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# Biosensors for Life Sciences

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## 1. Introduction into Research Problems

In the last period of time, the micro and nanotechnologies changed important fields of the molecular biology, in order to diagnose and treat at cellular and molecular level. In this area may be included also biosensors that use nanoparticles as immobilisation support (colloidal particles, carbon nanotubes, optic fibre) of the biological components that are used for cellular analysis *in vivo.*

The subject of this chapter propose as research the field of scientific investigation using biosensors based on the synergism of the knowledges from biophysics, biochemistry, electronics, biology, medicine, informatics and mathematic. Some of the classical and modern methods used in order to detect organophosphorus and carbamates pesticides, mycotoxins in the frame of EU regulations are presented, as partial results of two research programs for young researchers supported by MEdC – UEFISCSU Romania and a Balkan Environmental Association (B.EN.A.) fellowship supported by TUBORG-B.EN.A.

The new point of the work was to obtain and to optimize some new biosensors in order to be used for pesticide (organophosphorus and carbamates) and mycotoxins detection. The experimental part is still in work, so in this chapter will be presented some of the selected partial results.

A biosensor is a measurement system based on a combination of biochemical and electronic elements, which are in close contact each other and are incorporated in a single unit. A biochemical component (enzyme or biological material such as micro-organisms, plant or animal tissues and cells) is chosen for its selectivity toward the substrate or the inhibitor to

be determined (Andreescu & Marty, 2006). The electronic signal-transducing element (electrochemical, optical detector, gravimetric detector) converts the biochemical response into electric and optic signals, which are amplified, measured and decoded by an appropriate electronic unit.
For enzyme based biosensor, the enzyme is situated inside of insoluble support and so it obtains a biphasic system. The enzyme can be reused after catalyse. An advantage of this technique is that final product is without enzyme. Other advantage of immobilizing the enzyme is the higher stability and activity (Badea&Coman, 2007; Coman et al. 2005).
The pollutants detection using biosensors offered viable alternative for usual chromatographic methods, the sensibilities for these two methods have been comparable after the immobilisation processes. Biosensors present many advantages: easy handling, compatibility with standard commercial equipment's, miniaturized possibility, and automatic measurement.

## 2. Generalities about Toxicity of Pesticides and Mycotoxins and their Detection Methods

The use of acutely toxic pesticides and mycotoxins associated with a weak or absent legislative framework regulating pesticide and mycotoxins use is one of the major reasons for the high incidence of poisoning in some developing countries (Kondardsen et al. 2003). Additional factors such as lack of information, low literacy, and education levels of the rural population, poor and inadequate working conditions, inadequate protection during pesticide application, and inappropriate spraying technology have also been shown to play important roles in the intoxication scenario (Hurtig et al., 2003; Karlsson, 2004).

### 2.1 Pesticides

A pesticide is any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest. Though often misunderstood to refer only to insecticides, the term pesticide also applies to herbicides, fungicides, and various other substances used to control pests.
A pesticides may be a chemical substance or biological agent used against pests including insects, plant pathogens, weeds, mollusks, bird, mammals, fish, nematodes (roundworms) and microbes that compete with humans for food, destroy property, spread disease or are a nuisance. Many pesticides are poisonous to humans (Coman et al., 2000)..
Organophosphorus and carbamate compounds are rapidly absorbed through the respiratory tract and through the digestive route, and to a lesser extent through the skin. After absorption, these compounds act by inhibiting the action of esterases, especially of acetylcholineesterases, following the interaction with the hydroxyl group of serine, which may determine: accumulation of acetylcholine which stimulates muscarinic and nicotinic receptors, increase cholinergic activity, and induce paralysis and death (Mijanovic & Zaciragic, 2006).
Organochlorine pesticides act primarily by altering the movement of ions across the nerve cell membranes, thus changing the ability of the nerve to fire. Organophosphate and carbamate pesticides act primarily at the synapses, altering the regulation of the transmission of the signal from one cell to the next (Hink et al., 2007).
A third, newer class of insecticides are the synthetic pyrethroids. These were developed because of their lower toxicity than OP and carbamates. These chemicals alter normal

neuronal function by inhibiting ion movements across the nerve cell membrane, alterations in intracellular calcium ion concentrations and possibly by binding to GABA receptors.
Organophosphates are some of the most widely used pesticides in the world. They are used in agriculture, homes, gardens and veterinary practices, replacing the same uses as the organochlorines, many of which have been banned for years. In general, they are not persistent in the environment as they break down quickly. Because of their relatively fast rate of degradation, they have been a suitable replacement for the more persistent organochlorines.
Some of the early organophosphates were developed as nerve poisons for human warfare. The organophosphates recommended for non-residential uses are relatively toxic to vertebrate organisms. Their primary mode of action on insects and other animals is by phosphorylation of the acetylcholinesterase enzyme. This enzyme is necessary for controlling nerve impulse transmission between nerve fibres. A loss of this enzyme function results in an accumulation of acetylcholine, which causes unregulated nervous impulses. Higher levels of acetylcholine result in sensory and behavioural disturbances, incoordination and depressed motor function. Symptoms of acute poisoning develop during or after exposure, within minutes to hours, depending on method of contact. (Moser, 2007).
Carbamate pesticides are derived from carbamic acid and kill insects in a similar fashion as organophosphate insecticides. They are widely used in homes, gardens and agriculture. Like the organophosphates, their mode of action is inhibition of cholinesterase enzymes, affecting nerve impulse transmission. Because of carbaryl's relatively low mammalian oral and dermal toxicity and broad control spectrum, it has had wide use in lawn and garden settings.
In the literature it were performed different kind of analytical methods in order to detect organophosphorus and carbamates pesticides: liquid chromatography (Badea et al., 2004), immunoassay (Badea et al., 2004, Brun et al., 2004), biosensors (Schulze et al., 2003; Mulchandani et al., 2001; Pemberton et al., 2005, Badea et al, 2005; Badea et al., 2006; Ghosh et al., 2006).
In most countries, in order to sell or use a pesticide, it must be approved by a government agency. For example, in the United States, the EPA does so. Complex and costly studies must be conducted to indicate whether the material is effective against the intended pest and safe to use (Blasco et al., 2005; Neisheim 2002).

### 2.2 Mycotoxins

The ingestion of food containing mycotoxins, the toxic products of microscopic fungi (moulds), may have serious adverse health effects in humans and animals. Occasionally, occupational exposure to airborne mycotoxins may also occur. The mycotoxin contamination of foodstuffs may vary with geographical conditions, production and storage methods, and also with the type of food, since some food products are more suitable substrates for fungal growth than others (Pfohl-Leszkowicz & Manderville, 2007).
Ochratoxins are produced by several species of the fungal genera *Aspergillus* and *Penicillium*. These fungi are ubiquitous and the potential for contamination of foodstuffs and animal feed is widespread. Ochratoxin A, the major compound, has been found in more than 10 countries in Europe and the USA.
Ochratoxin A has been found in maize, barley, wheat, and oats, as well as in many other food products, but the occurrence of ochratoxin B is rare. Residues of ochratoxin A have

been identified in the tissues of pigs in slaughterhouses, and it has been shown, under experimental conditions, that residues can still be detected in pig tissues one month after the termination of exposure.

Field cases of ochratoxicosis in farm animals (pigs, poultry) have been reported from several areas of the world, the primary manifestation being chronic nephropathy. The lesions include tubular atrophy, interstitial fibrosis, and, at later stages, hyalinized glomeruli. Ochratoxin A has been found to be nephrotoxic in all species of animals studied so far, even at the lowest level tested (200 μg/kg feed in rats and pigs). It has also been reported to produce teratogenic effects in mice, rats, and hamsters (Gresham et al, 2006; Pfohl-Leszkowicz & Manderville, 2007).

