

Research summary report
(2011-2016 period)

EXPLORATORY RESEARCH PROJECT - PN-II-ID-PCE-2011-3-0784

Novel electrochemical micro-biosensors based on bio-catalytical nano-structures for clinical diagnostic of patients with neuro-psychiatric diseases

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Summary

Brain research, or - in a broader sense - neuroscience, has an important role in the improvement of health and quality of life. Neurotransmitters play a major role in how the brain works and consequently how we function, in terms of our emotions and our behavior. In the medical field there is a clear need to develop laboratory based methods for detection and monitoring of selected neurotransmitters for clinical diagnosis of brain disorders. A simple and efficient solution for detection of neurotransmitters will minimize the human brain disorders (social impact) and by this decreasing the cost of the treatment (economic impact). In our proposed technology for detection of neurotransmitters we will develop clinically relevant micro-biosensors which provide identification and detection of neurotransmitters in a much shorter time scale than classical techniques. If successful, the studies outlined in the Project may be expected to result in the development of new micro-biosensors devices for important analytes (neurotransmitters), which should present improved analytical performances compared with those described in literature, and the entry into an entirely new area of clinical diagnosis of neuro-psychiatric diseases based on electrochemical biosensors. Finally it would be possible to reach an approach that includes necessary and sufficient criteria for diagnosis and pursuit of treatment that addresses the specific mechanism underlying the neuro-chemical contribution to the pathology.

Objectives

The overall objective of the Project is to develop and demonstrate the functionality of new bio-analytical nano-systems based on novel electrochemical micro-biosensors for assessment of neurotransmitters in real clinical samples. The long-term goal of the proposed work is to establish a novel bioanalytical method based on micro-biosensors, whose surface is made from carbonaceous nano-structured materials (e.g. CNTs) modified with enzymes, electro-catalysts and/or electro-generated polymers, for assessment of neurotransmitters in real biological samples at the concentration level relevant from clinical point of view (10^{-7} - 10^{-9} M) and to demonstrate their applicability in clinical diagnosis of patients presenting different neuro-psychiatric diseases. A second aspect will be the validation of developed analytical methodology.

Another innovative aspect will be the examination of the neurotransmitters role in the neuro-psychiatric diseases (major and bi-polar depression, Parkinson disease; this could be

correlated with the possible clinical diagnostics of the patients presenting different neuropsychiatric disorders based on the data obtained by using the elaborated micro-biosensor and also with an adequate medical treatment.

Neurotransmitters-an introduction

The driving forces behind the European effort in neuroscience research are the desire to gain new insights into mental processes in general and the need to develop new and better means for diagnosis and therapy of neurological and psychiatric disorders and diseases. Chemical signaling underlies every function of the nervous system, from those of which we are unaware, for example, control of the heart, to higher cognitive functions, such as emotions, learning and memory. Neurotransmitters and neuro-modulators mediate communication between neurons and between neurons and non-neural cells such as glia and muscle. In the past, the means for studying the production and release of these signaling agents directly has been limited in its temporal and spatial resolution relative to the dynamics of chemical signaling and the structures of interest in the brain.

Now microelectrode biosensors are becoming available that give unprecedented spatial and temporal resolution, enabling, for the first time, direct measurement in real time of the chemical conversations between cells in the nervous system [1]. The brain is a challenging environment for chemical sensing because low concentrations of analytes must be detected in the presence of interferences, while disturbing the tissue as little as possible, and because various surface processes inherent to biological systems can affect sensor response [2].

In the medical field, the monitoring of the neurotransmitters in different neuro-psychiatric patients represents a major demand focusing of course on the health state of the patients. Because of the effect on affected individuals and due to the enormous healthcare costs, these disorders present a major health problem. These disorders can substantially worsen people's economic circumstances because of the cost of medical or traditional treatments.

The efficient control of the health state cannot be realized with the help of the traditional chemical and/or biochemical methods precise and selective, but expensive and laborious. Traditional methods for identification and detection and of neurotransmitters lack the speed and sensitivity to be of real usage since that they are not *real time* or even typically completed in a single day.

One possible solution is represented by the use of the micro-biosensors for assessment of neurotransmitters, whose construction means a multidisciplinary effort, based on developing the designing of nano-technologies for the integrated micro-nano-systems. The potential of using the micro-biosensors results from their capacity to measure the interactions between the nano-sensorial systems and the neurotransmitters. The micro-biosensors have the capacity to detect in a simple, rapid, selective and specific way the interest substances in complex matrices, and with the possibility to generate continuous information. The designing of the micro-biosensors is one of the most illustrative examples of the evolutions based on a multi-disciplinary concept.

Our approach to assess neurotransmitters is an innovative departure from conventional chemical analysis. Designing miniaturized devices that retain the high sensitivity and selectivity of sophisticated laboratory-based instruments represents a major analytical challenge. Resolving the problems regarding the designing and using the micro-biosensors is far from being over. It is required an increase of the sensitivity and specificity, the improvement of the response time and an increase of safety in functioning.

The obtained results based on realized micro-biosensor for detection of the neurotransmitters could contribute to clinical development in the field of diagnosis of neuro-psychiatric disorders by establishing of clinical correlates related to neurotransmitters concentrations range in real samples, by identification neurotransmitter deficits and malfunctions and optimization of drug dosing, and finally to a rapid identification of drug responders.

Electrochemical detection of neurotransmitters in the brain

The neurotransmitters (epinephrine, norepinephrine, dopamine and serotonin) are present in various organs, including the central nervous system and have an important role in preventing depression and anxiety. There are many possible associated abnormalities in depression, but neurotransmitter abnormalities are the best studied and best understood. It is accepted by most researchers that the neurotransmitter abnormalities are only a part of the biological pathology of depression. Data suggest a predominant role of altered neuronal plasticity and cellular resilience. In Figure 1 are presented the principal neurotransmitters involved in depression [3].

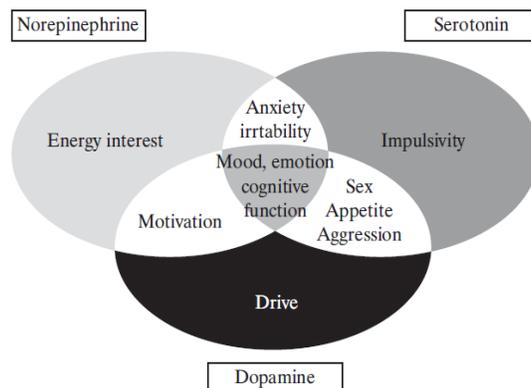


Fig. 1. Principal neurotransmitters involved in depression [3]

Abnormal concentrations level of dopamine were related to neurological disorders, such as Parkinson`s disease and schizophrenia [4,5]. High concentration levels of neurotransmitters in the human body were connected to different types of tumors, heart diseases and circulatory system diseases [6]. Clinical measurement of catecholamines and their analogs in biological samples is useful for clinical diagnosis of pheochromocytoma and neuroblastoma of Parkinson`s disease and in the investigation of stress systems [7].

With respect to electrochemical detection, neurotransmitters can be separated into three different categories; the first group is the electrochemically active compounds such as the tyrosine derivatives dopamine, norepinephrine, and epinephrine (Table 1). Many of their

metabolites are also electroactive such as 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), 3-methoxytyramine, or L-DOPA.

Tab. 1. Electrochemical oxidation of tyrosine and tryptophan derivatives [8]

Molecule	Redox reaction	Approx. ox potential in vivo
Tyrosine derivatives		+0.7 V
L-DOPA		+0.4 V
Dopamine		+0.2 V
Norepinephrine		+0.2 V
Epinephrine		+0.2 V
DOPAC		+0.2 V
Homovanilic Acid		+0.5 V
3-Methoxytyramine		+0.5 V
Tryptophan derivatives		+0.8 V
Serotonin		+0.35 V
5-Hydroxyindolacetic acid		+0.35 V

The neuroactive tryptophan derivatives are also electroactive and include 5-hydroxytryptamine (serotonin) precursors or metabolites like 5-hydroxyindolacetic acid, 5-hydroxyindoletryptophan or melatonin. Other electroactive neurotransmitters are histamine and adenosine. All of these compounds can be directly detected by electrochemical oxidation of the molecule. Furthermore, other electroactive substances in the brain like ascorbic acid, uric acid, nitric oxide, oxygen or hydrogen peroxide are also readily detectable by electrochemical methods. The second group of neurotransmitters is not inherently electroactive, and thus these compounds cannot be detected by traditional electro-analytical methods. However, those that can be oxidized by an enzymatic reaction can be measured electrochemically by coupling the enzyme reaction with an electrochemical reaction. Some of the neurotransmitters in this category are amino acid transmitters like glutamate and γ -aminobutyric acid (GABA), but also acetylcholine and its precursor choline have been detected with this approach. Glucose and lactate, compounds important in energy production in the brain, can also be detected with such indirect electrochemical approach. Neuropeptides and some amino acid neurotransmitters form the third

group of neurotransmitters. Neuroactive peptides cannot be detected directly in the brain with electrochemical biosensors. However, neuropeptides with inherently electrochemically active group can be detected off-line electrochemically. Inmate redox-active functionalities include tyrosine, tryptophan, methionine and cysteine residues [8].

