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Développement d'une procédure originale pour la multi-détection de composés toxiques utilisant des biocapteurs à base d'acétylcholinestérase

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DEVELOPMENT OF AN ORIGINAL PROCEDURE FOR TOXIC COMPOUNDS MULTI-DETECTION USING ACETYLCHOLINESTERASE-BASED BIOSENSORS

Abstract :

Investigations reported in this manuscript are focused on the development of an original approach for the detection of several toxic compounds, mainly aflatoxins and organophosphorus pesticides, using acetylcholinesterase (AChE)-based inhibitory electrochemical biosensors. In a first step, a new potentiometric biosensor using pH Sensitive Field-Effect Transistors (pH-FETs) as transducers was investigated for aflatoxin B1 (AFB1) determination and different elaboration and working parameters were optimized. The proposed biosensor was characterized by high operational stability and reproducibility of the signal during the work as well as during the storage. The biosensor was further evaluated for real samples analysis (wheat, sesame, walnuts and peas) and a mathematical simulation of the potentiometric biosensor response to aflatoxin B1 was proposed for the first time and validated. In a second step, a conductometric biosensor using interdigitated gold microelectrodes was developed. The sensitivity of the biosensor to aflatoxins and other classes of toxic substances, such as organophosphorus pesticides, heavy metals ions, glycoalkaloids, and surfactants, was determined. A new and original procedure, enabling the selective determination of multiclass toxins by applying successive reactivation solutions targeting either irreversible or reversible inhibitors, was finally proposed. Using this method, the electrochemical enzyme inhibitory biosensors could be applied to the analysis of aflatoxins and organophosphorus pesticides, as well as for the determination of total toxicity of the samples.

Keywords: electrochemical biosensors, acetylcholinesterase, inhibitory analysis, aflatoxins, organophosphorus pesticides, reactivation, mathematical simulation

DEVELOPPEMENT D'UNE PROCEDURE ORIGINALE POUR LA MULTI-DETECTION DE COMPOSES TOXIQUES UTILISANT DES BIOCAPTEURS A BASE D'ACETYLCHOLINESTERASE

Résumé:

Les travaux présentés dans ce manuscrit concernent le développement d'une approche originale permettant la détermination de plusieurs composés (principalement aflatoxines et pesticides organophosphorés), à l'aide de biocapteurs électrochimiques basés sur l'inhibition de l'acétylcholinestérase. Dans un premier temps, un nouveau biocapteur potentiométrique utilisant des transistors à effet de champ sensibles au pH (pH-FETs) comme transducteurs a été développé pour la détermination de l'aflatoxine B1 (AFB1) et différents paramètres d'élaboration et de fonctionnement du biocapteur ont été optimisés. Le biocapteur proposé est caractérisé par une stabilité opérationnelle élevée et une bonne reproductibilité du signal en cours d'utilisation et de stockage. Le biocapteur a ensuite été évalué pour l'analyse d'échantillons réels (blé, sésame, noix et pois) et une simulation mathématique de la réponse du biocapteur potentiométrique à l'AFB1 a été proposée pour la première fois et validée. Dans un deuxième temps, un biocapteur conductimétrique utilisant des microélectrodes interdigitées en or a été développé. La sensibilité de ce biocapteur aux aflatoxines ainsi qu'à d'autres classes de substances toxiques, tels que les pesticides organophosphorés, les métaux lourds, les glycoalkaloïdes, et les surfactants, a été déterminée. Une nouvelle procédure originale, permettant la détermination sélective de toxines multiclassées par application successive de solutions de réactivation visant spécifiquement des inhibiteurs irréversibles ou réversibles, a été finalement proposée. En utilisant cette méthode, il a été montré que les biocapteurs enzymatiques pouvaient être appliqués à l'analyse des aflatoxines et des pesticides organophosphorés, ainsi qu'à la détermination de la toxicité globale des échantillons.

Mots clés: biocapteurs électrochimiques, acétylcholinestérase, analyse inhibitrice, aflatoxines, pesticides organophosphorés, réactivation, simulation mathématique

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RÉSUMÉ ÉTENDU

La surveillance de la composition et de la contamination des aliments joue un rôle crucial dans la société d'aujourd'hui. Les aliments peuvent être contaminés à différentes étapes de la chaîne alimentaire, soit chimiquement par des molécules de petite taille telles que des toxines, des pesticides, des résidus pharmaceutiques, ou microbiologiquement par des bactéries pathogènes. Introduits de manière accidentelle ou volontaire dans les denrées alimentaires, ces contaminants représentent une menace majeure pour la santé humaine et animale, entraînant de graves conséquences sur le système de santé et la productivité économique. Parmi les toxines figurent les aflatoxines. Il s'agit de métabolites secondaires produits par certains champignons de type *Aspergillus*, très répandus dans l'environnement et qui se retrouvent de manière importante dans les aliments, la nourriture pour animaux et certaines matières premières. L'aflatoxine B1 (AFB1) est la plus toxique des aflatoxines, répertoriée dans le groupe I (« cancérogène pour l'Homme ») par le Centre International de Recherche sur le Cancer de l'Organisation Mondiale de la Santé. En raison de leur présence fréquente et de leur toxicité, de nombreux pays ont mis en place des réglementations pour régir le niveau maximal admissible des aflatoxines dans les denrées alimentaires. Certaines d'entre elles, comme le blé et d'autres céréales, peuvent être exposées à la contamination par d'autres substances toxiques telles que les pesticides, qui sont largement utilisés dans l'agriculture pour la lutte antiparasitaire.

Aujourd'hui, d'importants efforts sont consacrés au développement et à l'optimisation de nouvelles méthodes rapides et bon marché pour la détermination des différentes classes de composés toxiques présents dans les aliments. Les biocapteurs font partie de ces dispositifs analytiques. Un grand nombre de biocapteurs a déjà été proposé dans la littérature, mais la plupart d'entre eux ne sont pas adaptés à l'analyse d'échantillons réels qui constitue encore actuellement un véritable défi. Parmi les biocapteurs, les biocapteurs enzymatiques électrochimiques sont les plus largement proposés en raison de leur faible coût et leur facilité d'utilisation. Les biocapteurs basés sur l'inhibition des enzymes par les substances d'intérêt sont largement utilisés mais ils manquent de sélectivité, rendant difficile l'analyse d'échantillons réels pouvant contenir plusieurs classes différentes de substances inhibitrices.

Le présent travail est consacré à l'élaboration d'une procédure originale pour la multi-détection de contaminants alimentaires (principalement les aflatoxines et les pesticides

organophosphorés) à l'aide de biocapteurs basés sur l'inhibition de l'acétylcholinestérase. Des transducteurs de type Transistors à Effet de Champ sensibles au pH (pH-FETs) et des microélectrodes d'or interdigitées ont été utilisés dans ce travail.

Dans une première étape, un nouveau biocapteur potentiométrique à base d'acétylcholinestérase et utilisant les pH-FETs comme transducteurs a été évalué pour la détermination de la concentration totale en aflatoxines. Différents paramètres d'élaboration et de travail ont été optimisés. Le biocapteur proposé est caractérisée par une stabilité élevée et une bonne reproductibilité du signal en cours d'utilisation et de stockage.

Une attention particulière a été portée sur l'étude des caractéristiques analytiques de l'enzyme immobilisée et de son inhibition par l'aflatoxine B1. Une simulation mathématique de la réponse du biocapteur potentiométrique à l'AFB1 a été proposée pour la première fois et validée. A l'heure actuelle, l'utilisation de la simulation mathématique est une voie prometteuse pour aider au développement des biocapteurs et optimiser leurs performances. Elle peut permettre de simplifier la procédure de correction de calibration nécessaire avant toute analyse.

Le biocapteur potentiométrique à base d'AChE développé pour la détermination de l'inhibition des aflatoxines a été évalué pour l'analyse d'échantillons réels (blé, sésame, noix et pois). Les échantillons ont été analysés pour déterminer la teneur en aflatoxines en utilisant le biocapteur et une technique chromatographique classique (HPLC-MS).

Dans une deuxième étape, un biocapteur conductimétrique utilisant des microélectrodes interdigitées en or a été développé. La sensibilité du biocapteur aux aflatoxines et à d'autres classes de substances toxiques telles que les pesticides organophosphorés, les métaux lourds, les glycoalcaloïdes et les agents tensio-actifs, a été déterminée. Les paramètres optimaux pour la détermination des pesticides organophosphorés par le biocapteur conductimétrique ont également été optimisés. La possibilité de réactivation de l'enzyme par une solution de pralidoxime (MAP-2) après son inactivation par les pesticides a été démontrée.

Une procédure nouvelle et originale, permettant la détermination sélective de toxiques multiclassés par application successive de plusieurs solutions de réactivation ciblant spécifiquement les inhibiteurs -soit irréversibles ou réversibles-, a finalement été proposé. En utilisant cette méthode, les biocapteurs enzymes électrochimiques proposés peuvent être appliqués à l'analyse des aflatoxines et les pesticides organophosphorés, ainsi que pour la détermination de la toxicité totale des échantillons.

ABBREVIATIONS

A	AChCl	Acetylcholine chloride
	AChE	Acetylcholinesterase
	AF	Aflatoxins
	AFG1	Aflatoxin G1
	AFB1	Aflatoxin B1
B	BSA	Bovine serum albumin
D	DMSO	Dimethyl sulfoxide
	DNA	Deoxyribonucleic acid
E	ELISA	Enzyme-Linked ImmunoSorbent Assays
	EDTA	Ethylene diamine tetra acetate
G	GA	Glutaraldehyde
	GA	Glycoalkaloid
	GC	Gas chromatography
H	HPLC	High-performance liquid chromatography
I	IARC	International Agency for Research on Cancer
	ISFET	Ion-selective field-effect transistor
L	LOD	Limit of detection
M	MS	Mass spectrometry
N	NM	Nanomaterial
O	OTA	Ochratoxin A
	OP	Organophosphate pesticide
	OPH	Organophosphorus hydrolase
P	PAM-2	Pyridine-2-aldoxy methyl iodide
	PAT	Patulin
	PBS	Phosphate buffer solutions
S	SAA	Surfactant
T	TLC	Traditional thin layer chromatography

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GENERAL INTRODUCTION

Monitoring of food quality and safety has become a key issue in the society today. Food may be contaminated at various points of the food chain, either chemically by small molecules (e.g., toxins, pesticides, residues of veterinary drugs), or microbiologically by pathogenic bacteria. Introduced accidentally or deliberately in food commodities, chemical and biological hazardous contaminants represent a major threat for human health with serious consequences on the health-care system and economic productivity.

Among the various toxins, aflatoxins are highly toxic secondary metabolites produced by *Aspergillus* fungi growing on crops, mostly cereals (e.g. maize, wheat, barley and rice), tree nuts and groundnuts. Among the 18 types of aflatoxins identified, AFB1 is predominant and the most toxic, responsible for liver cancer in animals and listed as a Group I carcinogen by the International Agency for Research in Cancer of the World Health Organization. Due to the frequent occurrence and toxicity of aflatoxins, many countries have established the regulations to govern their level in food commodities. Some of them, like wheat and other grains, are exposed to other toxic substances such as pesticides, which are widely used in agriculture for pest control.

Nowadays, huge efforts are devoted to the development and optimization of new rapid and cheap methods for the determination of toxic compounds in food. In this context, biosensors are attractive analytical devices capable of fast, sensitive, selective and low-cost determination of a wide range of substances in foodstuff.

A number of biosensors have been already reported for the determination of various toxic substances, but most of them are not adapted to the analysis of real samples, which constitutes today an actual challenge for the analysts. Among the biosensors, electrochemical enzyme biosensors are very popular due to their specificity, cost-effectiveness and ease-of-use. Biosensors based on enzyme inhibition are largely proposed but they lack of selectivity, making them hardly applicable to the analysis of real samples susceptible for containing several inhibitors of different groups.

In this work, we developed a new original approach for the multidetection of several food contaminants, mainly aflatoxins and organophosphorus pesticides, using acetylcholinesterase (AChE)-based inhibitory electrochemical biosensors and applying an algorithm involving sequential and specific steps of enzyme reactivation. pH sensitive Field Effect Transistors (pH-FETs) and interdigitated gold microelectrodes were used as transducers.

The present manuscript will be organized in six chapters.

The **first chapter** will be dedicated to a bibliographic review on some main toxic compounds present in food (i.e., mycotoxins, pesticides, heavy metals) and their common methods of determination, on biosensors and more particularly enzyme-based electrochemical biosensors applied to food contamination analysis.

The **second chapter** will describe materials, methods and devices used in this work.

In the **third chapter**, an acetylcholinesterase-based potentiometric biosensor for inhibitory analysis of aflatoxins will be proposed and optimized, and its characteristics described.

A mathematical simulation of the developed potentiometric biosensor will be proposed and discussed in the **fourth chapter**.

The **fifth chapter** will report the development of a new acetylcholinesterase-based conductometric biosensor, investigating its sensitivity to different groups of toxic compounds (aflatoxines, organophosphorus pesticides, heavy metals, surfactants and glycoalkoloids) and the possibility of selective reactivation for multi-use purpose.

The **sixth chapter** will focus on the applicability of the proposed biosensors to real samples analysis. A new method of simultaneous analysis of toxic substances of different classes in multicomponent complex will be proposed.

The last section of the manuscript will summarize the main results of this work and end with a brief discussion of the perspectives.

1. BIBLIOGRAPHICAL REVIEW

1.1. Introduction

The rapid development of chemical industry and intensive use of various chemicals in agriculture and other fields of human activity has led to an important deterioration of environmental quality, urging the implementation of legislations and monitoring programmes to help defining suitable remediation and protection strategies.

Among the large variety of chemicals released in the environment, toxic compounds such as pesticides, heavy metals, microbial toxins, drug residues, glycoalkaloids are important contaminants of feed and food. Adequate methods of analysis are therefore absolutely needed to control their levels in commodities and safeguard the health and safety of the consumer. In this work, we particularly focused on three families of chemicals (aflatoxins and organophosphorus pesticides) which possess in common to degrade human health by inhibiting acetylcholinesterase enzyme.

1.2. Mycotoxins

Mycotoxins are toxic organic compounds, secondary metabolites produced by fungi [1]. So far, more than 400 mycotoxins generated by more than 250 species of fungi have been identified. Storage of food and feed at high temperature and high humidity promotes the growth of these fungi. Molds usually infect plant in the field, which are weakened and damaged by drought, pests, diseases, etc. Mycotoxins rapidly diffuse into the product. Their presence in food and feed, even in extremely small quantities, may be a cause of serious toxic effects, threatening human and animal health [2, 3]. Human exposure to mycotoxins occurs directly through the intake of contaminated agricultural products (cereals, corn, fruits, etc.) or indirectly through the consumption of products of animal origin (milk, eggs, etc.) prepared or obtained from animals that were fed with contaminated material. The co-contamination of foods/feeds with known or unknown mycotoxins is being reported at an increasing high rate but toxicological informations on the potent additive or synergistic toxic effects of simultaneous exposure are still very scarce and limited [4]. Chronic or acute toxicity has been reported for individual toxins [3]. Most mycotoxins are carcinogenic and mutagenic but other disorders, e.g., on central nervous, pulmonary, cardiovascular, reproductive and immune

systems, have been described. The main source of mycotoxins in the food chain is the agricultural products, including a wide variety of grains. The accumulation of mycotoxins in grain, feed and food is due to the violation of storage and/or transportation conditions. Economic losses due to mycotoxins are diverse and can be associated with reduction of quality foods for humans and animals, reduction in animal production due to feed refusal or diseases, increasing medical cost for toxicosis treatments, increased costs to find alternative foods, to design adequate management of contaminated supplies, to improve detection and quantification methods and to develop strategies that reduce toxin exposure [5].

A survey has shown that, by the end of 2003 [6], more than 100 countries (covering > 90% of the world's inhabitants) had specific regulations or detailed guidelines for mycotoxins but many countries still lack appropriate guidelines to manage these toxins (particularly in Africa and Latin America). At present, the issue of mycotoxins contamination control in food, feed and food raw materials is resolved not only within individual countries but also internationally, under the Food and Agriculture Organization (FAO) and World Health Organization (WHO) auspices. The mycotoxins, for which limits and regulations currently exist at special organizations and programs on the mycotoxin regulatory developments, include the naturally occurring aflatoxins, aflatoxin M₁, agaric acid, deoxynivalenol, diacetoxyscirpenol, the fumonisins B₁, B₂ and B₃, HT-2 toxin, ochratoxin A, patulin, phomopsins, sterigmatocystin, T-2 toxin and zearalenone [7,8]. Mycotoxins, however, differ in their chemical structure, toxicity and mechanism of action.

1.2.1. Aflatoxins

Due to their highly carcinogenic properties, aflatoxins are one of the most dangerous mycotoxins. Aflatoxins were discovered in the late 50's-early 60's. They were identified as the causative agent of the "turkey X" disease which caused the death of more than 100000 turkeys in England. Turkeys had been fed with Brazilian groundnut meals contaminated with *Aspergillus Flavus* [9]. Metabolites produced by the fungi, further called aflatoxins, were isolated and it was shown they were able to induce the "turkey X" syndrome. . Further investigation of contaminated peanut extracts confirmed that the agents can induce acute liver disease in ducks and liver cancer in rats [10]. Intense fluorescence of aflatoxins under ultraviolet light facilitates their detection in contaminated peanut extracts and peanut-based products [11].

Aflatoxins family contains several compounds all including coumarin and difuran moieties. The four main representatives produced in nature are aflatoxins B1, B2, G1 and G2 (fig.), but other compounds (metabolites or derivatives) such as aflatoxins M1, M2, V2A, G2a, GM1, P1, Q1, G2a, and others have been also identified. Aflatoxins are very stable and resist to ordinary cooking and food processing practices [1].

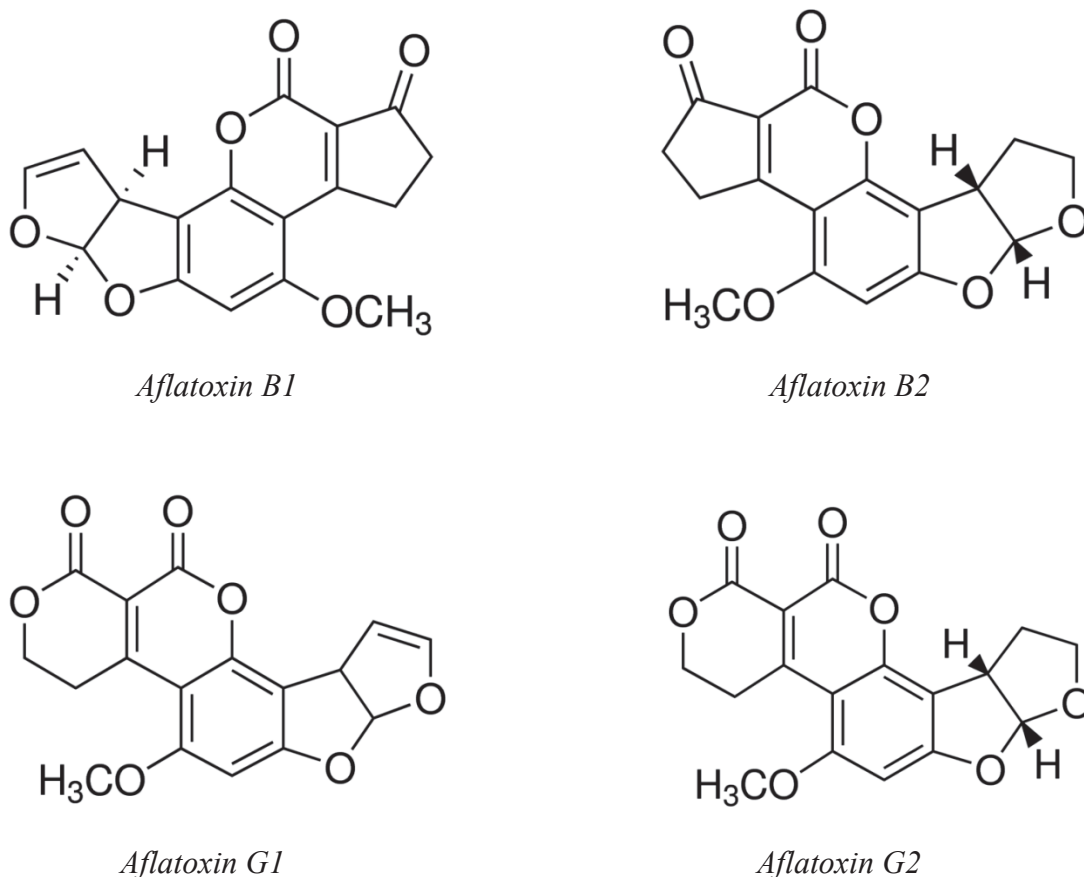


Fig.1.1. Chemical structures of natural aflatoxins B1, B2, G1, G2.

Members of the blue (B) fluorescent group (aflatoxins B1 and B2) are characterized by the fusion of a cyclopentenone ring with the lactone ring of the coumarin moiety, whereas green (G) fluorescent toxins (aflatoxins G1 and G2) contain a fused lactone ring [11]. Fluorescence underlies almost all physical and chemical methods of quantitative and qualitative detection of aflatoxins.

Aflatoxins are produced principally by three species of the fungi genus *Aspergillus* (*A. Flavus*, *A. Parasiticus* and *A. Nomius*) growing mostly on crops, such as grains and nutsbut

they can also accumulate in large quantities in wheat, barley, various oilseeds products, cocoa and coffee beans [12]. *A. Flavus* produces toxins of group B only, while *A. Parasiticus* produces toxins of both groups (B and G) and is more common in peanuts. Toxin production may start in various conditions, and is not observed *only in countries with tropical and subtropical climate*. 27-30 °C is an optimum temperature for toxigenesis, nevertheless aflatoxins synthesis is possible at lower (12-13 °C) or higher (40-42 °C) temperatures. Humidity of substrate and air is also an important factor affecting the growth of microscopic fungi and aflatoxins synthesis

Aflatoxins have been recognized as widespread contaminants of feed and food products. Even low levels of the toxins in the diet can have deleterious impact on humans and animals health. The way the toxins affect health depends on the type of molecule and on a variety of other parameters. Chronic outcomes such as hepatocellular carcinoma, as well acute effects causing sudden death, have been reported. Aflatoxins also increase the risk of liver cancer in people chronically infected with hepatitis B and induces different common adverse health effects, such as growth disorders in children [11].

Among the different types of aflatoxins identified, aflatoxin B1 is predominant, is a potent human carcinogen and responsible for liver cancer in animals. Ingested by cows via the feed, it is transformed into aflatoxin M1, which is further secreted in milk [12]. Aflatoxin M1 is found not only in whole milk but also in sour milk, cheese and yoghurt. Aflatoxin M1 contaminates dairy products which become environmentally hazardous to humans. One of the important evidences of the actual danger that aflatoxins represent for human health is the correlation found between the frequency and level of aflatoxin contamination of foodstuffs and the frequency of primary liver cancer in the population [11].

To control the levels of these highly toxic that contaminate staple foods all over the world, regulations have been implemented in many countries. Allowed limits depend on the type of food. In the European Union, the maximal allowed concentrations have been set at 8,0 ng g⁻¹ for AFB1 and 15,0 ng g⁻¹ for total aflatoxins in groundnuts (peanuts) and other oilseeds to be subjected to sorting, or other physical treatment before human consumption or use as an ingredient in foodstuffs. Le limits is much lower (2.0 ng g⁻¹ for AFB1 and 4.0 ng g⁻¹ for total aflatoxins) for groundnuts (peanuts) and other oilseeds and processed products thereof intended for direct human consumption or use as an ingredient in foodstuffs. The same limits are given for all cereals and all products derived from cereals, including processed cereal products, while maximal levels are 5,0 ng g⁻¹ for AFB1 and 10,0 ng g⁻¹ for total aflatoxins for

maize and rice to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs [8].

1.2.2. Patulin

Patulin (PAT) is the second of the most common mycotoxins (Fig 1.2). The chemical structure of patulin is shown in Fig.1.2. Its maximum of absorption lies in the ultraviolet region (276 nm).

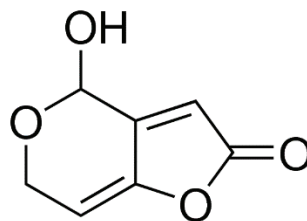


Fig.1.2. Chemical structures of patulin

In most cases, patulin occurs in moldy apples, sea-buckthorn, and other fruits, vegetables and berries [13]. Mold is hidden under the hard shell of stone in peaches, apricots and plums. Most frequently patulin infects apples, where the toxins concentration can reach up to 17.5 mg kg⁻¹. It is interesting that in apples patulin concentrates mainly in rotten parts, unlike tomatoes, where it is distributed evenly. Patulin is detected in high concentrations in processed fruits (juices, jams, prepared from moldy fruit). Citrus and some vegetables such as potatoes, onions, radishes, radish, eggplant, cauliflower, pumpkin and horseradish are naturally resistant to infection by patulin.

Patulin has been extracted for the first time in 1943 from cultures of *Penicillium patulum* fungus but other fungi of the *Penicillium*, *Aspergillus*, *Byssochlamys*, *Eupenicillium* and *Paecilomyces* genera are able to produce the toxin [14]. Among them *Penicillium expansum*, a common pathogen of brown rot in apples, pears, apple-quince, apricots, peaches and tomatoes, is the most common [14]. *Penicillium urticae* sometimes occurs in these same fruits and causes rotting, while *Bissochlamys nivea* is a heat-resistant fungus extracted from fruit juices.

Maximum of toxigenesis takes place at 21-30 ° C. Apart from the acute toxic effects (i.e., agitation, convulsions, dyspnea, pulmonary congestion, and others) observed in rodents, sub-acute effects such as gastrointestinal disorders (e.g., bloating, ulceration and bleeding, alteration of the intestinal barrier function) have been reported [14].

Patulin forms covalent adducts with essential cellular thiols, which explain its inhibitory effect on many enzymes and its cytotoxic, genotoxic and immunotoxic properties in mammals [14-15]. Some embryotoxic and teratogenic effects have been also reported. Recently, it has been shown that patulin causes Ca²⁺ entry into erythrocytes, an effect triggering suicidal erythrocyte death or eryptosis [16]. However, the toxin has been classified in the group 3 as « not classifiable as to its carcinogenicity to humans » by the International Agency for Research on Cancer (IARC). In view of its ability to trigger apoptosis of tumor cells, patulin has even been considered a candidate for the treatment of malignancy [14].