Ochratoxin A is a nephrotoxic mycotoxin which is carcinogenic to rodents and possesses teratogenic, immunotoxic and possibly neurotoxic properties. Further, it may be implicated as a factor in the human disease Balkan Endemic Nephropathy and the development of urinary tract tumours in humans. Human endemic nephropathy is a kidney disease of unknown etiology that has so far only been encountered in some areas of the Balkan Peninsula. The renal changes observed with this disease are comparable to those seen in ochratoxin A-associated nephropathy in pigs. Also, recent data from France and North Africa point towards a correlation between chronic interstitial nephritis and high exposure to ochratoxin A.

The effects of superoxide dismutase and catalase on ochratoxin A-induced nephrotoxicity were studied. Superoxide removes oxygen by converting it to hydrogen peroxide; this enzyme works in conjunction with catalase, which removes hydrogen peroxide within cells. Superoxide dismutase and catalase prevented most of the nephrotoxic effects induced by ochratoxin A, observed as enzymuria, proteinuria, and creatinaemia, and increased the urinary excretion of ochratoxin A (Sovoz et al., 2004).

Analytical techniques have been developed for the identification and quantitative determination of ochratoxin levels in the μg/kg range.

Ochratoxin A has been found in many commodities, including cereals, cereal products, coffee, grapes, grape juice, wine, cocoa and chocolate, beer, meat, pork products, pulses, milk and milk products, and spices. Several published analytical methods for the determination of ochratoxin A in maize, barley, wheat, wheat bran, wheat wholemeal, rye, wine, beer, and roasted coffee have been formally validated in collaborative studies. The methods are based on liquid chromatography (LC) with fluorescence detection, include a solid-phase extraction clean-up step with reversed-phase C18, silica gel 60, or immunoaffinity columns, and can guarantee detection of < 0.5 μg/kg (Pussemier et al, 2006).

The first LC method for determining ochratoxin A in maize and barley was validated in a collaborative study with materials spiked with ochratoxin A in the range of 10–50 ng/g. Ochratoxin A was extracted from grains with chloroform:aqueous phosphoric acid and isolated by liquid–liquid partitioning into aqueous bicarbonate solution that had been cleaned-up on a C18 (solid-phase extraction) cartridge. Identification and quantification were performed by reversed-phase LC with fluorescence detection.

The use of antibody-based immunoaffinity columns in the clean-up step has improved the analysis of ochratoxin A. Two methods based on immunoaffinity clean-up for determination of ochratoxin A in barley and roasted coffee have been developed and validated in collaborative studies under the auspices of the European Commission, Standard and Measurement Testing programme ( Entwisle et al, 2000).

Screening methods based on TLC are also available. These methods are used in only a few laboratories since they do not provide an adequate limit of quantification (LOQ). Enzyme-linked immunoabsorbent assays (ELISAs) have been developed for the detection of ochratoxin A in pig kidney, animal and human sera, cereals, and mixed feed. The results obtained with these methods require confirmation since the antibodies produced often show cross-reactivity to compounds similar to ochratoxin A.

The new elements will be comparations of different chromatographic, spectral and enzymatic methods, trying to detect ochratoxin A also using biosensors, with the help of our partners from Romania, France and Brasil.

Aflatoxins are a family of fungal toxins produced mainly by two *Aspergillus* species which are especially abundant in areas of the world with hot, humid climates. *Aspergillus flavus*, which is ubiquitous, produces B aflatoxins. *A. parasiticus,* which produces both B and G aflatoxins, has more limited distribution. Major crops in which aflatoxins are produced are peanuts, maize and cottonseed, crops with which *A. flavus* has a close association. Human exposure to aflatoxins at levels of nanograms to micrograms per day occurs mainly through consumption of maize and peanuts, which are dietary staples in some tropical countries.

Aflatoxin M1 is a metabolite of aflatoxin B1 in humans and animals. Human exposure to aflatoxin M1 at levels of nanograms per day occurs mainly through consumption of aflatoxin-contaminated milk, including mothers' milk. Measurement of biomarkers is being used increasingly to confirm and quantify exposure to aflatoxins. In large studies realized in China, it was observed that risk for hepatocellular carcinoma was elevated among people with aflatoxin metabolites in urine, after adjustment for cigarette smoking and hepatitis B surface antigen positivity (Huang et al, 2003; Yu et al, 2002).

Extensive experimental studies on the carcinogenicity of aflatoxins led to a evaluation of the evidence as follows: sufficient evidence for carcinogenicity of naturally occurring mixtures of aflatoxins and of aflatoxins B1, G1 and M1, limited evidence for aflatoxin B2 and inadequate evidence for aflatoxin G2. The principal tumours induced were liver tumours.

The use of resistant varieties of seed and of pesticides, and careful drying and storing procedures can reduce fungal infestation and thus diminish food contamination by aflatoxins. The toxin is not eliminated from foodstuffs or animal feeds by ordinary cooking or processing practices and, since pre-and post-harvest procedures do not ensure total protection from aflatoxin contamination, techniques for decontamination have been developed. The toxin is generally concentrated in a small proportion of seeds that are often different in colour.

Biological and chemical procedures have been developed for the detection and determination of aflatoxins and other mycotoxins. The bioassay techniques that are currently available are not suitable for routine screening purposes, their detection levels being not low enough. The chemical assay techniques, although more accurate and faster, are not always specific. The presence of a certain toxin is usually confirmed by derivative formation and its toxicity verified by bioassay.

The aflatoxins are concentrated by evaporation of the chloroform, and then separated by thin-layer chromatography (TLC). Aflatoxins are intensely fluorescent when exposed to long-wave ultraviolet radiation, which makes it possible to determine these compounds at extremely low levels. An analyst experienced in this field can detect 0.5 ng aflatoxin B1 on a TLC plate. In most methods, the intensity of fluorescence of the sample is compared with that of a standard (Stroka & Anklam, 2000).

The methods using high-pressure liquid chromatography become the methods of choice for mycotoxin analyses because of their sensitivity and improved accuracy, and because they can be applied to a number of mycotoxins including aflatoxins B1, B2, G1, and G2 (Stroka et al., 2000; Castegnaro et al., 2006).
Immunoassays are also important in the qualitative and quantitative detection steps of aflatoxins (Badea & Coman, 2004; Coman & Badea, 2004; Sapsford et al, 2006).
It were performed and are in progress studies in the frame of these research projects, by collaboration between representatives from Transilvania University of Brasov, (Romania) from Sanitary Veterinary Direction and for Food Safety, Brasov (Romania), from BIOMEM, University of Perpignan via Domitia (France) and .Universidade Federal do Maranhão, Sao Louis (Brasil).

