A clear discrimination between primary amine (CAD), secondary amine (DMA) and tertiary amine (TMA) is observed, also. The heterocyclic amine, HIS has a particular electrochemical behavior that permits to discriminate it from aliphatic amines and ammonia.

Fish freshness monitoring

Fish freshness has been monitored trough the global assessment of spoilage products (including biogenic amines) using a multisensor array based in Ppy. For this purpose, fishes were eviscerated, washed and stored at 4°C during 10 days in a closed box. Every day, muscle samples were prepared and measured with Ppy based sensors.

A characteristic pattern of the deterioration of fish stored in ice can be divided into four phases: i) fish is fresh and has a sweet, seaweed and delicate taste (highly fresh); ii) there is a loss of the characteristic odor and taste. The flesh becomes neutral but has no off-flavors (fresh); iii) there is sign of spoilage and a range of volatile, unpleasant-smelling substances are produced; iv) fish is spoiled and has a putrid odor.

Principal Component Analysis was used to analyze the degradation process measured with the array of sensors. Figure 4 shows the PCA score plot obtained using the electrochemical signals registered every day using Ppy-based sensors.

Figure 4. PCA score plot of fish freshness monitoring with Ppy –based sensor array

The PCA score plot of the three first principal components accounts for 79% of variance. Clearly discriminated clusters can be observed. The first cluster, that appears in the left side of the figure corresponds to samples analyzed days 1 and 2 and correspond to a highly fresh product. Samples analyzed in days 3 and 4 did not show any off odor and could be classified as fresh product. The clusters appear in the central part of the figure. Samples collected days 5 and 6 showed off odors (degraded product). The last clusters that appears on the right side of the figure corresponds to samples collected on days 7 to 10 (spoiled fish).

PLS-DA was used to classify the day of fish degradation from the sensor array response.

A supervised method, the Partial Least Squares-Discriminant Analysis (PLS-DA) was used to evaluate the classification capability of the system.

As presented in Table 2, the fully cross-validated PLS-DA model (using an optimal number of 6 latent variables), revealed a clear identification of the fish degradation phases. Table 2 collects the quantitative data derived from the PLS-DA regression model.

Table 2. Results of the calibration and validation of PLS-DA

As observed, both the calibration and the validation values involved a good-quality model performance are achieved (large correlations between sensors and categorized variables, and low root mean square errors of calibration and validation). These results indicate that this methodology is able to real time monitor the fish freshness during storage.

Sensors based on carbonaceous materials for detection of biogenic amines.

SPEs modified with three carbonaceous materials: CNT, GPH and CNF were used for the analysis of different biogenic amines in the solution. The PCA score plot is presented in the Figure 5.

Figure 5. PCA score plot of CV obtained with SPEs in amine solutions

The sensors array is able to discriminate among solutions of biogenic amines.

Application in detection of histamine

Different types of fish samples were analyzed with the developed biosensor. The purpose of this diversity of samples was to evaluate if the biosensor was able to quantify the amount of histamine in different fish species with good reliability.

The quantification of histamine was carried out with the biosensor based on DAO immobilized on carbon screen-printed electrodes modified with graphene. Histamine quantification was performed by means of direct interpolation in the histamine calibration plot. The standard addition method was also used in order to study the influence of matrix effect. Under the optimum established conditions, amperometric

measurements were carried out in an electrochemical cell containing 20 mL of phosphate buffer solution at pH 7.4 and applying a potential of 0.4 V vs. Ag. All amperometric measurements were done in triplicate. As can be observed in Table 3, there are relative small differences between interpolation and standard addition methods, concluding that the matrix effect was not significant in histamine detection.

Table 3. Amounts of histamine in different fish samples determined by means of biosensor and ELISA kit. Results obtained by amperometric measurements are expressed with their interval of confidence (n = 3, confidence level of 95%).

The evolution of histamine content in fish samples stored at 4 °C was studied initially and after 48h for all fish samples. Obtained results are shown in Figure 6.

Figure 6. Evolution of total histamine content in fish samples. Samples were stored at 4 °C. Values of histamine concentration are the average of all quantification methods.

For all samples there was a clear increasing of histamine content, demonstrating an increment of toxicity as the time of storage increases. For this reason, histamine concentration levels could be used as an indicator of fish quality or fish freshness. Control of quality and freshness is especially important to prevent scombroid syndrome, which results from eating spoiled fish.

As an additional proof of the proposed methods, a Student's paired samples t-test was performed between the results obtained with the amperometric biosensor and the ones obtained with the ELISA kit. In the case of measurements by interpolation and standard addition, obtained experimental t values was 0.4678, while the critical tabulated t value was 2.2621 (95% confidence level and 9 degrees of freedom). Therefore, there are no significant differences between the concentrations found by interpolation and standard

addition method. On the other hand, experimental t values were 0.1678 and 0.7263, respectively, when were compared interpolation method vs. ELISA and standard addition method vs. ELISA. Experimental t values are lower than the critical t value in both cases (2.2621 at 95% confidence level and 9 degrees of freedom). It was concluded that there are no significant differences between the concentrations found with the amperometric method and the ELISA kit method.

Use of ANOVA in data analysis

Electrochemical data were tested for statistical significance using one-way ANOVA routines running under Excel. The factor was fish species. Values of $p < 0.05$ were considered statistically significant. In Table 4 are presented the ANOVA significance results. ANOVA was based on honestly significant difference test (Tukey test). This test is based on pairwise comparison among means as presented in equation:

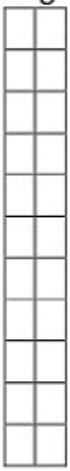
$M_i - M_j$ = difference between pair means

MSE = mean square error

n_h = the harmonized mean

As presented in Table 4 the population means are significantly different. The samples could be classified in groups in agreement with type of fishes. ANOVA showed significant differences among these groups based on the results obtained with biosensors.