Considering the toxic effects of patulin, the European Commission has set at 50 ng g⁻¹ the maximal admissible concentration of patulin in fruit juices, concentrated fruit juices as reconstituted and fruit nectars, 25 ng g⁻¹ for solid apple products, including apple compote, apple puree intended for direct consumption [8].

1.2.3. Fusarium toxins

Another important class of mycotoxins (Fusarium toxins) is produced by Fusarium filamentous fungi and affects cereal crops [1]. These mycotoxins are therefore commonly found in cereal food and feed and in other animal products consumed daily. Among this family of mycotoxins, zearalenone, some tricothecenes (i.e., deoxynivalenol, T-2 and HT-2 toxins), as well as fumonisins B1 and B2 are the most toxic and are regulated in Europe [8].

Zearalenone is the most important exponent of the Fusarium toxins class with regard to animal health implications and related economic losses. It was first extracted from moldy corn contaminated by *Fusarium graminearum* but it has been further evidenced that it may be produced by several other species of *Fusarium* fungus, including *F. culmorum*, *F. cerealis*, *F. equiseti*, *F. verticillioides* and *F. incarnatum*. Its structure is presented in Fig. 1.3 [1].

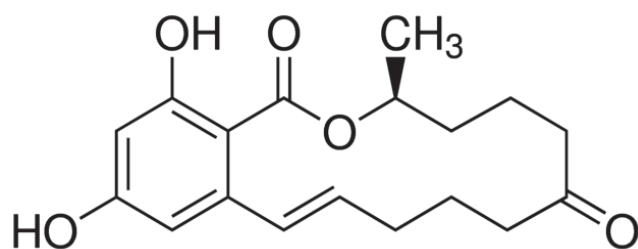


Fig. 1.3 Chemical structure of zearalenone.

It has three maxima of absorption in the ultraviolet region (236 nm, 274 nm, 316 nm) and exhibits blue-green fluorescence.

Contamination of the corn occurs in the field as well as during storage. Zearalenone infects primarily maize but occurs in lower concentrations in other cereals such as wheat, barley, oat and sorghum [17].

Zearalenone has been reported to induce a variety of adverse effects in farm animals. Due to its structural similarity with the estrogen hormones, it competitively binds to estrogen receptors and alters the reproduction and fertility, especially in pigs which is the more susceptible animal species. Chronic exposure of animals to zearalenone in diet induces carcinogenic, genotoxic, reproductive, endocrine disrupting and immunotoxic effects. Zearalenone may be possibly associated with reproductive issues in humans but IARC has classified the toxin in group 3 [18].

The European Commission has set at 100 ng g⁻¹ the maximal concentration of zearalenone admissible in unprocessed cereals other than maize, at 350 ng g⁻¹ in unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling, and at 75 ng g⁻¹ in cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption [8].

1.2.4. Trichothecene mycotoxins

Trichothecenes are compounds characterized by a common 12,13-epoxytrichotec-9-ene ring system. At present, about 170 trichothecene mycotoxins have been identified. They are divided in four groups, depending on whether they are non-macrocyclic (A and B groups) or macrocyclic (C and D groups). **Deoxynivalenol** and **T-2 toxin** are the most toxic

representatives of B and A groups, respectively (Fig.1.4). They are produced mainly by fungi of the *Fusarium* species (*F.graminearum*, *F. culmorum*, *F. cerealis* for deoxynivalenol, *F. sporotrichioide*, *F. langstsethiae*, *F. acuminatum* and *F. poae* for T-2 toxin) but also by some species of *Trichoderma*, *Stachybotrys* and *Myrothecium* to name a few. Deoxynivalenol is principally found in maize and wheat, while T-2 toxin and its main metabolite HT-2 occur in raw oats and barley [17].

Toxic effects caused by the consumption of food and feed contaminated by microscopic fungi producing trichothecene mycotoxins may be of different types according to the difference in toxins characteristics [3].

Numerous *in vitro* and *in vivo* studies have shown that trichothecene mycotoxins inhibit the synthesis of proteins and nucleic acids. In addition, they cause instability of lysosomal membrane and activation of lysosomal enzymes, which ultimately leads to cell death.

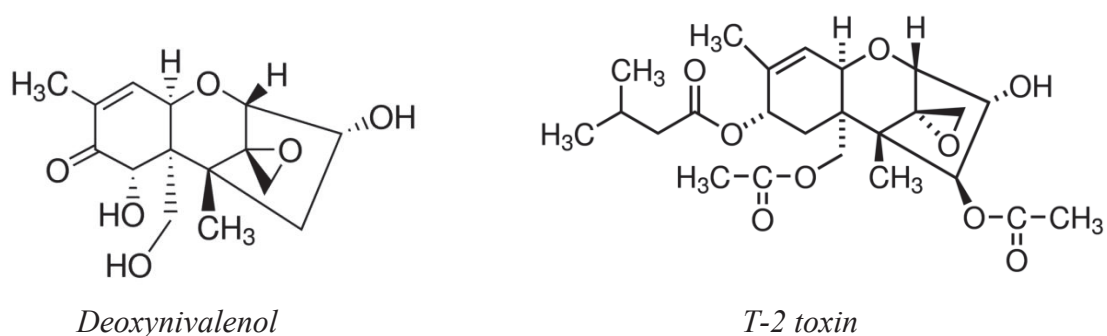


Fig.1.4 Chemical structures of deoxynivalenol and T-2 toxin.

These toxins have teratogenic, cytotoxic, immunosuppressive properties. They affect the blood-forming organs and central nervous system, cause leukopenia and hemorrhagic syndrome. Although deoxynivalenol is less toxic than other trichothecenes such as T2 and HT-2 and has been classified in Group 3 by the IARC, this is one of the most contaminants of cereals worldwide and it is highly resistant to food processing.

The European Commission has set the maximal authorized content of deoxynivalenol in unprocessed cereals other than durum wheat, oats and maize at 1250 ng g⁻¹, at 1 750 ng g⁻¹ the value in unprocessed durum wheat and oats and at 750 ng g⁻¹ the maximal concentration in cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption [8].

1.2.5. Ochratoxins

Ochratoxins A, B and C are mycotoxins produced by several *Aspergillus* and *Penicillium* fungi, specifically *A. Ochraceus* and *P. Viridicatum*. They all are based on an isocoumarin moiety linked with a phenylalanine moiety by an amide bond [19]. Ochratoxin A (OTA) (Fig.1.5) is the most toxic (B and C are an order of magnitude less toxic) and is the most prevalent and relevant fungal toxin of this group of mycotoxins [20]. Although the genotoxic status of OTA is still controversial, many other adverse effects such as hepatotoxicity, teratogenicity, immunotoxicity, and neurotoxicity have been demonstrated on several species of animals. OTA has been classified as a possible human carcinogen (category 2B) by the IARC. The toxin promotes oxidative DNA damage through the production of reactive oxygen species and generates DNA adducts [20]. OTA is highly resistant to acidity and temperature and is therefore quite impossible to remove from contaminated foodstuffs, which constitutes a real threat for human health.

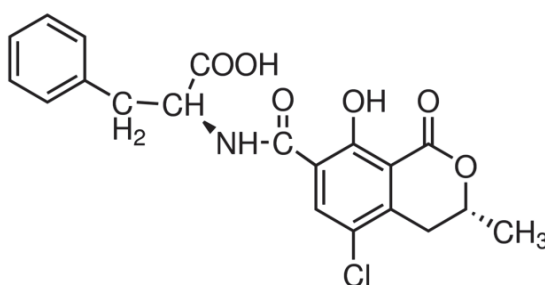


Fig.1.5 Chemical structure of ochratoxin A

Ochratoxin is known to occur in different grains such as corn, barley, oats, rye and wheat, and has been also reported in other plant products including coffee beans, spices, nuts, olives, grapes, beans, and figs. OTA is also found in animal products (milk, eggs, meat). This contamination is usually explained as a result of the digestive absorption of feed contaminated with OTA by the animals [21].

In view of the recognized negative effects and health risks caused by ochratoxin A, the European Commission has decided to limit its content in different foodstuffs. For example, the maximum admissible level of OTA is 5 µg/kg in unprocessed cereals and is 3 µg/kg in all products derived from unprocessed cereals [8].

1.2.6. Classical methods for the determination of mycotoxins

Taking into account all above-mentioned reasons, there is no doubt that mycotoxins constitute a real threat for human and animal health and should be monitored. Mycotoxins monitoring includes regular quantitative analysis of foodstuff. It allows evaluating the level of contamination and actual risks, identifying the foodstuffs, which are the most favorable substrate for microscopic fungi – mycotoxins producers, and verifying effectiveness of the measures on preventing and reducing mycotoxins contamination.

Mycotoxins control is of particular importance when the quality of raw materials and products imported from other countries is scrutinized. Mycotoxins control is particularly relevant in feed mills, livestock and poultry farms, flour-grinding factories.

The analysis of mycotoxins is challenging as these molecules are present in low concentrations in complex matrices, and they may occur in various combinations produced by a single or by several fungal species. In addition to reliability, cost, rapidity and simplicity are desired, as it will affect the amount of data generated and the practicality of the ultimate measures taken. To date, most analysis of mycotoxins are performed by skilled personnel in accredited laboratories using Enzyme-Linked ImmunoSorbent Assays (ELISAs), radioimmunoassays (RIAs) or more sophisticated and costful methods, mostly based on the separation of the toxins by high performance liquid chromatography (HPLC) or gas chromatography (GC) and their detection by fluorescence spectroscopy or mass spectrometry. Before the separation step, an extraction may be required, followed by a cleanup step to reduce or eliminate unwanted matrix components, and possibly concentrate the sample. Several papers have recently reviewed analytical methods for mycotoxins analysis [22-31], some of them focusing on specific foodstuffs [25], specific groups of mycotoxin such as trichotecenes [26] or aflatoxins [27-28], or new trends in chromatographic/mass spectrometric techniques and their coupling for the determination of mycotoxins and other contaminants [29-31].

The traditional thin layer chromatography (TLC) method is considered as a powerful screening tool for the presence of aflatoxins and a reliable quantification method when combined with densitometry. However, in spite of the new developments performed in this technology (i.e. high performance TLC, two-dimensional TLC, overpressured-layer chromatography), TLC has been largely replaced by HPLC for quantitative analysis of aflatoxins [27]. The most recent methods reported for the determination of aflatoxins in feed and food combine HPLC [32], microHPLC [33], ultrahigh performance liquid

chromatography (UHPLC) [34-36] or 2D-liquid chromatography [38] with MS or MS/MS detectors. Due to their high separative capacities, these methods enable the simultaneous separation of different aflatoxins but also other mycotoxins such as ochratoxin A, zearalenone, T-2, HT-2 or compounds of other classes (e.g. pesticides). Two recent reviews have addressed new trends in UHPLC-MS [38] and HUPLC-MS/MS [39] for multi-class contaminants in food. For patulin detection (alone or in mixture with other mycotoxins), HPLC-MS [40], UHPLC-MS/MS [41, 42] as well as GC-MS/MS [43, 44] are, at present, classically proposed.

In parallel, the interest for more easy-to-use and highly sensitive enzyme-linked immunosorbent assay methods (ELISAs) has increased in recent years. For example, ELISAs for deoxynivalenol analysis [45, 46], aflatoxin B1 detection [46, 47], zearalenone and ochratoxin determination [46], simultaneous determination of aflatoxin B1 and aflatoxin M1 [48] have been recently reported.

1.3. Organophosphate pesticides (OPs)

1.3.1. Exposure and toxic effects

Pesticides are widely used to improve the productivity in agricultural activities. This group of chemicals covers a variety of molecules which may be broadly classified into three categories (insecticides, herbicides and fungicides) on the basis of target organism. The extensive use of pesticides has resulted in their appearance as residuals in crops, livestock and poultry products. Among these, organophosphates (OPs) and N-methyl carbamates play an important role in environment and food contamination.

OP compounds were first developed by Schrader shortly before and during the Second World War. They were first used as agricultural insecticides and later as potential chemical warfare agents [49]. In the late 1990's and 2000's, nerve agents have gained prominence as weapons of mass destruction.

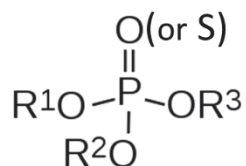
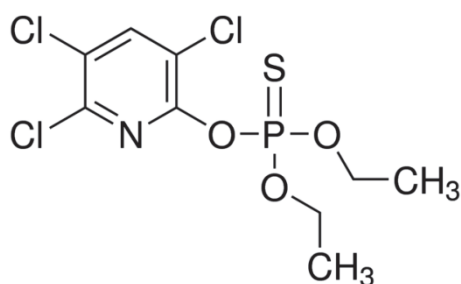
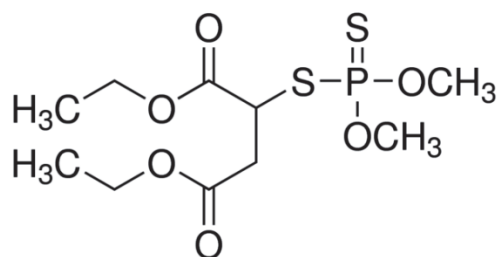


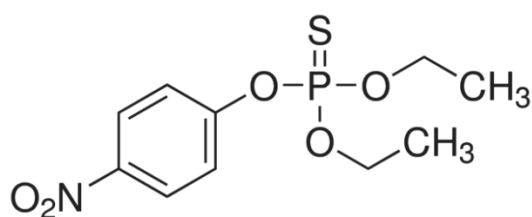
Fig.1.6. General chemical structures of organophosphate pesticides



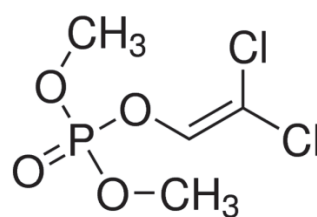
Chlorpyrifos



Malathion



Parathion



Dichlorvos

Fig.1.7. Chemical structures of some OP pesticides

Human exposure to pesticides occurs through a variety of pathways including consumption of foods and water containing OP residues, dermal absorption by direct contact or inhalation by working or living in close proximity to a farm that applies OPs [50]. Intensive use of OP contributes to morbidity and mortality in farmworkers and their families through acute or chronic pesticides-related illnesses. The principle action of OPs is the inhibition of acetylcholinesterase enzyme (AChE), which is essential for the function of central and peripheral nervous system in humans [51]. Basically, OP cause four important neurotoxic effects in humans: the cholinergic syndrome, the intermediate syndrome, delayed polyneuropathy, and chronic neuropsychiatric disorder [52]. Experimental and epidemiological relationships between pesticide exposure and Alzheimer's, Parkinson's, and Amyotrophic Lateral Sclerosis (ALS) diseases have been also found [51]. Neurodevelopmental effects in children have been reported [53].

1.3.2. Classical methods for the determination of OPs

Classical analysis of pesticides, especially OPs, relies heavily on chromatographic separation techniques [54-57], including HPLC [58, 59], GC [60-66], and, to a lesser extent, capillary electrophoresis (CE) [67] or supercritical fluid chromatography (SFC) [68], in combination with different detectors, such as nitrogen phosphorus detector (NPD) [65], flame photometric detector (FPD [60-62], UV-Vis detector [58-59], fluorescence detector, [67] or mass spectrometry (MS) detector [63-64, 66, 68]. ELISAs have been also reported for the determination of organophosphate pesticides [54].

In addition to the sample extraction step, generally performed using solvent assisted solid-liquid techniques, a clean-up/pre-concentration procedure is often needed to remove matrix interferences analytes and concentrate the analyte. In comparison to the conventional strategies, such as liquid–liquid extraction (LLE), solid-phase extraction (SPE) [59, 61-62, 64], solid-phase microextraction (SPME) [65-66], liquid-phase microextraction (LPME) [63], dispersive liquid–liquid microextraction (DLLME) has attracted much attention in the past few years, due to the simplicity of the method, the short extraction times, low solvent consumption, and the high enrichment factors achieved [58, 60].

1.4. Other food contaminants

✓ Heavy metals

Heavy metals (i.e., cadmium, mercury, lead, arsenic, tin, selenium, antimony, copper, nickel, chromium, tellurium, thallium) are another class of environmental pollutants. The main sources of contamination are cosmetic products, automobiles, effluents released from electroplating and from various other industries. Because they are non-degradable, they persist in the environment and penetrate into living organisms, contributing to a wide spectrum of adverse health effects [69]. Heavy metals can cause malfunctioning of the cellular processes via displacement of essential metals from their respective sites. Oxidative deterioration of biological macromolecules has been found to be primarily due to binding of metals to DNA and nuclear proteins. Symptoms that arise as a result of metal poisoning include intellectual disability in children, dementia in adults, central nervous system disorders, kidney diseases, liver diseases, insomnia, emotional instability, depression and vision disturbances. These symptoms, of course, depend not only on the route and duration of

exposure and the person's individual susceptibility but also on the nature of metal species. For example, the possible role of Hg^{2+} , Pb^{2+} , and As^{3+} in the impairment of the central nervous system has been recognized. Likewise, the kidney and liver can be damaged by Cu^{2+} , Cd^{2+} , Hg^{2+} , and Pb^{2+} , while skin, bones, and teeth can be damaged by Ni^{2+} , Cu^{2+} , Cd^{2+} , and $\text{Cr}^{3+/6+}$. As regards the carcinogenic effects of heavy metals, only arsenic, cadmium, chromium, and nickel are classified in group 1 (carcinogenic to humans) by the International Agency for Research on Cancer [70].

Due to these adverse effects, maximal levels of lead, cadmium, mercury and tin admissible in various foodstuffs have been set by the European Commission [8]. The values are between 0.02 and 200 mg/kg depending on the metal and on the foodstuff type.

Conventional analytical techniques available for heavy metals determination include atomic absorption or emission spectroscopy, inductively coupled plasma mass spectrometry, cold vapor atomic fluorescence spectrometry, X-Ray fluorescence spectrometry, neutron activation analysis, chromatographic or electrochemical methods [71-73]. These techniques can be used to detect single and/or multiple metals at low concentrations, some of them being directly applicable to solid samples analysis, the others requiring a preliminary extraction step [71].

✓ Glycoalkaloids (GAs)

Glycoalkaloids (GAs) are a group of nitrogen-containing compounds that are naturally produced in various cultivated and ornamental plant species of the Solanaceae family [74]. This family of plants is represented by vegetables such as potatoes, eggplants, peppers, tomatoes, as well as non-food plants, such as tobacco, petunia, and climbing or bittersweet nightshade. The main glycoalkaloids are α -solanine and α -chaconine which together account for 95% of the total glycoalkaloid content. The GA α -chaconine is considered more toxic than α -solanine.

Glycoalkaloids are toxic to humans if consumed in high concentrations and they are not destroyed during the cooking. GAs may induce gastro-intestinal and systemic effects, by cell membrane disruption and acetylcholinesterase inhibition [75]. For food safety purposes, an upper limit for glycoalkaloid content of 20 mg per 100 g fresh weight in potato is generally established [76]. The main analytical methods available for glycoalkaloids determination are mainly chromatographic techniques [77-79].

✓ **Surfactants**

Surface Active Agents (SAAs) constitute a group of compounds which contain in molecules hydrophobic and hydrophilic parts [80]. Surfactants are widely applied in household detergents, personal care products and as vital components in a multitude of industrial and institutional sectors household which possessing specific properties (washing, wetting, emulsifying, and dispersing). The one of the largest end users for surfactants industry is food industry [80]. Due to contamination and potential toxicity of surfactants their usage is limited.

For the surfactants analysis in environmental samples – spectrophotometry [81, 82], tensammetry [83] or electrophoresis [80] and chromatography [83] may be used. Nowadays, high-performance liquid chromatography (HPLC) [80, 83, 84] is usually coupled with a universal mass spectrometry detector (MS) (or tandem mass spectrometry detector MS–MS).

1.5. Biosensors

1.5.1. Generalities on biosensors

Conventional analytical methods described above provide high reliability and very low limits of detection but are time-consuming, require skilled personnel and are based on costful instrumentation. The need of alternative rapid, sensitive and easy-to-use methods of qualitative and quantitative determination of toxic compounds is therefore actual and of uppermost importance.

Among the emerging and innovative technologies proposed, biosensors are presented to date as promising rapid and low-cost alternatives, or at least complementary techniques, to conventional analytical methods for many types of applications, including food safety [85]. As screening tools, biosensors can help selecting a rather limited number of suspect samples that would be further analysed for confirmation by conventional techniques, reducing the cost and time of analysis [86].

Biosensors are self-contained devices primarily made up of two basic components: a *biorecognition element* or *bioreceptor*, which constitute the sensing part of the biosensor, and a *physical transducer* [87]. Biosensors are capable of transforming the modification of

physical or chemical properties of the bioreceptor, induced by specific interactions with a target analyte, into an electrical signal directly related to the analyte concentration. Biosensors therefore combine the specificity of the biorecognition element with the capacity of physical transducers to convert biological events into electrical measurable signals exploitable for quantitative analysis (Fig.1.8.).

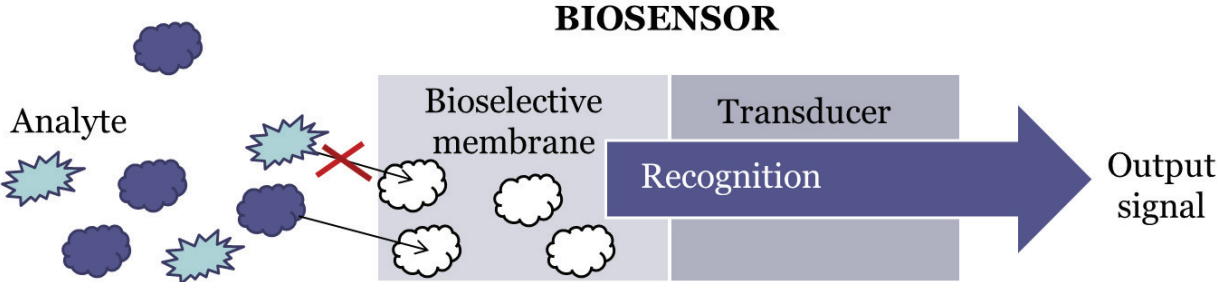


Fig.1.8. Schematic diagram of biosensor

Biological components of the biosensors can be divided into two main groups: catalytic and non-catalytic (Fig. 1.9). The catalytic group includes enzymes, tissues and microorganisms, whereas the non-catalytic group includes antibodies, antigens, receptors, DNA and nucleic acids.

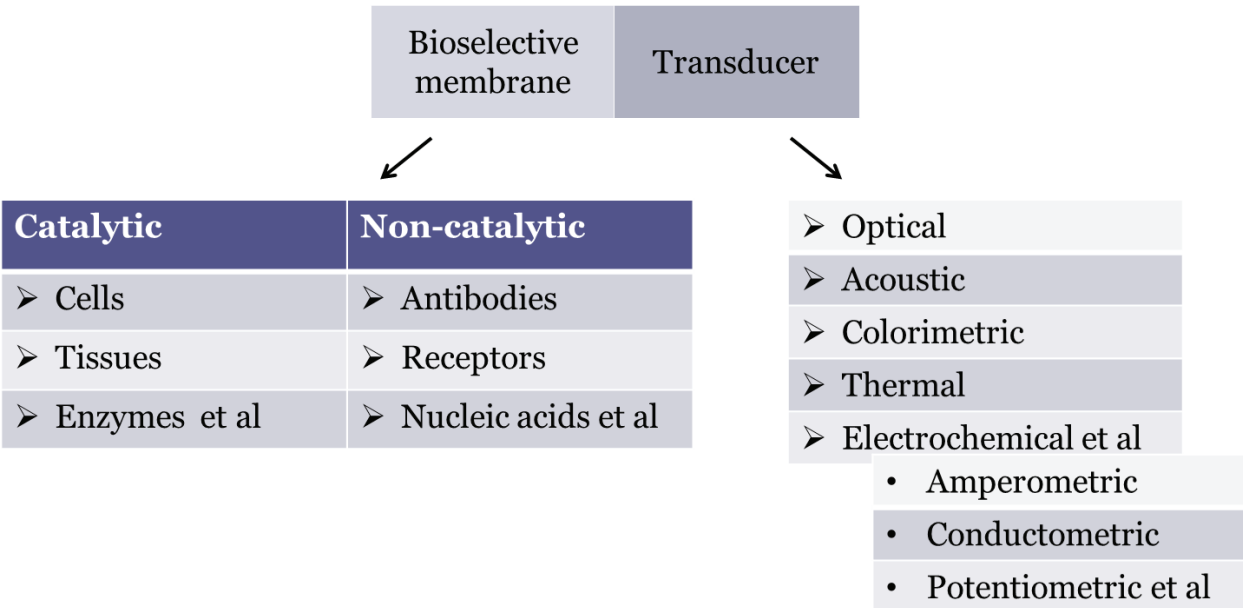


Fig.1.9. Main possible components of biosensor

For their part, transducers can be classified into the following categories (Fig. 1.9):

- Electrochemical – they measure the electrical signal changes [88].
- Impedimetric – they measure the resistive and capacitive properties of the system upon perturbation by a small amplitude sinusoidal ac excitation [92].
- Optical – they measure optical properties changes [93],
- Calorimetric – they measure small temperature changes [94],
- Acoustic – they measure changes in the acoustic properties of the sensor [95].

The high specificity of enzyme-substrate interactions, the usually high turnover rates of biocatalysts, their commercial availability at high purity levels are at the origin of the development of a large number of enzyme-based biosensor devices for a wide range of applications, including environmental [96] or food analysis [97].

1.5.2. Electrochemical detection

Among possible modes of detection, electrochemical transduction offers high sensitivity and specificity and the possibility to miniaturize the required instrumentation providing compact and portable analysis devices [88].

Electrochemical techniques are generally organized into three main categories of measurement: current, potential and conductive.

- ✓ Amperometric transducers measure leaking currents at constant voltage [89];
- ✓ Potentiometric transducers measure potential changes at constant current (usually zero) [90];
- ✓ Conductometric transducers measure electrical conductivity changes between two electrodes [91].