Biosensor concept

In recent years, considerable effort in bio-electrochemical research has centered on the development of a new family of monitoring devices that are collectively referred to as "biosensors". The primary function of these devices, which can be defined as systems that "incorporate a biochemical or biological component as a molecular recognition element and yield an analytical signal in response to biomolecules" [9], is to provide highly selective chemical detection for various biological molecules and their substrates. To accomplish this, electrochemical biosensors utilize synergistic couplings of separate biological and electrochemical processes at the surface of an electrode probe. Conventionally, the approach involves the incorporation of the desired bio-component -usually an enzyme but potentially a nucleic acid, an immuno-agent, a binding protein, or even an intact cell - onto a functioning electrode surface where the molecule's biological activity can be maintained and monitored by electrochemical techniques.

Project background

Metallo-phthalocyanines are a possible choice for preparing voltammetric modified sensors due to their catalytic activity for a wide range of redox processes [10-12]. The sensitivity and the selectivity of the (bio)sensors can be greatly improved as a result of the electrocatalysis by metallo-phthalocyanines [13,14]. Recently, immobilization of phthalocyanines and metallo-phthalocyanines at the surface of carbon nanotubes has been tested [15,16]. Carbon nanotubes (CNTs) working electrodes have been proved beneficial helping the electron exchange reaction in redox processes of different species like DA, epinephrine, and 5-HT among other compounds [17,18].

We developed a novel assay for the enhanced electrochemical detection of low dopamine concentrations in the presence of serotonin in deproteinized serum samples based on the electrocatalytic action of carbon nanotubes paste electrodes (CNTPEs) modified with iron(II) phthalocyanine (FePc) [19]. The developed FePc-MWCNTPE electrodes were able to detect dopamine with a detection limit of $2.05 \cdot 10^{-7} \text{M}$ in the presence of 5-HT. There was no electrochemical response for ascorbic acid (AA) added in the sample and electroactive species like uric acid and paracetamol did not interfere in dopamine determination due to a good peak separation at the developed sensor. Thus, the strengths of the chemically modified electrode approach include its sensitivity and selectivity. The monoamine neurotransmitter measuring method has been tested in analyzing deproteinized serum clinical samples with very good results. The electrochemical approach is helpful in early diagnosis of neurodegenerative diseases, is used to detect from serum samples compounds like dopamine and serotonin which play a key role during excessive oxidative stress events in humans.

Our group developed a modified carbon paste electrode that incorporates the anionic surfactant SDS in the paste (SDS-CP) [20]. The tests performed by cyclic voltammetry have shown that the SDS-CP electrode discriminates effectively between the cationic form of dopamine and the anionic electroactive species existing normally in biological fluids at the physiological *pH* value of 7.4. On this principle, a differential pulse voltammetry method has been developed. It has the detection limit comprised in the submicromolar range and the capability to remove completely the interference of the ascorbic acid and to diminish significantly the interference of the uric acid. The method has been tested with good results on real samples of deproteinized serum and is appropriate for important applications. This encouraging result gives us the hope that the reported electrode can be useful, in a very simple manner, in important applications (for instance, in the analysis of cerebrospinal fluid).

Project design

Our research was focused on the development of new methods of analysis based on chemical sensor and biosensor technology for the detection of some neurotransmitters to give reliable information of risk to some neuro-psychiatric diseases and to improve existing control and prevention strategies of such diseases. A second aspect will be the development and validation of analytical methodologies to ensure in real time a complete traceability in clinical analysis.

According to the proposed objectives the developed work includes the following:(i) The use of electrocatalytic chemically modified electrodes (CMEs) in biosensor devices to decrease their dependence on solution composition (e.g., on O₂ levels). The CMEs were selected from among several such systems developed and characterized in recent years [19,21,22]. It is suggested that the biosensors obtained by coupling these enzymes and electrodes together will provide sensitive and selective analytical response toward phenolic compounds in general and neurotransmitters in particular; (ii) The design and characterization of novel biosensors containing enzymes exhibiting activity toward neurotransmitters; electrochemical biosensor devices making use of enzymatic systems and catalytic chemically modified electrodes will be designed and evaluated. The specific enzyme species considered will include glucose oxidase, a flavoprotein which is responsible for the oxidation of glucose, peroxidase a hemprotein responsible for reduction of hydrogen peroxide and tyrosinase, a copper-containing protein responsible for the oxidation of mono-phenols to the corresponding quinones; (iii) Validation of CME-based biosensors.

The sequence of experiments conducted began with solution-phase cyclic voltammetry studies to characterize the electrode behavior of each of the mentioned species in solution. Subsequently, CMEs containing first the mediator species by itself and then the enzymes as well were utilized to attempt the oxidation or reduction of the phenol substrate or hydrogen peroxide. After optimization at this level, the performances of the electrode systems as biosensors for detection of the substrates or clinical applications were examined. Novel materials with unique physical and chemical properties and their introducing into the construction of biosensors with a high performance were the subject of great research interest. The Project is concerned in

preparation of nanostructured electrochemical enzymatic, tissue and nucleic acid based biosensors. SEM, AFM, voltammetric techniques and electrochemical impedance spectrometry (EIS) were utilized for morphological characterization of the nanostructured sensors and biosensors. Chromatographic and other independent methods will be used for verification and validation. Finally rapid methods of analysis based on biosensor technology will be developed to monitor in real time the neurotransmitters (bio-markers of some neuro-psychiatric diseases), with low cost effective instrumentation. If successful, the studies outlined above may be expected to result in the development of new biosensor devices for important analytes (neurotransmitters), the improved analytical performance of new and old biosensor devices alike, and the entry into an entirely new area of clinical (medical) applications for enzyme-based biosensor systems. All the developed screening methods will be proposed to the public and private clinical laboratories which will use established reference procedures for validation.

Stage 1 and 2 (5.10.2011-15.12.2012): Design, construction and characterization of micro-biosensors based on carbonaceous nano-structured materials (laboratory experimental models) for assessment of neurotransmitters in complex biological matrices.

During the Project researches, new chemical sensors and biosensors with improved characteristics of sensitivity, stability and selectivity were constructed, assembled and used for neurotransmitters detection and clinical control. The design and construction (development) of an enzymatic sensor for neurotransmitters detection consists in several stages:

◆ **(1) Surface modification and enzyme immobilization**

- ▶ involves enzyme orientation and packing density considerations (there are problems related neurotransmitters access at the active center of the enzyme;



◆ **(2) Enzymatic reaction at the solution/electrode interface**

- ▶ problems related to enzyme kinetics (process ensuring the selectivity, response time, sensitivity and reversibility of the biosensor)



◆ **(3) Detection and analysis of information content**

- ▶ detection of analytes or other interfering compounds by electrode relies on the signals generated by binding events; improving the detection limit

Developed sensors and biosensors

1 – **Development of new CMEs** for application to direct detection of neurotransmitters. These sensors should be able to detect minute changes in current which occurs when neurotransmitters interact with the mediator immobilized on the transducer surface. Efforts were done to improve the sensitivity of these CMEs.

Based on our previous experience [19,21], during the Project research we developed a cobalt(II) phthalocyanine modified multi-wall carbon nanotubes paste electrode in order to construct enzymatic sensors for neurotransmitters detection. Multi-wall CNTPEs were prepared by thoroughly hand mixing the CNTs powder with paraffin oil (60:40 w/w) in a mortar and

pestle. Chemically modified CNTPEs were prepared in a similar fashion except that the CNTs powder was first mixed with the desired weight of modifier (CoPc). The content of the phthalocyanine in the CME is described on a percent basis as the weight of the CoPc added to the 1 g of CNTs powder. Both pastes were packed into 1.0 mL polyethylene disposable syringes with a copper wire being used for electrical contact. The initial CMEs activity could always be immediately restored by simply removing the outer layer of paste by briefly smoothing the newly exposed portion. Mixed CNTPEs renewed in this manner exhibited stable and reproducible electrochemical behavior over weeks.

The unmodified and modified CNTPEs were pretreated by performing 10 cyclic voltammetric scans from -0.2 V to 3.0 V at a scan rate of 500 mV s⁻¹, in acetate buffer solution pH = 4.0, this step assuring the electrode activation and stabilization. Cyclic voltammetric experiments were performed in the potential range 0.70–1.10 V with a scan rate of 100 mV s⁻¹. The electrochemical pretreatment of unmodified and modified CNTPEs was examined. The degree of reversibility for K₃[Fe(CN)₆] redox system was enhanced by the electrochemical pretreatment procedure. This electrode will be used and characterized further in our studies for neurotransmitters detection.

2 - Development of molecularly imprinted polymer (MIP) sensors for application to direct detection of neurotransmitters. The regeneration of the electrode surface would be a real improvement to make the MIP sensors more reliable for clinical application.

Molecularly imprinted polymers (MIPs) have been recognized as optimal elements to construct sensor with specific binding sites to target molecule [23]. In principle, molecular imprinting techniques can be useful to build up specific host sites on a solid, because of the easy preparation, good selectivity, and robustness in different conditions [24]. A major challenge in developing chemical sensors based on MIPs is to find an effective way of transducing selective recognition process into analytical signal. Sensors based on molecularly imprinted materials, which improve the selectivity of the electrochemical method, for DA detection have been reported [25-30].