## 3. Importance and Relevance of the Scientific Content

The research projects propose the analysis and the optimisation of some detection possibilities of several bioactive compounds with toxic potential (mycotoxins, organophosphorus and cabamates pesticides) from water samples, foods and from biological samples, using enzymatic, chromatographic and spectral methods. Analysis using the biosensor technology is part of this area of research and offers the advantages such as miniaturization, easy sample manipulation, and the possibility of *in-situ* determination which further substantially diminishes the errors resulted from sample processing operations, with simple and low-cost instrumentation, fast response times, minimum sample pre-treatment, and high sample throughput. Biosensors are devices consisting of biological active protein species immobilized on the surface of physical transducers.
In the last period of time, there were reported also several enzymatic methods that may possible the detection of pollutants (pesticides, mycotoxins) from different samples using oxidoreductase and hydrolase. The use of enzyme-based biosensors is presented also for other fields as: medicine, agriculture, food industry, biotechnology.
Our research group intend to add some original contributions to the developing of this kind of methods, using free or immobilised enzymes (biosensors).
The existence of an experimental nucleus also in the frame of Transilvania University of Brasov, Romania, makes possible a large use of this technique and also the implementing of some subjects in courses and laboratory practice in the curricula of the students from specialisation general medicine, medical college, physics-chemistry and not only their theoretical discussions of this processes.
All these reference elements will constitute the base for the theoretical and experimental research, the young team of specialists being eager to bring new contributions to the knowledge level in the field of life and earth, by studies of some toxic compounds and analysis of food hygiene with impact over human and animal public health.
It is important to dedicate considerable time and energy to planning of the activities for detection of some toxic compounds (pesticides, mycotoxins) from water, foods, and biological samples, using enzymatic, chromatographic and spectral methods, as good planning makes work much easier in the long run and helps to avoid problems and misinterpretations. After the optimization studies using references samples, it will be tested the presence of the bioactive compounds with toxic effects from real samples (waters, foods, biological samples) in order to report the exceeded the maximum limits admitted by European Union environmental regulations.

The objectives of the theoretical and experimental research will be attended by well-established activities that will be performed during the project financing.

## 4. Experimental Procedures

### 4.1 Principle of the Experimental Method

There were obtained enzyme-based biosensors that were tested for detection of some pollutants compounds from reference and real samples, using amperometric detection. There were compared the experimental results for different commercial and mutants acetylcholinesterase and different pollutants compound.

### 4.2 Reagents and Equipments

#### Reagents

- Acetylcholinesterase (AChE) *Electric eel* – commercial enzyme Sigma Aldrich Co (St. Louis, MO, USA).
- Acetylcholinesterase (AChE) - *Drosophila melanogaster* wild type and genetic modified (E107W, E107Y, G406, I199V), obtained by genetic engineering using recombinant DNA - PBS Company (Toulouse, France)
- Electrochemical Mediator 7,7,8,8-tetracyanoquinodimethane (TCNQ), hidroxyethyl-cellulose (HEC) - Sigma Aldrich Chemie GmbH, (Steinheim, Germania)
- Substrate acetylthiocholine chloride (ATCh), pyridin-2-aldoxyme metachloride (2-PAM) - Sigma Aldrich Co (St. Louis, MO, USA).
- Polyvynil alcohol with stirylpiridinium groups SPP-S-13(bio) (PVA-SbQ), polymerization degrees 1700 and 2300 bio were provided by Toyo Gosei Kogoyo Co., Ltd. (Tokyo, Japan).
- Graphite - TIMREX TAS Graphite, M-058 - from TIMCAC LTD., Graphites and Technologies (Bodio, Switzerland).
- The plastic bed used for transducer obtaining – Electrodag PF-410, 423SS, 6037SS-Acherson (Plymounth, UK)
- Chlorpyriphos methyl oxon, Diazinon - CHEM SERVICE, West Chester, PA (USA) 99% purity. Pesticide stock solution was prepared in acetonitrile.
- Methyl paraoxon (98% purity) – Dr Ehrenstorfen GmbH, D86199 (Augsburg, Germania)
- The precursors that were used in sol-gel immobilisation: TMOS (tetrametoxysilane) (99% purity) and MTMOS (methyl thiometoxisilane) (98% purity) - Sigma Aldrich Chemie GmbH, (Steinheim, Germania). The precursors hydrolysis was realized in acid medium (HCl 1mM), and for immobilization was used also PEG600
- All other reagents used have had analytical purity

#### Equipments and Consumables

- Equipment for screen printed transducer obtaining – DEK 248, UK
- System with 3 screen printed electrodes, obtained in University of Perpignan via Domitia France
- Amperometric measurements were realized using a potentiostat METROHM 641 VA DETECTOR (Metrohm, Sweden), working potential being 100mV
- The signal was measured using BD40 (Kipp & Zonen, Flatbed Recorder, Olanda) equipment

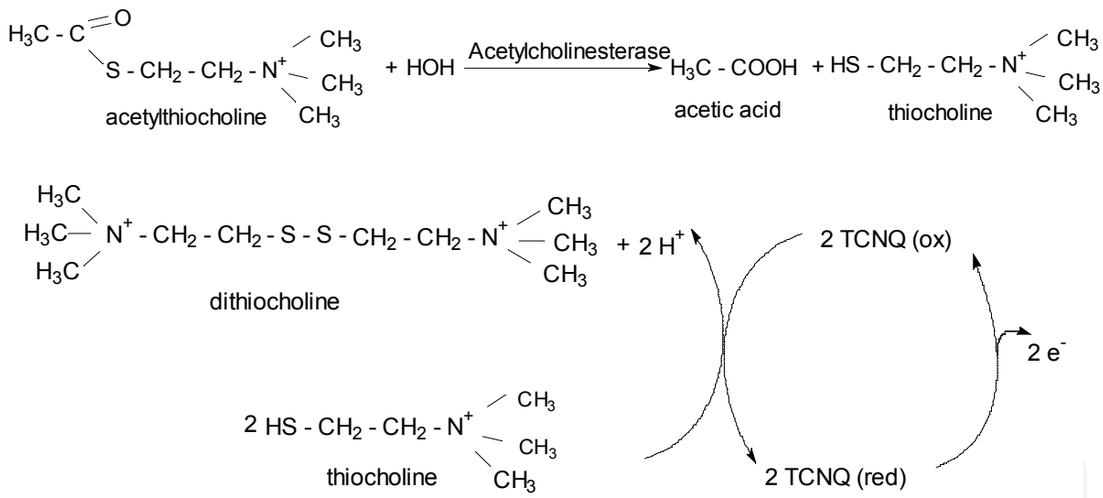
- pH measurement were performed using PHM 220 MeterLab, Radiometer Copenhagen.
- Neon lamp for photopolymerization
- Waterbath –Buchi Waterbath B-480

### 4.3 Working Procedure

The transducer was realised in University of Perpignan via Domitia, BIOMEM, France, using screen-printed procedure. The reference electrode is considered Ag/AgCl, and auxiliary electrode –graphite. Working electrode contained mediator layer deposed to a graphite layer. The enzyme could be immobilised using different procedures.

There were tested two immobilisation methods frequently used in enzymatic biosensors field research: the method that use PVA-SbQ and sol-gel methods. The immobilised enzymes were *Electric eel* AChE (Sigma) and wild-type and genetic modified *Drosophila melanogaster* AChE.

Amperometric determinations are based on the measurement of electric current intensity generated in redox processes of an electrochemical species, working at a constant potential. An example is the transformation of acetylthiocholine in acetic acid and thiocholine, in presence of acetylcholinesterase. Thiocholine forms dithiocholine in presence of TCNQ mediator, liberating two protons and two electrons.




After the biosensor optimisation (stability, reproducibility, calibration), there were tested the influence of different pollutants (organophosphorus and carbamates pesticides, aflatoxins) (Badea et al, 2005; Gurban et al, 2005; Sikora et al, 2005). The obtained results were presented as degree of inhibition or residual enzymatic activity for each experimental condition.

### 4.4 Results and Discussions

*(a) Enzyme immobilisation using PVA-SbQ*

The PVA-SbQ enzyme immobilisation method presents the advantage that doesn't involve covalent binding, which determine the variation in enzyme conformation. It

doesn't appear intermediate product, which may determine the enzyme denaturation. Around the enzyme it is formed a polymer network, the enzyme being included in the polymer cavities (Fig.1.)