Advances in nanotechnology, microelectronics and microfluidics have permitted the miniaturization of electrochemical biosensors and the fabrication of high density arrays, particularly interesting for the real-time parallel monitoring of multiple chemicals or biological parameters, or the monitoring of one single parameter in several samples [96]. The miniaturization of sensing platforms can provide a number of benefits including a reduction in both bioreceptor and sample amounts, an increased of sensitivity and high throughput analysis. Additionally, the small size is well-suited for the design of portable biosensors, especially for in-site monitoring applications. In recent years, a particular attention has also been paid to the integration of nanomaterials, typically gold nanoparticles, magnetic beads

and carbon-based NMs (e.g. carbon nanotubes, graphene), for the elaboration of electrochemical biosensors. The nanoscale modification of electrode surfaces is likely to provide novel and sometimes unique properties due to the ability of the intrinsic properties of the NMs employed, the ability to control the architecture of the electrode interface at the nanoscale, or both [98,99].

In this work, we focused on the detection of some food contaminants by an enzyme-based conductometric and potentiometric biosensors.

1.5.2.1. Conductometric transducers.

In conductometric method, the most important property of an electrolytic solution is its conductivity, which varies in accordance with quite a wide range of enzymatic reactions [100]. The liquids analyzed are mostly considered to have significant background conductivity which is easily modified by different factors, therefore the selectivity of this method is presumed to be low and consequently its potential use for different applications, rather doubtful. However, in the case of an integral microbiosensor, most of these difficulties can be overcome using a differential measuring scheme which compensates for changes in background conductivity, the influence of temperature variations, and other factors [101].

The conductometric transducer is a miniature two electrode device to measure the conductivity of the thin electrolyte layer adjacent to the electrode surface. The interdigitated structure of electrodes is commonly utilized for the development of conductometric electrodes. The various electrode materials have been tested in order to select proper material for the conductometric interdigital transducers [102]: platinum, gold, aluminum, nickel, copper, titanium, chromium, Ta₂O₅, silver, and carbon. All these materials are suitable, especially when the high-frequency current is used.

Microelectronic techniques, such as photolithography [103] and vacuum spraying [102] are usually used for the conductometric transducers manufacturing. The low cost manufacturing is the main advantages of transducers of such a type.

1.5.2.2. Potentiometric transducers.

In potentiometric detection the potential across an interface (often a membrane) measures. Traditionally in the measurement, zero-current or little current is involved [104].

Potentiometric biosensors make use of ion-selective electrodes in order to obtaining the potential signal for specific ions, such as H^+ , K^+ , and Ca^{2+} .

A recent development from ion-selective electrodes is the production of ion-selective field effect transistors (ISFETs). ISFET typically consists of three terminals, namely source, drain and reference electrode that replace of a voltage gate. When the ion concentration near the sensing area is change, the source-drain current changes accordingly.

Complementary metal oxide semiconductor fabrication (CMOS Fabrication) is used to the ISFET fabrication. These processes are summarized by following steps [105]:

1. Field oxide growth
2. Photolithography for gate oxide definition
3. Gate oxide growth
4. Sensitive inorganic membrane deposition
5. Photolithography of the sensitive membrane
6. Photolithography of contacts
7. Metal deposition
8. Photolithography for metal patterning
9. Passivation deposition
10. Photolithography for passivation opening over bonding pads and ISFET gates

These transducers can be turned into biosensors by attached thereto a biological element such as an enzyme that catalyzes a reaction that forms the specific ions.

1.5.2. Enzyme-based electrochemical biosensors

Enzyme biosensors consist of enzymes immobilized at the surface of the transducer. Immobilization step is very important as it affects the sensitivity, selectivity and robustness of biosensors by influencing enzyme orientation, stability and activity. Various immobilization strategies have been reported including adsorption, covalent binding, entrapment or cross-linking [106]. Enzyme-based electrochemical biosensors have been developed for the detection of a large number of substrates of biological importance such as glucose [107-109], urea [110, 111], creatinine [112-114], arginine [115,116], acetylcholine [117,119] [97] [98] among others. Biosensors of this type have been also proposed for the determination of feed and food contaminants, e.g. mycotoxins, OPs and heavy metals.

They rely on either activation or inhibition of the enzyme by the chemicals. Enzyme products or by-products may be electroactive, meaning their activity may be followed by amperometry. Other enzymes produce or consume protons, meaning their activity can be monitored through pH changes. Other ions may also be produced; in this case, the enzyme activity may be monitored through conductimetric measurements.

1.5.2.1. Electrochemical biosensors based on enzyme activation for the determination of toxic compounds

- **Mycotoxins**

A range of electrochemical biosensors have been reported in the literature for mycotoxins analysis, most of them targeting ochratoxins and aflatoxins (more particularly OTA and AFB1) and harnessing the affinity of the molecules for specific antibodies or aptamers [119,120]. Very few biosensors, based on enzyme activation mechanism, have been proposed [121-124].

- **OPs**

Enzyme-activation biosensors proposed for OP pesticides analysis rely on the hydrolysis of OPs such as paraoxon, parathion, coumaphos, diazinon, chlorpyrifos, and methyl parathion by organophosphorus hydrolase (OPH, isolated or overexpressed at the surface of bacteria), leading to the production of organophosphorus acid and alcohol as a result of the cleavage of the P–O, P–F, P–S, or P–CN bonds. The detection can be performed by potentiometry or amperometry [125]. Although OPH-based biosensors can detect only some OPs and offer a lower sensitivity compared to the Acetylcholinesterase (AChE)-based biosensors, which will be described in the next section, they are more selective. Some examples of recent works published on NM-based biosensors using OPH as bioreceptor can be found in [125-127].

- **Heavy metals**

Many types of biorecognition elements, i.e., antibodies, proteins, peptides, nucleic acids, DNA-zymes, whole cells and enzymes, may be used for the elaboration of heavy metals electrochemical biosensors [128,129]. However, as no heavy metal is able to act as enzyme

substrate, all enzyme based biosensors reported in the literature rely on inhibition mechanisms.

1.5.2.2. Biosensors based on enzyme inhibition

- **Generalities on enzyme inhibitors**

Inhibitors are molecules that can bind to (interact with) enzymes. As a result, the activity of the catalyst is decreased or abolished. Because they are able to control enzyme activity, inhibitors have been used in a wide range of applications. A large number of drugs, including those for the treatment of HIV infection, cancer and heart disease, are enzyme inhibitors [130-132]. Enzyme inhibitors are also used in agriculture as pesticides and herbicides [133]. Most inhibitors are products of natural origin [134] but a lot of them are also synthetically prepared. Inhibitors differ in their mechanism as demonstrated by the analysis of enzymatic kinetics. There are two large groups of enzyme inhibitors: irreversible and reversible.

- ✓ **Irreversible inhibitors**

Irreversible inhibitors form strong stable interactions that can be removed only chemically. First, the inhibitor binds non-covalently to the enzyme (EI or ESI). Then, the inactivation of the enzyme is achieved when the covalent complex EI* is formed (fig 1.10).

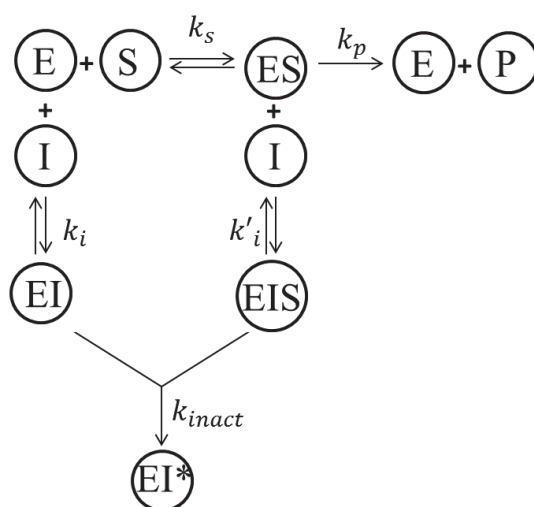


Fig.1.10. Kinetic scheme for irreversible inhibition

Typically, irreversible inhibition is the consequence of alkylation or acylation of an active site residue. Irreversible inhibitors can act as poisons. For example, organophosphates, used as insecticides, herbicides and nerve gas, function by irreversible inhibition of acetylcholinesterase. Aspirin, familiar with its use as an analgesic drug, works by irreversible acetylation of active-site serine residue of cyclooxygenases (COX), preventing the synthesis of prostaglandins and thromboxane [135].

✓ **Reversible inhibitors**

Reversible inhibitors form weak interactions with their target enzymes and are easily removed (substrate excess, dialysis, and others). The inhibitor comes to equilibrium with the enzyme, to form an enzyme-inhibitor complex. The equilibrium constant for the dissociation of this complex is mentioned as K_i . Also known, inhibition constant, K_i is an indication of how potent an inhibitor is.

Reversible inhibitors can be further classified according to their mechanism of action: competitive, uncompetitive, non-competitive (pure and mixed types).

a) Competitive inhibition type

In the classical model, the inhibitor (I) competes with the substrate (S) for binding to the active site of the enzyme (E) (Fig.1.11). Alternately, binding sites of the substrate and the inhibitor are different but the binding of the inhibitor does not allow the substrate interaction with the enzyme (for example, the steric hindrance of the inhibitor prevents the binding of the substrate). In both models, the inhibitor blocks the entry of the substrate in the active site.

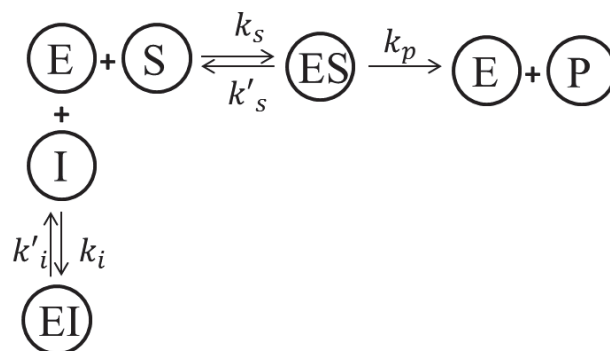


Fig.1.11. Kinetic scheme for reversible inhibition of competitive type

In this case the initial rate v_0 and apparent Michaelis-Menten constant K_m^{app} are next:

$$v_0 = V_{max} \frac{[S]}{\left(1 + \frac{[I]}{K_i}\right) K_m + [S]} \quad (1.1)$$

$$K_m^{app} = \left(1 + \frac{[I]}{K_i}\right) K_m \quad (1.2)$$

The structure of competitive inhibitors is often similar to that of substrate. The enzyme affinity to the substrate decreases in the presence of inhibitors and the value of Michaelis-Menten constant increases accordingly. The value of maximum velocity (V_{max}) does not change, since saturating concentrations of substrate displace the inhibitor from its complex with the enzyme (Fig1.12).

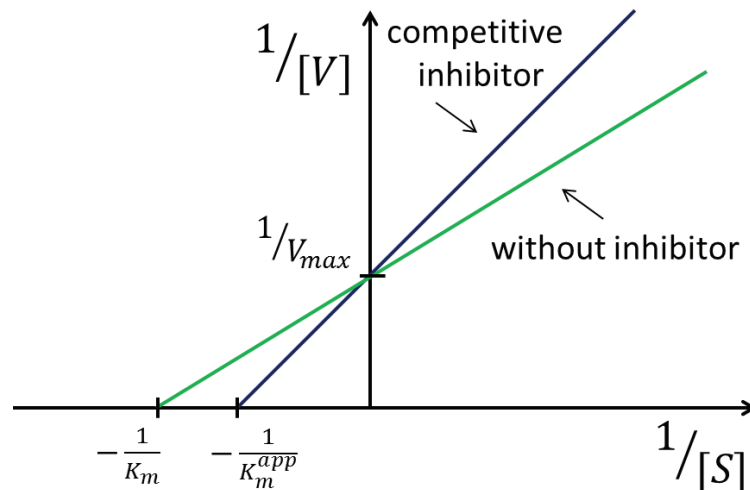


Fig1.12. Double-reciprocal plot of competitive inhibition

b) Uncompetitive inhibition type

In uncompetitive inhibition, inhibitor (I) only binds to the enzyme-substrate complex (ES) and not to free enzyme (E) (fig 1.13). Indeed, the inhibitor binding site is only exposed when the enzyme and the substrate interact each other. The ternary complex (ESI) does not form any product.

c) Mixed non-competitive inhibition type

Mixed inhibition combines the effects of competitive and uncompetitive inhibitions. In this case, the inhibitor (I) can bind to the enzyme (E) and/or to the enzyme-substrate complex (ES) (fig 1.15). However, the inhibitor does not have the same affinity for (E) and (ES), resulting in different equilibrium constants, K_i and αK_i , respectively. Similarly, substrate affinity is different for free enzyme (E) and for enzyme-inhibitor complex (EI), with associated equilibrium constants K_m and αK_m , respectively.

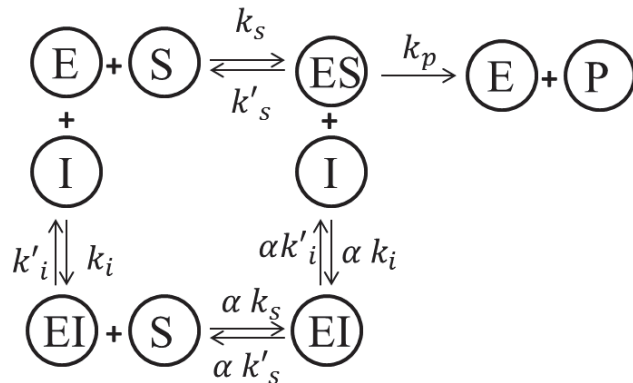


Fig.1.15. Kinetic scheme for reversible mixed inhibition type

In this case the initial rate v_0 and the apparent Michaelis-Menten constant K_m^{app} and the apparent maximum rate V_{max}^{app} are next:

$$v_0 = \frac{V_{max}}{\alpha'} \cdot \frac{[S]}{\frac{\alpha}{\alpha'} K_m + [S]}, \quad (1.6)$$

$$\text{where } \alpha = 1 + \frac{[I]}{K_i}, \quad \alpha' = 1 + \frac{[I]}{K'_i} \quad (1.7)$$

$$K_m^{app} = \frac{\alpha}{\alpha'} K_m \quad (1.8)$$

$$V_{max}^{app} = \frac{V_{max}}{\alpha'} \quad (1.9)$$

Like uncompetitive inhibitors, mixed inhibitors usually affect both K_m and V_{max} . The value of V_{max} decreases, the value of K_m can either increase (fig.1.16) or decrease.

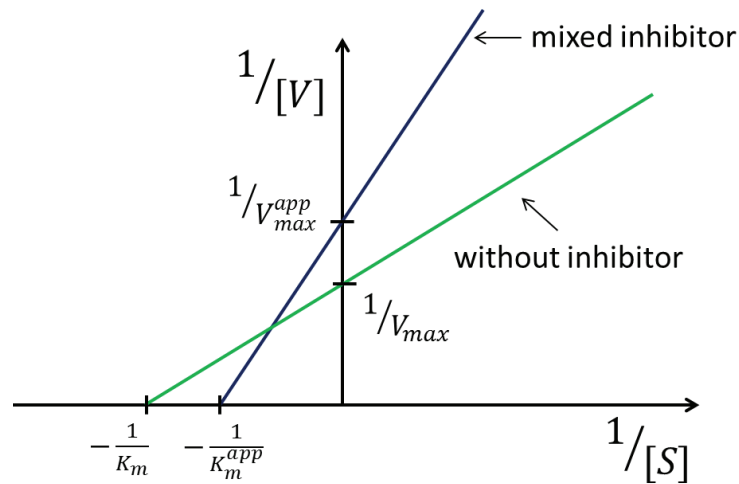


Fig1.16. Double-reciprocal plot of mixed inhibition

d) Pure non-competitive inhibition type

In non-competitive inhibition, the inhibitor binds to the enzyme at a location other than the active site in such a way that the inhibitor and substrate can simultaneously be attached to the enzyme (fig.1.17). The substrate and the inhibitor have no effect on the binding of the other and can bind and unbind to the enzyme in any order. Non-competitive inhibition type is a special kind of mixed inhibition.

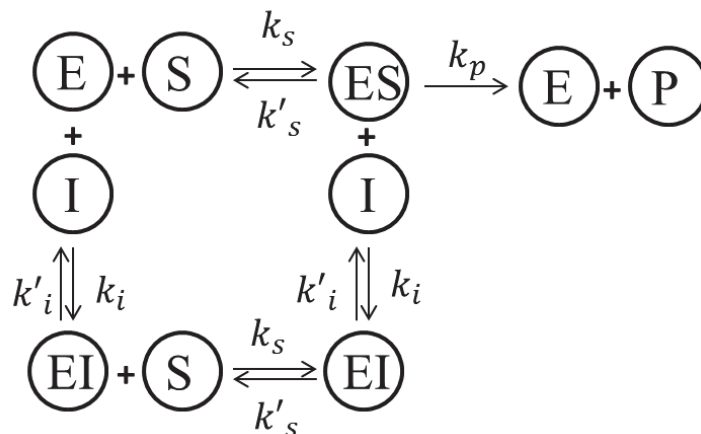


Fig.1.17. Kinetic scheme for reversible non-competitive inhibition type

In this case the initial rate v_0 and the apparent maximum rate V_{max}^{app} are next:

$$v_0 = \frac{V_{max}}{1 + \frac{[I]}{K_i}} \cdot \frac{[S]}{K_m + [S]} \quad (1.10)$$

$$V_{max}^{app} = \frac{V_{max}}{1 + \frac{[I]}{K_i}} \quad (1.11)$$

Noncompetitive inhibitors decrease V_{max} but leave K_m unaffected (fig.1.18).

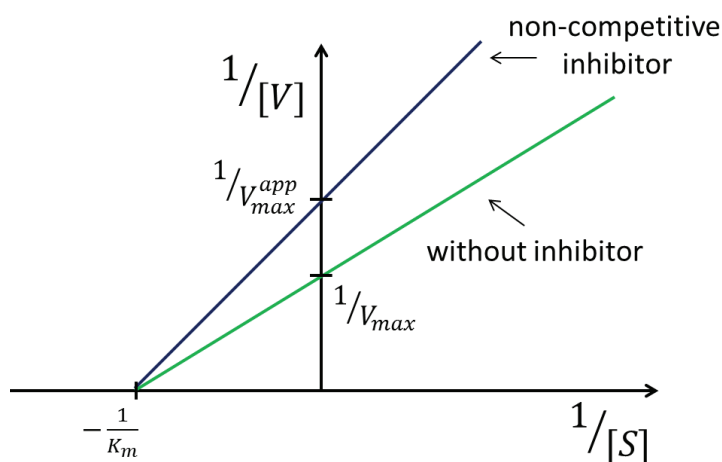


Fig1.18. Double-reciprocal plot of non-competitive inhibition

▪ **Application to biosensors elaboration for toxic compounds**

In recent years, interest for the determination of compounds that are enzyme inhibitors has significantly increased [136].

The first enzyme inhibition based biosensor was developed in 1962 for the determination of organophosphorus compounds such as Systox, Sarin, parathion and malathion [137]. The biosensor principle was based on cholinesterase inhibition by organophosphates.

Since then, numerous biosensors based on inhibition analysis of various toxic substances have been developed. Several reviews have been published recently on that topic [136, 138]. The choice of enzyme/analyte system is determined by the ability of the toxic substance to inhibit the enzyme.

The evolution of the number of publications dedicated to biosensors based on enzyme inhibition and published in the 1990-2014 period is presented in the review [136]. These data were obtained by searching for the items by keywords "biosensor" and "enzyme" and "inhibition" using ISI Web of Knowledge database, Thomson Reuters and Scopus. The article reports that during the period of 1990-1996, the number of publications was about 100, in 1997-2002 it increased almost twice, in 2003-2008 reached nearly 400 and continued to grow, so that in 2009-2014 almost 500 articles were published on the topic.

For biosensors of this type, enzyme activity is measured before and after inhibition, which enables assessing the inhibition level, which is proportional to the toxin concentration.

In the case of irreversible inhibition, after the response to the substrate (A_0) is obtained, the biosensor is incubated in the inhibitor solution for some time, then washed from the inhibitor excess and the response to the substrate (A_i) is measured again (fig.1.19).

As irreversible inhibitors strongly bind to the bioselective element, before the biosensor can be reused, it must be incubated in a reactivator solution. Enzyme reactivators are compounds which restore enzymatic activity by removing an inhibitory group bound to the reactive site of the enzyme.

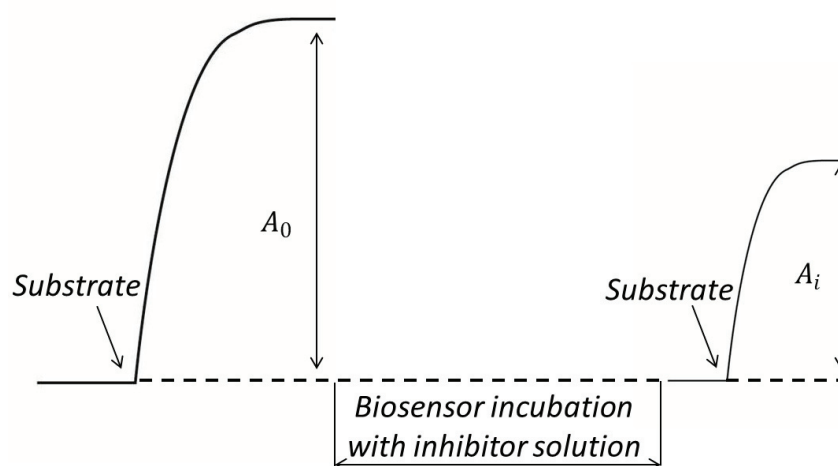


Fig.1.19. Scheme of inhibitory analysis at irreversible inhibition

In case of reversible inhibition, for example, by aflatoxins or glycoalkaloids, the inhibitor is added directly to the working cell (fig.1.20). For the repeated procedure only washing with the working buffer is needed.

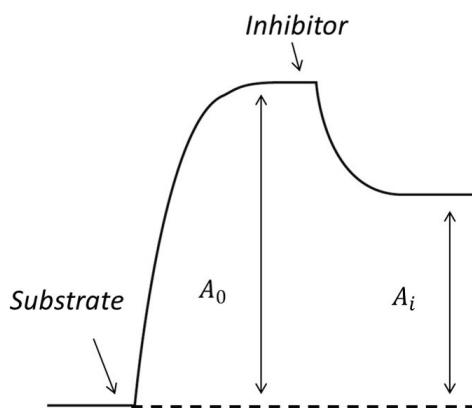


Fig.1.20. Scheme of inhibitory analysis at reversible inhibition

The level of inhibition can be assessed according to the following formula:

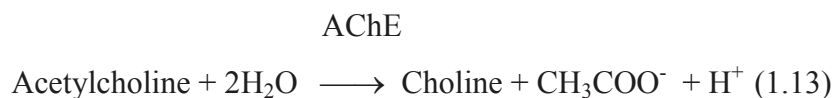
$$I = \frac{A_0 - A_i}{A_0} \times 100\% \quad (1.12)$$

A variety of enzymes, including alkaline phosphatase, tyrosinase, glucose oxidase, urease and cholinesterases among others, have been proposed for the elaboration of inhibition biosensors [136, 138].

For example, alkaline phosphatase was reported for the determination of vanadium [139, 140], carbofuran [140], phosphates [141] and caffeine [140], catalase for inhibitory determination of nitrite ions [143]. Aflatoxin B1 was determined by an elastase-based biosensor [144]. Glucose oxidase was used to develop biosensors for different heavy metals ions [145,146]. Chromium was measured by a peroxidase-based biosensor [147]. Tyrosinase and phenoloxidase biosensors were proposed for benzoic acid monitoring [148, 149], while urease was hardnessed for the determination of mercury ions [150], arginine [116] or atrazine [151].

Among the large number of enzymes proposed for inhibition biosensors, cholinesterases are very frequently used due to their high sensitivity to a variety of toxic substances, in particular pesticides [136, 138]. Acetylcholinesterase (AChE) has a very high catalytic activity - each AChE molecule degrades about 25000 molecules of acetylcholine per second, approaching the limit allowed by diffusion of the substrate [152].

AChE stabilizes the levels of the neurotransmitter acetylcholine by catalyzing the hydrolysis of acetylcholine to thiocholine:



The reaction catalyzed by AChE is required for decontamination of acetylcholine in the synaptic cleft and transition target cells in a resting state (for example, relaxation of muscle cells). Therefore, AChE inhibitors are powerful toxins whose effects on the human body usually lead to death from respiratory muscles court. Poisoned substances of nerve action (sarin, soman, tabun, VX), some medicines for Alzheimer's disease (galantamine, huperzine, donepezil, rivastigmine), pesticides (carbofuran, trichlorfon, paraoxon, malaaxon), heavy metals and natural toxins (aflatoxin, pirydostyhmin, glycoalkaloids) can inhibit AChE activity. It has been shown that inhibition of AChE by aflatoxins, glycoalkaloids, and some other natural toxins is of reversible type and the inhibition is irreversible for pesticides and heavy metals [153,154]. Regeneration may be performed by ethylenediaminetetraacetic acid (EDTA) or cysteine for heavy metals, by some oxime, such as pralidoxime for OPs. AChE has therefore been successfully applied to the development of a large number of electrochemical biosensors, mostly targeting pesticides [155-165], but in a lesser extent aflatoxins [166] or heavy metals [166]. Several reviews dedicated to this topic has been recently published [155-158].

A major issue, however, comes from the possible presence of several AChE inhibitors in the analyzed samples, making difficult the direct determination of the specific analytes with required accuracy.

1.6. Mathematical simulation for biosensors optimization

Mathematical simulation is widely used for a better understanding of biochemical processes occurring in the biosensors and for their optimization.

Various mathematical models have been developed and successfully applied to optimize biosensors since the '70s until today [167-168]. For example, over the past five years, S. Loghambal, L. Rajendran et al. suggested several mathematical models for amperometric electrode with immobilized enzyme based on nonlinear differential equations

that describe Michaelis-Menten kinetics and diffusion [170,171], moreover mathematical model of amperometric and potentiometric biosensor were proposed [1172]. The method of homotopy perturbation was used for solving the systems of equations in stationary conditions. Ašeris and colleagues described a mathematical model of biosensors [173,174], where the input parameters (such as concentration of reagents, kinetic constants and thickness of the membrane) are changed. The sensitivity of the developed biosensors was improved by using the mathematical simulation. The finite difference method was used for solving the system of equations in these models.