During the Project research our group developed a simple and reliable method for preparing a selective dopamine (DA) sensor based on a molecularly imprinted polymer of ethacridine(MIPET) [31]. Thin, non-conductive films of polyethacridine molecularly imprinted with dopamine have been obtained by cyclic voltammetry from acetate buffer (pH=5.0) solutions onto the surface of a glassy carbon electrode (GCE). By Raman and FTIR spectra it has been shown that the oxidative polymerization develops free low molecular weight oligomers with specific morphology in plate-like forms, reach in finger print bands. FTIR and Raman spectroscopy also proved the presence of phenazine-like units in ethacridine oligomers. After a conditioning step having the role to extract the dopamine template from the film, the MIPET-DA GCEs responds very sensitively and selectively to dopamine, as revealed by voltammetric studies. In optimized DPV conditions the oxidation peak current was well-proportional to the concentration of DA in the range from 2.0 x 10⁻⁸ M up to 1 x 10⁻⁶ M. The limit of detection (3σ)

of DA was found to be as low as 4.4 nM, by the proposed sensor that could be considered a sensitive marker of DA depletion in Parkinson's disease.

3 – Development of new DNA based biosensors for application to direct detection of neurotransmitters. These sensors should be able to detect changes in the current which occurs when neurotransmitters bind to DNA immobilized on the transducer surface. Efforts should be put to improve especially the selectivity and sensitivity of these biosensors.

Biosensing is a field of interest for analytical chemists for more than 40 years, with excellent results in fields like environment, clinical and food analysis. Although there were resulting excellent biosensors, their applications did not penetrate routine laboratories as expected [32]. It is therefore a challenge for researchers to diminish the gap that exist between academic expectations and commercial achievements in this regard. Biotechnology related to DNA immobilization on electroactive surfaces was used for the determination of a wide range of biomolecules such as norepinephrine [33], dopamine, uric acid [34], cytochrome c [35], adenine, guanine, and thymine [36].

Since two centuries ago, carbon played an important role in material science, and has become widely and intensively studied for diverse electrochemical applications. Nowadays carbon allotropes e.g. synthetic monocrystalline diamond [37,38], fullerenes C₆₀ [39-42] and carbon nanotubes [43], are amongst the most popular matrices for construction of biosensors, due to their unique electrochemical features, such as high electrical conductivity [44], chemical stability and catalyst support [45]. Since the discovery of carbon nanotubes [46] and fullerenes [47] the need to miniaturize electronic devices down to nanometer scale became more relevant.

We studied the influence of double-stranded DNA (dsDNA) physical immobilization on the electrocatalytic behavior of different carbon matrices (Figure 2) using three neurotransmitters: dopamine, epinephrine and norepinephrine as model analytes. The carbon matrices considered for the study were: multi-walled carbon nanotubes (MWCNT), synthetic monocrystalline diamond (DP) and fullerenes C₆₀ (BTP).

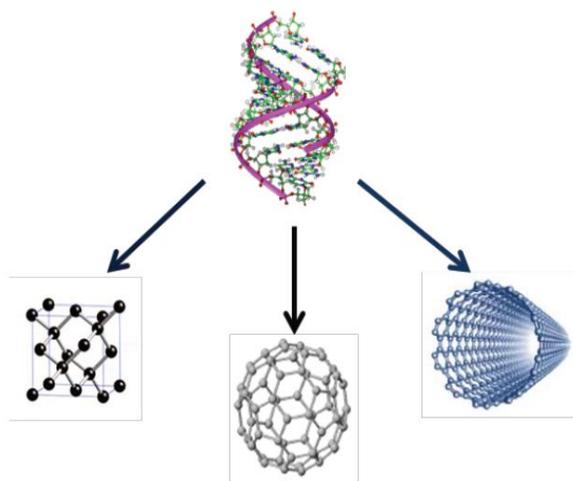


Fig. 2. The influence of physical immobilization of dsDNA in diamond paste, fullerene paste and carbon nanotubes, on the behavior of biosensors was shown using three neurotransmitters: dopamine, epinephrine and norepinephrine

Accordingly, electrochemical sensors based on unmodified paste of MWCNT, DP, and BTP, respectively, and modified paste (MWCNT, DP, and BTP, respectively) with dsDNA were designed and evaluated. This study will help the researchers in the field to select the best carbon based matrix for the bioanalysis of the compounds of their interest.

The study proved that the arrangement of atoms in the matrix – Bucky balls, multi-walled carbon nanotubes, and diamond is having a high influence on the behavior of the modified dsDNA biosensors. The response characteristics, selectivity and recoveries of model analytes were compared between unmodified and modified matrices. As a result, for C₆₀ and carbon nanotubes matrices there were improvements only when dopamine was assayed; no significant improvement was recorded for the assay of epinephrine, and a decrease in sensitivity was recorded for the assay of norepinephrine. While no response for the assay of dopamine, epinephrine and norepinephrine was recorded with diamond paste based sensor, modification with dsDNA of diamond paste made possible the assay of epinephrine and norepinephrine at very low concentration, and decreased limits of quantification and detection for the same analytes, although the sensitivity of this biosensor was lower than 100nA/nmol/L (value recorded for e.g., CNT and dsDNA based biosensor). In terms of selectivity, utilization of dsDNA has not improved the performance of the sensors based on plane pastes. This behavior was also reflected in the recovery tests (when the interference occur), when one can make a selection of the applications of the sensors for pharmaceutical or clinical field. These studies helped to understand the importance of correlating the arrangement of carbon atoms in the molecules used as matrices with the behavior of the sensors, and also that addition of molecules such as dsDNA will not always improve the selectivity and sensitivity of the sensors. Although the DNA has a strong affinity towards CNTs and fullerenes, not the same effect can be recorded for diamond material.

Stage 3 (16.12.2012-15.12.2013): Design, elaboration and optimization of the technology for the construction of the micro-biosensors based on nano-structured materials (e.g. CNTs) with increased sensibility and selectivity towards neurotransmitters. Analytical evaluation and optimization of the response characteristics of the micro-biosensors towards neurotransmitters by using electrochemical methods.

Developed sensors and biosensors

4 - **Development of amperometric tissue based biosensors** for application to direct detection of neurotransmitters. Amperometric probes based on tissues can be used to measure neurotransmitters based on their metabolic redox reactions generating an amperometric response to the electrode. Efforts in this direction were devoted to the improvement of the relatively poor selectivity, of the long response time and the lack of sensitivity when these probes are to be used to monitor different analytes.

Following the pioneering steps of Clark Jr. [48], the research in the field of developing and application of biosensors has been an intensive cultivated area of analytical chemistry. The function of the earliest developed group of biosensors is based on catalytic action of different enzymes used in immobilized form. The stability of enzymes in their natural environments is

usually much higher than in extracted preparations or in immobilized form. It was obvious to prepare enzyme sensors by using native biological tissue as catalytic layer. Several plant tissue based biosensors were made for measurements of phenolic compounds like catechols [49-52]. One of them is the dopamine (DA) measuring biosensor that uses the catalytic action of banana pulp that was nicknamed “bananatrode” [53]. In case of this biosensor, the banana pulp was used as a very thin slice (membrane form) mechanically attached to the active surface of an electrode [54] or was incorporated, by a well-known procedure, into carbon paste [55]. There are two ways for obtaining amperometric signal. One is following the oxygen concentration with a Clark oxygen cell [54]. The other one is recording the concentration dependent reduction current of the quinone formed in catalytic oxidation [56].

Regardless of cost efficient nature and good stability of biosensors prepared with native enzyme sources, their application in every day practice of analytical chemistry is not frequent. Most likely the relatively small specific enzyme activity of their reaction layer is responsible for this. To provide the necessary measuring range and sensitivity higher enzyme activity and well-tailored reaction layers would be needed. Since the early times of biosensor research, several attempts have been made to describe the concentration profiles inside biocatalytic layers or to explain the function of different kinds of enzyme sensors [57-59]. For improving analytical values of biosensors, fundamental studies of the enzymatic reaction and that of the concentration values in close proximity of the electrochemical transducer is of vital importance. It is hoped that the measuring performance of biosensors could be optimized knowing the concentration profiles of reactants and products of the biocatalytic process as well as finding the right parameters for detection.

Scanning electrochemical microscopy (SECM), invented by Bard [60], is the powerful tool in investigating concentration profiles of different species in microenvironments of a special model biosensor. SECM studies have been carried out over biocatalytic surfaces in liquid phases [61,62]. SECM measurements were used for investigation of concentration profiles of different species inside the model of the reaction layer of enzyme sensors by Csóka et al. [63,64].

One of our research goals was to investigate the possibility of improving the performance of a banana tissue biosensor for dopamine detection [65].

A model biosensor based on a reaction layer containing banana pulp as native enzyme source was constructed. Dynamic voltammograms (DVs) were used to find the optimal electrode potential for DA detection. In our study, the concentration profiles of different species involved in the enzyme catalyzed reaction were investigated inside the reaction layer of the model biosensor by using SECM. The model biosensor consists of the amperometric SECM measuring tip as transducer and the enzyme layer in a short glass tube (length=2 cm, ID=15 mm) as shown in Figure 3. The enzyme layer is made of phosphate buffer suspended banana tissue (1:5) that is kept inside the short glass tube. The bottom end of the tube is sealed by a 50 μm thin dialysis membrane.