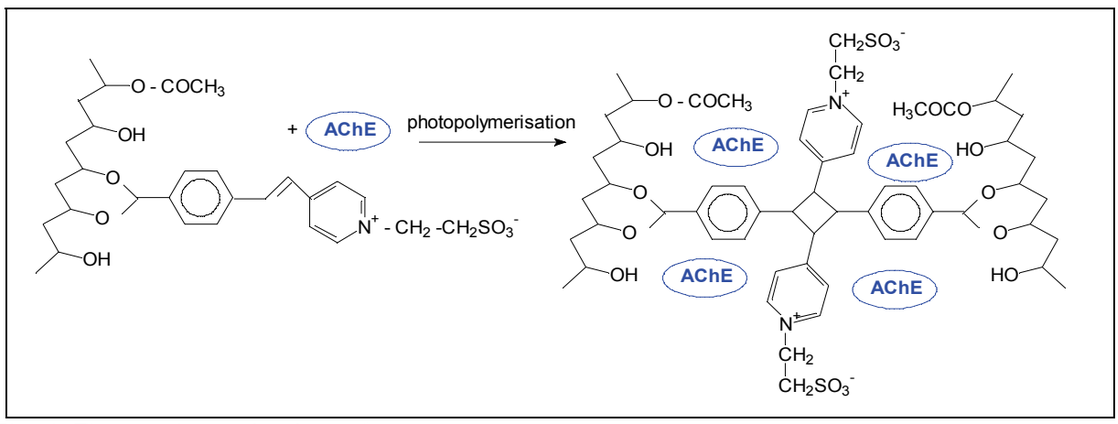



Fig. 1. Photopolymerisation schema

The values of electric signals for immobilisation of E107W and *Electric eel* are bigger than in case of AChE Dm wild type using. For E107W and Electric eel immobilised enzyme it was observed a signal decrease after the firsts assays (Fig. 2.).

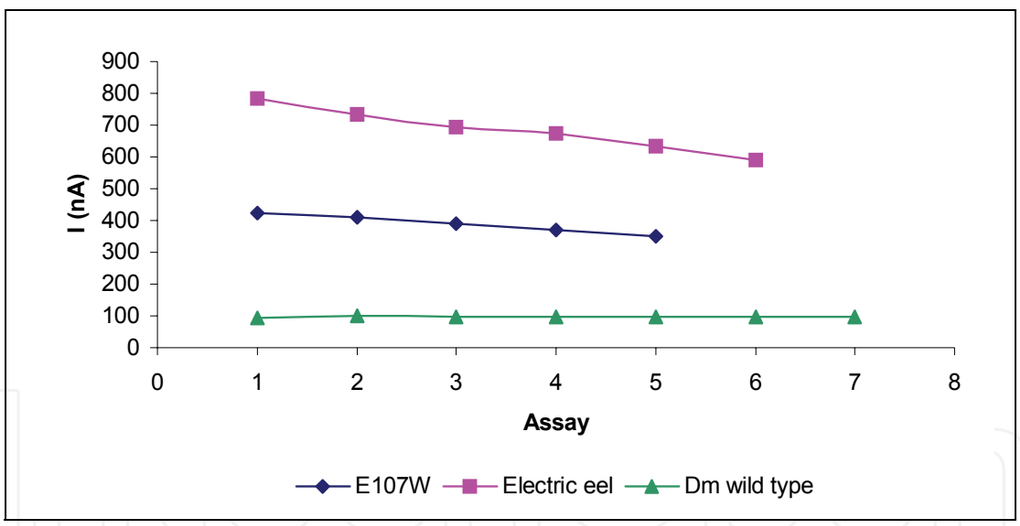



Fig. 2. The operational stability after 2 days drying of biosensors which use immobilised AChE Dm wild type, E107W and *Electric eel* enzymes using PVA-SbQ method; work conditions: buffer solution pH 7; 33% PVA (type PVA-SbQ 2300) in enzymatic mixture; work potential 100mV vs Ag/AgCl; [ATCh]=1mM

For the biosensors containing AChE Dm wild type it was obtained the best stability, the standard deviation representing 1,4% from the average of the electric signal. When it was used enzyme from *Electric eel*, it was immobilised a higher enzyme quantity and that determine the leaking the enzyme from the PVA network.

Two days after the immobilisation of 2mU AChE Dm wild-type and respectively PVA-SbQ 2300 (2:1), the sensor presented 85.5 % stability after 10 min incubation in buffer. The change

of the mixture ratio (1:2) indicated a standard deviation of the experimental values representing 23,21% from the mean of all values obtained for 150 min analysis (double percentage then accepted value 10%).
For these experimental conditions the electric signals versus the enzyme activity (EA) have been presented in Table 1.

| Enzyme | EA / electrode (mU/electrode) | I ± STDEV (nA) |
|---|---|---|
| AChE Dm wild type | 2.24 | 97.13 ± 1.36 |
| Electric eel | 6;75 | 685.83 ± 69.60 |
| E107W | 0.74 | 389.00 ± 30.08 |

Table 1. Electric signal for 2 days storing of the biosensors containing AChE Dm wild type, E107W and *Electric eel*; immobilization using PVA –SbQ method; working conditions: buffer solution pH 7; 33% PVA (type PVA-SbQ 2300) in enzymatic mixture; working potential 100mV vs Ag/AgCl; [ATCh]=1mM

For studying the influence of PVA percent from mixture over the enzymatic biosensor stability, there were utilised in the experimental studies electrodes that contain 50% PVA-SbQ (type PVA-SbQ 1700), too. Analysing the biosensor answer after successively preservation in buffer system (20 min each), followed by washing steps with distilled water, the experimental values were plotted.
It was observed that the electric signal decrease in time for the same acetylthiocholine substrate concentration (1mM) in the reaction mixture. The standard deviation calculated for the experimental values represent 20,27% from the average value obtained in the experimental case previously analysed in 160 minutes, double percent over the literature accepted value for the relative variations of biosensor signals.
The failure of a good operational stability observed for using of PVA method for enzyme immobilisation enforce the testing and optimisation of other possibilities to obtain enzymatic biosensors that will be used in organophosphorus pesticide monitoring.

*(b) Enzyme immobilisation using sol-gel method*
The enzymes immobilisation on the measuring electrode using the sol-gel method involves the obtaining of a $SiO_2$ network and the precursor's polymerisation, resulting a network that includes the enzyme. The method involves two steps: precursor's hydrolysis and the condensation in the presence of enzyme. The sol-gel material which is obtained gives important properties to the biosensor: rigidity, thermal and photochemical stability, chemical inertia, functionality in water and organic environment. It presents the advantage of a single immobilisation stage. Processes are achieved at low temperatures comparable with optimum temperature for enzyme action. It still presents the disadvantage of a diffusing barrier, as the toxicity of intermediary reaction products.
In our experiments it were analysed the properties of biosensors obtained using different hydrolysis time of the precursors, mixture method of precursors with enzyme solution, drying time before their utilisation, type of acetylcholinesterase, to find an optimum method for pesticide analysis from different environmental matrices (water, food).
The precursors solutions were prepared (Table 2.).

| Precursors | TMOS (µL) | MTMOS (µL) | Deionised water (µL) | HCl 1mM (µL) | PEG600 (µL) |
|---|---|---|---|---|---|
| 1 | 5 | 15 | 44 | 40 | 4 |
| 2 | 10 | 10 | 44 | 40 | 4 |
| 3 | 15 | 5 | 44 | 40 | 4 |
| 4 | 20 | 10 | 44 | 40 | 4 |