The significant majority of developed mathematical models describe enzyme biosensors for direct determination of substrates. Nevertheless, recently it has been observed a growing interest in biosensors based on inhibition analysis [138,166]. To a greater extent these biosensors are used in environmental monitoring to detect toxic substances such as pesticides, heavy metal ions, aflatoxins, etc. [166,175]. Up to now, only a small number of mathematical models of biosensors of this type have been developed. The mathematical model of glucose oxidase biosensor for mercury ions determination should be highlighted [176]. In this model, the system of equations describes diffusion and enzymatic reactions related to nonlinear Michaelis-Menten kinetics and modified considering irreversible inhibition. This model will be used in this work to describe the proposed biosensor.

1.7. Conclusions

Application of biosensors for environmental monitoring is a promising approach due to their capability of fast and cheap determination of various toxic compounds. Today, a number of enzyme biosensors based on inhibition analysis have been developed. Diverse transducers, measuring circuits and enzymes have been used to create enzyme biosensors for toxic compounds detection. Acetylcholinesterase has a very high catalytic activity, and there is a wide range of toxic compounds, which inhibit this enzyme. Therefore, AChE is often used to create biosensors based on inhibition analysis.

However, selectivity of one biosensor is usually insufficient to identify the specific toxin among others and to determine its concentration in the test sample. These biosensors often can be used to determine only one toxic substance, the analysis of multi-component mixtures is challenging.

Thus, taking into account the analysis of the above data, the aim was to develop a new original approach for the multidetection of several food contaminants, mainly aflatoxins and organophosphorus pesticides, using acetylcholinesterase (AChE)-based inhibitory electrochemical biosensors and applying an algorithm for analysis of real samples.

2. MATERIALS AND METHODS

2.1. Materials

The following reagents were used in this work.

Bioselective membranes contained:

- enzyme acetylcholinesterase (AChE) from electric eel (EC 3.1.1.7), activity 426 U/mg (Sigma-Aldrich Chemie, Germany);
- bovine serum albumin (BSA) (fraction V) (Sigma-Aldrich Chemie, Germany);
- 50% aqueous solution of glutaraldehyde (GA) specially purified for use as an electron microscopy fixative or other sophisticated use (Sigma-Aldrich Chemie, Germany);
- glycerol (purity 99%, Sigma-Aldrich Chemie, Germany).

Acetylcholine chloride (AChCl) (purity 99%) (Sigma-Aldrich Chemie, Germany) was used as enzyme *substrate*.

The following substances were used as *inhibitors*:

- aflatoxin B1 (purity 98%, Sigma-Aldrich Chemie, Germany);
- aflatoxin G1 (purity 98% , Sigma-Aldrich Chemie, Germany);
- trichlorfon (analytical standard) (purity 98% Riedel de Haën, Germany);
- cationic surfactant benzalkonium chloride (purity 95.0%) (Fluka, Sweden);
- crystalline α -solanine from sprouts of *Solanum tuberosum* (purity 98% , Sigma–Aldrich Chemie GmbH, Germany);
- copper nitrate was of p.a. grade (Helicon, Russia).

The following substances were used as *reactivators*:

- pyridine-2-aldoxime methyl iodide (PAM-2) (purity 98%, Sigma–Aldrich Chemie, Germany);
- ethylene diamine tetra acetate (EDTA) (analytical standard) (Sigma–Aldrich Chemie, Germany).

- *Phosphate buffer solutions* were prepared from KH_2PO_4 salt (purity 98.5%, Helicon, Russia) and NaOH (purity 99%, Helicon, Russia).

Compounds for the *buffer "Polimix"* preparation were next: (purity 98.5-99%, Helicon, Russia):

- 2,5 mM Na-Tetraborate ($\text{Na}_2\text{B}_4\text{O}_7$) (anhydrous) (pH 7.8-9,2);
- 2,5 mM Tris (pH 7.0-9.2);
- 2,5 mM K-P (pH 6-8);
- Citric Acid (2,5 mM);
- NaCl (150 mM).

The following substances were used as *solvents*:

- acetonitrile (purity 99,8%, Sigma–Aldrich Chemie, Germany);
- methanol (purity 99.9%, Sigma–Aldrich Chemie, Germany);
- DMSO (purity $\geq 99.5\%$, Sigma–Aldrich Chemie, Germany);
- Ethanol (purity $\geq 99.8\%$, Sigma–Aldrich Chemie, Germany);

2.2. Conductometric transducers and measuring device

The conductometric transducers (Fig. 2.1), used in this work, were produced at the Lashkarev Institute of Semiconductor Physics of National Academy of Sciences of Ukraine (Kyiv, Ukraine). They are 5 mm x 30 mm in size and composed of two identical pairs of gold interdigitated electrodes deposited on a ceramic base, one of them serving as working electrode and the second as reference electrode. The sensitive surface of each electrode pair is approximately 1.0 mm x 1.5 mm. The width of the transducer fingers and the distance between them were 20 μm .

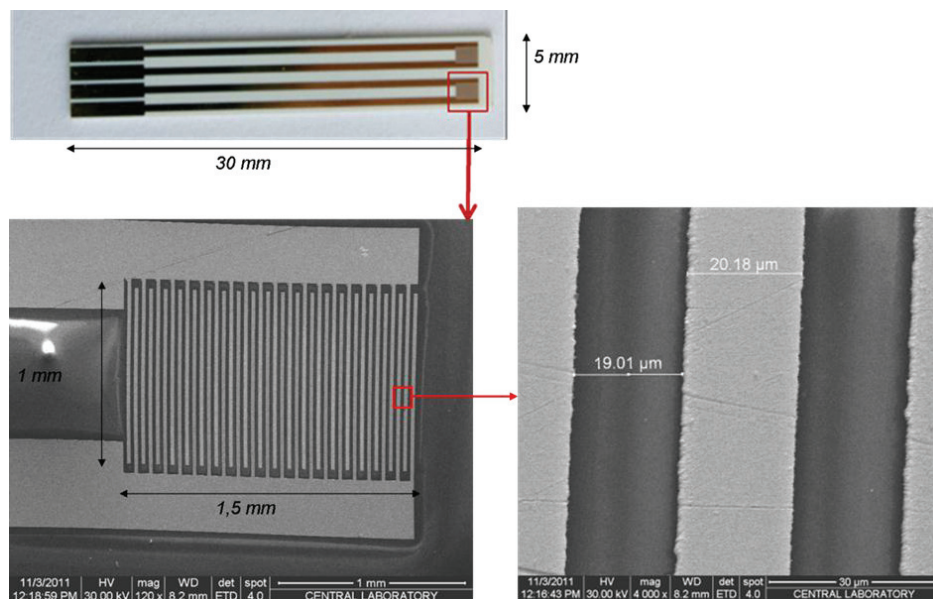


Fig. 2.1. General view of the conductometric transducer and images of the gold interdigitated electrodes obtained using Scanning Electron Microscopy.

The conductometric transducers were connected to the measuring setup as described in Fig. 2.2. The electrodes, placed into the measurement cell filled with the tested solution, were supplied with alternating voltage (100 kHz frequency, 10 mV amplitude) from the low-frequency signal generator G3-118 (Radiopribor, Russia). A differential mode was used to increase the sensor sensitivity and minimize the nonspecific signals. The sensor electrodes were coupled with a 1 k Ω load resistance. The signal issued from the electrodes through the differential amplifier Unipan-233-6 (Poland) enters the selective nanovoltmeter Unipan-233-6, and then is registered by a recording device.

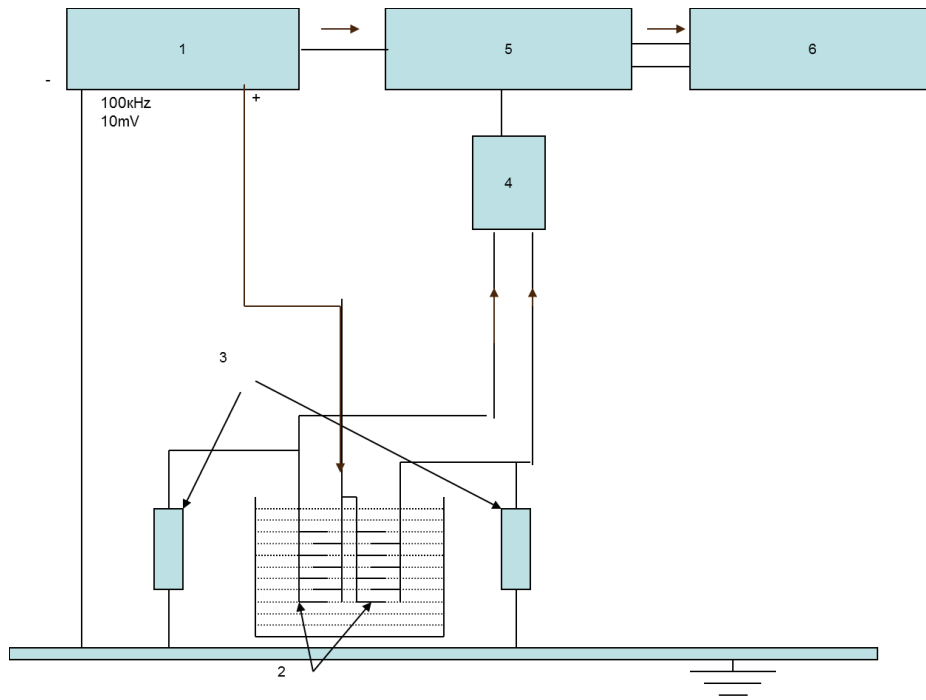


Fig. 2.2. Diagram of measuring setup (1 — generator, 2 — electrodes with enzyme and reference membranes, 3 — load resistances (1 k Ω), 4 — differential amplifier, 5 — phase-sensitive nanovoltmeter, 6 — recording device).

2.3. Potentiometric transducers and measuring portable device

The potentiometric transducers used in this work, were produced at the Lashkarev Institute of Semiconductor Physics of National Academy of Sciences of Ukraine (Kyiv, Ukraine). The sensor chip consists of two identical pairs of p-channel transistors ($\text{SiO}_2/\text{Si}_3\text{N}_4$ -ISFETs) on a monocrystalline silicon substrate of $8 \times 8 \text{ mm}^2$ total area. One pair serves as working electrode and the second one is used as reference electrode. Sensor elements used in this work demonstrated intrinsic pH-sensitivity of approximately 40 mV/pH and transconductance of 400–500 $\mu\text{A}/\text{V}$, thus providing pH-sensitivity of the transistor channel current of 15–20 $\mu\text{A}/\text{pH}$. pH-FET sensors response was measured by means of a current-to-voltage converter circuit with the sensors working in the current source mode with active load. Threshold voltage of the pHFETs was about 2.5 V. Measurements were performed with the initial channel current magnitude of approximately 500 μA , drain-to-source voltage of approximately 2 V, transistor bulk connected to the source. Fig. 2.3. shows the general view

of the sensor and a focus on the biomembranes deposited on the electrodes obtained by optical microscopy.

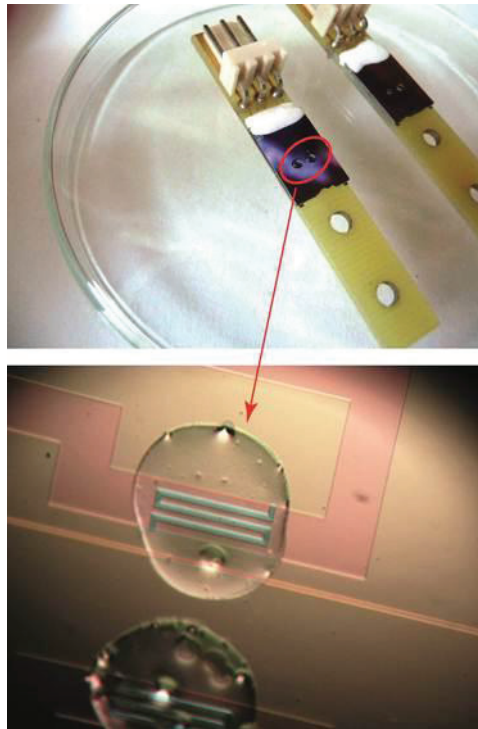


Fig. 2.3. General view of the potentiometric transducers and focus on the enzyme and reference membranes.

The measurements were performed using a portable device, developed and manufactured at the Lashkarev Institute of Semiconductor Physics of NAS of Ukraine (Fig. 2.4). The device operates by measuring the surface potential of the transistor gate. The tracking circuit was used with a negative feedback supporting a constant current value of 0.3 mA in the channel of the field-effect transistor at a constant source–drain voltage of about 2 V. The output signal corresponds to the gate potential. The device allows the operation in a differential mode (with 10- or 100-fold multiplication of the signal) as well as in a single mode of monitoring (i.e., it measures the difference of signals issued from the two pairs of electrodes or separate signals from each of the two channels). Informations from the transducers are imported to a computer and processed using the MSW_32 software (V.Ye. Lashkarev Institute of Semiconductor Physics of NAS of Ukraine).

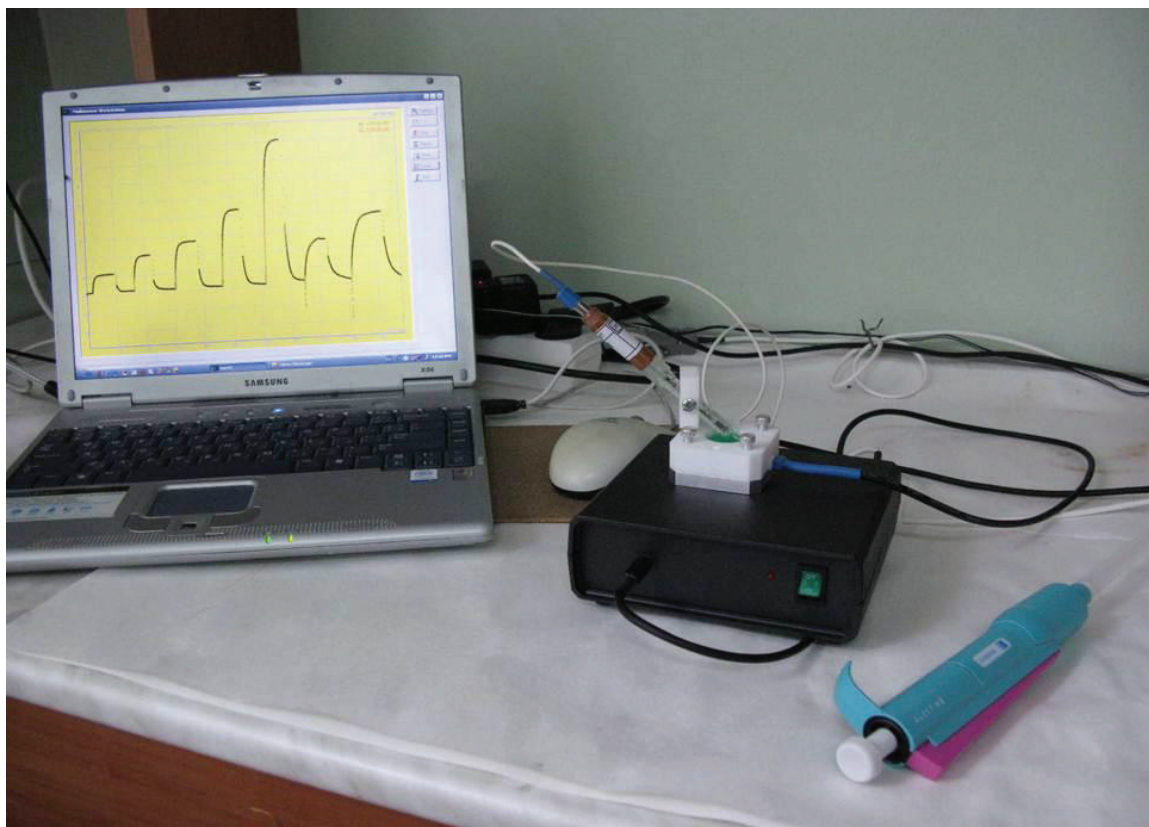


Fig. 2.4. General view of the portative device for measurements

2.4. Preparation of the bioselective membranes

Biologically active membranes were formed by cross-linking acetylcholinesterase with bovine serum albumin (BSA) on the transducer surface in a saturated glutaraldehyde vapor. The solution for working bioselective membranes consisted of 1% acetylcholinesterase (w/w), 1% BSA and 10% glycerol in 20 mM phosphate buffer, pH 7.0. The mixture for the reference membrane consisted of 2% BSA and 10% glycerol in the same buffer. After deposition of the prepared solutions on the working surfaces of the potentiometric transducers, the latter were placed in saturated glutaraldehyde vapor for 20 min, afterwards kept for 10-15 min in the air at room temperature. The membranes were finally washed with the buffer solution to remove unbound components.

2.5. Electrochemical measurements

Potentiometric and conductometric measurements were carried out after placing the microelectrodes in a glass cell filled with 5 mM phosphate buffer, pH 7.0. The solution was stirred vigorously. All experiments were carried out in two or three replicates. Nonspecific changes in the output signal related to the fluctuations of temperature, pH of environment, etc., were suppressed and avoided due to the differential mode of measurement.

After stabilization of the differential output signal, a small aliquot of a concentrated substrate solution was added to the cell. Then, small volumes of inhibitor solutions were injected. Responses to the working substrate concentration were measured before (A_0) and after (A_i) inhibition. The method of inhibition of the bioselective element varied depending on the type of inhibition (reversible or irreversible). In case of reversible inhibition, the inhibitor was added directly to the working cell. The biosensor could be re-used by only washing with the working buffer. For irreversible inhibition, after the response to the substrate (A_0) was obtained, the biosensor was incubated in the inhibitor solution for some time, then washed from the inhibitor excess and the response to the substrate (A_i) was measured. The biosensor could be re-used by incubation for 30 min into EDTA or PAM-2 reactivator solution.

The level of inhibition was calculated according to the equation (1.1).

2.6. Real samples preparation

Two types of real samples were used in this study. The first ones (sesame, walnut, peas) were bought in a supermarket and dry milled. 1g of the materials was mixed with 4 ml acetonitrile/water (80:20 v/v) and the suspensions were placed into an horizontal shaker (TH 15 model, Edmund Bühler) for 2 h. Then, sample extracts were recovered by centrifugation at 15,000g for 15 min (2-16K model, Sigma).

The second type of sample was prepared by growing AFB1 producer (*Aspergillus flavus*) on wheat substrate for 21 days (Fig. 2.5), then dried at a temperature lower than 60 ° C and crushed until a flour was obtained, adding a small amount of 4% KCl. Extraction was further performed using acetonitrile as extractant for 30 minutes with vigorous stirring. Extracts were evaporated under reduced pressure without light to dryness. The obtained

samples were dissolved in 3 ml of methanol and solid impurities that may mechanically damage the working biosensor membrane were removed by filtration.

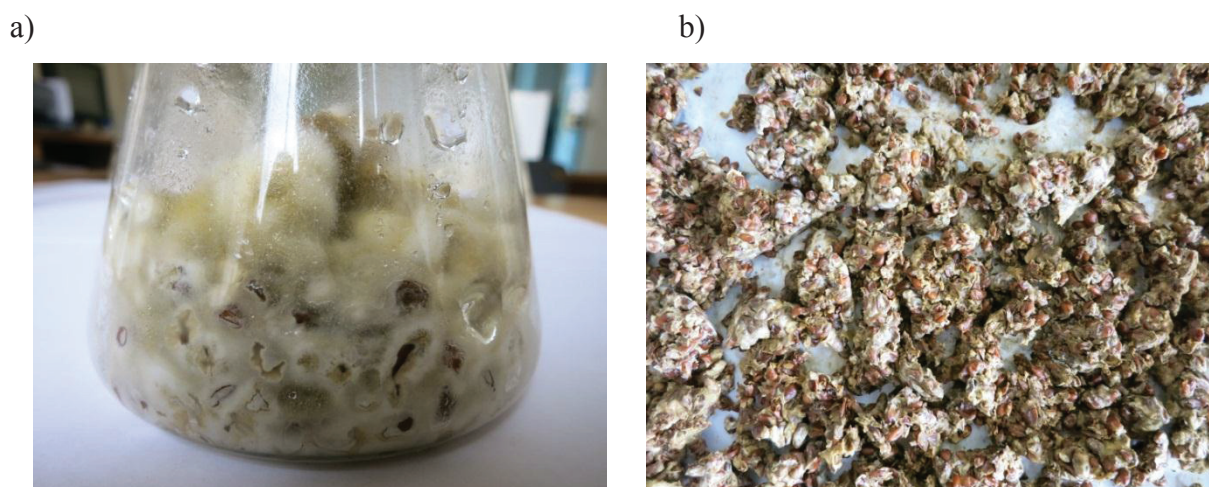


Fig. 2.5. *Wheat infected by Aspergillus mold during growth (a) and before the sample preparation (b).*

2.7. HPLC analysis

HPLC analysis was carried out in the Institute of Microbiology and Virology DK Zabolotny NASU using the Agilent 1200 liquid chromatography system (Agilent technologies, USA) equipped with G1315 diode array, G1321A fluorescence and G1956B mass-spectrometry detectors. The separation was performed at a 0.25 ml/min flow-rate using a Zorbax SB-C18 column (2.1 mm × 150 mm, 3.5 μm) in the isocratic elution mode. The mobile phase was MeOH/ACN/H₂O 40/10/50 (v/v). UV detection was carried out in the 200-400 nm range with special focus on 230 and 365 nm. Fluorescence detection was carried out at 365 nm for excitation and 455 nm for emission. To confirm the presence of aflatoxins, the mass spectrometry detector was configured to fix the m/z values in SIM mode. The samples were ionized by the method of electrostatic spray ionization (ESI) in a positive mode.

2.8. Mathematical simulation

Kinetic equations method. In order to simulate biosensor operation the kinetic equations method was used. Kinetic equations are used to describe a variety of phenomena in different fields, ranging from rarefied gas dynamics and plasma physics to biology and socio-economy, and appear naturally when one considers a statistical description of a large particle system evolving in time [177].

We have described the biochemical process in the biomembrane by the system of ordinary differential equations. Such a system consists of seven unknown functions x_i , and all of these functions depend on a single “independent variable” t , which is the same for each function.

Differential Equation Solving in Mathematica. Solving of the system of differential equations in mathematical modeling of the potentiometric biosensor operation was carried out using the Wolfram Mathematica 10 computational software. Numerical solution of the system of rate equations was found using *NDSolve* built-in algorithm.

The Mathematica function *NDSolve* is a general numerical differential equation solver. It can handle a wide range of ordinary differential equations (ODEs).

Finding numerical solutions to the system of ordinary differential equations (*eq*) is as following:

$$NDSolve [eq, \{n_e[t], n_s[t], n_i[t], n_{es}[t], n_{ei}[t], n_{esi}[t], n_p[t]\}, \{t, t_{min}, t_{max}\}]$$

This function finds numerical solutions for several functions n .

NDSolve represents solutions for the functions x_i as *InterpolatingFunction* objects. The *InterpolatingFunction* objects provide approximations to the x_i over the range of values t_{min} to t_{max} for the independent variable t .

In general, *NDSolve* finds solutions iteratively. It starts at a particular value of t , then takes a sequence of steps, trying eventually to cover the whole range t_{min} to t_{max} .

In order to get started, *NDSolve* has to be given appropriate initial conditions for the x_i and their derivatives. These conditions specify values for $x_i[0]$ at $t=0$.

3. DEVELOPMENT OF A POTENTIOMETRIC ACETYLCHOLINESTERASE-BASED BIOSENSOR FOR INHIBITORY ANALYSIS OF AFLATOXINS

1. Biosensor principle
2. Choice of AChCl substrate concentration
3. Choice of enzyme concentration in the biomembrane
4. Optimization of working pH
5. Influence of AFB1 concentration on biosensor level of inhibition
6. Biosensor reproducibility
7. Investigation of biosensor storage stability
8. Study of possible interferences with aflatoxins of other groups
9. Conclusions

3.1. Biosensor Principle

As already mentioned in the bibliographic chapter, acetylcholinesterase (AChE) is an enzyme inhibited by a range of chemicals including aflatoxins, organophosphorus and carbamates pesticides, as well as heavy metals. The inhibition of acetylcholine decomposition catalyzed by AChE, (reaction 1.13. in chapter 1), may be harnessed to develop electrochemical biosensors sensitive to the presence of such inhibitors in various types of samples. In this first part of our work, we propose a new potentiometric biosensor using ISFET microtransducers specially designed and fabricated at the Lashkarev Institute of Semiconductor (Kiev). The working electrodes have been modified by an AChE biomembrane deposited on the transducer surface and cross-linked using glutaraldehyde vapors.

AChE substrate (acetylcholine), added to the measurement cell, is decomposed by AChE into choline and acetic acid according to reaction 1.13. Acetic acid dissociates, thus increasing the local concentration of protons in the working membrane. Changes in the solution pH close to the transducer surface are then detected by ISFET transducers, leading to an increase of biosensor signal. Further addition of AChE inhibitors in the cell, e.g., aflatoxins, decreases the number of protons formed as a result of the enzymatic reaction, and the biosensor response decreases (Figure 3.1).

By comparing the biosensor signal intensities recorded before and after substrate addition, it is possible to calculate the inhibition percentage. It induced by the addition of AChE inhibitor (eq. 1.1 in the Materials and Methods chapter). The inhibition level is directly proportional to the inhibitor concentration in the measuring cell. Figure 3.1. compares the typical responses obtained following the injections of 4 $\mu\text{g/ml}$ (12.8 μM) and 10 $\mu\text{g/ml}$ (32 μM) aflatoxin B1 (AFB1) for 4 mM acetylcholine chloride (AChCl). Aflatoxins family was targeted for further development of the biosensor.