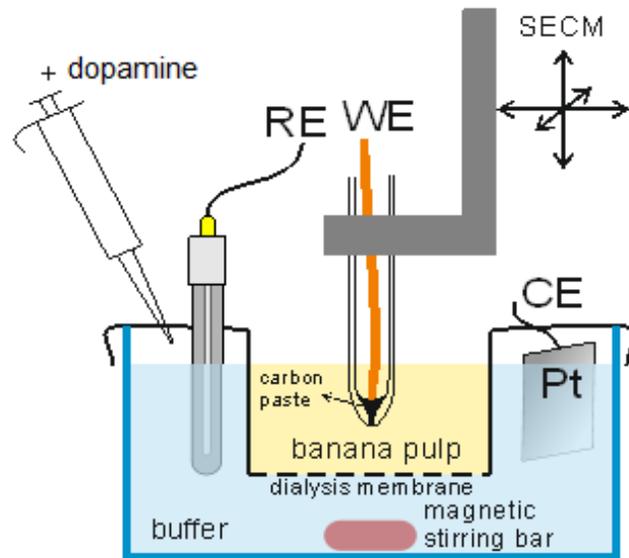


Fig. 3. Setup of the SECM measurements

In order to prepare the “bananatrode” for DA measurements, a homemade GC electrode was covered with spacer ring approx. 130 μm of thickness forming a well-defined space for the enzyme reaction layer on the surface of the GC electrode. The banana pulp was spread into this space on the surface of the glassy carbon disc and it was fixed with membrane to ensure the tight attachment of the reaction layer. Besides dialysis membrane, in some experiments Tecoflex membrane layer was also applied. In the latter case, 10 μL of Tecoflex solution (1% in tetrahydrofuran) was spread on the surface of the spacer ring surrounded pulp. After the quick evaporation of the solvent a thin membrane was obtained coating the banana layer. The schematic drawing of the electrode is shown in Figure 4. When the biosensor was not in use it was kept in buffer solution at room temperature.

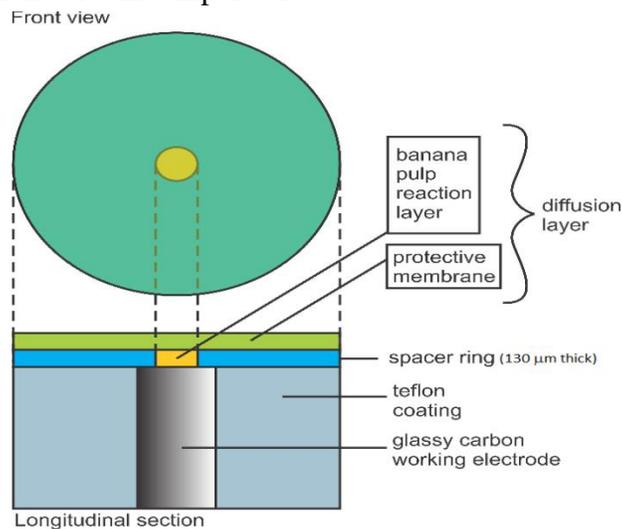
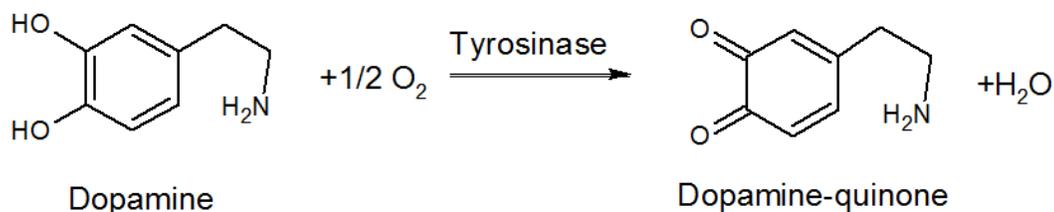


Fig. 4. Sketch of the “bananatrode”

In each experiment large surfaced platinum wire as auxiliary electrode and saturated calomel electrode (SCE) as reference electrode were used. All measurements were carried out at room temperature and all potentials were measured vs. SCE.

Polyphenol oxidase (PPO) enzymes in the banana tissue [66,67], often called tyrosinases, are the active biocatalyst in amperometric sensors for selective measurements of polyphenolic compounds, including dopamine. Polyphenol oxidases catalyze the interaction of ortho or para-diphenols with molecular oxygen resulting the corresponding quinones [68]. The function of the “bananatrode” is based on the tyrosinase catalyzed reaction of dopamine with dissolved oxygen. The dopamine from the sample solution diffuses through the dialysis membrane into the banana pulp. It interacts with tyrosinase being transformed into dopamine-quinone, the enzyme being transformed in its reduced form. The dopamine-quinone formed, gets by diffusion into sample solution as well as to the close vicinity of electrode surface, where it is reduced back to dopamine by means of an electrode reaction if the appropriate electrode potential is applied; the reduced form of the enzyme is oxidized back to its natural form by the dissolved oxygen. The generated current depends on the analyte concentration.



The thickness of the enzyme containing layer has basic effect on the working condition of the electrode. It influences the response time as well as the signal.

On the basis of dynamic voltammograms it was found that the optimal potential for DA detection should be -0.2 V vs. SCE, potential where the oxygen interference was avoided. The concentration profiles of different species involved in enzyme catalyzed reaction were investigated inside the reaction layer of the model biosensor by using SECM. Local maximum of dopamine-quinone profile was found at approximately 130-160 μm far from the analyte/banana tissue boundary in SECM experiment. Enzyme layer thickness optimization resulted in decreased response time and relatively higher reduction current. It was selected the optimal potential to be applied as resting potential in PIA detection mode which should fall between -0.02 and -0.03 V vs. SCE, since at these potentials no measurable anodic or cathodic current could be observed. For doing experiments in PIA mode an optimum value of 0.5 s for resting time (t_3) was also selected. Considerable increase of the “bananatrode” sensitivity and improvement of the LOD towards dopamine were achieved by using optimal electrode potential and reaction layer thickness, together with the measuring technique of PIA. A detection limit (LOD) of 1.05×10^{-5} M was obtained for DA by using conventional amperometric technique, while in case of PIA studies the LOD was 2×10^{-6} M. The banana pulp reaction layer of the model biosensor could be rapidly and reproducibly renewed. Cost efficient way of preparation and relatively long life time are important advantages of the elaborated model biosensor. Further experimental improvements

and analytical evaluation including selectivity studies of this type of biosensor are currently in progress with particular emphasis on rapid neurotransmitter measurements. The sensor might be useful for measurements in urine, where dopamine is found in micromolar levels.

Continuing our work, we made SECM measurements inside a reaction layer existing in a close vicinity of a cellulose acetate (dialysis) membrane which separates the banana tissue, kept in a glass tube, from an electrolyte solution containing H₂Q as electroactive species [69]. Two kinds of studies concerning the redox activity of enzyme Tyr-ase belonging to its natural environment (i.e. banana pulp) were performed. First experiment, based on a Pt UME disk-shaped, was dedicated to SECM investigation of the biocatalytic effect of Tyr-ase on hydroquinone contained in the bulk solution. The local changes of the concentrations of hydroquinone and oxygen within a very thin reaction layer in vicinity of the dialysis membrane were recorded by SECM technique using the approaching curve at two working electrode potentials -0.2 V and -0.6 V, respectively. From the data measured and interpreted one can advance that a reaction layer thickness is approximately 70 μm. Based on SECM results, in the second experiment, a model “bananatrode” biosensor was used to detect H₂Q by means of DPA technique. The response time of the tissue biosensor was estimated to be in between 1-2 min. The banana pulp reaction layer of the model biosensor could be rapidly and reproducibly renewed. Cost efficient way of preparation and relatively long life time are important advantages of the elaborated model biosensor. Further experimental improvements and analytical evaluation including selectivity studies of this type of biosensor are currently in progress with particular emphasis on rapid H₂Q measurements. The present sensor might be useful for measurements in tap water, where H₂Q is found in micromolar levels.

Stage 4 (16.12.2013-15.12.2014): Experimentation and evaluation of micro-biosensors performances for neurotransmitters determination on real samples.

Developed sensors and biosensors

5 – **Development of new amperometric enzyme electrodes** mainly based on oxidoreductase enzymes coupled with appropriate mediators and electrodes to measure neurotransmitters; they are able to transduce the enzymatic redox reactions into a quantifiable electrical signal. Efforts were oriented to the improvement of the stability and sensibility of the enzyme sensors. These devices offer a real-time output, simplicity of use and cost effectiveness.

The ability of certain enzymes to catalyse biochemical reactions has led to their use to detect important analytes. This approach should be quite attractive to detect neurotransmitters due to its extremely high specificity. In this stage we developed a multiwall carbon nanotubes-cobalt phthalocyanine-amphiphilic polypyrrole composite for the construction of a tyrosinase-based amperometric dopamine biosensor.

Among the numerous procedures of biosensor construction, the entrapment of biomolecules within electropolymerized films constitutes a simple, versatile and efficient method that does not require additional chemicals which could denature the biomolecules. Moreover, the electropolymerization of pre-adsorbed amphiphilic pyrrole protein coatings offers specific advantages such as control of the real amount of entrapped biomolecules. Furthermore, the

stability and permeability of electrogenerated polymer films in organic solvents are attractive properties for the elaboration of organic-phase biosensors. The properties and thickness of the host polymeric films can be easily modulated by the choice of the monomer and the electrolysis charge. In particular, the electropolymerization of pre-adsorbed amphiphilic pyrrole-protein coatings offers specific advantages such as an extremely economical expenditure of biomolecules for the fabrication step and the control of the real amount of entrapped biomolecules. Furthermore, this method allows the construction of controlled architectures based on spatially segregated multilayers, exhibiting complementary biological activities. Our studies were done in order to test different protocols for tyrosinase immobilization at glassy carbon electrode surface with the aim to obtain biosensors with application in detection of dopamine and neurotransmitters in general.