Table 2. Precursors volumes used to test sol-gel method for enzyme immobilisation

It was deposed a final mixtures that have 1mU enzymatic activity / electrode before immobilisation.
The ratio between the precursors was presented in Table 3. There were tested different immobilisation methods, following the signal stability (repeatability) and reproducibility for the same experimental conditions.

| Method | Precursors | Mixture 1 (HEC+graphite +TCNQ) (µL) | Mixture 2 (precursors) (µL) | Ratio Enzyme: Mixture 1 : Mixture 2 |
|---|---|---|---|---|
| M.1.1. | 1 | 50 | 50 | 1:1:1 |
| M.1.2. | | 25 | 50 | 1:1:2 |
| M.2.1. | 2 | 50 | 50 | 1:1:1 |
| M.2.2. | | 25 | 50 | 1:1:2 |
| M.3.1. | 3 | 50 | 50 | 1:1:1 |
| M.3.2. | | 25 | 50 | 1:1:2 |
| M.4.1. | 4 | 50 | 50 | 1:1:1 |
| M.4.2. | | 25 | 50 | 1:1:2 |

Table 3. Reagents volumes used to test sol-gel immobilisation method for E107Y

It was tested ***biosensor stability***. For mutant E107Y, there were tested the previous methods, for different drying times. Using statistics, the medium values and their corresponding standard deviations were presented in Fig. 3.

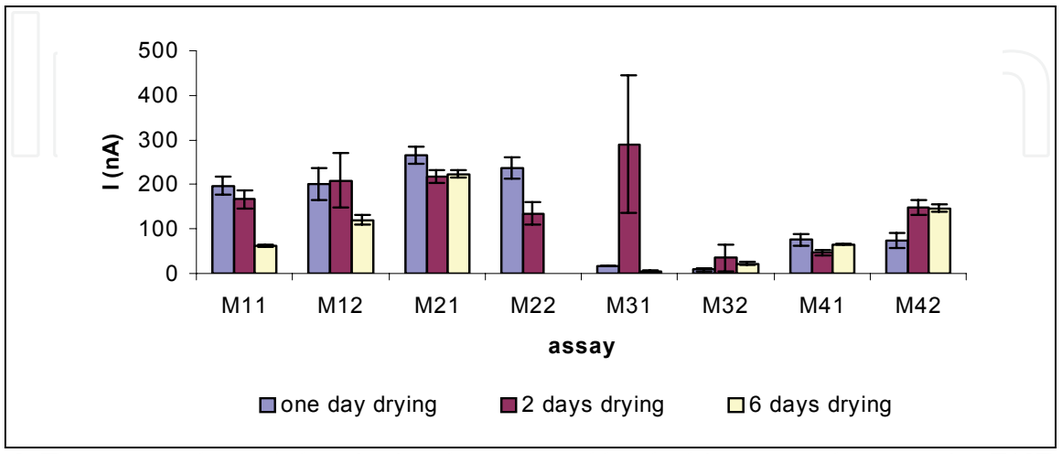



Fig. 3. The mean values of electric signals generated by the biosensors that contains E107Y immobilised using different sol-gel methods (12h hydrolysis time of the precursors)

For some experiments, the standard deviations of the signals represent values higher than 10% from the mean of these determinations (Table 4.).

| % | M11 | M12 | M21 | M22 | M31 | M32 | M41 | M42 |
|---|---|---|---|---|---|---|---|---|
| one day drying | 10,65 | 17,91 | 7,13 | 10,10 | 3,44 | 38,41 | 18,25 | 23,35 |
| 2 days drying | 12,36 | 28,64 | 6,45 | 19,25 | 53,27 | 85,24 | 13,46 | 11,28 |
| 6 days drying | 3,78 | 8,30 | 3,54 | nd | 36,47 | 14,86 | 1,55 | 6,12 |

where nd – undetermined

Table 4. The percentile values of the standard deviations corresponding to the mean values of electric signals generated by the biosensors containing immobilised E107Y using different sol-gel methods (12h-hydrolysis time of the precursors)

It was observed an increase of the signals stability during the increase of drying time, even that the mean values are lower than in the first analysis. The transducers obtained using M.3.1. and M.3.2. methods present, also for 6 days drying time, big values of the experimental values (bigger than 10%), so they cannot be used in the next experiments. It may be recommended the use of biosensors obtained by M.4.1 and M.4.2. methods, these presenting the smallest values.

The similar studies were realised for 6h-hydrolysis time of the precursors. It is not recommended in any case the use of transducers that contains the mutant E107Y immobilised using M.3.1 and M.3.2 methods in the next experimental studies, also because of the low signals, but especially because of the big variations of the currents obtained for the same experimental conditions.

The ***electrode calibration*** was realised following the current intensity variations from analysis system. The current intensities resulted from successively injection, in the same quantity of buffer system, of known amounts of acetylthiocholine were plotted versus the final concentration of enzymatic substrate from reaction mixture.

The calibration of the biosensors containing cholinesterase from Dm E107Y is presented in Fig.4.

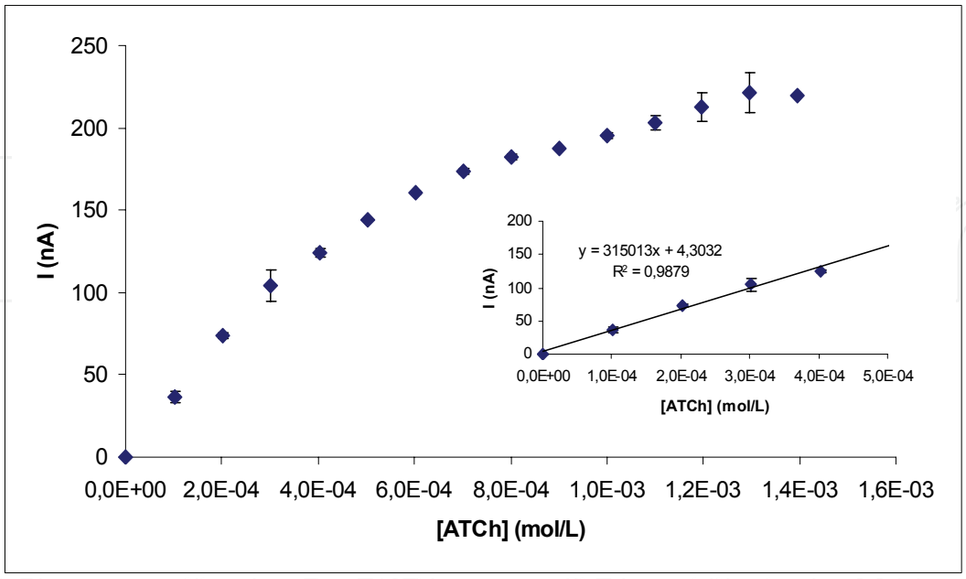



Fig. 4. Biosensors calibration; Dm E107Y mutant AChE immobilised using M.4.1. sol-gel method; working potential 100mV vs Ag/AgCl

Michaelis-Menten allure can be seen, with a linear dependence for concentration of substratum smaller then $4 \cdot 10^{-4}$M, characterised by a correlation coefficient close to the unitary value. It was characterized the kinetic of the reaction, corresponding to immobilised acetylcholinesterase. For this, it was realised the Lineweaver-Burk representation (Fig. 5.).