Table 4. ANOVA significance results



* $p < 0.05$ (significant).

** $p < 0.01$ (highly significant).

*** $p < 0.0001$ (extremely significant).

Basic statistics

Ty/PO4-Ppy/Pt biosensor was applied to the determination of tyramine in salted sauerkraut samples. Total tyramine content of the sample, expressed in tyramine equivalent units, was analyzed using the standard addition method.

Amperograms were recorded under the optimum conditions (applied potential −0.250 V, constant stirring of the sample, pH 7.0). A volume of 100 μL of the extracted tyramine was placed into the electrochemical cell, containing 5 mL of phosphate buffer solution 0.01 M of pH 7.0. After that, successive additions of 100 μL of a 10 μM tyramine solution were carried out. Current vs. concentration regression parameters were evaluated using the XLstat software. The concentration of tyramine found was 15.56±0.42 μM ($n = 5$, $\alpha = 0.05$), with a RSD of 2.16%.

Therefore, concentration level found in sauerkraut sample was 264.52±7.14 mg kg^{-1}, value according to relative low content of biogenic amine in sauerkraut sample. To check the viability of this procedure in the determination of tyramine in salted sauerkraut samples, recovery studies were also performed. The total amount of tyramine in a spiked sample, 54.78 μM, was determined by standard addition. Five replicates were carried out. The concentration of tyramine found was 54.51 ± 1.60 μM (n = 5, a = 0.05, RSD = 2.36%), with an average recovery of 99.32%.

Multivariate regresions

Changes in pH in the fish samples throughout the storage time are summarised in Table 5. Partial least squares (PLS1) regressions were performed to model the relationships between biosensor signals and the pH values of the samples. The initial pH of fish flesh was close to 7.49, which is a typical value in very fresh products. In the solution obtained by procedure beforementioned the pH is 6.81, which indicate that the KCl aqueous solution do not influence the pH of the fish flesh.

During the storage is registered a decrease in the pH as a consequence of the enzymatic and chemical processes occurred in the beginning the post-mortem stage. After 5 days the pH of the solution started to increase as consequence of the biogenic amine generation. An excellent correlation was obtained using a model with 5 latent variables and validated by full cross validation method. The regression coefficient was 0.973 in calibration and 0.961 in validation. Additionally, low values of RMSEC (0.042) and RMSEP (0.051) were accomplished.

Table 5. pH evolution during fish storage at 4°C

Classification by SIMCA

Soft Independent Modelling of Class (SIMCA) has also been applied to classify the samples. This method calculates several PCA models for each class in a training set (full cross validated with an optimal number of principal components). In this case, the models were constructed using the healthy people (class 1) and seek people (class 2). Then, external unknown samples or replicates are compared to the class models and assigned to classes (seek or health) according to their likeness to the original training set. To display the results obtained with SIMCA, Coomans plots have been used, in which the unknown data is recognized or not by a model with a confidence interval of 95%.

As observed in the Coomans graphic presented in Figure 7 a 100% classification of clinical samples was achieved.

Figure 7. Coomans plot of clinical samples classification

Two type of database were constructed: one for quantification of specific analyte, and the second for classification of the samples in function of their characteristics.
Good correlations have been found between the signals obtained from the expert sensory system and the biogenic amine content by means of PLS regression models. Using SIMCA or PLS-DA models, correlations between (bio)sensor responses and heart diseases were established.

In conclusion, a great varieties of methods were developed and applied for data analysis with good results facilitating the establishment of data significance.

3. Analysis of biogenic amines in meat products, cheese and fermented beverages
For this purpose we have developed a multibiosensor system coupled with a multivariate data analysis for detection and/or quantification of biogenic amines. Analyses of samples were carried out by means of amperometric and/or voltammetry techniques.
Analysis of biogenic amines in meat products.
This work describes the development of a bioelectronic tongue for the quantification of biogenic amines in meat products. The second application is the monitoring of meat products quality in time, in accelerate degradation conditions. The bioelectronic tongue includes an array of four biosensors developed and optimized in the laboratory. The biosensors are based on carbon screen-printed electrodes modified with single-wall carbon nanotubes and enzymes. The enzymes were tyrosinase, diamine oxidase, peroxidase and monoamine oxidase. The measurements with biosensors were carried out by amperometry and cyclic voltammetry. Cyclic voltammograms show redox processes related to the electrochemical activity of the compounds from samples or formed in the reactions catalyzed by enzymes (i.e. biogenic amines, hydrogen peroxyde, hydroquinone derivatives). Data analysis were carried out by means of Principal Component Analysis, Partial Least Squares Discriminate Analysis, Partial Least Squares regression, and Analysis of Variance.
For instance, in Table 6 are presented the performance characterists of a biosensor used in the quantification of biogenic amines in meat products.

Table 6. Quantification of biogenic amines in meat products

It was found that bioelectronic tongue system is able to quantify the biogenic amine content in meat products (Italian ham, Sibiu salami, Banatean salami). Images of samples used in these studies are presented in the Figure 8.