Also, the effect of iron and cobalt phthalocyanine on the electrochemical properties of the modified electrode was evaluated. Ratio between enzyme and amphiphilic pyrrole in the solution utilized for immobilization was varied to reach optimal results. Among all the used immobilization protocols, best results were obtained when enzyme and cobalt phthalocyanine were immobilized in a single electropolymerization step from a solution that contained also amphiphilic pyrrole. Utilizing this method, the difference in between oxidation and reduction peaks potentials obtained in a catechol solution decreased in comparison with the electrode modified only with carbon nanotubes.

The fabrication of the dopamine biosensor was based on the entrapment of tyrosinase into polypyrrole-cobalt phthalocyanine (CoPc) at multiwalled carbon nanotubes (MWCNTs) electrodes. CoPc acts as a mediator for tyrosinase (Tyr) reaction and MWCNTs provides a highly porous conductive network enhancing the enzyme immobilization and the electrochemical transduction of the enzyme reaction by boosting the amplification phenomenon involved in the biosensing of catechol and dopamine. The comparison of the performance of CoPc-tyrosinase electrodes with and without MWCNTs film clearly indicates the improvement in sensitivity and maximum current brought by the combination of MWCNTs and mediator (CoPc). These nanostructured hybrid bioelectrodes were tested in chronoamperometry; they exhibit high sensitivity (LOD = 3.6 μ M) for the detection of dopamine. The biosensors had very low response time, less than 10 seconds. Further studies to improve the response characteristics of this type of biosensor are in progress.

6 - Development of screen printed electrodes SPE

A major important aspect of the electrochemical detection of neurotransmitters is the construction of disposable transducers to be assembled as enzymatic biosensors for screening procedures.

In order to develop screen printed (bio)electrodes for neurotransmitters detection, we initially realized a simply and high selectively electrochemical method for simultaneous determination of hydroquinone (HQ) and catechol (CC) at an electrochemically activated screen-printed carbon electrode (SPCE) modified with Prussian Blue (PB) [70]. The PB acted as a

mediator and thereby enhanced the rate of electron transfer in chemical reaction (Figure 4). Various optimization studies such as the pH of the measuring solution, linear range of response, sensitivity and detection limit, were conducted to obtain maximum amperometric responses for analytes measurement. Differential pulse voltammetry (DPV) was used for the simultaneous determination of HQ and CC in their mixture, and the peak-to-peak separation for HQ and CC was about 0.11 V. The two corresponding well-defined oxidation peaks of HQ and CC at activated Prussian Blue-modified screen-printed carbon electrode (PB-SPCE) occur at -0.012 V and +0.094 V, respectively. Under the optimized condition in DPV, the oxidation peak current of HQ and of CC is linear over a range from 4.0×10^{-6} M to 9.0×10^{-5} M HQ and from 1.0×10^{-6} M to 9.0×10^{-5} M CC. The obtained detection limit for HQ and CC was 1.17×10^{-7} M and 4.28×10^{-7} M, respectively. DPV can be used for individual or simultaneous determination of HQ and CC.

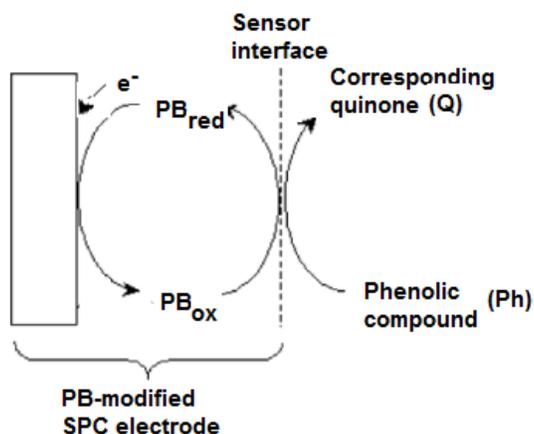


Fig. 5. Schematic diagram of HQ/CC and PB mediated redox reaction at the WE surface.

The proposed activated PB-SPCE was successfully applied to the simultaneous determination of HQ and CC in spiked tap water. Using the standard addition method, the average recovery of the proposed method based on activated PB-SPCE was 99.03% and 95.87% for HQ and CC, respectively. A successful elimination of the fouling effect by the oxidized product of HQ on the response of CC has been achieved at the activated PB-SPCE. Moreover the PB-SPCE exhibited good selectivity towards ascorbic acid, phenol and uric acid. The response of PB-SPCE was remarkably high compared to the negligible current responses of the studied interferences. This research has developed a cheap, sensitive, and rapid method for the electrochemical determination of HQ and CC in aqueous pH 6.64 PBS solution without previous separation.

7 - Development of sensors for L-dopa

Levodopa [(-)-3-(3,4-dihydroxyphenyl)-L-alanine], an unusual amino acid, is an important neurotransmitter, and has been used for the treatment of neural disorders such as Parkinson's disease (PD)[71] PD is believed to be related to low levels of dopamine (DA) in certain parts of the brain. Levodopa (L-dopa) is considered the most effective treatment available

for PD. When L-dopa is taken orally, it crosses through the “blood–brain barrier”; once it crosses, it is converted to DA. The resulting increase of DA concentration in brain is believed to improve nerve conduction and assist the movement disorders in PD. Therefore the success of DA replacement therapy by its precursor, L-dopa, is a major landmark in the field of neurology[72]. L-dopa can alleviate the symptoms of Parkinson’s disease and can also decrease muscular rigidity and tremors [73,74] So the research about L-dopa has an important practical significance. Nevertheless, elevated levels of DA also cause adverse reactions such as psychosis, nausea, emesis, hypotension and dyskinesia, vomiting and cardiac arrhythmias [74-76]. Therefore, in order to achieve a better curative effect and a lower toxicity, it is very important to rapidly control the content of L-dopa and its inhibitors and impurities in biological fluids and pharmaceutical formulations. In vitro, L-dopa is a lethal toxin to the culture of neurons and a few animal studies have shown that chronic L-dopa may be toxic in vivo, too [77-80]. Chronic L-dopa treatment in PD patients is frequently associated with some side effects such as nausea and vomiting results from the increases of plasma L-dopa level. Clearly the process of L-dopa detection and its concentration determination is an important feature in pharmaceutical and clinical procedures [81,82].

We developed a carbon nanotubes paste electrode modified with iron-phthalocyanine for the sensitive voltammetric determination of L-dopa [83]. The measurements were carried out using cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The results showed an efficient catalytic activity of the electrode for the electrooxidation of L-dopa, which leads to lowering its potential by more than 200 mV. Under the optimum conditions the electrode provides a linear response versus L-dopa concentrations in the range of 10 μ M and 80 μ M using DPV. The modified electrode was used for determination of L-dopa in commercial dosage forms (Madopar) without any pre-treatment with satisfactory results.

Stage 5 (16.12.2014-31.12.2015): Data validation of the biosensor

8. Development of a chromatographic method for biosensor data validation

In this stage of the Project, in order to validate the data obtained with the biosensor, some neurotransmitters were investigated by using liquid chromatography with reverse phase following a mechanism based on the formation of ionic liquid pairs (RP-IPC). We studied the influence of the main elution parameters upon chromatographic behavior for several compounds with neuronal importance under ion-pairing (IP) mechanism [84]. The principle of this mechanism is based on the fact that the analyte in ionic form will interact with a contra-ion forming ions pairs which would be distributed in between the two phases. The ions pairs will be characterized by an enhanced hydrophobicity, the contribution being usually brought by the contra-ion participating in the ionic pair formation [85]. The biogenic amines investigated in this study include dopamine, norepinephrine and epinephrine, and serotonin.

In order to obtain small times of chromatographic analysis without losing the characteristics of a satisfactory chromatographic separation initially, we optimize the

experimental parameters for analytes separation. By using RP-IPC we obtained the chromatographic separation of the four analytes as shown in Figure 6. It can be seen that for mobile phase composition of 40, 35 and 30% MeOH the epinephrine and norepinephrine peaks are overlapped.

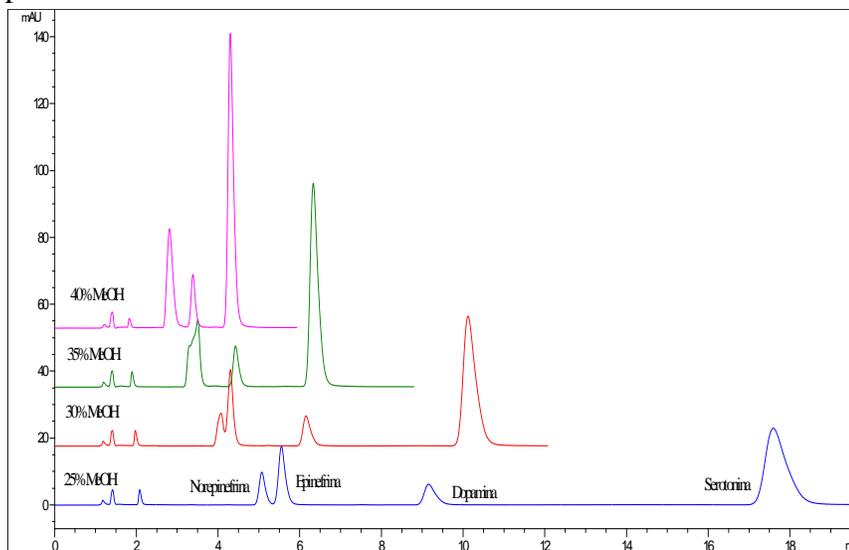


Fig. 6. Chromatograms of analytes mixture solutions, Zorbax Eclipse XDB C18 column, M.F. Aq (0.1% H_3PO_4 + 10 mM $C_7H_{15}SO_3Na$) + MeOH, isocratic elution.