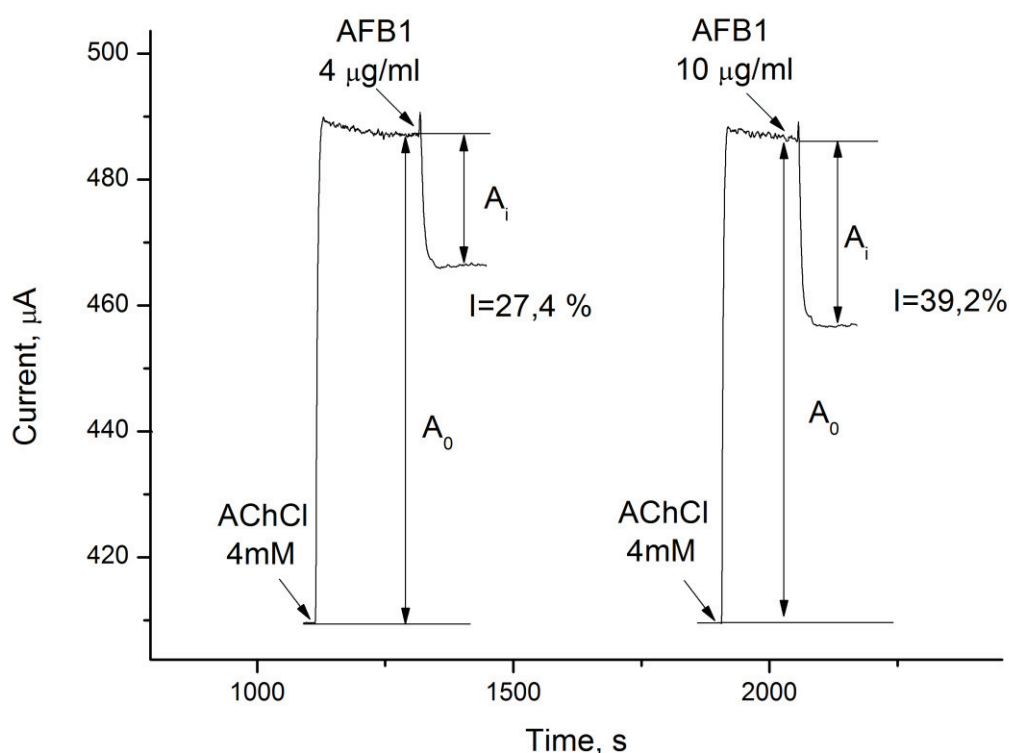


Fig.3.1. Potentiometric biosensor responses to 4 mM AChCl before and after inhibition by two different concentrations AFB1. Measurements were performed in 5 mM phosphate buffer, pH 6.5. $I = A_i/A_0 * 100$

3.2.Choice of AChCl substrate concentration

In the first stage of the work, a series of experiments was carried out at various AChCl concentrations in order to define the most suitable AChCl concentration to be used in further

experiments. Aliquots of a 500 mM AChCl concentrated solution were introduced in the 2 ml cell in order to achieve final concentrations in the 0.5 to 8 mM range. AChE enzyme concentration in the biomembrane was chosen to be 1 % (w/w). The biosensor responses to the different substrate concentrations were measured before and after inhibition by 10 mg/ml of AFB1. The inhibition was performed by adding small aliquots of a 400 mg/ml AFB1 concentrated solution to the working cell when the enzyme-substrate reaction achieved its dynamic equilibrium (i.e., when the response to the substrate was stabilized and reached the plateau).

After each step of the process, the biomembrane was washed for 5 min with fresh working buffer in order to remove the excess of substrate, inhibitor and products. The evolution of biosensor response to AChCl and level of inhibition by AFB1 with AChCl concentration are presented in Figure 3.2. Average values and standard deviations were obtained from one biosensor and replicate measurements at each AChCl concentration.

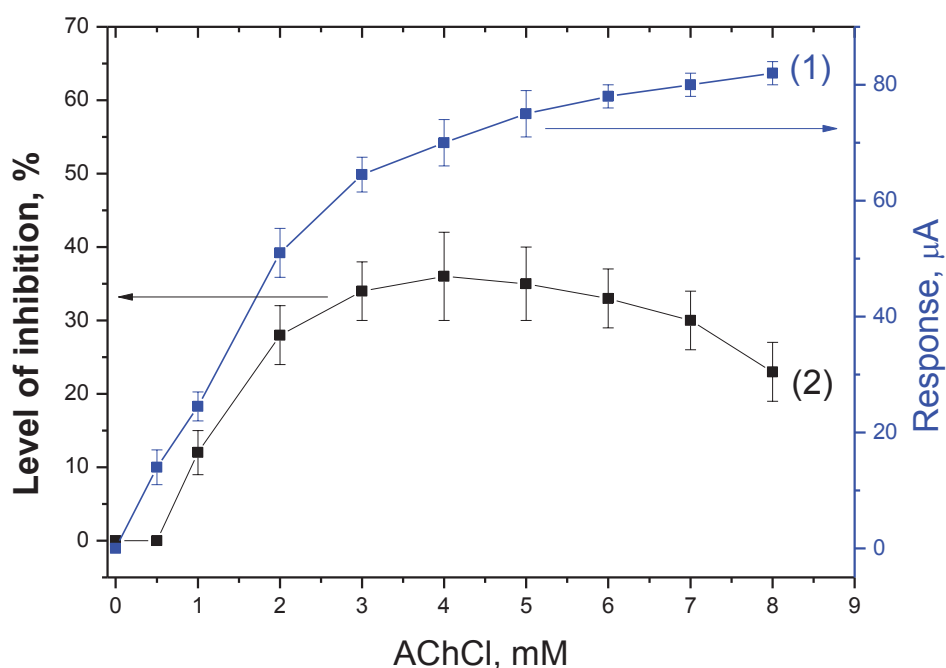


Fig. 3.2. Influence of AChCl concentration on AChE-based potentiometric biosensor response (1) and on the level of biosensor inhibition by 10 $\mu\text{g/ml}$ of aflatoxin B1 (2) . Measurements were performed in 5 mM phosphate buffer, pH 6.5.

As seen in Figure 3.2., the biosensor signal increased linearly with substrate concentration up to 2 mM and tended progressively to a plateau beyond this value. The highest inhibition level was observed at 4 mM AChCl, a slow decrease being observed at higher substrate concentrations. Such a pattern is typical for competitive inhibition where substrate and inhibitor compete for the same binding site. 4 mM AChCl was therefore chosen as working substrate concentration for further experiments.

3.3.Choice of enzyme concentration in the biomembrane

In a second stage, we investigated how the changes in AChE concentration in the biomembrane affect the biosensor performances. The biosensors were prepared with different AChE and BSA concentrations (w/w) in the membrane but keeping a ratio AChE over BSA of 2. Tested concentrations were 0.25%, 0.5%, 1%, 2%, 5% for AChE and 0,5%, 1%, 2%, 4%, 10% for BSA, respectively. Biosensors were prepared for each ratio and replicates were carried out for each AChE concentration. The responses to 4 mM AChCl were measured before and after the addition of 4 mg/ml AFB1 and the curves representing the influence of enzyme concentration in the biomembrane on the biosensor response as well as on the inhibition level were plotted (Fig. 3.3).

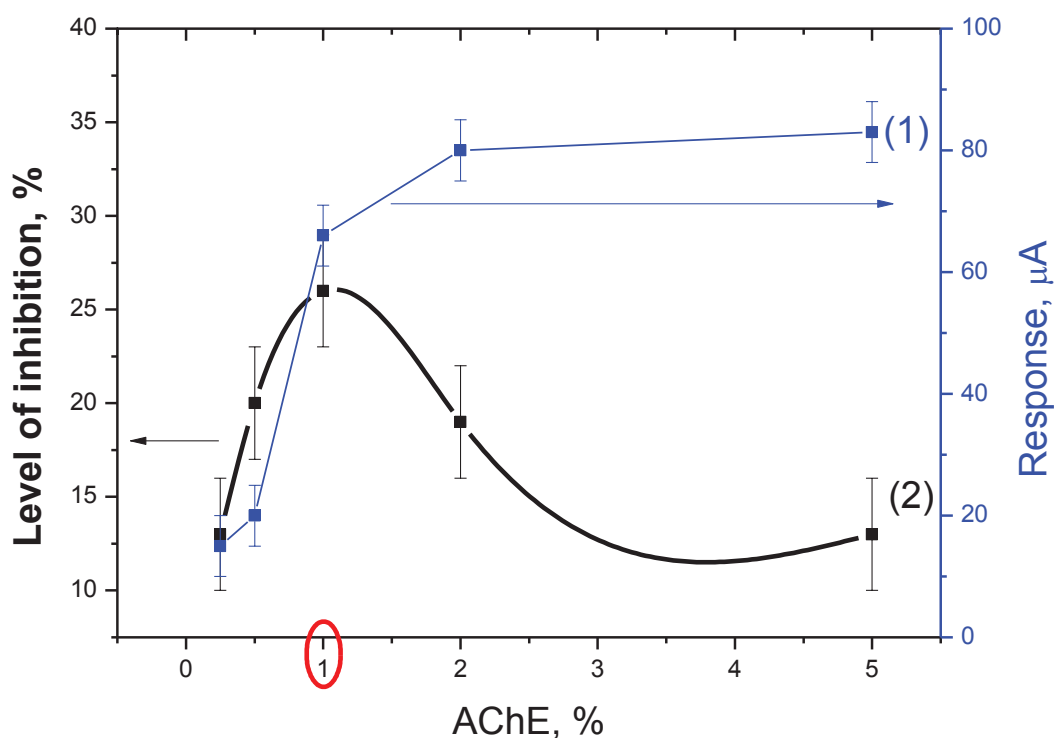


Fig. 3.3. Influence of AChE concentration in the biomembrane on the potentiometric biosensor response to 4 mM AChCl (1) and on the level of inhibition by 4 $\mu\text{g/ml}$ AFB1 (2). Measurements were performed in 5 mM phosphate buffer, pH 6.5.

As shown in the figure, the biosensor response value increases with enzyme concentration up to 2% (w/w) and reaches a plateau, while the level of inhibition was maximal for 1 % (w/w) AChE concentration. The best compromise to achieve high response and high level of inhibition was obtained for 1% AChE. This was therefore chosen as optimal enzyme concentration in the biomembrane for further experiments.

3.4. Optimization of working pH

pH of the working buffer is a key parameter that is expected to affect the biosensor performance, since it is known to have a major impact on enzymes stability and biological activity. In this study, a 2.5 mM «polymix» working buffer was used to regulate pH value in the 4.5 to 9.5 range. 2.5 mM was chosen as buffer concentration as it allowed to get sufficient biosensor responses whereas the signals decreased significantly at higher concentrations. The

biosensor responses to 4 mM AChCl and the level of inhibition by 2 $\mu\text{g/ml}$ AFB1 were measured changing pH by 0.5 unit at each measurement. The results are depicted in Fig. 3.4.

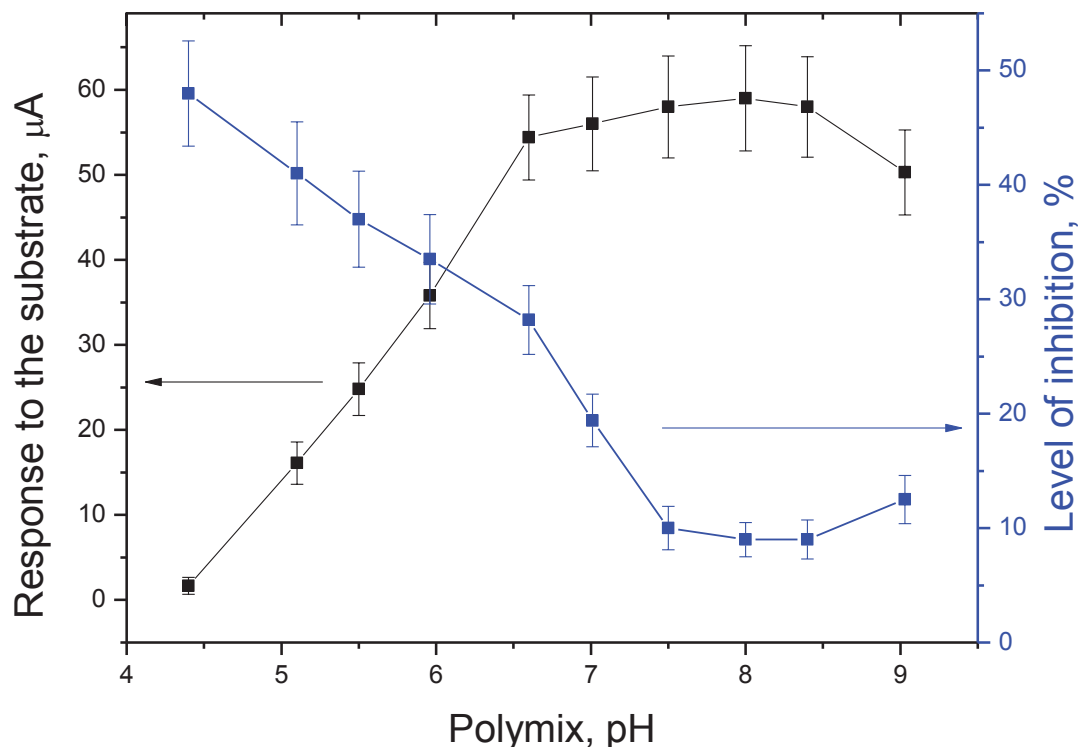


Fig. 3.4. Influence of pH on the operation and inhibition of immobilized AChE in the presence of 2 $\mu\text{g/ml}$ aflatoxin B1 and without it. Measurements were carried out in 2.5 mM "polymix" buffer at room temperature.

As can be seen, the optimal pH values for biosensor operation were observed in the 6.5 - 8.5 range whereas the highest levels of inhibition were detected between pH 4.5 and 6.5. The best compromise was therefore achieved at pH 6.5.

3.5. Influence of AFB1 concentration on biosensor level of inhibition

In the next step of the work, the sensitivity of the proposed biosensor to different AFB1 inhibitor concentrations was evaluated under the optimum conditions previously defined. As before, 4 mM AChCl was introduced into the measuring cell before AFB1 addition. Different biosensors were tested and replicate measurements were performed at each

AFB1 concentration. As shown in Fig. 3.5, the biosensor response, when plotted as level of inhibition (in%) vs log[AFB1] (in $\mu\text{g/ml}$) was linear in the 0,4 to 40 $\mu\text{g/ml}$ with a sensitivity of 26.642 % of inhibition per log of AFB1 concentration (in $\mu\text{g/ml}$) and limit of detection was 0,28 $\mu\text{g/ml}$.

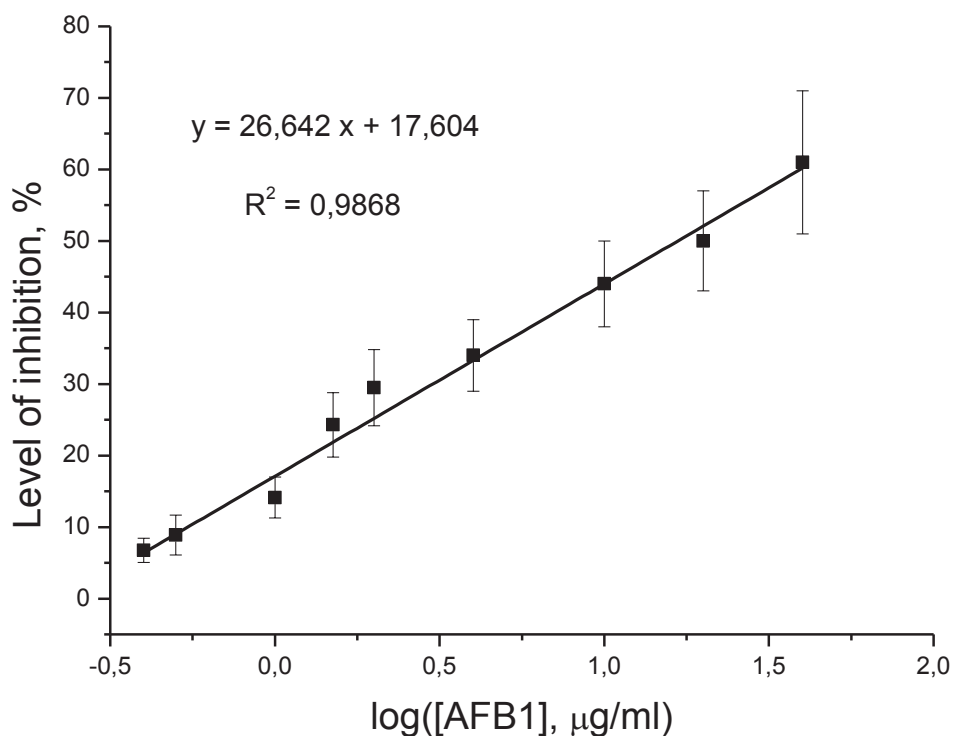


Fig.3.5. Influence of AFB1 concentration on the potentiometric biosensor response. Measurements were performed in 5 mM phosphate buffer, pH 6.5, AChCl concentration : 4 mM.

3.6. Biosensor reproducibility

We also studied the short-term reproducibility of the 1% AChE-biosensor. For that, the biosensor responses to AChCl and AFB1 (4 mM and 2 $\mu\text{g/ml}$, respectively) were measured over a period of one working day. In the intervals between measurements, the working buffer in the measuring cell was replaced several times and the biosensor was washed in the working buffer for 3 min to remove completely the substrate, inhibitor and products. Fig. 3.6 shows the evolution of the biosensor response to AChCl and the level of inhibition by 2 $\mu\text{g/ml}$ AFB1 with the number of measurement.

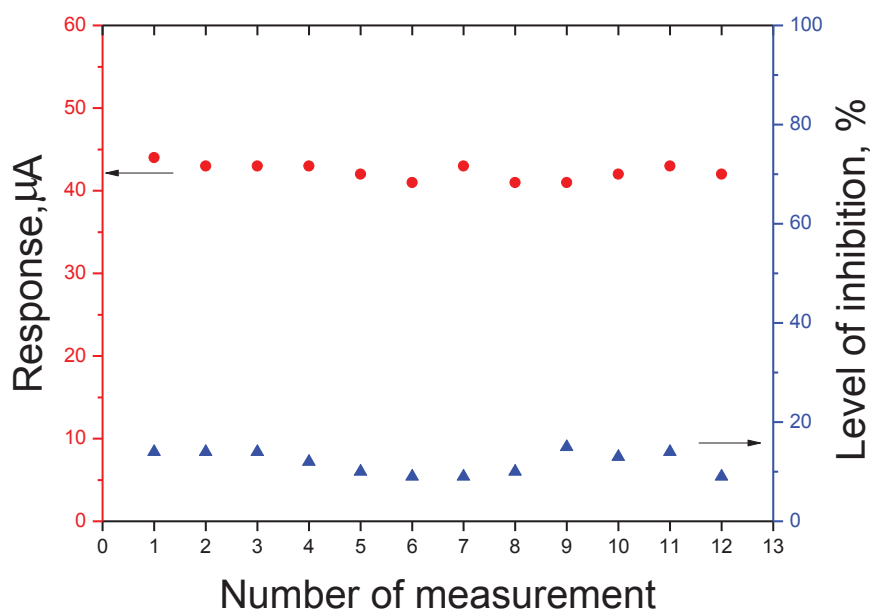


Fig. 3.6. Reproducibility of the potentiometric biosensor responses to 4 mM AChCl and 2 $\mu\text{g/ml}$ aflatoxin B1. Measurements were conducted in 5 mM phosphate buffer, pH 6.5, at room temperature.

As seen, both curves are very stable, indicating the excellent reproducibility of the biosensor during one working day.

3.7. Investigation of biosensor storage stability

Another important biosensor characteristic is storage stability. In order to investigate it, biosensor responses to 4 mM substrate were measured at different days during storage in buffer solution, two different temperatures of storage being tested. One biosensor was stored at room temperature and another one was stored in the fridge (+2°C- +5°C). Results (i.e., average values and standard deviations obtained from replicate measurements) are summarized in Fig. 3.7.

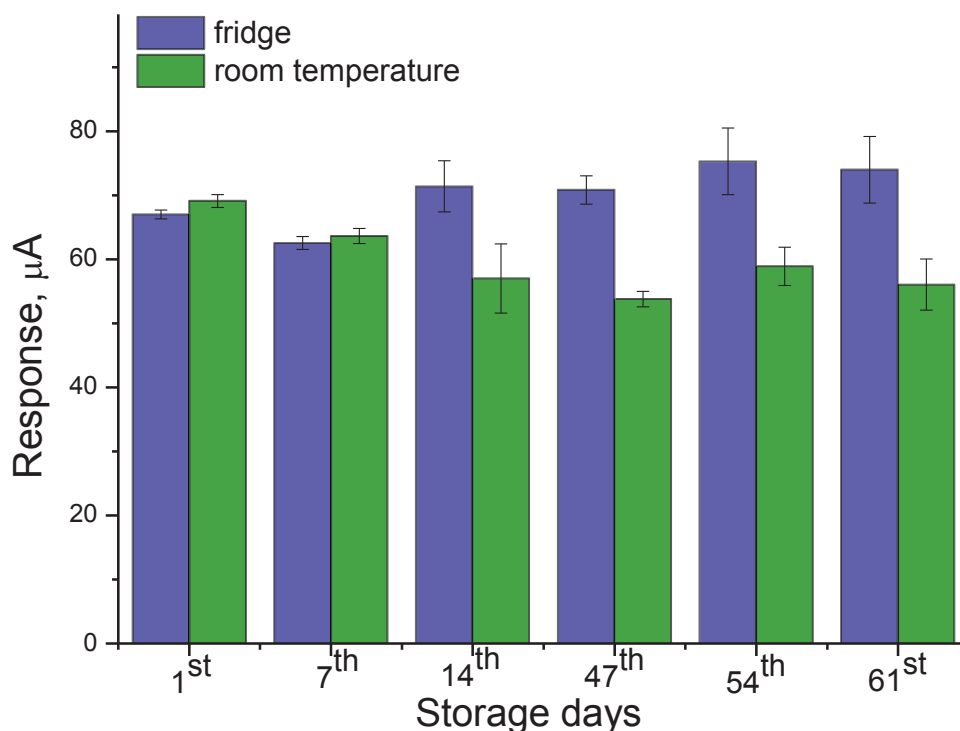


Fig. 3.7. Potentiometric biosensors responses during storage in buffer solution at room temperature and in the fridge (+2°C- +5°C)

Experiment results show that AChE-biosensors based on ISFET can be stored more than two months and storage in the fridge is more suitable for our purposes.

3.8. Study of possible interferences with aflatoxins of other groups

The sensitivity of the proposed biosensor to aflatoxins belonging of another group (group G) was further studied. Group G is, along with group B, a group of naturally produced AFBs. AFG1 was chosen as representative of group G. In the experiment, 4 mM AChCl was added to the measuring cell, and then AFB1 or AFG1 of various concentrations injected in the cell. The biosensor responses before and after inhibition were analyzed and the inhibition level was calculated. As shown in Fig. 3.8, the biosensor sensitivity to AFB1 and AFG1 are very similar (the curves are not different taking into account experimental errors). It is quite reasonable to assume that AFB2 response would be also close to that of AFB1, and that the biosensor sensitivity to AFG2 is similar to that of AFG1. This result is interesting, since some

molds of *Aspergillus* genus produce toxins of both groups (B and G) and only these ones. If AFBs and AFGs have an additive effect, the whole toxicity coming from aflatoxins may be simply determined in these samples from the AFB1 calibration curve and the total biosensor inhibition signal recorded.

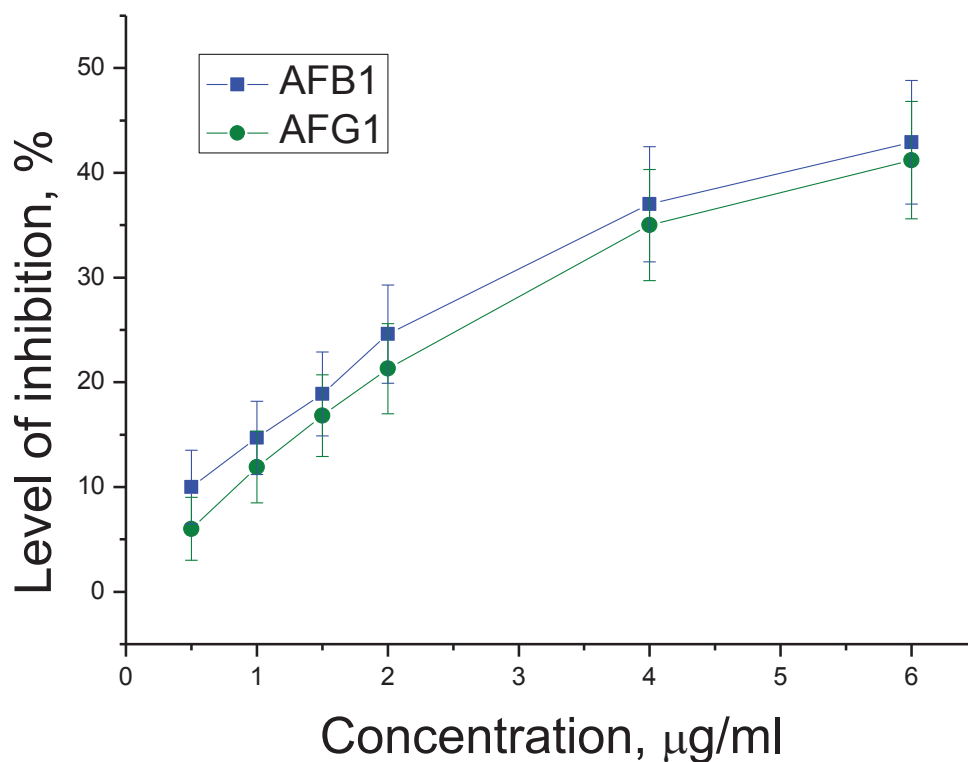


Fig. 3.8. Influence of AFB1 and AFG1 concentration on the potentiometric biosensor response. Measurements were performed in 5 mM phosphate buffer, pH 6.5, AChCl concentration – 4 mM.

In order to determine the the effects of AFB1 and AFG1 on the inhibition of AChE immobilized on the surface of the transducers, an isobole diagram approach [178] was adopted. Isobole diagrams are commonly used for the identification of synergistic, additive or antagonistic toxic effects in mixtures of several compounds. A typical isobole diagram is represented in Fig. 3.9. To built this diagram in our case, different experiments were carried out with individual toxins or mixtures containing various ratios but constant total concentrations of 2 or 10 µg/ml. Each component was converted to integrated units of toxicity. The unit of toxicity for each mixture was determined as the ratio of inhibition effect of each component alone to the inhibition effect of their mixture. The obtained units of

toxicity were plotted on X- and Y-axes. If the points are below the line of concentration addition, it corresponds to a synergistic effect, i.e. the combined action of the substances mixture is higher than the separate effect of individual substances. The points above the line of concentration addition correspond to an additive effect, i.e. the combined effect of the mixture equals the sum of the effects of individual components. The points outside the limits of components independence from each other are considered to correspond to the antagonistic effect, i.e. the combined effect of the mixture is smaller compared to the effects of individual components.