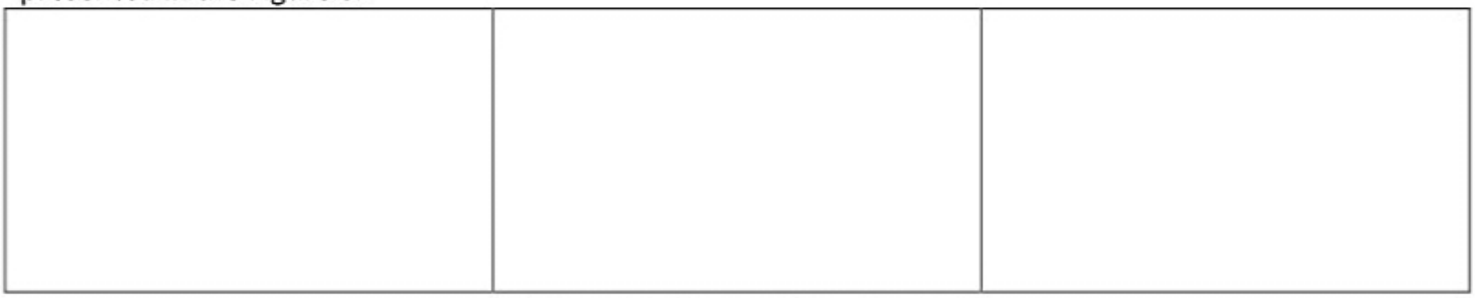


Figure 8. Meat products under study

The values are in the range 380-450 mg×kg^{-1}, values below the maximum limit permitted in this type of food products. In the accelerate conditions of degradation, the increasing of biogenic amine amount is easy to follow by using the models based on bioelectronic tongue measurements.

Analysis of biogenic amines in beef meat extracts

In another study PPy based sensors were used for detection and quantification of biogenic amines in beef meat extracts. The performances of sensors towards amonia and putrescine are presented in the Table 7.

Table 7. Performance characteristics of PPy based sensors

Ppy/FCN sensor had the lowest limits of detection. The recovery is near to 100% for all sensors highlighting the viability of sensors to detect putrescine and ammonia in beef extract solution.

Analysis of biogenic amines in pickled and smoked fish samples

The tyramine level in different pickled and smoked fish samples was in the range of 16.7-61.8 mg×kg^{-1} (Table 8). These levels of contamination are below the acceptable level of tyramine in foods.

Table 8. Quantification of biogenic amines in fish samples

In other study the histamine content in carp fish samples was quantified during 5 days. In Table 9 are presented the results obtained.

Table 9. Histamine content in carp fish samples

The histamine content increasing during storage in the fridge at 4 Celsius degree. The histamine content after 4 days can have negative influences in the human health.

Analysis of biogenic amines in cheeses.

This paper describes the designing and optimization of an electrochemical biosensor for the determination of biogenic amines in blue cheese samples. The developed biosensor is based on a carbon screen-printed electrode, modified with Prussian Blue, which detect hydrogen peroxide produced by the reaction catalyzed by the diamine oxidase immobilized onto the surface of the electrode. Therefore, the biosensor detection mechanism is based on the electrochemical reduction of hydrogen peroxide.

The experimental conditions that influence the biosensitive properties of biosensors were optimized. In the optimal conditions of pH and potential applied, the biosensor performance characteristics were quantified. The measurements with the biosensor were carried out by amperometry, the current flowing through biosensor at –0.06 V was measured as a function of concentration of H_2O_2 in 100 mM phosphate buffer (pH 7.4).

The biosensor shows a low detection limit (0.045 μM), and a linear range from $2x10^{-6}$ to $4x10^{-5}$ M. The biosensor fabrication is reproducible, relative standard deviation being 3.2%. Furthermore, the biosensor has good repeatability, and high affinity to biogenic amines typically found in blue cheeses.

Images of samples used in these studies are presented in the Figure 9.

Figure 9. Chesses samples under study

Quantification of biogenic amines in cheese samples (Gorgonzola, Brie, Danish blue, and *Brânză de burduf*) was validated by standard addition method.

The results obtained are summarized in the table 10.

Table 10. Results of biogenic amine quantification in cheese samples

Analytical recovery of added BAs in the cheese samples are presented in the table 11.

Table 11. Analytical recovery of biogenic amines in cheese samples

The efficiency of biosensor for determinate BAs in cheese samples has been demonstrated.

Analysis of biogenic amines in fermented beverages.

Wine and beer have been reported as a cause for headaches with patients susceptible to migraines. Histamine in alcoholic drinks could cause of allergic and allergic like adverse responses. Wine has been a more common source than beer. Principal biogenic amines that could be found in wines were detected and quantified in red wines and beers using biosensors developed in this project.

In the case of wines several red wine samples were analyzed. Samples were analyzed with the biosensors array by triplicate. Results obtained are included in Table 12.

Table 12. Biogenic amines in wine samples

Beer samples were analysed by means of biosensors array and the results obtained are included in Table 13. RSD of the biogenic amines detection was 3.4%.

Table 13. Biogenic amines in beer samples

Other fermented food analyzed was the sauerkraut samples. Recovery (%), RSD and 95% confidence interval for mean for extracted tyramine from salted sauerkraut samples are presented in table 14.

Table 14. Biogenic amines in sauerkraut samples

	Tyramine	Recovery (%)	RSD (%)	95% confidence mean
Tyramine spiked lev				
Spiked sauerkraut s				
39.22				

The efficiency of biosensor for determinate tyra6mine in sauerkraut samples has been demonstrated. Biosensors developed in this project have shown excellent performance characteristics and were successful employed for detection of biogenic amines in a great variety of samples.

In this year were carried out all the activities from the work plan and the results obtained were in agreement with the objectives of the research. The objectives of this year were completely accomplished.