In all gradient elution experiments the time of chromatographic analysis was decreased without affecting the efficiency, resolution and the selectivity of the peaks obtained in case of isocratic elution.

The chromatographic peak area dependence on the quantity of the analyte injected in the chromatographic column (using RP-IPC) is presented in Figure 7. A linear dependence was obtained presenting a very good correlation factor.

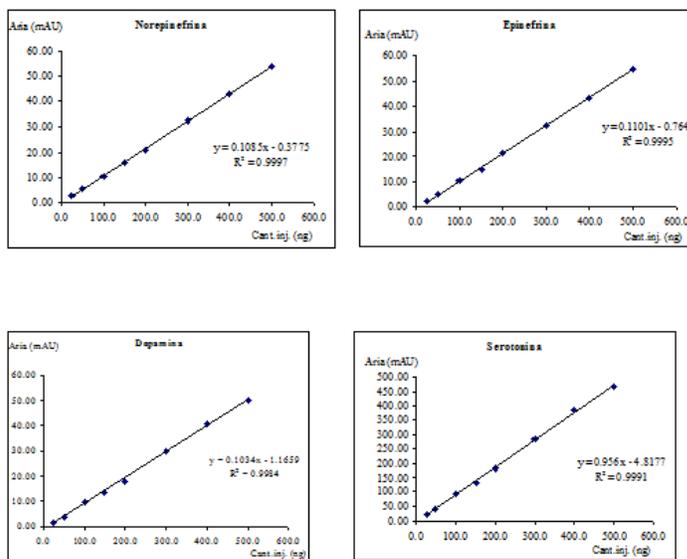


Fig. 7. Dependence of the peak area vs. the quantity of the injected analyte in the chromatographic column by RP-IPC technique.

In Table 2 the detections limits and of identification of the studied compounds are presented.

Tab.2. The limits of detection and identification for the analyzed compounds

	Norepinephrine	Epinephrin e	Dopamine	Serotonin
Detection limit (ng)	18.4977	24.1378	40.5885	31.4664
Identification limit (ng)	5.5493	7.2413	12.1765	9.4399

Two main models are describing the retention process in ion-pairing liquid chromatography, namely the partition and electrostatic models, which are very useful in understanding this complex process and also for the analytical method development [84]. According electrostatic model, the retention factor of an analyte electrically charged $A^{\pm z_A}$ with charge z_A , in presence of an API, presenting -1 charge (for this case), which is present in a mobile phase in a certain concentration, C_{API} , could be correlated with its retention factor in the absence of API, k_A^0 , by equation:

$$\ln k_A^{IPA} = \ln k_A^0 + \left(\frac{z_A}{2}\right) \left[\ln \left(\frac{n_0 K_{IPA} C_{IPA}}{\kappa} \right) + \ln \left(\frac{F^2}{RT \epsilon_0 \epsilon_m} \right) + 1 \right] \quad (1)$$

where k_A^{IPA} represent the retention factor in the presence of API, κ – inverse Debye length (m^{-1}), n_0 - is a parameter related to the adsorption of IPA- as a monolayer of the stationary phase surface (mol/m^3), C_{API} - concentration of API mobile phase, K_{API} – adsorption constant; ϵ_0 permittivity of vacuum, ϵ_r is the dielectric constant of the mobile phase, F –Faraday constant (C/mol); T – absolute temperature (K); R – gas constant (J/mol K).

According to the partition model the retention factor of an analyte (A) in the mechanism of ion pairing formation mechanism depends on the pH value of the aqueous component through its two contributions: one due to the partition of the un-charged specie (k_A^0), between mobile and stationary phase, and the second one due to the A^+ ionic specie find as an ionic pair A^+IPA^- , in between the two phases ($k_{A^+IPA^-}$):

$$k_A^{IPA} = k_A^0 + k_{A^+IPA^-} \frac{K_b \times 10^{-pH}}{K_w + K_b \times 10^{-pH}} \quad (2)$$

where K_b represent the basicity constant for the analytes with basic character. A sigmoidal dependence should be obtained presenting an inflexion point at pH values equal with equal to the pK_b of the basic compound.

Experimental parameters which influence the chromatographic separation of the neurotransmitters were investigated such as the hydrophobicity and the concentration of the ion-pairing agent added to the aqueous phase, the organic modifier content in mobile phase, temperature, ionic strength and pH value of the mobile phase on the retention process [84]. We present the temperature influence on the retention process only. Temperature has a significant influence on chromatographic separations. Elevated temperatures decrease viscosity and increase

solubility and diffusivity, thus the retention, peak shape, column efficiency, and total analysis time are affected. Particularly, in IP mechanism the main influence of increasing temperature is to decrease the interaction between the ion pairs and the stationary phase, in accordance to the van't Hoff equation [84]. Linear $\ln k$ vs $1/T$ relationships have been obtained within the interval 20 - 55°C, as can be seen in Figure 8 for the neurotransmitters investigated.

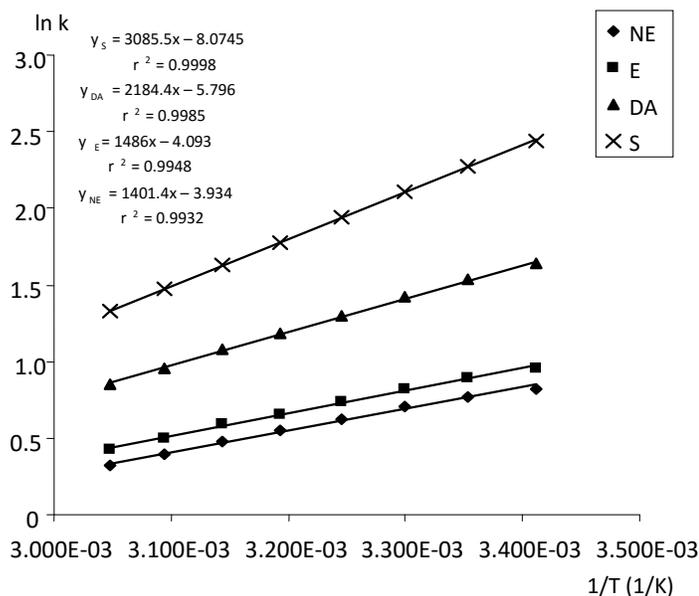


Fig. 8. Dependence of the $\ln k$ of the neurotransmitters on $1/T$ (experimental conditions: 25% MeOH and 75% aqueous component consisting of 10 mmol/L $C_7H_{15}SO_3Na$, pH = 2).

This study included the investigation of some major experimental parameters, such as the organic modifier content in mobile phase, the hydrophobicity and the concentration of the ion-pairing agent added to the aqueous phase, temperature, ionic strength and pH value of the mobile phase on the retention process. The influences of different experimental parameters for the studied compounds by using ionic pair formation mechanism could be explained by both models: electrostatic and partition. The effects of hydrophobicity API, its concentration in the mobile phase and of ionic strength also are well explained by electrostatic model; good correlations were obtained for dependences between experimental parameters and chromatographic results foreseen by theoretical model. By using partition model, the effects of organic modifier concentration and of pH of the aqueous solution are well explained and good correlation between experimental parameters and chromatographic results were obtained. These correlations could be used for estimation of some extrapolated obtained chromatographic data which can be used in the characterization of the formed analyte ions pairs with API regarding their hydrophobicity. It is well known that chromatographic methods are the most often ones in data validation, and so the obtained data will be used for comparison and validation of the data obtained by means of the biosensor in the case of the studied neurotransmitters.

In this phase of the Project we also developed a novel assay for the electrochemical detection of L-dopa based on an electrochemically pretreated screen-printed carbon electrode (SPCE). We demonstrate the voltammetric analysis of L-dopa in BRB solution of pH 2.21 using a disposable screen-printed carbon electrode coupled with DPV for low sample volume and rapid L-dopa determination in Isicom® pharmaceutical formulation. The proposed method significantly reduced the over potential for L-dopa oxidation on activated SPCE and offers the advantage of simple electrode preparation steps with no need to use chemical modifiers [86].

Stage 6 (1.01.2016-15.10.2016): Construction of a micro-biosensor prototype for neurotransmitters determination.