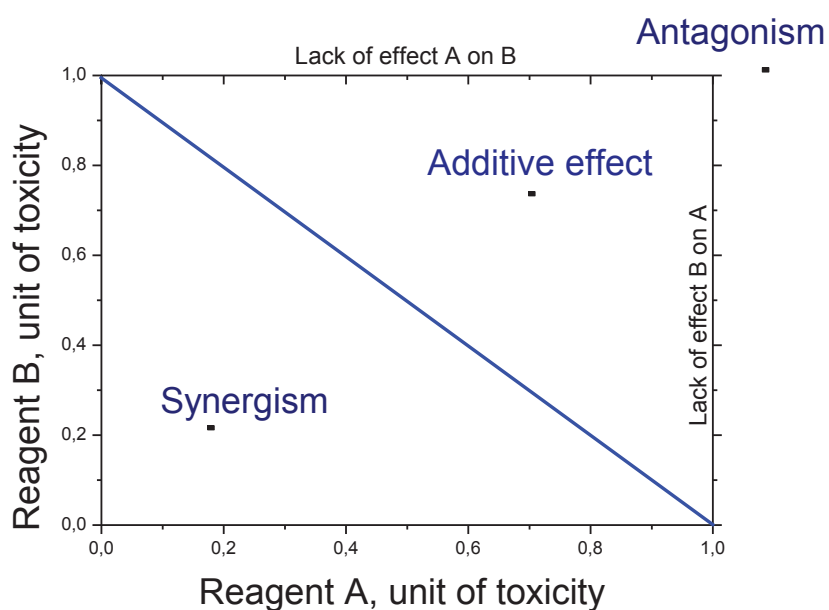


Fig. 3.9. A typical isobole diagram to determine the effects of synergism, additive effect or antagonism.

Isobole diagrams obtained for the different AFB1 to AFG1 ratios and for the 2 and 10 $\mu\text{g/ml}$ total (AFB1+AFG1) concentrations are shown in Fig. 3.10.

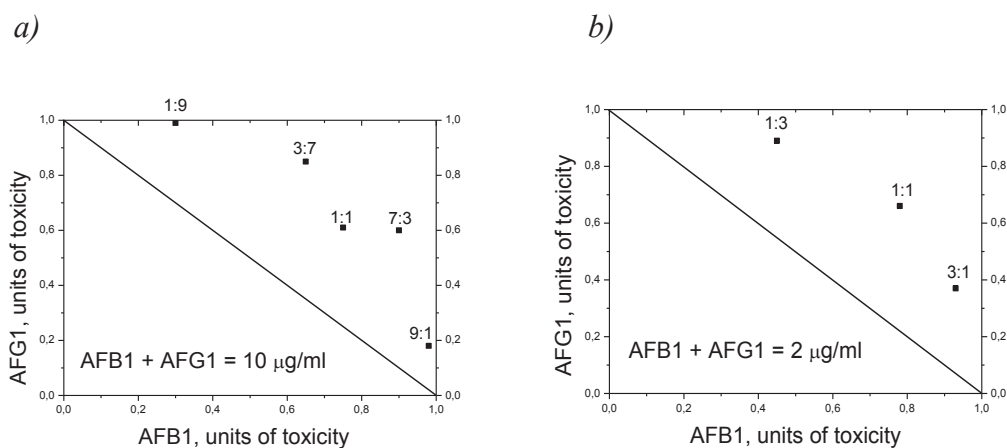


Fig. 3.10. *Isobole diagrams for the determination of interaction effects for AFB1 and AFG1 inhibition of AChE immobilized at the surface of pH-FET transducers. Each compound has been converted to toxic units (TUs) along the x and y axes.*

Figures clearly show that in all cases, the inhibition of immobilized AChE by various mixtures of aflatoxins is the result of an additive effect, i.e. inhibition effect of the mixture equals the sum of effects of each component alone. Therefore, the AChE-biosensor can be used for measurement of the total aflatoxins concentration in the sample.

3.9. Conclusions

The laboratory prototype of AChE biosensor based on ISFET was developed for inhibition determination of aflatoxins. To achieve this goal, working parameters of AChE-biosensor were studied and optimized for inhibitive determination of AFB1. The bioselective membrane contained 1% AChE, and 4 mM AChCl was chosen as working substrate concentration. The optimum pH level for inhibitory analysis was 6.5. The linear range of AFB1 determination was 0.4 µg/ml to 40 µg/ml in semi-logarithmic coordinates and limit of detection was 0.28 µg/ml. The developed biosensor was characterized by an excellent signal reproducibility over one working day, and could be stored in buffer solution more than two months by keeping it in the fridge between two measurements. The biosensor sensitivity to AFG1 was similar to that to AFB1 and additive effects between the two aflatoxins were demonstrated, showing the possibility to measure the whole toxicity coming from aflatoxins in the samples by the biosensor developed.

4. OPTIMIZATION OF ACETYLCHOLINESTERASE-BASED BIOSENSOR FOR AFLATOXINS INHIBITORY ANALYSIS BY USING MATHEMATICAL SIMULATION

1. Introduction
2. Experimental determination of the inhibition parameters of immobilized acetylcholinesterase by aflatoxin
 - a. Determination of the inhibition type by using method "degree of inhibition"
 - b. Determination of the inhibition type by using Lineweaver-Burk method
3. Development of mathematical model of biochemical interaction into bioselective membrane
4. Application of the simulation for real experimental parameters
5. Investigation of different input parameters changes
 - a. Study of rate constant changes
 - b. Assessment of enzyme concentration in the biosensor membrane by using simulation
 - c. Study of the inhibitory coefficient α and its effect on the system behavior
6. Validation of mathematical simulation
7. Conclusions

4.1.Introduction

The aim of this chapter was to develop a mathematical model to simulate the potentiometric biosensor developed in the previous chapter. A mathematical description of the biochemical reactions that occur in the biosensor membrane is necessary to create such a model. In the proposed biosensor, AChE enzyme catalyzes acetylcholine to choline and acetic acid. The number of protons generated by this reaction is measured, producing the biosensor signal. Acetylcholinesterase is further inhibited by AFB1, which results in a decrease of the number of protons produced, and then in a decrease of the biosensor signal. Thus, the mathematical model of biosensor operation has to monitor the protons number during all enzymatic processes in the membrane.

4.2. Experimental determination of the parameters characterizing the inhibition of immobilized acetylcholinesterase by aflatoxins

In order to create the mathematical model, it was first necessary to determine the type of inhibition of immobilized AChE by aflatoxin (reversible or irreversible, competitive, uncompetitive, noncompetitive or mixed). The inhibition is obviously of reversible type as the biosensor response, and thus the biomembrane activity, can be completely restored by rapid washing with the working buffer after inhibition by AFB1, which indicates weak aflatoxin-enzyme interaction. However, the competitive or uncompetitive nature of the reversible inhibition had to be determined.

4.2.1. Determination of the inhibition type by using the "degree of inhibition" method

A graphical method that allows for the determination of the type of reversible inhibition has been recently reported in the literature [179]. In this method, a number of calibration curves are plotted, demonstrating the dependence of the degree of the biosensor inhibition on different concentrations of the inhibitor. Each curve is plotted at a fixed value of substrate concentration, for instance, the substrate concentration equal to the K_m value or the saturating concentration. For each curve, the degree of inhibition I_{50} is determined. It is numerically identical to the inhibitor concentration, at which the degree of biosensor inhibition is 50%. Next, an analysis of the changes in the calibration curve and the inhibition degree I_{50} allows for the conclusion regarding the type of inhibition. If I_{50} increases with substrate concentrations, and the calibration curve shifts to the higher concentration range, it means that the *inhibition* is of *competitive type*. If I_{50} decreases with increasing substrate concentration and the calibration curve shifts to the lower concentration range, it corresponds to the *uncompetitive type of inhibition*. In the case of the *noncompetitive type of inhibition*, I_{50} and calibration curve do not change. Finally, in the case of *inhibition of mixed type*, I_{50} increases with increasing substrate concentration likely to the competitive type of inhibition, but the calibration curve shift is significantly lower. For example, a 10-fold increase in the substrate concentration causes an increase of I_{50} for the competitive type by 5.5 times whereas for mixed type - by only 1.5 times.

Prior to the experiment on the determination of the type of reversible inhibition, it was necessary to evaluate the apparent Michaelis constant K_{mapp} for immobilized AChE. Basically, the Michaelis constant for native enzymes is determined as half the maximum initial rate of the enzymatic reaction. With regard to the immobilized enzymes as the biosensor constituents, the estimation of this rate is almost impossible. It should be taken into account that it is inconsistent to equate the speed of biosensor response with the rate of biochemical reaction in the membrane, as the former is defined by certain electrochemical peculiarities of the registration method and diffusion properties. Considering the fact that the response time of any biosensor is almost the same, we assume that it would be appropriate for plotting the graphs to apply directly the value of biosensor response as it is proportional to the initial rate of the enzymatic reaction with use of an immobilized enzyme. Therefore, to determine the Michaelis constant for the enzyme immobilized on the surface of the potentiometric transducer, the curves of dependence of the biosensor response on the AChCl concentration were plotted. The data obtained are presented in both direct (Fig. 4.1) and inverse (Fig. 4.2) coordinates.

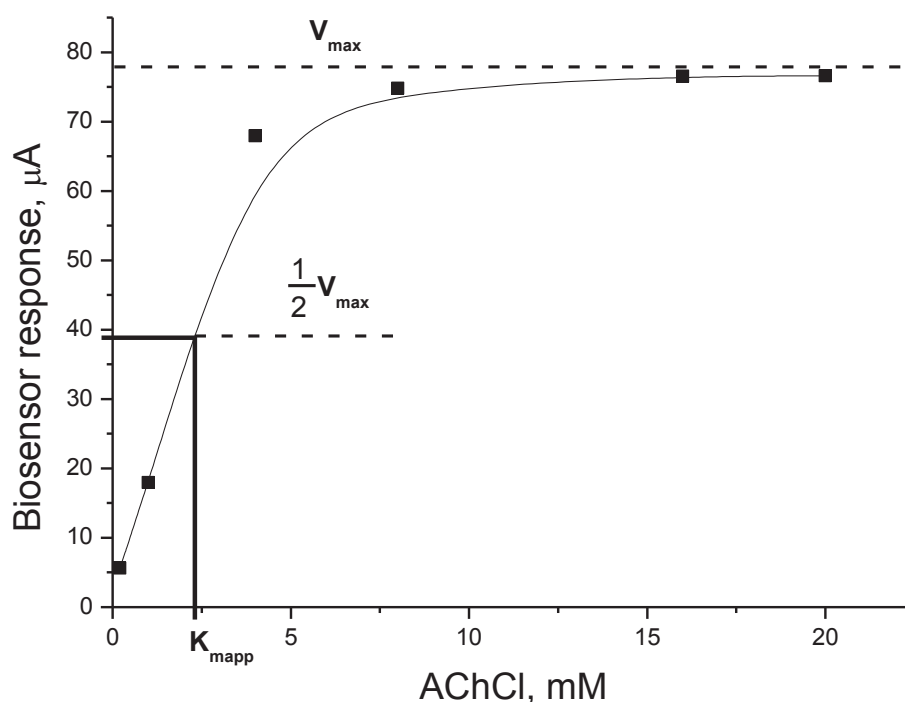


Fig. 4.1. Dependence of the AChE-biosensor response on acetylcholine chloride concentration in direct coordinates. Measurements were performed in 5 mM phosphate buffer at room temperature, pH 6.5

The graph shows (Fig. 4.1) that maximum biosensor response is 77 μA , i.e. the maximum initial rate V_{max} is also proportional to this value. Determining the apparent Michaelis constant as half the maximum rate, we obtain $K_m^{app} = 2.3 \text{ mM}$. Another method of the Michaelis constant determination suggests the presentation of the same data in inverted Lineweaver-Burk coordinates (Fig. 4.2). The obtained straight line cuts off on the X- and Y-axes the reciprocal values K_m and V_{max} , respectively. The equation of the straight line obtained (Fig. 4.2) is: $y = 0.0334x + 0.0116$. From this equation, the reciprocal value $1/K_m^{app} = 0.3475$. The apparent Michaelis constant determined by this method $K_m^{app} = 2.8$.

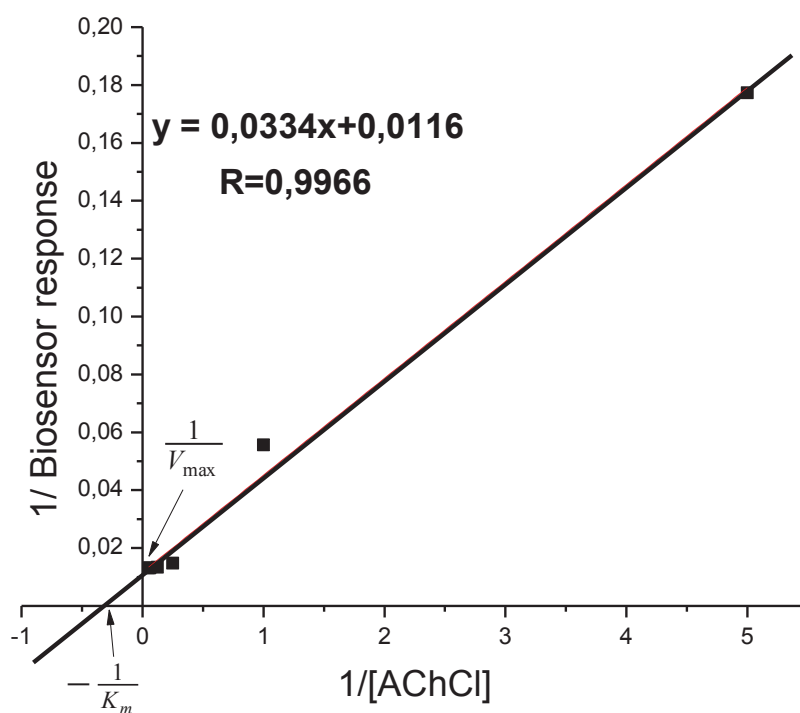


Fig.4.2. Dependence of the AChE biosensor response on acetylcholine chloride concentration in inverted Lineweaver-Burk coordinates. Measurements were performed in 5 mM phosphate buffer at pH 6.5, at room temperature

Thus, the obtained apparent Michaelis constant of acetylcholinesterase immobilized on the surface of the potentiometric transducer was 2.3 mM or 2.8 mM, depending on the method of determination. For convenience, the average $K_m^{app} = 2.5 \text{ mM}$ was used further on.

For the next experiment on determination of the type of inhibition of immobilized AChE by the “degree of inhibition” method, the substrate concentrations of 2.5 mM and 20 mM were fixed. They corresponded respectively to the apparent Michaelis constant K_m^{app} and $8K_m^{app}$, which is saturating concentration. The calibration curves of aflatoxin B1 determination (dependence of degree of inhibition on the inhibitor concentration) were plotted for two fixed substrate concentrations (Fig. 4.3).

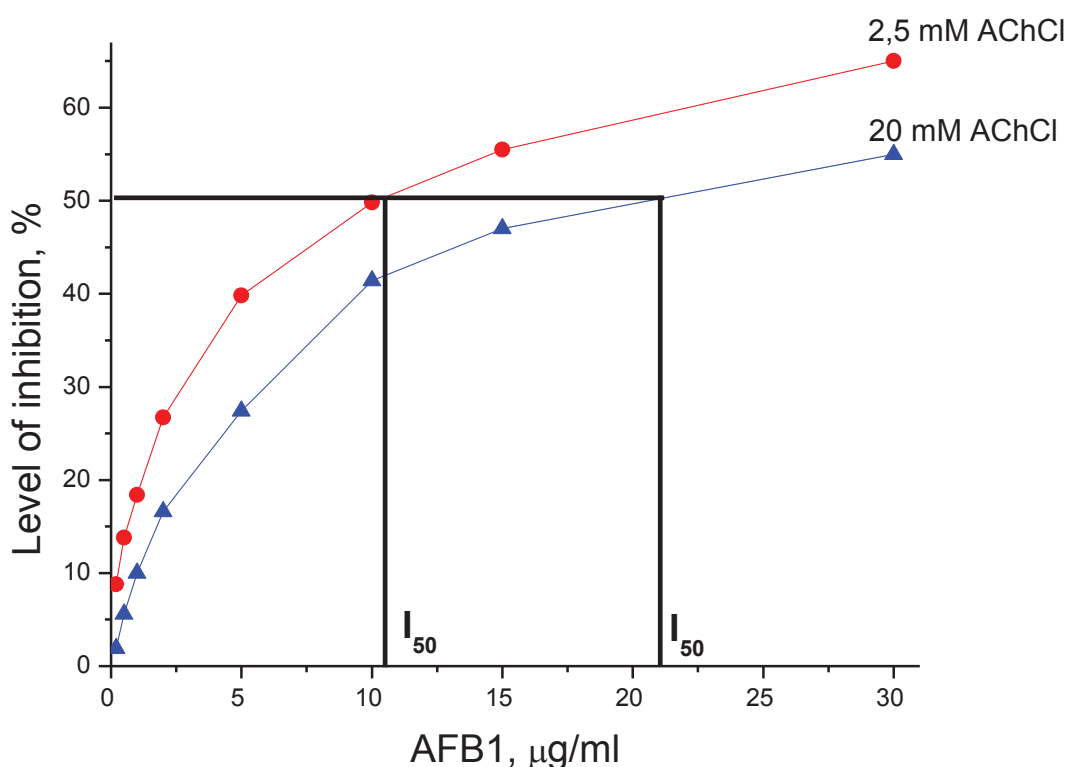


Fig.4.3. Dependence of the degree of inhibition of immobilized AChE on aflatoxin B1 concentration in the presence of 2.5 mM and 20 mM AChCl. Measurements were performed in 5 mM phosphate buffer, pH 6.5, at room temperature

As seen, with an increase of the substrate concentration, I_{50} also increases. Graphically, this corresponds to a rightward shift of the calibration curve. Since the shift is slight and the ratio of I_{50} at substrate concentration $8K_m$ ($I_{50} = 21 \mu\text{g/ml}$) to I_{50} at substrate concentration K_m ($I_{50} = 10.5 \mu\text{g/ml}$) is 2, the conclusion can be made that the inhibition is of the mixed type.

4.2.2. Determination of the inhibition type by using Lineweaver-Burk method

It was interesting to confirm the obtained results using one of the traditional methods of identifying the type of inhibition, such as the Lineweaver-Burk, Dixon, Cornish-Bowden, Eadie-Hofstee methods. They are based on the construction of the Michaelis-Menten equation in different coordinate systems: $1/v$ versus $1/[s]$, v versus $v/[s]$, $1/v$ versus $[i]$ and $[s]/v$ ($[i]$) respectively, where v - an initial rate of the enzymatic reaction, $[s]$, $[i]$ - concentrations of substrate and inhibitor respectively. We chose the Lineweaver-Burk method as the most commonly used. To invert the Michaelis-Menten equation into the Lineweaver-Burk plot it is necessary to determine the initial rates of the enzymatic reaction at different substrate concentrations in the presence of inhibitor and without it and to plot the $1/v$ vs $1/[s]$ graphs. The type of inhibition can then be identified by the analysis of these straight lines. For *competitive inhibition*, the lines intersection should be on the Y-axis ($1/v$), meaning that the maximum rate does not change, and the apparent Michaelis constant increases. For *noncompetitive inhibition*, the lines intersection should be on the X-axis ($1/[s]$), showing that in this case, on the contrary, the apparent Michaelis constant remains unchanged, and the maximum rate decreases. For *uncompetitive inhibition*, both the maximum rate and apparent Michaelis constant decrease by the same times, graphically it looks like parallel straight lines. And finally, for *mixed inhibition*, the maximum speed decreases and the apparent Michaelis constant can either increase or decrease, therefore graphically the lines intersection should be not on X- and Y-axes.

During the experiment, the changes in biosensor responses to different substrate concentrations were investigated with no inhibitor as well as in the presence of various aflatoxin B1 concentrations (5 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$). The experimental results were presented in the double-inverted Lineweaver-Burk coordinates (Fig. 4.4).

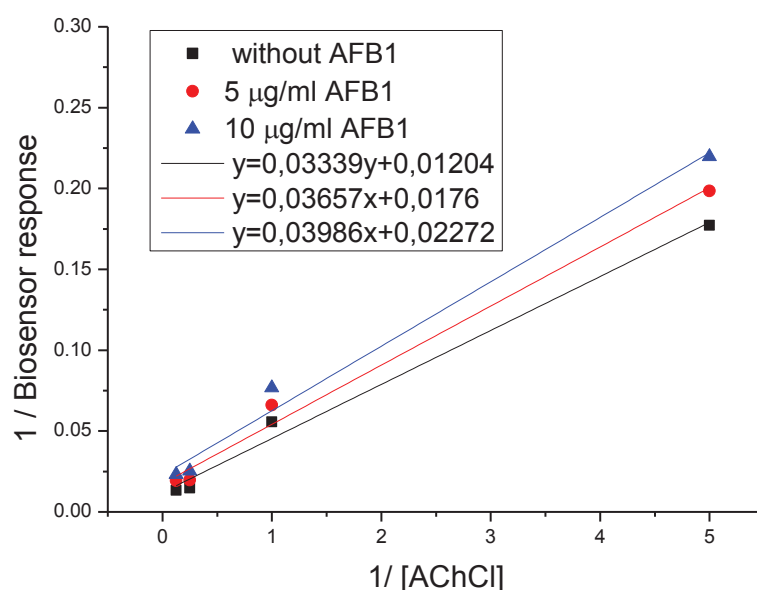


Fig.4.4 Dependence of the biosensor responses on acetylcholine chloride concentration without inhibitor and in the presence of 5 µg/ml and 10 µg/ml of aflatoxin B1 in inverse Lineweaver-Burk coordinates. Measurements were performed in 5 mM phosphate buffer, pH 6.5, at room temperature.

From the equations of the regression lines (Fig. 7), the apparent Michaelis constants of immobilized AChE can be calculated in absence of inhibitor ($K_m^{app} = 2.8$) and in the presence of 5 µg/ml inhibitor AFB1 ($K_m^{app} = 2.1$) and 10 µg/ml AFB1 ($K_m^{app} = 1.8$). The fact that the lines do not intersect on any of the axes and that at increasing inhibitor concentration the values of both K_m^{app} and the maximum biosensor response (proportional to V_{max}) decreases, indicates that aflatoxin B1 is a reversible acetylcholinesterase inhibitor of mixed type.

4.3. Development of mathematical model of biochemical interaction in bioselective membrane

There are several stages (fig. 4.5) of aflatoxin B1 inhibitory analysis by AChE-based ISFET biosensor (hereafter – AChE/ISFET biosensor): obtaining the baseline (0), obtaining the response to the working concentration of acetylcholine chloride (AChCl) as a substrate (I), and obtaining the response to FB1 as an inhibitor (II).

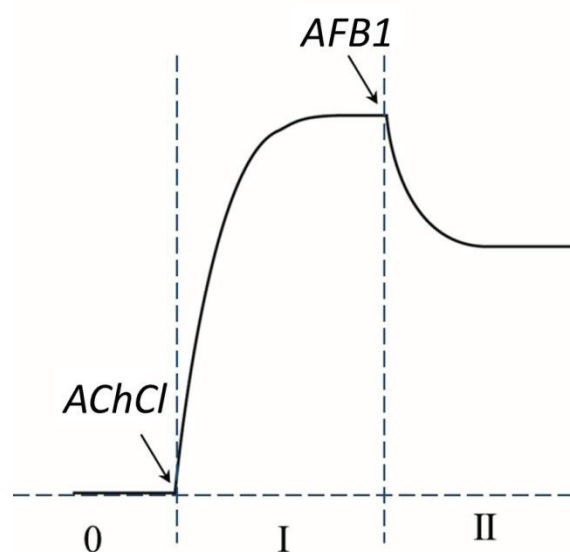


Fig.4.5. Schematic representation of aflatoxin B1 inhibitory analysis using AChE/ISFET biosensor

At the zero stage, when the bioselective membrane is in contact only with the working buffer, no enzymatic reactions occur in the membrane, and the biosensor signal corresponds to the "baseline" (Fig.4.5, stage 0).

At the first stage, the enzymatic reaction (1.13) occurs in the membrane after substrate addition to the working cell. The product (proton) is formed as a result of the reaction, which leads to changes in pH of solution close to the transducer surface. These changes are visualized as a response to the substrate and registered by a potentiometric transducer (Fig.4.5, stage I).

At the second stage the enzyme is inhibited by AFB1 after its addition to the working cell. As established earlier, AFB1 is a reversible AChE inhibitor of mixed type, which can be schematically represented in the following way (Fig. 4.6).