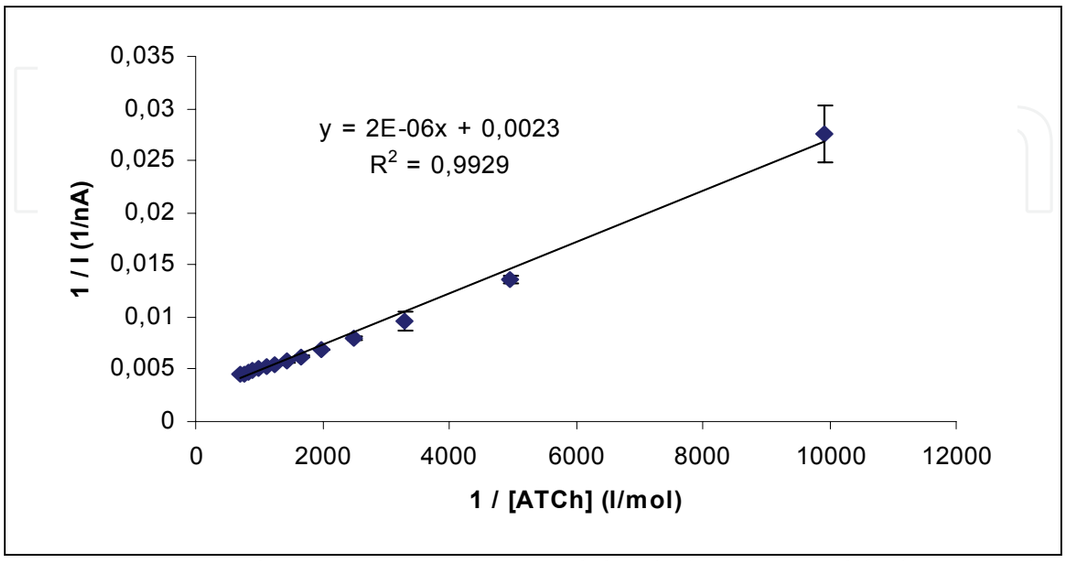



Fig. 5. Lineaweaver-Burk representation for immobilised of genetic modified Dm E107Y acetylcholinesterase using M.4.1. sol-gel method; working potential 100mV vs Ag/AgCl

The same steps were followed for mutant of AChE (*Drosophila melanogaster*) E107W and AChE from *Electric eel*. Using the information from calibration curves and from Lineweaver-Burk equations, there were determinate biosensors sensitivities and apparent Michaelis-Menten constants (Table 5.).

| Parameter | AChE Electric eel | Mutant AChE *Drosophila melanogaster* | |
|---|---|---|---|
| | | E107W | E107Y |
| $K_M$ (mM) – for free enzyme | 0,21 | 0,55 | 0,51 |
| $K_M$ (mM) – for immobilised enzyme | 0,29 | 0,66 | 0,87 |
| Biosensor sensitivity (mA·L/mol) | 245,75 | 80,53 | 315,01 |
| I max (nA) | 144,92 | 82,64 | 434,78 |

Table 5. Michaelis-Menten constants for AChE from Electric eel and mutants *Drosophila melanogaster* E107W and E107Y and the slopes of the linear dependencies from the calibration curves

A lower Michaelis-Menten constant for *Electric eel* AChE indicates a higher affinity of enzyme for their substrate (Coman et al, 2003) and the slope indicates a higher transformation rate of the substrate in reaction product, comparing with mutant *Drosophila melanogaster* AChE (E107W and E107Y).

***The influence of organic solvents over the enzyme biosensors answers*** was also tested. Depending by the solvent used and their quantity used in the experimental studies, it was necessary to study the enzyme behaviour in these organic media .
For the inhibition tests in presence of organophosphorus pesticides there were used small volumes of pesticide solutions in acetonitrile (2-20 μL) in 5 mL buffer system pH 7.
Biosensors response was tested adding controlled volumes of acetonitrile in the reaction mixture, studying the influence of this solvent over the enzymatic activity.
Over a certain value of the percent of the organic solvent in the system, it was observed a decrease of the electric signal. This fact may be explained by the enzyme inhibition, because of the changes from reaction media, in presence of the tested solvent.
The experimental results were statistically analysed and the residual enzymatic activity was plotted for each situation (Fig. 6.).

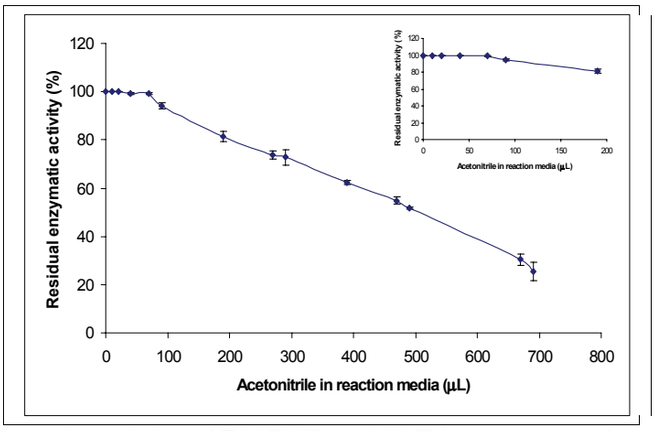



Fig. 6. Residual enzymatic activity of E107Y Dm AChE in presence of acetonitrile; work conditions: sol-gel immobilisation method M.2.1.; 12h precursors hydrolysis 6 days drying

For the values that there will be used in the following studies, it wasn't present a significant inhibition due by the presence of this organic solvent. So, in the following inhibition studies it may be assumed that only the influence of the organophosphorus pesticide from the synthetic samples or the organophosphorus and carbamates pesticides are important. The similar results were also obtained by other researchers for other immobilised enzymes, using different immobilisation method and different working procedure ( Avramescu et al., 2002; Montesinos et al., 2001).
The ***inhibition tests*** regard the organophosphorus pesticides action over different origin acetylcholinesterase. It was analysed the biosensor signals obtained before inhibition and compared them to those obtained after a certain inhibition period with different pesticide solutions (after inhibition).
The pesticide selection for the experimental studies was realised term by their preponderant use in Romania (CODEX, 1996). The mutant enzyme systems selection followed to use those AChE that have inhibition constants different by the commercial AChE (Sigma), for the studied pesticides.
The degree of inhibition increases with the increase of the concentration of organophosphorus pesticide because of the binding of the pesticides to the serine hydroxyl function, which inhibits the enzyme. For concentration lower then $10^{-7}$M methyl paraoxon,

the possibility of linearisation the dependence of inhibition degree versus the pesticide concentrations is observed for Electric eel AChE.
This dependency allows the estimating of the detection limits of the biosensor, pesticides concentration which cause inhibits levels of 10%, 20%, respectively 50%.
It was studied also the influence of methyl paraoxon over the genetic modified acetylcholinesterase activity. So, different pesticide concentrations were added in analysis system, maintaining the same sol-gel immobilisation method (M.4.1.) (Fig. 7, Fig. 8).