Developed sensors and biosensors

9 - Development of sensors for dopamine and L-dopa

Based on experimental data analysis obtained during the Project regarding the possibility of neurotransmitters determination in real clinical samples, we finally decided to use the molecular imprinted (MIP) polymer electrode due to its performances (low detection limit and good selectivity) [31]. The MIP electrode was prepared through electrodeposition of polyethacridine-dopamine (PET-DA) film on the glassy carbon electrode and then removing DA from the film via chemical induced elution. The selectivity of the prepared MIP-modified glassy carbon electrode (GCE) was tested for three interfering molecules, ascorbic acid (AA), uric acid(UA) and paracetamol at the same initial concentration for each molecule, 10^{-5} M. These molecules may interfere with the determination of dopamine, particularly AA which has higher concentration than dopamine and is oxidized at the same potential. The oxidation peak currents at MIPET-DA-GCE in phosphate buffer solutions (PBS) containing 1.0×10^{-7} M DA and 100 times that of each interferent obtained by differential pulse voltammetry (DPV) showed that in all of the cases, there were no changes in the current responses. Polyethacridine is a non-conductive polymer and its pore size is smaller [87]. So, the responses of the larger molecules such as AA, UA and paracetamol are suppressed at this polymer-coated GC electrode. This allows voltammetric determination of DA in presence of the mentioned electroactive species. Beside this, the high specificity is also due to the cavities which match the shape and functional groups of DA formed during the procedure that template molecules were embedded in the material. These results confirmed that the MIPET-DA-GCE had an excellent selective recognition capacity toward the template molecule as a result of the imprinting effect produced in the presence of DA. The modified sensor was validated for the analysis of DA in deproteinized human serum (DHS) samples using DPV technique. Dopamine level in human serum ranges from 8.5×10^{-9} to 7.9×10^{-8} M [88]. The linear DA response from 2.0×10^{-8} M up to 1.0×10^{-6} M based on MIPET-DA-GCE is suitable for its practical application. DHS was obtained from the blood samples collected in the morning at clinical hospitals in Bucharest from three different patients and processed as described previously [19]. The method proved applicable in the analysis of DHS after the removal of proteins. Three DHS probes were analyzed by the developed DPV method using the developed MIPET-DA-GCE. All measurements were performed at room temperature. DA recovery experiments were carried out via standard addition method by adding

10.0×10^{-9} , 30×10^{-9} and 50×10^{-9} M DA to the samples, respectively. In all analyzed samples, the added standard was found in good per cents that varied between 96 and 106.6% with a RSD of 3.36 to 4.43% (Table3). The results of the determination show that the prepared sensor is reliable and feasible in the detection of DA in real samples.

Tab. 3. Results of the determination of DA in human's serum and recovery

Sample number	Found DA 10^{-9} M	Added DA 10^{-9} M	DA found after standard addition, 10^{-9} M	Recovery (%)	RSD (%)
1	23	10	33	100	1.74
		30	52	96.6	
		50	72	98	
2	29	10	39	100	3.36
		30	60	103.3	
		50	77	96	
3	33	10	43	100	4.43
		30	65	106.6	
		50	82	98	

Average of three measurements

Under the optimized conditions, experimental results revealed that the concentration molar ratio of 1/100 for AA, uric acid and acetaminophen do not interfere in the determination of DA by the MIPET-DA-GCE. The excellent sensitivity and selectivity of the MIPET toward DA can be attributed to the recognition cavities within the MIPET film. These recognition cavities are complementary in terms of molecular shape, size, and functionality of the template molecule, resulting in the specific rebinding ability of DA. The chemically modified electrode prepared and the DPV method developed performed well in analyzing deproteinized human sera samples. The recovery of 96–106.6% resulted from the specific recognition to DA against the structural analog and coexisting interferences, indicated that the new electrochemical sensor based on MIPET-DA-GC performed well for the measurements of trace DA in real samples analysis. The result shows the practicability of the sensor applied in the DA detection.

Continuing our research for L-dopa determination we develop a rapid and efficient DP voltammetric method for simultaneous evaluation/determination of levodopa (L-dopa) and benserazide (Bz) in real samples[86,89]. This is the first example of an electrochemically pretreated screen-printed carbon electrode (SPCE*) used in evaluation of the mentioned compounds in pharmaceutical samples. Preliminary CV experiments were carried out to study the electrochemical behaviour of L-dopa and Bz on SPCE*. L-dopa electrochemistry at untreated

and electrochemically pretreated SPCE was discussed elsewhere [86]; it was showed that in acidic medium the voltammogram of L-dopa exhibits two well-defined and sharp peaks at the SPCE* with $E_{pa}=0.244$ V and $E_{pc}=0.161$ V and a second reduction peak at -0.043 V. In case of Bz an irreversible process with anodic peak potential at 0.156 V is observed. The oxidation peak potential of L-dopa and Bz shifted at about 273 mV [86] and 213 mV to a less positive potential, respectively, compared with an untreated SPCE [89]. For a 5.0×10^{-4} M equimolecular mixture of L-dopa and Bz the observed anodic peak to peak separation between the two compounds is around 0.073 V which is sufficient enough to permit their determination in presence, while in case of the same equimolecular mixture no separation of the mentioned compounds occurred at an non-activated SPE (Figure 9).

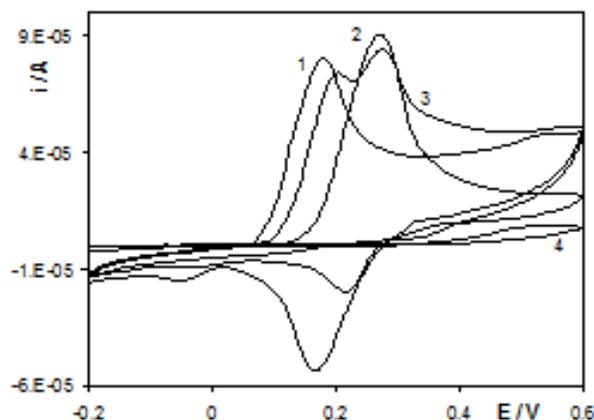


Fig. 9. Cyclic voltammograms of a 5.0×10^{-4} M Bz (1), 5.0×10^{-4} M L-dopa (2) and for 5.0×10^{-4} M an equimolecular mixture of L-dopa and Bz (3) obtained at SPCE* and of a 5.0×10^{-4} M equimolecular mixture of L-dopa and Bz at SPCE (4); BRB solution pH 2.21; scan rate 50 mVs $^{-1}$.

The validity of the proposed DPV method for the simultaneous determination of L-dopa and Bz in pharmaceutical preparation samples was investigated. After sample preparation and adequate dilution steps, as described in experimental section, the method was applied for the direct determination of L-dopa and Bz in Madopar tablets using the standard addition method (Figure 10). The corresponding obtained oxidation peaks currents were measured and used to calculate L-dopa and Bz, content in the Madopar tablets. Inset Figure 10 calibration graphs for L-dopa and Bz are presented.

To check the accuracy of the investigated method in pharmaceutical preparations the recovery studied were carried out. The L-dopa and Bz content per Madopar tablet estimated by the present method (mean of four determinations) using the SPCE* (203.75 mg for L-dopa and 49.39 mg for Bz) was in close agreement with that declared on the label (200 mg and 50 mg respectively per tablet) within acceptable error limit. Recovery of $101.87 \pm 2.14\%$ of L-dopa with a relative standard deviation (RSD) lower than 3% was obtained for the pharmaceutical formulation sample ($n = 4$); in case of Bz the recovery was 98.79% with a RSD of 2.73% . The relative errors calculated considering the values claimed by manufacture were 2.41% for L-dopa and 2.32% for Bz showing a good agreement.

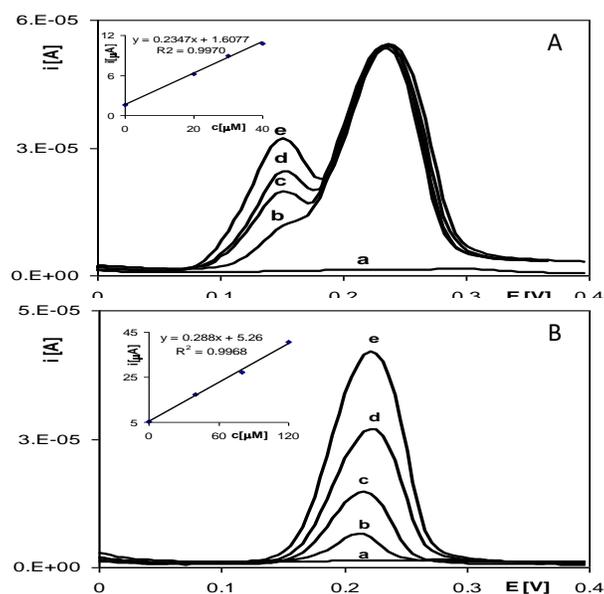


Fig. 10. Differential pulse voltammograms for a Madopar sample diluted in BRB solution pH 2.21 obtained at SPCE*. A: (a) BRB solution pH 2.21, (b) Madopar sample and additions of 2 μL (c); 4 μL (d); 6 μL (e) of 10^{-3} M Bz. B: (a) BRB solution pH = 2.21, (b) Madopar sample and additions of 2 μL (c); 4 μL (d); 6 μL (e) of 10^{-3} M L-Dopa. Inset, the calibration graphs.

The proposed method was compared with a chromatographic one [90]. The obtained chromatogram for a mixture of Bz and L-Dopa in a real sample of Madopar is presented in Figure 11.