Scientific report regarding the project implementation during V stage

January 2016 – June 2016

In the period January- June 2016 were carried out the activities included in the research plan of the project. These activities were necessary in order to finalize the project and to accomplish of the project main objective, an electronic sensory system based on chemical sensors and biosensors for the analysis of biogenic amines.
In the next pages it will be presented the activities carried out and the accomplishment of the objectives of this stage.

The objective of this stage was: **Test of sensors and biosensors in real samples. Applicative studies.** For this purpose, sensors and biosensors developed in this project were applied in the analysis of real samples taking into account most of the important aspects regarding the analysis of the foods, pharmaceutics, and clinical samples.

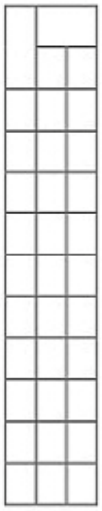
1.1. Monitoring of food freshness

Different studies regarding food freshness monitoring were carried out during this stage of the project.

Freshwater fish freshness monitoring

In this study, we present a novel biosensor for the accurate quantification of histamine. The sensitive element is based on diamine oxidase immobilized into a nanostructured nPt/GPH/chitosan thick film (nPt – platinum nanoparticle, GPH – graphene) of a modified carbon screen-printed electrode (CSPE). For this purpose, several experimental parameters have been studied with the aim of identifying the most favorable ones that improve its electrochemical signal characteristics. In addition, the novel biosensor has been validated in the laboratory regarding the quantification of the histamine amount in several freshwater fish samples to evaluate the applicability in practice.
Different freshwater fish samples were analyzed with the DAO/nPt/GPH/chitosan/CSPE biosensor for histamine detection and quantification. The purpose of this relatively high diversity of samples was to evaluate if the quantification of the histamine amount in different freshwater fish species could be carried out with good reliability.
Histamine quantification was performed by interpolation of biosensor response in the calibration plot. For the recovery studies and for the influence of the matrix effect in the detection of histamine, the standard addition procedure was implemented. In the optimal experimental conditions, amperometric measurements with biosensors were performed in triplicate. The results are presented in Table 1.

Table 1. Quantities of histamine in freshwater fish samples under study



[1] n = 3; 95% confidence level.

As can be noticed in Table 1, there are relatively small differences between direct interpolation and addition methods. Therefore, the matrix interfering effect is not significant in histamine detection in freshwater fish samples.

The freshness evolution was followed by the quantification of histamine amount in all freshwater fish samples stored at 4 °C. Histamine contents were determined initially (very fresh samples) and after 48 h of storage for all fish samples. The results obtained are revealed in Figure 1 in the form of a bar diagram.

Figure 1. Increments of total histamine amount in freshwater fish samples. Values reported are the average of all quantification methods. S1—Carp (fresh); S2—Carp (48 h); S3—Tench (fresh); S4—Tench (48 h); S5—Prussian carp (fresh); S6—Prussian carp (48 h); S7—European perch (fresh); S8—European perch (48 h); S9—Wels catfish (fresh); S10—Wels catfish (48 h).

For all samples in the study, the histamine contents increased after 48 h of storage. It is recognized that histamine amount levels could be considered specific and reliable indicators of fish freshness. The quality of fish is also related to histamine content. Quality control and freshness are important for fish samples in order to prevent scombroid syndrome, which is related to the ingestion of spoiled fish. It could be successfully implemented with this novel biosensor.

As a supplementary confirmation of the method based on a DAO-nPt/GPH/chitosan/CSPE biosensor, was based on a Student's paired samples *t*-test (95% confidence level, nine degrees of freedom). The Student's *t*-test com method. When comparing on method, the experimen 2621. Therefore, the differe method and the standard a btained *t*-values were 0.16 tandard addition method *vs* oretical critical *t*-value of 2. by means of the biosensor sensor.

Applying the sensor array in monitoring of beef freshness

This study has developed, characterized and used voltammetric sensors based on carbon modified by bisphthalocyanines and polypyrrole doped with various dopants for the detection and quantification of putrescine and ammonia. In optimal experimental conditions, sensors array have been used for the detection and quantification of putrescine and ammonia in beef extract powder. Another study dealt with monitoring the freshness of beef preserved in refrigeration conditions.

The beef freshness was monitored using the sensor array based on polypyrrole and bisphthalocyanine. To this effect, the beef (cutlet and chine) was cut in small-sized portions and refrigerated at 4 °C for ten days in an air-sealed plastic box.

Every day, 5 g of beef was blended, adding 45 mL KCl solution. The mix was ultrasonicated for 5 minutes and centrifuged at 4000 rpm for 5 minutes. Then the cyclic voltammograms with the sensors array in supernatant solution were recorded. The experimental protocol was repeated daily for 10 days, resulting 7 replicated for each sensor every day of the study.

The scheme of the experimental procedure is presented in Figure 2.

Figure 2. Experimental setup for extraction of biogenic amines

The electrochemical results were processed and organized in a matrix where the variables (20 values for each sensor) are presented vertically, and horizontally, the 7 replicates of all samples. The matrix had 6 sensors × 20 coefficients (120 variables) and 7 replicates × 10 days (70 samples).

For beef freshness monitoring, the principal component analysis was applied. Figure 3 depicts the plot of PCA scores obtained from the electrochemical signals recorded daily with the sensors array.

The scores graph of the three principal components explains 81% of variance. It may be seen that there is a good separation between the clusters specific to each monitoring day. Clusters groups corresponding to days 1 and 2, 3 and 4, 5 and 6, 7, 8-9 and 10 were observed. These clusters are correlated with the evolution phases of the degradation of beef process.