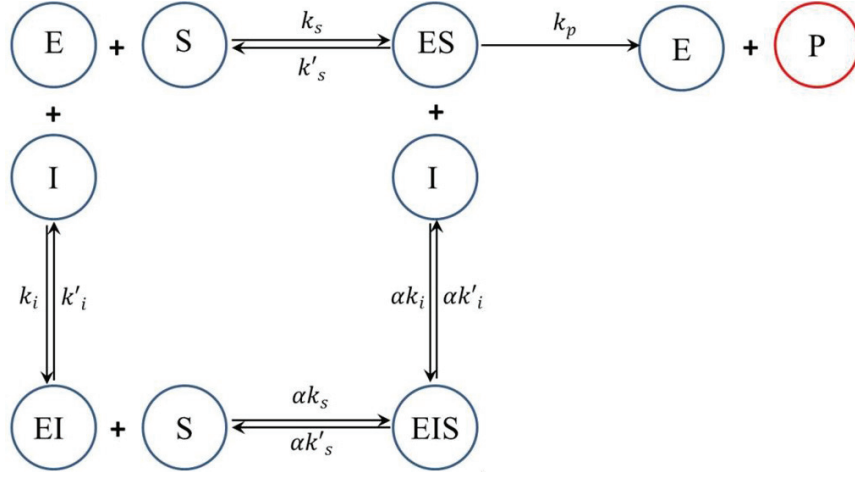


Fig. 4.6. Schematic representation of enzymatic reaction in the membrane of AChE/ISFET biosensor during AFB1 analysis, where E – enzyme, S – substrate, I - reversible inhibitor of mixed type

In Fig. 4.6 k_s and k'_s are the rate constants of direct and reverse reactions of the complex (ES) formation, k_p is the rate constant of the product (P) formation, and k_i and k'_i are the rate constants of direct and reverse reactions of the complex (EI) formation. The biochemical interaction in bioselective membrane can be described by the following system of differential equations:

$$(4.1) \frac{dn_e(t)}{dt} = -k_s n_e(t) n_s(t) - k_i n_e(t) n_i(t) + k'_s n_{es}(t) + k'_i n_{ei}(t) + k_p n_{es}$$

$$(4.2) \frac{dn_{es}(t)}{dt} = k_s n_e(t) n_s(t) - k'_s n_{es}(t) - \alpha k_i n_{es}(t) n_i(t) + \alpha k'_i n_{esi}(t) - k_p n_{es}(t)$$

$$(4.3) \frac{dn_{ei}(t)}{dt} = k_i n_e(t) n_i(t) - k'_i n_{ei}(t) - \alpha k_s n_{ei}(t) n_s(t) + \alpha k'_s n_{esi}(t)$$

$$(4.4) \frac{dn_{esi}(t)}{dt} = \alpha k_i n_{es}(t) n_i(t) - \alpha k'_i n_{esi}(t) + \alpha k_s n_{ei}(t) n_s(t) - \alpha k'_s n_{esi}(t)$$

$$(4.5) \frac{dn_s(t)}{dt} = -k_s n_e(t) n_s(t) - \alpha k_s n_{ei}(t) n_s(t) + k'_s n_{es}(t) + \alpha k'_s n_{esi}(t)$$

$$(4.6) \frac{dn_i(t)}{dt} = -k_i n_e(t) n_i(t) - \alpha k_i n_{es}(t) n_i(t) + k'_i n_{ei}(t) + \alpha k'_i n_{esi}(t)$$

$$(4.7) \frac{dn_p(t)}{dt} = k_p n_{es}(t)$$

where k_s , k'_s , k_i , k'_i and k_p – are appropriate rate constants of the reaction of complex formation, α is constant, the numerical values of which determine the enzyme inhibition or activation;

n_e , n_s , n_i , n_p , n_{es} , n_{ei} , n_{esi} – are the concentrations of the enzyme, substrate, inhibitor, product, and the enzyme-substrate, enzyme-inhibitor, enzyme-substrate-inhibitor complexes respectively, which change over the time. The change in product concentration n_p over the time is proportional to the biosensor response.

Additionally, it was taken into account that the total enzyme concentration E_0 in the system remained fixed. Thus, the sum of concentrations of free enzymes (E) and enzymes in the complexes (ES), (EI), (ESI) is constant at any time and equals $(E) + (ES) + (EI) + (ESI) = E_0$.

To simulate the biosensor operation, the system described above was solved numerically by using built-in NDSolve algorithm of “Wolfram Mathematica” software. The simulation results are shown in Fig. 4.7.

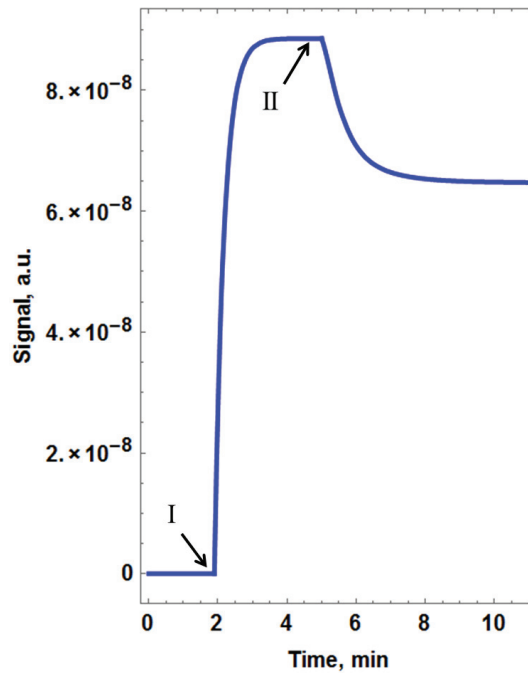


Fig. 4.7. Simulation of enzymatic reactions in membrane of AChE/ISFET biosensor, using kinetic equations (1.1 – 1.7) and random parameters.

The following initial conditions are specified at stage 0 of the modeling: $n_s(0) = n_i(0) = n_p(0) = n_{es}(0) = n_{ei}(0) = n_{esi}(0) = 0$. That is, the system contains neither the substrate nor the inhibitor, but only the initial enzyme concentration in the biosensor working membrane (n_e).

At stage 1, under the initial conditions given by the solution from stage 0, we specify the initial concentration of substrate that is added to the working cell (n_s).

At stage 2, the system is solved while using the solutions from the previous stage; in addition, the initial concentration of inhibitor (n_i) is specified.

The system solution is computed using the given initial conditions and the input parameters, and the derivative $n_p(t)$ is calculated and plotted.

The derivative of proton concentration is proportional to ISFET-based biosensor signal because the working scheme of the measuring device provides for measurement of the current in the transistor channel.

It is well known that the current is given by the following formula

$$I(t) = \frac{dQ(t)}{dt} \quad (4.8),$$

where $\frac{dQ(t)}{dt}$ is the derivative of the charge over time.

4.4. Application of the simulation for real experimental parameters

The substrate and inhibitor concentrations in the measuring cell, as well as the enzyme concentration in the biosensor membrane, are the important input parameters of the simulation.

In the real experiment, 4×10^{-3} M AChCl was used as the working substrate. The inhibitor (AFB1) concentrations in the model were 0.2 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$, 2 $\mu\text{g/ml}$, 3 $\mu\text{g/ml}$, 4 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$. To normalize the input parameters, these values were converted to molar concentrations (see the Table 4.1).

Table 4.1. *Aflatoxin B1 concentration values in different units*

Aflatoxin B1 concentration	
$\mu\text{g/ml}$	M
1	3.2×10^{-6}
2	6.4×10^{-6}
3	9.6×10^{-6}
4	12.8×10^{-6}

The maximum possible enzyme concentration in the biosensor membrane was estimated in the following way. The membrane volume is approximately 0.03 ml, which corresponds to 0.03 mg. As the membrane contains 1% AChE, the enzyme mass in the membrane is 0.3×10^{-6} g. The molar mass of AChE is known to be 280 kDa, or 280×10^3 g/mol (1 Da = 1 g/mol). Now, the enzyme mass and molar mass being known, the substance amount can be calculated, it is 1.0×10^{-12} mol. Being divided by the membrane volume, it gives the molar concentration of the enzyme 2×10^{-5} M, which can be used in simulation.

The rate constants of biochemical reactions are also significant input parameters of the simulation. Their estimation is difficult, rather almost impossible. In this study, the experimental concentrations were taken as the simulation input parameters and the measured model signals were compared with the signals obtained in the experiment (Fig. 4.8).

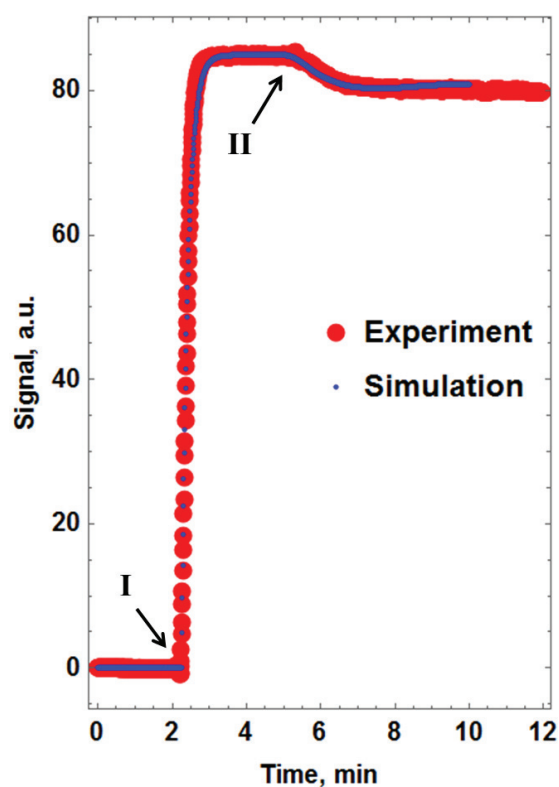


Fig.4.8. Simulation of enzymatic reactions in the membrane of AChE/ISFET biosensor using kinetic equations (1) and experimental input parameters; I – addition of 4×10^{-3} M AChCl, II – addition of 6.4×10^{-6} M AFB1 (blue dots) and comparison with the experimental signal (red dots).

As seen, the results of experiment and simulation were in good agreement at the input parameters: $k_s = 5 \times 10^2$, $k'_s = 0,01k_s$, $k_i = 2 \times 10^4$, $k'_i = 0,0001k_i$, $k_p = 1 \times 10^{-1}$, $\alpha = 0,2$. All these parameters as well as the enzyme concentration in the membrane ($n_e = 1 \times 10^{-6}$) have been chosen for the particular experimental biosensor responses to 4 mM AChCl and 6.4 μ M AFB1.

4.5. Investigation of changes in input parameters

When selecting the input parameters it was established that the stable biosensor operation (at specified concentrations of inhibitor, substrate and enzyme) was achieved at the definite balance between the rate constants. In our case, the inhibitor-enzyme interaction is stronger than the substrate-enzyme interaction by 100 times ($k_i \sim 100 k_s$). The rate of decomposition of complexes (EI) and (ES) is much less than the rate of their formation ($k_i' = 10^{-4} k_i$, $k_s' = 0.01 k_s$).

4.5.1. Study of changes in rate constants

Some physical meaning of the rate constants was found during their choosing. Thus, the rate constant (k_s) of the complex (ES) formation was responsible for the shape of the substrate response curve: when (k_s) increased the response curve became sharper and when (k_s) decreased - more rounded (fig.4.9).

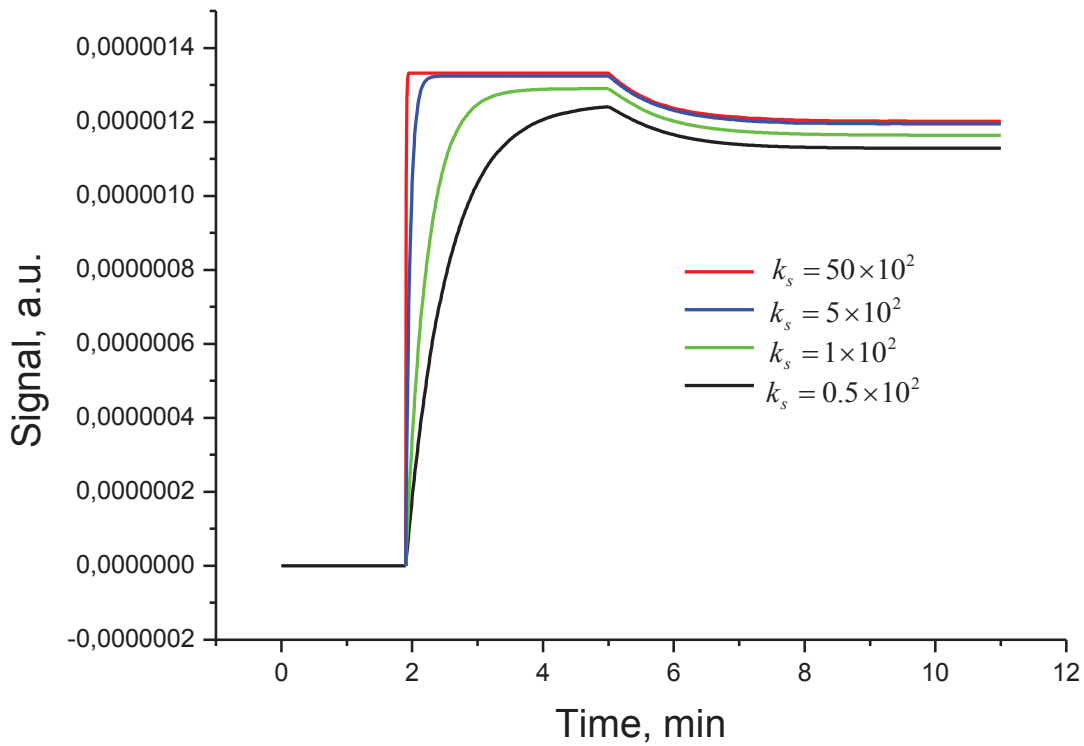


Fig. 4.9. Simulation of enzymatic reactions in the membrane of AChE/ISFET biosensor using kinetic equations (1), experimental input parameters and different values of constant k_s .

The rate constant k_i of the complexes (EI) and (EIS) formation was responsible for the shape of the inhibitor response curve (Fig. 4.10). Similarly to the constant k_s , when k_i increased, the response curve became sharper and when its value decreased - more rounded.

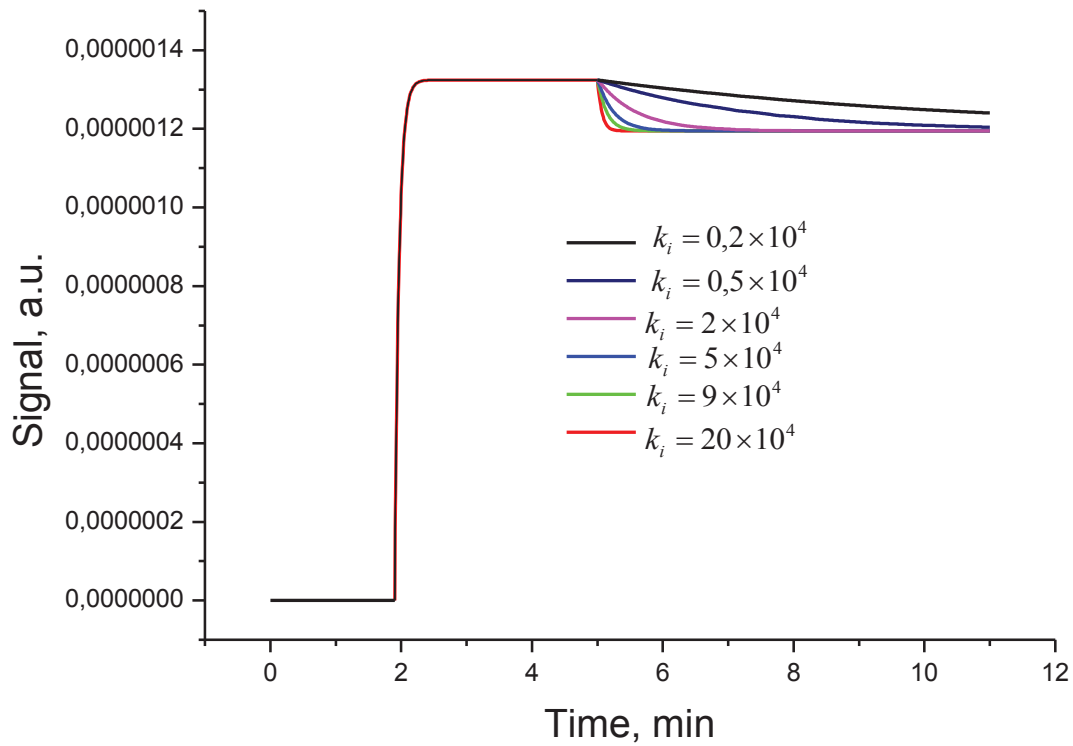


Fig. 4.10. Simulation of enzymatic reactions in the membrane of AChE/ISFET biosensor using kinetic equations (1), experimental input parameters and different values of constant k_i .

Concerning the constant of product formation k_p , when its value increased the response increased accordingly (fig. 4.11). At the same time, an insignificant change in the inhibition level was observed. The levels of inhibition were 9.771%, 9.781%, 9.787% and 9.794% for $k_p = 1 \times 10^{-2}$, 5×10^{-2} , $7,5 \times 10^{-2}$ and 10×10^{-2} respectively.

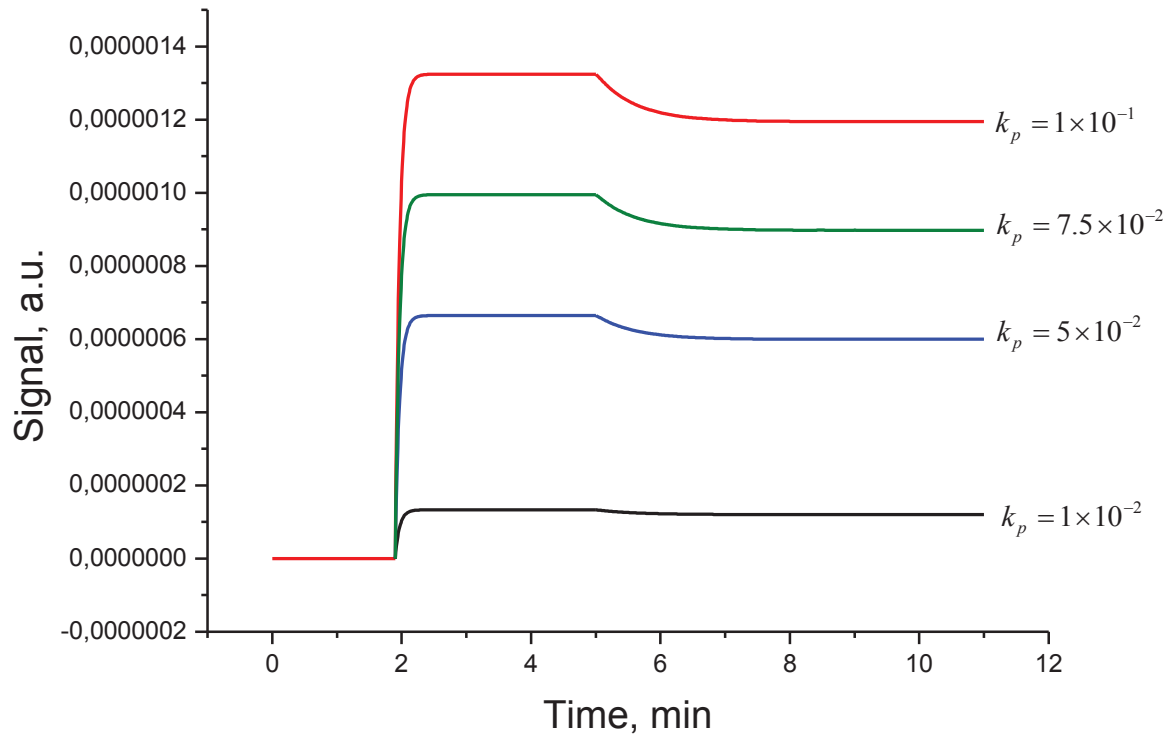


Fig. 4.11. Simulation of enzymatic reactions in the membrane of AChE/ISFET biosensor using kinetic equations (1), experimental input parameters and different values of constant k_p .

With the further increase of k_p (more than 1×10^{-1}) the model responses became inconsistent with the experimental values.

4.5.2. Assessment of enzyme concentration in the biosensor membrane by simulation

As has been mentioned earlier, the enzyme concentration in the biosensor membrane was experimentally estimated to be approximately 2×10^{-5} M. It was the maximum possible concentration because the enzyme molecules lose their activity during immobilization and the concentration of the immobilized enzyme should be considerably lower than the calculated one. To study the effect of enzyme concentration in the membrane on the biosensor work, the dependence of the inhibition level on the enzyme concentration was plotted (fig. 4.12)

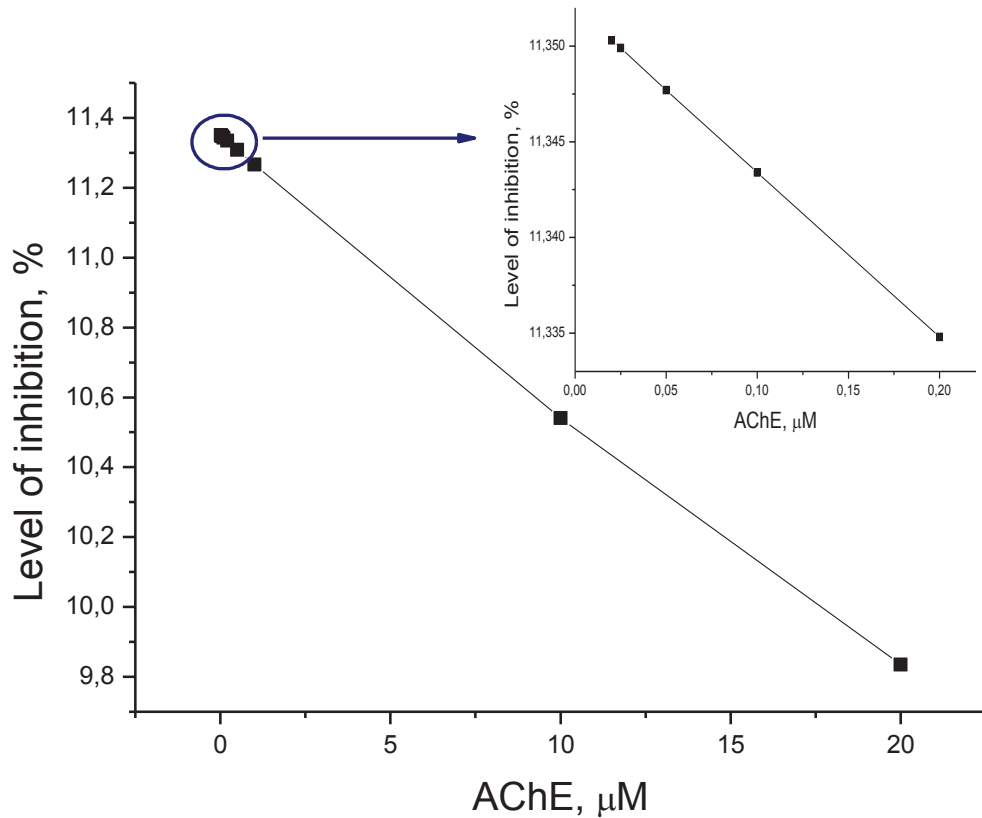


Fig. 4.12. *Dependence of inhibition level on different acetylcholinesterase concentrations in the membrane.*

As seen from the graph, the inhibition level is inversely proportional to the enzyme concentration in the membrane: when the latter decreases the level of inhibition increases. When the enzyme concentration in the membrane reached $0.01 \mu\text{M}$ an atypical surge was observed at the beginning of the inhibitor response curve.

With the further decrease in the enzyme concentration, the similar surge occurs before the substrate response.

4.5.3. Study of the inhibitory coefficient α and its effect on the system behavior

The coefficient α is responsible for the nature of the inhibitory interaction. As known, $\alpha = 1$ corresponds to the non-competitive inhibition, $\alpha = 0$ - to the competitive type, and $\alpha > 1$ - to the enzyme activation.

As has been previously found from the experiment (Chapter 3), the inhibitory interaction between AFB1 and immobilized AChE is characterized by a bell-shaped curve of dependence of the inhibition level on the different substrate concentrations. To investigate an influence of the inhibitory coefficient α on the system behavior, the graphs of dependence of inhibition level on substrate concentration were plotted for different values of inhibition coefficient α (fig.4.13).

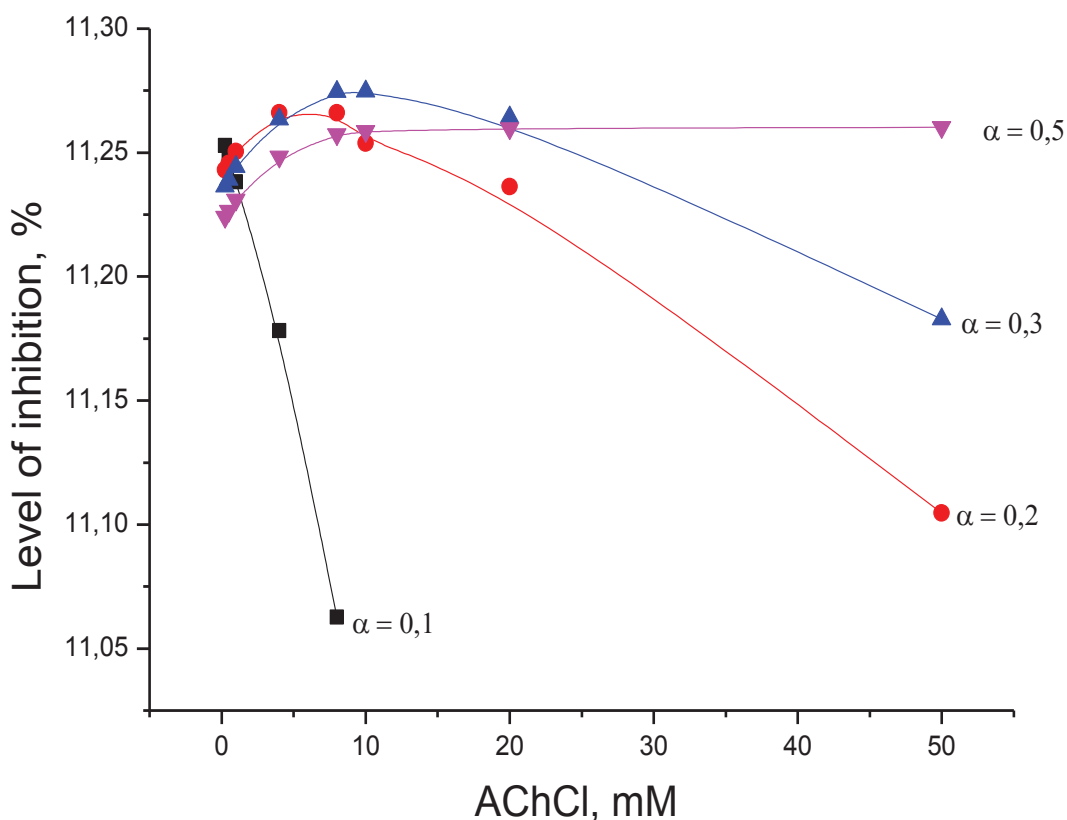


Fig. 4.13. Dependence of inhibition level on different AChCl concentrations for different values of inhibition coefficient α

As can be seen, at $\alpha = 0,1$ when the substrate concentration increases the inhibition level decreases. This dependence is typical for competitive inhibition. At $\alpha = 0,5$ when the substrate concentration increases the inhibition level increases too. This dependence is typical for non-competitive inhibition. At $\alpha = 0,2$ and $\alpha = 0,3$ the bell-shaped dependence is observed, which is typical for our case (when aflatoxin B1 inhibits AChE).