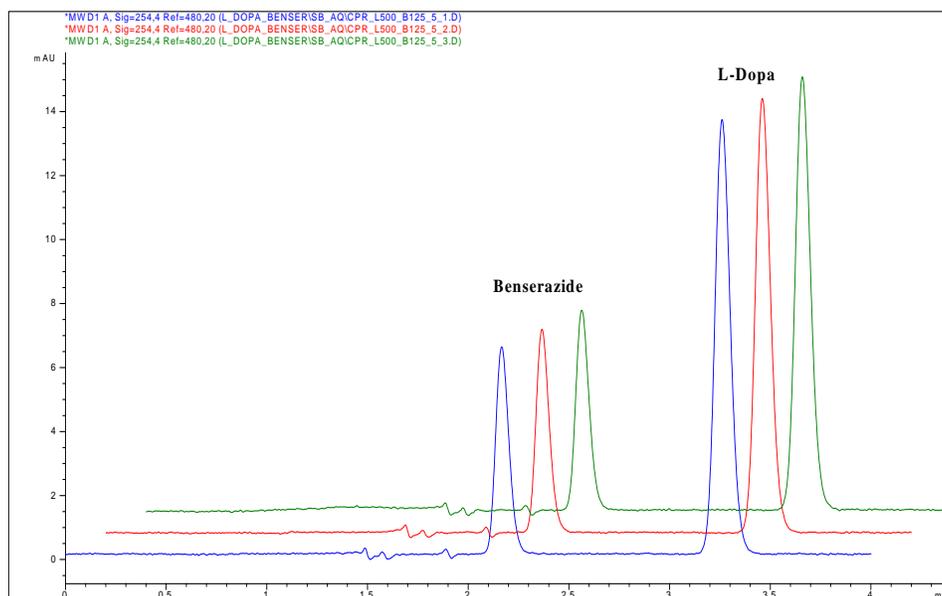


Fig. 11. Chromatograms in triplicate of a mixture of 125 ppm Bz and 500 ppm L-Dopa in a real sample of Madopar. Retention times: Bz 2.165 min ($k = 0.15$); L-Dopa 3.259 min. ($k = 0.74$); chromatographic resolution: 8.27.

The amounts of L-dopa and Bz are fairly close to the labeled amounts for both techniques. The summarized results for the analysis are shown in Table 4.

Tab. 4. Comparison of the results obtained by DPV and chromatographic methods for L-dopa and Bz determination in Madopar tablets

Claimed content (mg)		Found content by DPV \pm SD (mg)	
L-dopa	Bz	L-dopa	Bz
200.00	50.00	203.75 \pm 4.27	49.39 \pm 1.37
Recovery, R \pm SD, %		RSD, %	
L-dopa	Bz	L-dopa	Bz
2.10	2.76	2.10	2.76
HPLC assay (mg)			
L-dopa		Bz	
202.5		49.8	

The obtained recovery results in Table 4 indicates that SPCE* can be successfully used for the simultaneous determination of L-dopa and Bz in pharmaceutical preparations of Madopar. Furthermore, all percentages found are in accordance with the Pharmacopeia requirements for the assay (90.0–110.0% of the labeled amount). These results demonstrate the capability of SPCE* in voltammetric determination of L-dopa and Bz in real samples with good recoveries of the spiked L-dopa and Bz. On the other hand, one can conclude that both mentioned methods (DPV and HPLC) give congruent results.

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Dissemination of the Project results

2012

Communications

1. A. Ciucu, O. Covaci, I.G. David, V. David, Development of Novel Chemically Modified Electrodes for Assessment of Neurotransmitters Involved in Neuro-Degenerative Diseases, XIIth Conference with International Participation: Present State and Perspectives of Analytical Chemistry in Practice, May 8th-11th, Bratislava, 2012, Slovak Republic, poster presentation.
2. I. G. David, G. Cracea, A. M Florea, A. A. Ciucu, V. David, Electrochemical preparation of polyTiron modified pencil graphite electrode for dopamine voltammetric detection, Analytical Chemistry for a Better Life, 1st International Conference on Analytical Chemistry, Targoviste, Romania, September 18th- 21st, 2012, oral presentation.

3. A. A. Rabanca, O. I. Covaci, A. Ciobanu, J. Ciurea, S. Cosnier, A. A. Ciucu, Carbon nanotubes/iron phthalocyanine/ionic liquid paste electrodes with application in catecholamines detection, Analytical Chemistry for a Better Life, 1st International Conference on Analytical Chemistry, Targoviste, Romania, September 18th- 21st, 2012, poster presentation.

4. L.A. Gugoasa, R.I. Stefan-van Staden, A.A.Ciucu, J. Frederick van Staden, Influence of the carbon matrices on the behaviour of the dsDNA based biosensors, Analytical Chemistry for a Better Life, 1st International Conference on Analytical Chemistry, Targoviste, Romania, September 18th- 21st, 2012, poster presentation.

2013

Papers

1. S. Iordache, R. Cristescu, A.C. Popescu, C.E. Popescu, G. Dorcioman, I.N. Mihailescu, A.A. Ciucu, A. Balan, I. Stamatina, E. Fagadar-Cosma, D.B. Chrisey, Functionalized porphyrin conjugate thin films deposited by matrix assisted pulsed laser evaporation, Appl. Surf. Sci. 278(2013)207–210. **IF 2.538**

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1. A. Ciobanu, A. Surdu, A. Ciucu, Particularities of depression in women with polycystic ovary syndrome, 17th International Congress of Comparative Endocrinology, July 15th-19th, 2013, Barcelona, Spain, poster presentation.

2. A. Ciobanu, I. Baciuc, A. Ciucu, Hypoactive sexual desire disorder impact on partners and repercussions on family life, 17th ICCE 2013 - International Congress of Comparative Endocrinology, July 15th-19th, 2013, Barcelona, Spain, poster presentation.

3. A. Ciucu, Novel developments in (bio)electrochemical detection of neurotransmitters - specific applications in biomedicine, Pécs University, Department of Physical Chemistry, Hungary, 2013, oral presentation

2014

Papers

1. L.A. Gugoasa, R. I. Stefan-van Staden, A.A. Ciucu, J. F. van Staden, Influence of physical immobilization of dsDNA on carbon based matrices of electrochemical sensors, Curr. Pharm. Anal. 10 (2014) 20-29. **IF 1.08**

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2. I.G. David, M.A. Florea, O.G. Cracea, D.E. Popa, M. Buleandra, V. David, I.A. Badea, E.E. Iorgulescu, A.A. Ciucu, Voltammetric determination of vitamin B₁ and vitamin B₆ on a disposable electrode, *Chem. Pap.*69 (2015) 901–910. **IF 1.193**

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Communications

1. A. Ciucu, Bioanalytical applications of DNA based biosensors, EMN Meeting, on DNA & RNA, Energy Materials Nanotechnology, November, 9-12, 2015, Istanbul Turkey, Plenary lecture-invited speaker. (<http://www.emnmeeting.org/DNA/DNA-based-hybrid-materials/>)

2016

Papers

1. L. Kiss, V. David, I.G. David, P. Lazăr, C. Mihailciuc, I. Stamatina, A. Ciobanu, C.D. Ștefănescu, L. Nagy, G. Nagy, A.A. Ciucu, Electropolymerized molecular imprinting on glassy carbon electrode for voltammetric detection of dopamine in biological samples, *Talanta* 160 (2016) 489–498. **IF: 4,035**

2. A.A. Rabinca, M. Buleandra, F. Tache, C. Mihailciuc, A.M. Ciobanu, D.C. Stefanescu, A.A. Ciucu, Voltammetric method for simultaneous determination of L-dopa and benserazide, *Curr. Anal. Chem.* 12, (2016)000-000DOI: 10.2174/15734110126661606011617 03. **IF: 1,132**

3. M. Buleandra, A.A. Rabinca, E.C Mitran, A.A. Ciucu, Differential pulse voltammetric method for 4-nonylphenol determination in water samples, *Materiale Plastice* 53(3) (2016) 491-494. **IF: 1,132**

Submitted for publication:

1. M. Buleandra, A.A. Rabinca, F. Tache, Z.Moldovan, A.A. Ciucu, Rapid electrochemical detection of kojic acid at a multi-walled carbon nanotubes screen-printed electrode, *Sens. Actuat. B: Chem.* – under revision (2016). **IF: 4,758**
2. M. Buleandra, A.A. Rabinca, I.A. Badea, I.Stamatin, A.A. Ciucu, Simultaneous electrocatalytic voltammetric determination of dihydroxybenzene isomers using a cobalt-phthalocyanine modified pencil electrode”, submitted to *Electrochimica Acta*– under revision (2016). **IF: 4,803**

Communications

1. A.A. Ciucu, M. Buleandra, Chemically modified electrodes based on carbon nanotubes used in bioanalysis, Energy Material Nanotechnology Meeting on Meeting on Organocatalysis, Biosensors Workshop, Kona, Hawaii, USA, March 21 - 24, 2016, plenary lecture-invited speaker.
2. A.A. Ciucu, M. Buleandra, A.A. Rabinca, I. Stamatin, Analytical applications of carbon nanotubes based electrochemical sensors, EMN Meeting on Carbon Nanostructures, Honolulu, Hawaii, USA, March, 27 - 31, 2016, plenary lecture-invited speaker.
3. A.A. Ciucu, Biomedical applications of sensors and biosensors based on carbonaceous nanostructured materials, The International Conference on Biomedical and Biological Engineering July 15th - 17th, 2016, Shanghai, China, invited keynote speaker.
4. A.A. Ciucu, A.A. Rabanca, M. Buleandra, Sensors Development for Neurotransmitters Determination, 67th Annual International Society of Electrochemistry, Hague, Netherlands, 21-26 August 2016, poster.
5. A.A. Ciucu, M. Buleandra, A.A. Rabinca, I. Stamatin, Simultaneous electrocatalytic voltammetric determination of dihydroxybenzene isomers using a cobalt-phthalocyanine modified pencil electrode, 2nd International Conference on Sensors and Electronic Instrumental Advances, 22-23 September 2016, Barcelona, Castelldefels, Spain, oral presentation.