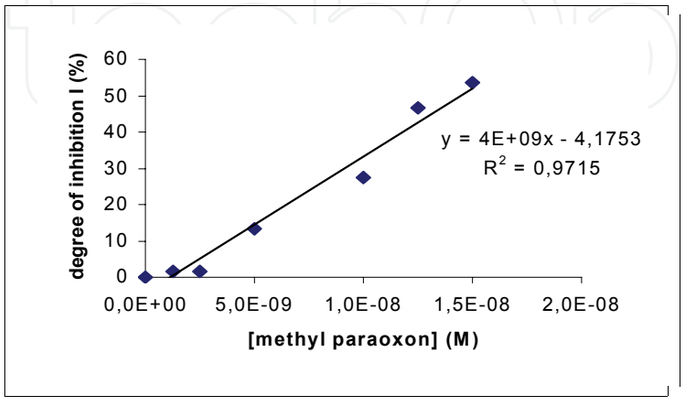



Fig. 7. The linearity between degree of inhibition of E107W acetylcholinesterase and different methyl paraoxon concentrations

Using this dependency there might be obtained I20 and respectively I50 with the values $6.6 \cdot 10^{-9}$M, respective $1.4 \cdot 10^{-8}$M methyl paraoxon, versus E107W, the results being in concordance with literature data (Andreescu et al., 2002) The detection limit for organophosphorus pesticides analysis is around $10^{-9}$ - 5 $10^{-7}$ M, depending by the pesticide and by biosensors obtaining protocol (Evtugyn et al., 1996; Ivanov et all, 2003).
The same increase of inhibition degree was also observed for immobilised mutant Dm acetylcholinesterase E107Y, using the same procedure sol-gel M.4.1. (Fig. 8.).

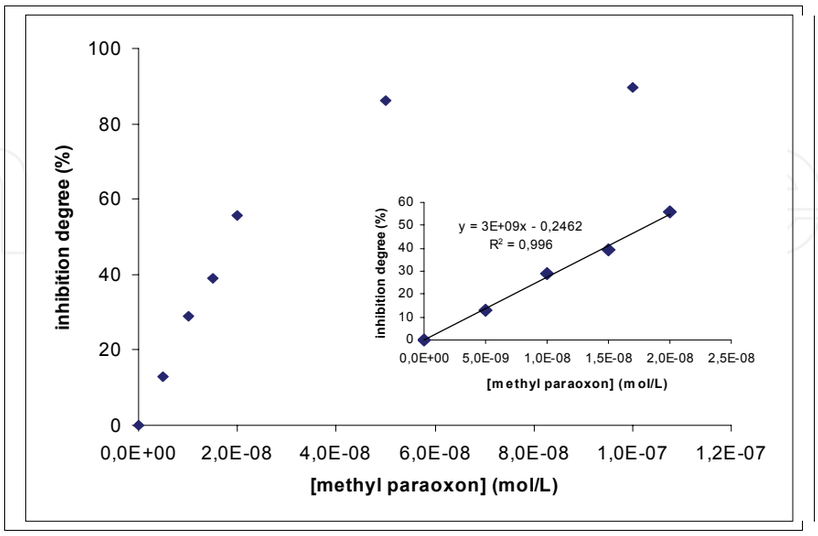



Fig. 8. The dependence of inhibition degree of E107Y acetylcholinesterase in presence of different methyl paraoxon concentrations

Using these dependence there were obtained I20 and I50 with values $6{,}75 \cdot 10^{-9}$M, and respectively $1.67 \cdot 10^{-8}$M methyl paraoxon, versus E107Y. The results are comparable with the data obtained for E107W enzyme.
But, comparing the slopes of the calibration lines for both enzyme it was observed a value higher for E107W AChE than E107Y enzyme (Table 6).

| Enzyme | Inhibition constant for methyl paraoxon | Sensitivity (slope of dependency I% versus methyl paraoxon concentration) |
|---|---|---|
| E107W | 3.52 | $4 \cdot 10^{-9}$ |
| E107Y | 1.00 | $3 \cdot 10^{-9}$ |

Table 6. The slopes of linear dependence between enzymatic inhibition degree and methyl paraoxon concentration

These observation resulted from experimental studies may be explained whereas by the inhibition constant. A high value of inhibition constant represents high enzyme sensitivity for the organophosphorus pesticide in the system, explaining the quick increase of the inhibition degree for the same pesticide concentration (the slope of the dependence is higher).
For E107W analysis, the sensitivity and the specificity for methyl paraoxon is more accentuated than other two studied acetylcholinesterase. It may be recommended E107W enzyme for methyl paraoxon analysis, being more sensitive for testing of low concentration of this pesticide in the analysed matrices.
The same experiments were done for aflatoxin detection using biosensors. For a concentration of aflatoxin $10^{-6}$M the inhibition of acetylcholinesterase from *Electric eel* was higher than the inhibition in the presence of mutant AChE Dm I 199V.
For ***reactivation studies***, there were analysed the influence of methyl chlorpyrifos (MCP) samples over enzymatic biosensors containing E107Y immobilised using method sol-gel M.2.1. After incubation steps with pesticide solutions for 10 min, there were analysed the electric signals generated for adding of 1mM acetylthiocholine. After measurement of inhibition (inhibition degree, residual enzymatic activity), the transducers were incubated for different times with different concentration of 2-PAM reactivator. It was studied the possibilities of enzymatic biosensor reactivation.
The enzymatic inhibition in presence of methyl chlorpyrifos (MCP) was compared with the situation in which was used chlorpyrifos methyl oxon (MCPO), the product of the first pesticide metabolisation (obtained after water treatment with hypochloride solutions).
For a good appreciation of the experimental results, there were calculated and plotted the dependencies of inhibition degree for each experimental situations (Fig. 9.)
It was observed an increase of E107Y inhibition degree versus the chlorpyrifos methyl concentration increasing in analytical system. It was not observed an inhibition for pesticide concentration lower than $10^{-12}$M. The reactivation increases versus incubation time in presence of 2-PAM increasing (5 or 10 min) and with reactivator concentration increasing (2mM or 10mM). Chlorinated sample presents a higher enzyme inhibition because of oxon derivative obtaining, with a pronounced toxic activity.
The tests were repeated with 3 different electrodes, the averages of inhibition degrees and their standard deviation being presented in Fig. 10.

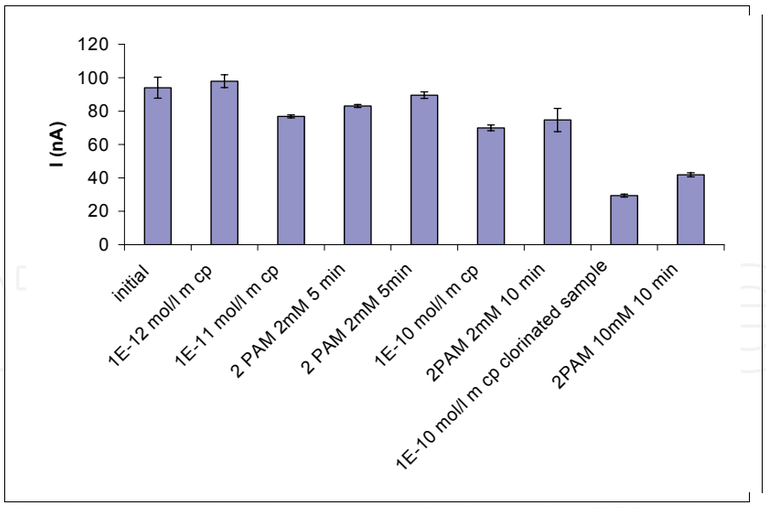



Fig. 9. Electric signals generated at the enzymatic electrodes (M.2.1.sol-gel method ; AChE type *Drosophila melanogaster* E107Y immersion in samples