Figure 3. PCA scores plot

In order to determine the classification capacity of the sensors array, we used PLS-DA. The PLS-DA method is supervised, so that a sample's belonging to a group is verified and the error is calculated both at the calibration and validation of the model. The PLS-DA model was built and validated, and the quantitative results are depicted in Table 2.

Table 2. Quantitative results of the calibration and validation of PLS-DA

RMSEC (Root Mean Square Error of Calibration)
RMSEP (Root Mean Square Error of Prediction)

On calibration, the samples analyzed in the 10 days of the study are 100% correctly classified in the four groups observed during PCA. The validation of the PLS-DA model was performed using the leave-one-out fully cross validation method, obtaining in all cases more than 96% levels of correct classification with higher than 97% sensitivities and more than 96% specificities.
The PCA and PLS-DA models have proved useful in the discrimination and classification of beef storage. By screen-printing technology, the preparation of miniaturized devices is possible, which is highly promising for the mass-production of low-cost, single-use sensors, with applicability in food industry.

Applying of bioelectronic tongue in monitoring of pork meat freshness

Bioelectronic tongue system includes an array of biosensors coupled with appropriate software for multivariate data analysis. This study describes the development of a bioelectronic tongue for the quantification of biogenic amines in pork meat products. The second application was the monitoring of pork meat freshness, in accelerate degradation conditions.
Developing of a novel array of biosensors based on carbon screen-printed electrodes modified with single-wall carbon nanotubes and enzymes. The enzymes were tyrosinase, diamine oxidase, peroxidase and monoamine oxidase. The measurements with biosensors were carried out by cyclic voltammetry in order to detect the biogenic amines in pork meat products.
Monitoring of the meat products freshness in time was carried out for 12 days. The procedure for processing of the sample is similar with those used for beef. The bioelectronic tongue system is able to follow the degradation process and the model could be used for the monitoring of pork meat freshness, assigning the period of storage. This work has demonstrated the high quality performances of a novel biosensors array coupled with appropriate software for data analysis in detection and quantification of biogenic amines. Biosensors based on enzymes immobilized on SWCNT-SPE have shown high sensibility and selectivity towards principal biogenic amines. Determination of biogenic amines by using biosensors is a useful, rapid and sensitive method with applicability in food industry: quality of meat products and freshness monitoring.

Applying of hybrid electronic tongue in monitoring of pear ripening

An array formed from sensors and biosensors, presented in Table 3, was used for the monitoring of pear ripening based on detection of biogenic amines. The method of detection employed was the cyclic voltammetry.

Table 3. Sensors and biosensors included in the array

The sample treatment consists in weighting of 10 g of pear pulp, addition of 40 mL of buffer solution 0.1M of pH 7.4 and heating at 40°C for 10 minutes. The heating was necessary in order to deactivate the polyphenol oxidase present in fruit pulp.

The voltammetric signals of the sensors and biosensors were registered daily during 10 days keeping the fruits at 25°C in humid atmosphere. Each day, a representative sample was processed and the measurements were carried out with (bio)sensors in 7 replicated. The data was structured in a matrix and multivariate data analysis methods were applied.

The PCA score plot has shown that the ripening process could be followed and this complex process is related to biogenic amine content.

In order to establish the capacity of the system to classify the samples in function of ripening stages PLS-DA model was developed. The quantitative data of the model are summarized in Table 4.

Table 4. Quantitative data resulted from PLS-DA model

1.2. Analysis of biogenic amines in fruits

Biogenic amines are synthesized and degraded during normal metabolism of animals, plants and microorganisms. Some fruits are particularly rich in biogenic amines, especially dopamine.

Detection of dopamine in fruits

Development of novel biosensors, which combine biological recognition through enzyme (tyrosinase) specificity with construction simplicity (screen-printing technology), have been carried out.

Cyclic voltammetry has been applied to study the detection of dopamine in fruits.

The response dependences and amperometric characteristics comprising sensitivity, kinetics, linear range, limits of detection and stability of the prepared enzyme electrode in the detection of dopamine have been investigated.

The images of samples used in this study are presented in Figure 6.

Figure 6. Image of Cavendish Banana, Avocado and Plantain samples

The results obtained with the biosensor are included in Table 5.

Table 5. The results obtained with biosensor based on tyrosinase detecting dopamine in fruits

CV – coefficient of variation
SD – standard deviation

This study has demonstrated the possibility of developing a SPE based biosensor for monitoring dopamine in aqueous medium. Furthermore, it has confirmed that the carbonaceous material can be utilized as an appropriate matrix for the immobilization of enzyme, tyrosinase. The biosensor exhibits fast response, excellent sensitivity and stability for the amperometric detection of dopamine. The suitability of the amperometric method for determinate dopamine content in fruit samples has been demonstrated.

Application of the biosensor to determination of serotonin in walnut samples

The proposed biosensor (Ty/CoPc-CPE – carbon paste electrode modified with cobalt phthalocyanine and tyrosinase) was tested in the determination of serotonin in the walnut samples. Thus, Table 6 shows the results obtained in the biosensor application for serotonin determination, obtained through the standard addition method in the walnut samples.

Table 6. Values of serotonin concentration, obtained with the proposed biosensor, through standard addition method

In order to evaluate the matrix effect Table 7 shows the results obtained in the recovery experiments of five different samples. The results obtained suggest that this proposed biosensor can be applied enough well in biological samples with no significant influence of the matrix. The recoveries obtained were very good giving an average recovery of 102±2%.