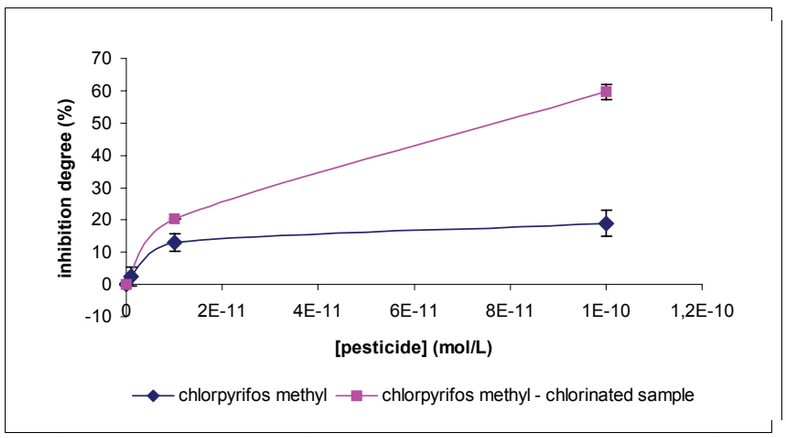



Fig. 10. Inhibition degree of enzymatic biosensors which contains immobilised AChE E107Y using sol-gel method M.2.1., in presence of chlorpyrifos methyl and chlorinated sample

It was observed a different inhibition for the same concentration of pesticide on the system for these two analysed samples. For $10^{-10}$M chlorpyrifos methyl the difference between the two-inhibitors increase significantly, the chlorinated sample presenting a inhibition 3 times higher versus the sample untreated with hypochloride. Normally, chlorpyrifos methyl sample should not determine the acetylcholinesterase inhibition, the oxon form being the compound with affinity to the active site of AChE.

But, the existence of 99% purity of MCP may be a possible explanation for the inhibition existence, the remaining 1% probably being the active form MCPO, that modify the activity of AChE.

So, the "good intentions" of hypochlorides adding in water samples for their purification, also favourites the oxonic metabolites of organophosphorus thioderivatives obtaining, compound that has an inhibition activity over acetylcholinesterases, producing neurotoxic effects more important to the living organisms from this media, versus untreated samples.

The study of inhibition action of oxon was studied also for small concentration in the system. The increase of reactivator concentration and the increase of reactivation time determine an increase of biosensor reutilization possibilities, increasing their lifetime.
The representation of inhibition degree may me realised versus the pesticide concentration or versus decimal logarithm of these values Fig. 11.

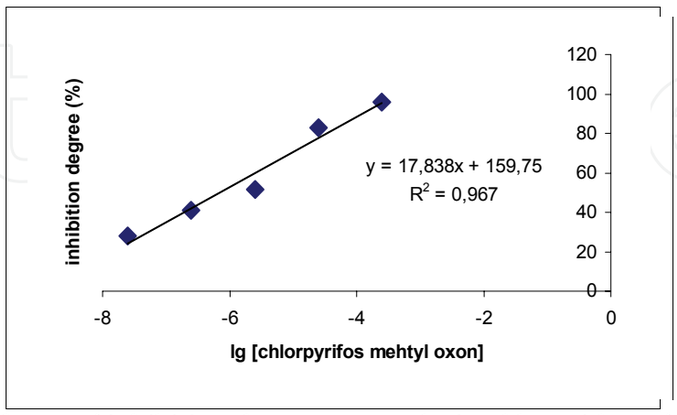



Fig. 11. The dependency of inhibition degree versus decimal logarithm of pesticide concentration

It was observed the possibility of linearization of this dependency, that permit the detection of the unknown concentration in the same experimental conditions.
***Applications in real samples analysis*** was done for water sample testing: water from Villeneuve Lake and Rear River (Perpignan, France) and Timis River (Brasov, Romania). There were used for beginning the biosensors that used immobilised E107W and B107Y acetylcholinesterase using method M.4.1. The experimental data obtained for samples testing were statistically analysed. It was distinguished a more sensitive action of biosensor that use E107W enzyme versus the other that use E107Y enzyme, the values of inhibition degree for tested samples being higher in the first case. For E107W immobilised based biosensor, the inhibition effect is more accentuated, obtaining values higher than the similar situations that use E107Y cholinesterase. River waters, in both cases, presents compounds with inhibitory activity over the two-studied acetylcholinesterases (Badea et al., 2005).
Using the equation of regression lines obtained for analysis of methyl paraoxon standard solutions, it was calculated the equivalent concentration on this pesticide. The equivalent methyl paraoxon concentrations from these three water samples are in the admitted limit by the regulation of the European Economic Community (EEC) and U.S. Environmental Agency (EPA) (EPA Procedures, 1998).
There were tested also baby food products using biosensors for organophosphorus and carbamates detection (Nunes et al., 2005; Badea et al, 2005). These values are lower than the maximum admissible limits from agro-food, so the tested product respects the national legislation. The recovery degree was variable between 40-55%, the matrices being very complex and in the extraction steps it may be possible to loss part of the active compounds.
To increase the coefficient of efficiency of the organophosphorus pesticides (methyl paraoxon and diazinon), there were also tested other solvents (Badea et al., 2004): hexane, and dichloromethane for diazinon and methyl paraoxon extraction from water and fruit juice. (apple), using biosensors and HPLC-UV (Badea et al., 2005). The recovery degree for methyl paraoxon using biosensors was a little bit lower than the similar result obtained by

chromatographic method, maybe due by the fact that this new extracted compound from juice may have an activator action over the acetylcholinesterase Dm E107Y.
The methods that use biosensors with immobilised acetylcholinesterase may be used for pesticide screening, being rapid and low cost techniques. Methods were compared with the chromatographic techniques, the difference between the results being explained.

## 5. Conclusions and Future Work

The biosensors offer the possibility to toxicological investigations from different matrices: water, biological liquids, foods, detecting specific and with very good sensitivities low concentrations of organophosphorus and carbamates pesticides. The applicability of the method is also in environmental protection (Coman et al., 2000), but also in analysis with medical interest (Nivolini et al., 1995; Akyilmaz et al., 2006).
The biosensors obtained and characterised have been used PVA-SbQ and sol-gel immobilisation method. Sol-gel method was optimised for immobilisation of different kind of acetylcholinesterase to be able to be use in sensitive detection of some organophosphorus pesticides (methyl paraoxon, clorpyrifos methyl oxon, diazinon) from synthetic samples. The methods were also applied for real sample testing (water, baby food, juice), testing different extraction methods from initial matrices. The extracts were tested also using HPLC-UV, for method validation and for the explanation of the phenomenon that appear during the extraction step. It was concluded that biosensors might be used successfully for rapid, sensitive and specific determination of the organophosphorus and carbamates pesticides and some times mycotoxins (still in work for optimisation procedures) from different media.
An important area of research in ChE biosensors is directed toward the development of automated and continuous systems for measuring ChE inhibitors in flow conditions. The automation can be obtained by a computer controlled-programmable valve system which allows reproducible pumping of different reagents including buffers, substrate and inhibitor solutions, reactivating agents and real samples. Further improvement in sensitivity and selectivity can be obtained with the use of sensitive multienzymes which also allow discrimination between the insecticides and other interferences.
In the future, compact and portable devices specifically designed for in-field analysis and development of arrays of multiple sensors will constitute another area of intensive research for ChE biosensors. A ChE biosensor array could be designed with each sensor containing a different immobilized enzyme (wild type and mutants ChEs extracted from different sources). This could allow sensitive detection and differentiation of multianalyte mixtures. Considerable progress is expected in genetic engineering for the production of more selective and sensitive ChEs.
The future work would like to make the integration of a fully autonomous electrochemical biosensor with pattern recognition techniques for the detection and monitorize the pollutants level in different matrices. The system will provide a continuous, real-time monitoring of immobilized enzymes activities upon exposure to pollutants compounds (Karasinski et al, 2005).
Our research group have also in work some animal studies, testing the modification of some enzymes activity after the aflatoxin and ochratoxin innoculation, intending in future to use also cell models.

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