Table 7. Recovery % obtained with the biosensor after serotonin addition (50μM) in each walnut samples

[a] Relative standard deviation for three replicates

The biosensor presented a linear response range for serotonin determination in the range between 4 to 140 μM of serotonin. Considering that in walnut the serotonin level is around 87±20 $\mu g \times g^{-1}$, therefore it could be employed for serotonin determination in food samples.

1.3. Analysis of biogenic amines in clinical samples

Study of biogenic amines in saliva samples

Malodor of mouth cavity is a widespread problem affecting a high percent of the adult human population. This health problem is fundamentally related to the putrefaction processes that take place inside of the mouth cavity due to bacterial proliferation. The processes starts from organic substrates such as food traces, traces of blood, epithelial cells etc. The existence of biogenic amines inside of the mouth cavity is related to two main processes. One is the hydrolysis of proteins, polipeptides and oligopeptides into amino acids and the other is the decarboxilation of the amino acids, process biocatalysed by enzymes resulted from the bacterial activity.

Several aliphatic polyamines, principally putrescine, cadaverine, spermine and spermidine, are present in soluble and non-volatile form at the physico-chemical environment of the mouth cavity; these are related to halitosis phenomenon. Additionally, it is plausible that their amounts in saliva could increase in different pathological processes.

This work describes the developing and optimization of a novel amperometric biosensor for the quantification of biogenic amines existent in saliva. The receptor element of the biosensor is developed from a carbon screen-printed electrode modified with polypyrrole doped with Prussian Blue and diamine oxidase. Sensing target molecule is the hydrogen peroxide formed in the enzymatic process biocatalysed by the diamine oxidase immobilized onto polypyrrole matrix.

The measurements with the biosensor were carried out by connection of biosensor with a special cable to potentiostat/galvanostat. Detection technique was fixed potential amperometry using a drop of sample. The optimizations of supporting electrolyte properties and of detection technique parameters were carried out. Assessment of the biosensor capacity to detect different biogenic amines demonstrates higher sensitivity for putrescine. Therefore, it was used as reference biogenic amine. For the putrescine detection a low limit of detection of 7.4×10^{-8} M and a wide linearity range between 1×10^{-6} and 2×10^{-4} M were achieved. The interferences and matrix effect were studied by standard addition method obtaining an excellent average recovery of 100.4%. The validation of the biosensor was carried out by quantification of biogenic amines in saliva samples and the values compared with standard reference method based on ELISA.

Application in DAO-CNF/C-SP in pharmaceutical samples

DAO-CNF/C-SP biosensor was applied to the quantification of norepinephrine in pharmaceutical samples. A measured volume of pharmaceutical sample was introduced into the electrochemical cell, containing PBS 100 mM of pH 7.4. Amperometric signals were recorded in the optimal conditions (applied potential −0.60 V, constant stirring, pH 7.4). Three replicates were carried out for each sample. The contents in norepinephrine of pharmaceutical formulations determined with DAO-CNF/C-SP biosensor are presented in Table 8.

Table 8. The norepinephrine concentration values (mean of three replicates) founded in pharmaceutical formulations by amperometry.

As observed in Table 8, the values obtained with the biosensor were close to labeled content demonstrating the applicability of biosensor in pharmaceutical analysis. The interference effect of excipients in detection of norepinephrine is insignificant.

Analysis of dopamine in pharmaceuticals by means of biosensor

The addition method has been used for real samples analysis. To the pharmaceutical solution 50 μM of dopamine has been added. The biosensor has been immersed in phosphate buffer solution 0.01 mol×L-1 (pH=7.0) and 5 cyclic voltammograms have been registered. After stabilization, measured volumes of samples have been added and 5 cyclic voltammograms have been registered. Using the calibration curve, the quantity of dopamine added, and the dilution factor the concentration of dopamine in pharmaceutical samples has been calculated.

Table 9 shows the results obtained for the analyses of pharmaceutical formulation using the biosensor (ITO-AA-$DyPc_2$/Ty LB).

The results obtained by cyclic voltammetry using the biosensor have been in very good agreement with the values included in the labels of both pharmaceutical products. Furthermore, the biosensor is able to detect selectively dopamine in presence of interfering compounds such as sodium disulfide, maleic acid, sodium chloride, ethanol, and propylene glycol (excipients of pharmaceutical products). This result is related to specificity of tyrosinase towards the phenolic groups present in dopamine structure. It exists a good correlation between the results obtained by biosensor and labeled value. In consequence, the biosensor proposed here can be used successfully in real samples analysis.

Table 9. Determination of dopamine in formulation by using biosensor

Capability to detect creatinine in model and real sample analysis.

Sensors based on carbonaceous materials were employed for detection and quantification of creatinine. Optimization and establishing of suitable measurement procedures were carried out.

Study of sensitivity, kinetics, linear range, limits of detection and stability of the sensors in the detection of creatinine in model and real sample solutions were performed. The responses of sensors towards creatinine solution are presented in Figure 7.

Figure 7. Cyclic voltammograms of CNF-SPE, CNT-SPE, and GPH-SPE immersed in 10^{-4} M creatinine solution

Calibration curve for CNT-SPE towards creatinine is presented in Figure 8.

Figure 8. Calibration curve towards creatinine

The sensors based on carbonaceous materials exhibit linear responses to creatinine over concentration ranges from 1 µM to 500 µM with detection limits in the range of 0.015-0.053 µM.
CNT-SPE sensor was employed for detection of creatinine in plasma samples and the results are presented in Table 10.

Table 10. Quantification of creatinine in plasma samples

The screen-printed based sensors were successfully used in quantification of trace amounts of creatinine in plasma samples. The simple instrumentation, short time need for analysis, minimal sample pre-treatment and small quantity of sample recommends this method as a valuable screening analytical method.

1.4. Validation of the systems by the establishment of correlations between the results obtained with sensors and biosensors and the results of physico-chemical, sensory, biochemical and medical analyses

Application of the biosensor to determination of dopamine and epinephrine in pharmaceutical formulas

The UV spectrophotometric methods indicated in the X Romanian Pharmacopoeia suggest the measurement of dopamine at 280 nm, in 1 g×L^{-1} Na_2SO_3, and the measurement of epinephrine at 279 nm, in presence of 10^{-2} M HCl.

Calibration curves were constructed using pure dopamine, and epinephrine, respectively. These were used for quantification of dopamine and epinephrine, respectively, in different of pharmaceutical formulations.

Table 11 shows the results obtained for the analyses of pharmaceutical formulations using the UV spectrophotometric procedure and biosensor (Ty/GPH-C/SPE), respectively (at +0.025 V for DA and -0.025 V for EP).

Table 11. Determination of DA and EP in pharmaceutical formulations by UV spectrophotometry and biosensor

a - Amount of catecholamine in the sample (mg).

b - Amount of catecholamine obtained by the proposed method (mg) ± RSD (n=5).

c - Amount of catecholamine obtained by the spectrophotometric method (mg) ± RSD (n=5).

RSD - Relative standard deviation

The biosensor has been employed for the determination of dopamine and epinephrine in pharmaceutical formulations and the results are satisfactory, which suggests that biosensor can act as a promising biosensor for the determination of dopamine and epinephrine. The concentration results have been in a good agreement with the Pharmacopoeia method.

Analysis of histamine in freshwater fish samples

Different freshwater fish samples were analyzed with DAO/nPt/GPH/chitosan/CSPE biosensor for histamine detection and quantification. The purpose of this relative high diversity of samples was to evaluate if the quantification of histamine amount in different freshwater fish species could be carried out with good reliability.

Histamine quantification was performed by interpolation of biosensor response in the calibration plot. For the recovery studies and for the influence of matrix effect in detection of histamine the standard addition procedure was implemented. In the optimal experimental conditions, amperometric measurements with biosensor were performed in triplicate. The results are presented in Table 12. Data are based on biosensor method and ELISA method, respectively.

Table 12. Quantities of histamine in freshwater fish samples under study

[1] n = 3; 95% confidence level.

As it can be noticed in Table 12, there are relatively small differences between direct interpolation and addition methods. Therefore, matrix interfering effect is not significant in histamine detection in freshwater fish samples.

Correlation with the sensorial analysis

Correlation of the results obtained with the (bio)sensors with the sensorial analysis was carried in different studies.

The sensorial parameters were determined by 3 persons using a scale of five points, 0 means the lower intensity of one parameter and 5 for the higher intensity of the same parameter, in all cases.

For the freshwater fish freshness monitoring the parameters quantified were: color of the skin, color of the eye, color of the bronchia, rigidity of the flesh, smell of fish, smell of ammonia/amine and overall smell. The results were correlated with the stages of the evolution of the freshness. Better correlations, R^2 greater than 0.85 were obtained for the color of the bronchia and overall smell.

In the case of pear ripeness monitoring the parameters taken into account are: green color, yellow color, uniformity of color, rigidity of the pulp, sweet taste, astringency, and fruity aroma. The best correlations, with R^2 greater than 0.85, were obtained for the yellow color, astringency and fruity aroma.

Analysis of creatinine in plasma samples

The results obtained with sensors based on different carbonaceous materials were compared with those obtained by means of standard method, which involve the use of picric acid (Jaffe reaction) and spectrophotometric determination of orange-red compound obtained in alkali solution. The scheme of the reaction is presented in Figure 9.

Figure 9. Jaffe reaction for the spectrophotometric determination of creatinine

The creatinine contents obtained for a group of plasma sample by spectrophotometric method (standard method) and those obtained with GPH-SCPE are presented in Table 13.

Table 13. Amounts of histamine determined in plasma samples

As can be seen, the results are very similar. In order to determine if the differences are significant or not, ANOVA method was applied. The results shown that at 99% confidence levels the results are not significantly different. This result demonstrates that the sensors developed are a viable method for detection of creatinine in plasma samples.

Models for diagnosis based on detection of biogenic amines

Biogenic amines are compounds of fundamental importance in the function of human body. Furthermore, the relationship among amount of biogenic amines in the body fluids is relevant for diagnosis. For this purpose we have developed a multibiosensor system coupled with a multivariate data analysis for detection and/or quantification of biogenic amines. Moreover, the chemical fingerprinting of clinical samples was carried out by means of differential pulse voltammetry technique. The novel biosensors developed are based on screen-printed electrodes modified with single walled carbon nanotubes functionalized with carboxyl groups and different oxidase enzymes: tyrosinase, diamine oxidase and monoamine oxidase. Multibiosensor system is able to detect all categories of biogenic amines. Biosensors were developed by self-assembling method. Firstly, the single walled carbon nanotubes functionalised with carboxyl groups were deposited onto commercial carbon screen printed electrodes. Subsequently, the enzyme was deposited. The presence of carboxyl groups facilitates the interaction between electrode and enzyme by means of hydrogen bonds increasing the stability of the sensitive layer. The sensitive element of biosensors was characterized by FT-IR spectrometry, atomic force microscopy and electrochemistry.

The combined effect of nanostructured material and detection method employed give rise biosensors with excellent performance characteristics. The biosensors system as whole is able to detect dopamine, epinephrine, norepinephrine, serotonin, putrescine, cadaverine, and histamine, at the levels found in clinical samples. The limits of detection are in the range 10-50 $ng \times mL^{-1}$.

Signals registered by means of differential pulse voltammetry were used as input for multivariate data analysis. Classification model based on Partial Least Square Discriminant Analysis was developed and employed in medical diagnosis. The model was validated by classical diagnosis tools and the results were in agreement in 99% of the cases.

Models for diagnosis based on detection of catecholamines and creatinine in biological samples

This study is based on principal heart failure biomarkers, which could be found in plasma (catecholamines and creatinine) and in urine (creatinine).

The sensors and biosensors used in this study are presented in Table 14.

Table 14. Sensors and biosensors used for models development

Two type of database were constructed: one for quantification of specific analyte, and the second for classification of the samples in function of their characteristics.
By using the quantitative model (PLS), the quantity of catecholamines and creatinine is established. The values predicted by the model are not significantly different that those obtained by classical methods (spectrophotometry, chromatography or ELISA). Correlation between measured and predicted values are good, with R^2 greater than 0.98. In conclusion, good correlations have been found between the signals obtained from the expert sensory system and the biogenic amine content by means of PLS regression models.
Using SIMCA model, correlations between (bio)sensor responses and heart diseases were established. The SIMCA model has demonstrated that one sample could be accurately assigned to a group with similar characteristics, which is associated with a diagnosis. The correctness of assignation is 99.5% proving the applicability of sensors and biosensor system in diagnosis.

Sensors and biosensors developed in this project have been demonstrate excellent performance characteristics and were successfully employed for the detection of biogenic amines in a great variety of real samples. In this stage (stage V, 2016) were carried out all the activities included in the research plan and the results obtained were in agreement with research plan. The objectives proposed in this year were totally accomplished.

Dissemination of research results

Dissemination of the research results of the project was carried out by publishing articles in ISI journals, publishing articles in BDI journals, publication of chapters in monographs published by prestigious editorials and participation with papers at international or national conferences, papers published in other journals and the habilitation thesis of the project manager.

Publication of papers in ISI journals

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; http://dx.doi.org/10.1016/j.foodres.2012.06.010

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

3. 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

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9. I.M. Apetrei, C. Apetrei, *Detection of virgin olive oil adulteration using a voltammetric e-tongue*, **Computers and Electronics in Agriculture** 108 (2014) 148–154, http://dx.doi.org/10.1016/j.compag.2014.08.002

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2. I. M. Apetrei, C. Apetrei, Y. El Rayess, ***Characterization of Red Wines Polyphenolics Employing Sensors and Biosensors*** (Chapter 2), pp. 41-70. in **Wine: Phenolic Composition, Classification and Health Benefits**, Editor Youssef El Rayess, 2014, ISBN: 978-1-63321-059-2, Nova Publishers, https://www.novapublishers.com/catalog/product_info.php?products_id=50003&osCsid=647a25d9d412d07c8690696cea0ed681

3. I. M. Apetrei, C. Apetrei, ***Biosensor Based on Nanostructured Sensitive Material for the Detection of Epinephrine*** (Chapter 5), pp. 55-74. in **SENSING - MONITORING - TELEDIAGNOSIS FOR LIFE SCIENCES, Vol. II, FOOD AND ENVIRONMENT** (e-book), Editors: L. Floroian, M. Badea, M. Moga, 2014, Editura Universităţii Transilvania din Braşov, ISBN: 978-606-19-0388-7 gen, ISBN: 978-606-19-0390-0 Vol. II

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5. I. M. Apetrei, C. Apetrei, *Quality analyses and authentication of coconut oil*, In **Corn and Coconut Oil: Antioxidant Properties, Uses and Health Benefits**, Editor C. Apetrei, ISBN: 978-1-63483-420-9, Nova Publishers, 2015, pp. 131-158.https://www.novapublishers.com/catalog/product_info.php?products_id=55691&osCsid=a58c860a7ae1f4d5f714272f3d819203

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Publication of papers in BDI journals

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, http://www.med.ugal.ro/annals_files/1%20_2013/art%202.pdf

Publication of other papers

1. 1. Constantin Apetrei. Senzori voltametrici pe baza de polimeri organici electroconductori, Buletinul S. Ch. R., nr. 3, 2015, 16-37. http://www.schr.org.ro/doc/BSCR-2015-3.pdf

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Conclusion

In this research project were fully carried out all the activities included in the implementation plan, the results are very good which allowed a wide dissemination activity. Thus, there has been developed a variety of sensors and biosensors with sensitivity and selectivity for various biogenic amines on the basis of sensitive materials and enzymes. Data analysis was performed using a variety of statistical methods starting from descriptive statistics to multivariate data analysis. Sensors and biosensors have been successfully used for the analysis of biogenic amines in foods, pharmaceuticals and biological samples.
The dissemination of the publication included 14 articles in ISI journals, 7 chapters in books / monographs, one article in BDI journals, 2 papers in other journals, 28 participation in international conferences and 3 participation in national conferences. There were published 7 papers in journals from the first quarter of the journal category (red zone), 4 papers in the second quarter of the journal category (yellow area). Chapters in monographs, where the authors were invited, were published by prestigious publishing houses: Elsevier, Academic Press, Nova Publishers, Bentham Science Publishers. It can be said that the dissemination was very good overtaking the plan of the project proposal and of its addenda.

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