4.6. Validation of mathematical simulation

For validation of the developed simulation, the parameters of biosensor operation at a constant concentration of the working substrate (4 mM) and different concentrations of inhibitor (3.2 μM , 6.4 μM , 9.6 μM and 12.8 μM) (fig. 4.14, a) was compared with the simulation (fig. 4.14, b) at the same concentrations.

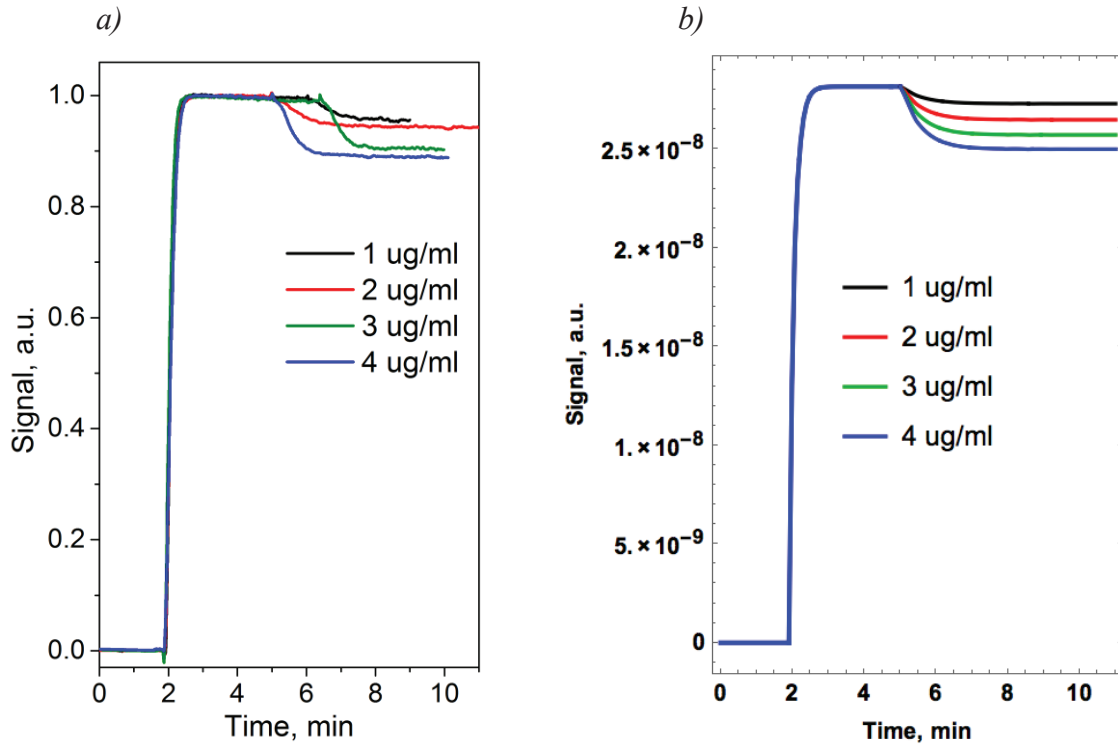


Fig. 4.14. Experimental AChE/ISFET biosensor operation (a) and its simulation (b) using 4mM AChCl as a substrate and 3.2 μM , 6.4 μM , 9.6 μM and 12.8 μM AFB1 as an inhibitor.

The experimental and simulated values of the inhibition levels at different aflatoxin B1 concentrations are listed in the Table 4.2.

Table 4.2. The inhibition level comparison of simulation and experiment

AFB1 concentration, μM	Level of inhibition, %	
	Experiment	Simulation
3.2	3.88	3.07451
6.4	5.82	5.96419
9.6	8.92	8.69504
12.8	11.16	11.2585

The correlation chart was plotted (fig. 4.15). Pearson coefficient was calculated to be 0.995, which showed a positive linear correlation of experimental and simulated data.

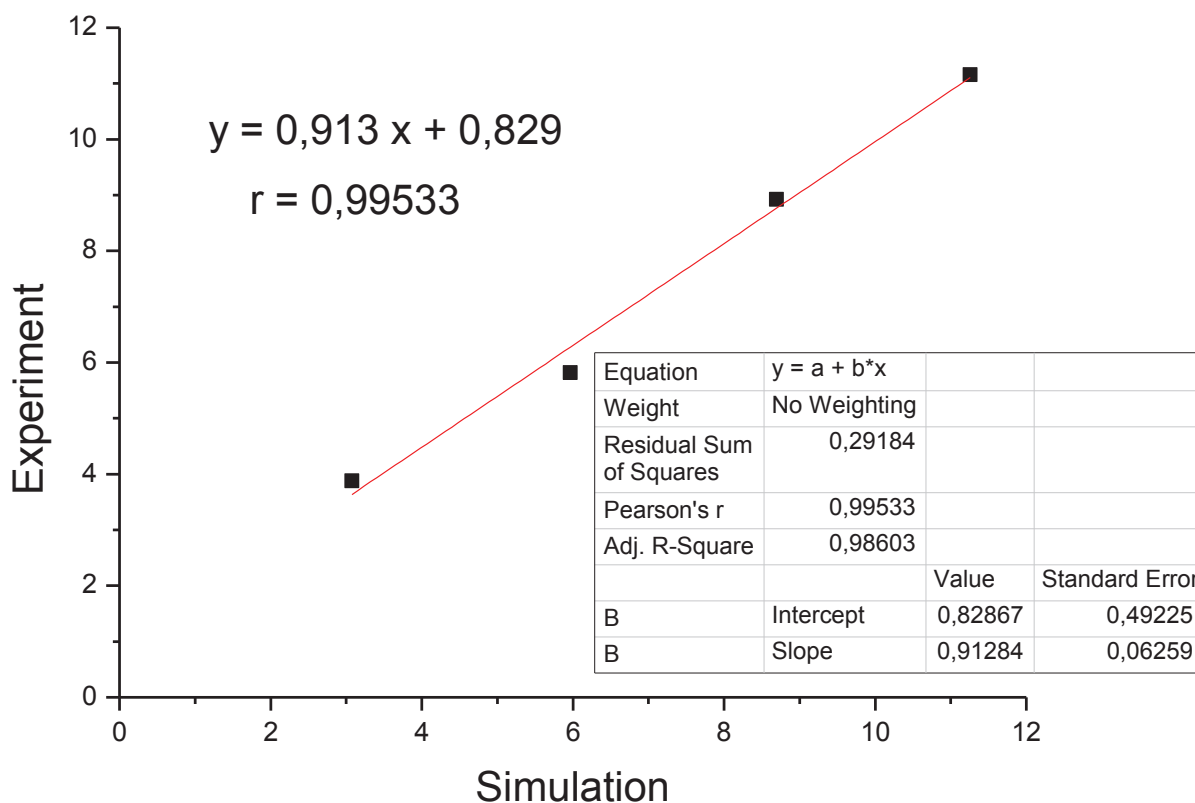


Fig. 4.15. Correlation between experimental inhibition levels of AChE/ISFET biosensor and simulation at different inhibitor concentrations (3.2 μM, 6.4 μM, 9.6 μM and 12.8 μM AFB1).

The positive linear correlation indicates that the developed simulation can be successfully applied to the construction of AFB1 calibration curves for AChE/ISFET

biosensor using only one experimental response. This permits to diminish the use of aflatoxin B1, which is expensive and harmful for human health.

4.7. Conclusions

The mathematical simulation of the potentiometric AChE-biosensor operation during aflatoxin B1 inhibitory analysis was developed. The analytical aspects of acetylcholinesterase inhibition by AFB1 were established. Using a new “degree of inhibition” method and Lineweaver-Burk plots, it was experimentally shown that AFB1 is a reversible inhibitor of mixed type. The mathematical model was described by a system of rate equations, presenting the dynamics of biochemical reactions in the biosensor. Each of the equations includes concentrations of the enzyme, substrate, inhibitor, product and their complexes over the time. The system was solved numerically using Wolfram Mathematica software. The initial concentrations of the enzyme, substrate and inhibitor act as boundary conditions for the system of rate equations. These concentrations, calculated from the experimental conditions, were: 2×10^{-5} M acetylcholinesterase, 4×10^{-3} M acetylcholine chloride, and 6.4×10^{-6} M aflatoxin B1. The rate constants have been chosen to fit the experimental response. The physical meaning of the rate constants has been shown. The developed model allows reproducing the performance of an actual potentiometric biosensor based on immobilized acetylcholinesterase, which was used for comparison with the model for its validation. The obtained positive linear correlation between experimental and simulated data gives a ground to state that mathematical simulation can be successfully applied in the future to optimize the biosensor work. It could be the opportunity to diminish the use of aflatoxin B1, which is expensive and harmful for human health.

CHAPTER 5. DEVELOPMENT OF A CONDUCTOMETRIC BIOSENSOR BASED ON ACETYLCHOLINESTERASE INHIBITION FOR TOXIC COMPOUNDS DETECTION

1. Biosensor principle and operation
2. Choice of substrate concentration
3. Influence of toxic compounds concentration on the biosensor level of inhibition
4. Reactivation of AChE enzyme after analysis
5. Conclusions

5.1. Biosensor principle and operation

Along with the potentiometric detection, we also evaluated in this work the potentiality of conductometric transduction for the determination of AFB1, and this time we investigated the detection of other AChE inhibitors belonging to different groups, i.e., organophosphorous pesticides, heavy metals, surfactants and glycoalkaloids. AChE was immobilized on the conductometric transducers, fabricated at the Lashkarev Institute of Semiconductor, using the same method as for the ISFET transducers.

As for ISFET biosensor, the conductometric biosensor operation involves: (i) first, the addition of AChE substrate in the measurement cell, (ii) second, the addition of the inhibitor. AChCl substrate decomposition generates acetate ions and protons, leading to an increase of ions and therefore conductivity of the solution, measurable by conductometry. Inhibition of the reaction will induce a decrease in ions produced, and then a fall in biosensor response. The level of inhibition may be calculated, as before, from the biosensor responses before and after inhibitor addition.

5.2. Choice of substrate concentration

First, we determined the optimal concentration of AChCl substrate for inhibitory analysis, i.e. AChCl concentration at which the biosensor has a maximal sensitivity to the inhibitor. Theoretically, this concentration should be in the region of enzyme saturation with the substrate, where each enzyme molecule is involved in the substrate conversion into final products, which changes the conductivity, and thus maximum response is generated. As seen

in Fig. 5.1, the saturation was observed beyond 1 mM AChCl, so this value was taken as the substrate working concentration in further experiments.

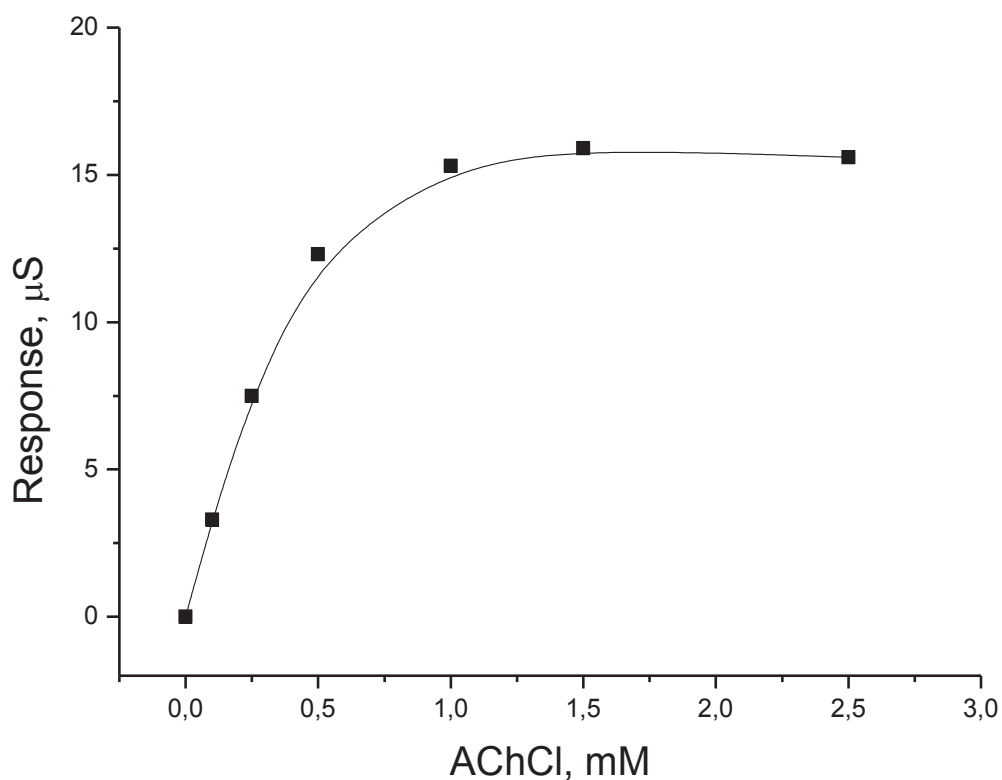


Fig. 5.1. Influence of substrate (AChCl) concentration in the measuring cell on AChE-based conductometric biosensor respons. Measurements were performed in 5 mM phosphate buffer, pH 6.5.

To be sure that the decrease in biosensor signal observed in the further steps of inhibition by the toxic molecules really comes from inhibition and not from measurement errors, an operational stability study was performed. For that, the biosensor responses to the same substrate concentration (1 mM AChCl) were measured for one working day with 15-min intervals. Between two consecutive measurements, the biosensor was kept in the buffer upon continuous stirring. As seen in Fig. 5.2, the biosensor signal was very stable with time, the the measurement root-mean-square error being less than 2.5%.

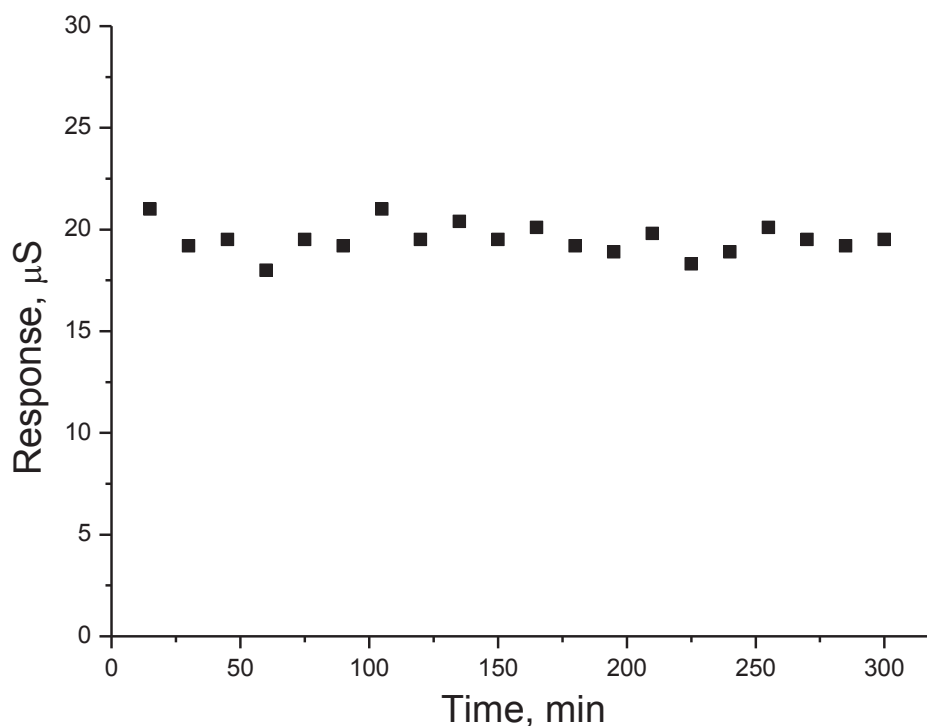


Fig. 5.2. Signal reproducibility of the conductometric acetylcholinesterase-based biosensor. Measurements were carried out in 5 mM phosphate buffer, pH 6.5. AChCl substrate concentration: 1 mM.

5.3. Influence of toxic compounds concentration on the biosensor level of inhibition

In the next step of the work, we studied the effect of toxic substances of different classes, i.e., aflatoxins, organophosphorus pesticides, heavy metal ions, surfactants, and glycoalkaloids - on the inhibition level of AChE immobilized at the surface of the conductometric transducers. One representative molecule of each category, i.e., AFB1, trichlorfon, copper nitrate, benzalkonium chloride and α -solanine, respectively, were chosen to perform the experiments. Fig.5.3. presents the influence of inhibitors concentration on the level of inhibition. Each point was the average of replicate measurements obtained from the biosensor.

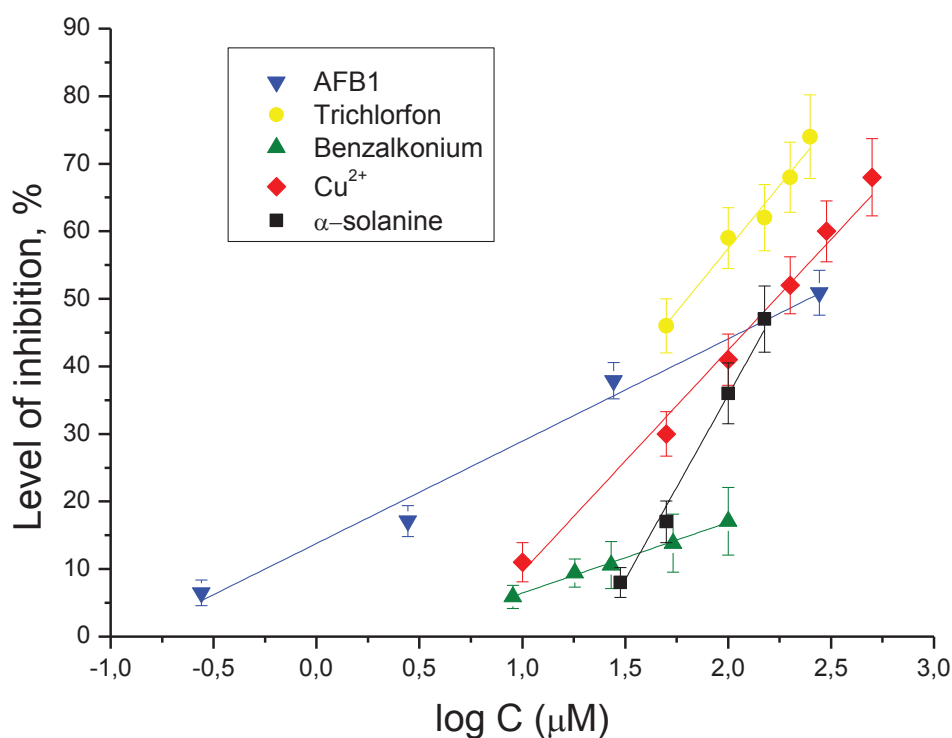


Fig. 5.3. Influence of trichlorfon, Cu^{2+} ions, AFB1, benzalkonium chloride, and α -solanine concentration on the inhibition level of AChE measured by the conductometric biosensor. Measurements were performed in 5 mM phosphate buffer, pH 6.5, at room temperature. AChCl: 1 mM

As shown in the figure, the sensitivity and linear range of the biosensor highly depends on the toxic molecule. Parameters obtained by fitting the curves $I(\%)$ vs $\log C(\mu\text{M})$ with a linear regression model are gathered in Table 5.1. We can see that the biosensor sensitivity decreases in the order: α -solanine > trichlorfon > Cu^{2+} > AFB1 > benzalkonium ions. The widest linear range was obtained for AFB1 (0.28-280 μM , corresponding to 0.09-90 $\mu\text{g/ml}$), but additional experiments should be performed at lower concentrations for trichlorfon and higher concentrations for all the molecules, since the lower and upper parts of the curves were not investigated. The very low sensitivity and biosensor responses achieved for benzalkonium salts (maximum 15-20 % level of inhibition) render the biosensor obviously not suitable for the reliable determination of surfactants in the wide concentration range. However, one positive point is that surfactants, if present in the sample with substances from the other classes, will not drastically interfere with their determination. The

linear range for Cu^{2+} ions is 10 - 500 μM , but other enzyme systems, such as urease-based [180] or three-enzyme (glucose oxidase/mutarotase/invertase) based systems [181], are more sensitive to heavy metal ions. For its part, α -solanine glycoalkaloid can be determined by the proposed AChE-based biosensor only at concentrations over 30 μM , whereas concentrations of 1-2 μM are more likely to be found in real samples. A butyrylcholinesterase (BuChE) biosensor offering higher sensitivity has been reported and is therefore more adequate for the determination of glycoalkaloids [182].

As a conclusion, the proposed AChE biosensor is an efficient tool for the determination of total toxicity or for the quantification of aflatoxins, heavy metals, organophosphorus pesticides and steroid glycoalkaloids (for concentrations given in the Table 5.1) if present individually in contaminated (and for some of them highly contaminated) samples. The linear range and the sensitivity of the conductometric biosensor to AFB1 are respectively wider and higher than the analytical characteristics obtained for the potentiometric biosensor proposed in the second chapter.

Table 5.1. Parameters of the conductometric biosensor calibration curves in response to different toxic compounds. *I*: inhibition level (in %), *C*: concentration of the substance (in μM)

Substance	Equation	R^2	Linear range (μM)
Trichlorfon	$I = 37.784 \log C - 18.106$	0.9780	50-250
AFB1	$I = 15.386 \log C + 13.589$	0.9853	0.28-280
Benzalkonium chloride	$I = 10.464 \log C - 4.0779$	0.9956	9-60
Cu^{2+}	$I = 33.87 \log C - 25.067$	0.9915	10-500
α -solanine	$I = 56.76 \log C - 77.328$	0.9922	30-150

5.4. Reactivation of AChE enzyme after analysis

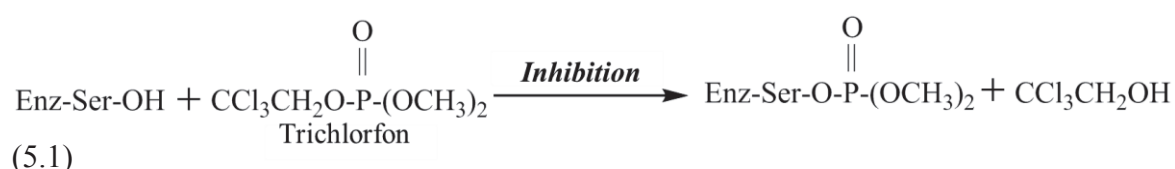
To be able to reuse the proposed biosensor, based on enzyme inhibition, it is necessary to restore the biological activity of the biomolecule after analysis. As already discussed in the bibliographic chapter, enzyme inhibition may be of reversible or irreversible type. AChE

inhibition by aflatoxins, surfactants and glycoalkaloids is reversible, whereas inhibition by organosphosphorus pesticides and heavy metals are irreversible.

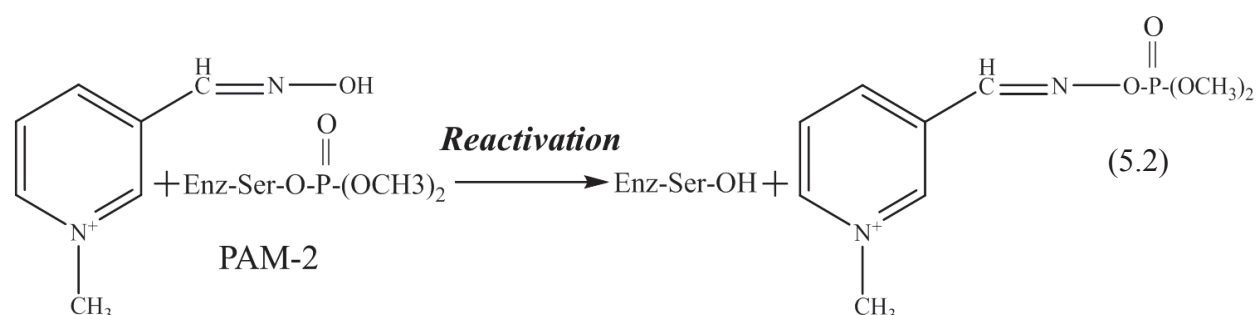
In this work, we showed that the biomembrane working activity (i.e., the response to substrate) could be restored after inhibitory analysis, whatever the inhibitor tested (AFB1, trichlosulfon, Cu^{2+} , α -solanine, benzalkonium chloride). However, the reactivation procedure depends on the toxic substances present in the sample tested. For example, even for reversible inhibitors which are commonly removed from the membrane by routine washing using the working buffer solution, the protocols may differ. In this work, we showed that only 3-5 min washing, i.e. about the same time as the response time, was enough when the enzyme membrane was exposed to alkaloids and aflatoxins. The largest number of reactivation cycles could be carried out for glycoalkaloids. On the contrary, 30-40 min washing of the biomembrane were required to recover its characteristics after exposure to surfactants.

After exposure to irreversible inhibitors, enzymes properties can be restored only by using special substances, called enzyme reactivators. To reactivate cholinesterases inhibited by heavy metals, ethylene diamine tetra acetate (EDTA), is commonly used. Effective reactivation with EDTA could be achieved by washing the biomembrane for 30 min with a 5 mM EDTA solution pH 6.5. The biosensor could be reused at least 4 times with less than 5 % loss of the initial value of the signal.

The enzyme inhibition process by trichlorfon relies on the phosphorylation of serine groups of the protein (5.1):



The enzyme activity can be restored only by special reactants, which displace the phosphoryl moiety bound to cholinesterase, thus releasing the enzyme molecule to interact with the substrate. An example of the AChE reactivation by pyridine-2-aldoxime methyl iodide (PAM-2), that we used in our work, is given below:



To optimize the reactivation procedure, four series of experiments were carried out at different PAM-2 concentrations. In each series, the biosensor was first inhibited for 20 min by trichlorfon of various concentrations to achieve inhibition levels between 0 and 100%. Then, the biosensor was incubated for 30 min in 250 μ l of phosphate buffer solutions containing 0.01 mM, 0.1 mM, 1 mM, 10 mM of PAM-2. After reactivation biosensors were washed from reactivator residues in working buffer solutions for 2-3 minutes.

The results of reactivation are shown in Fig. 5.4.

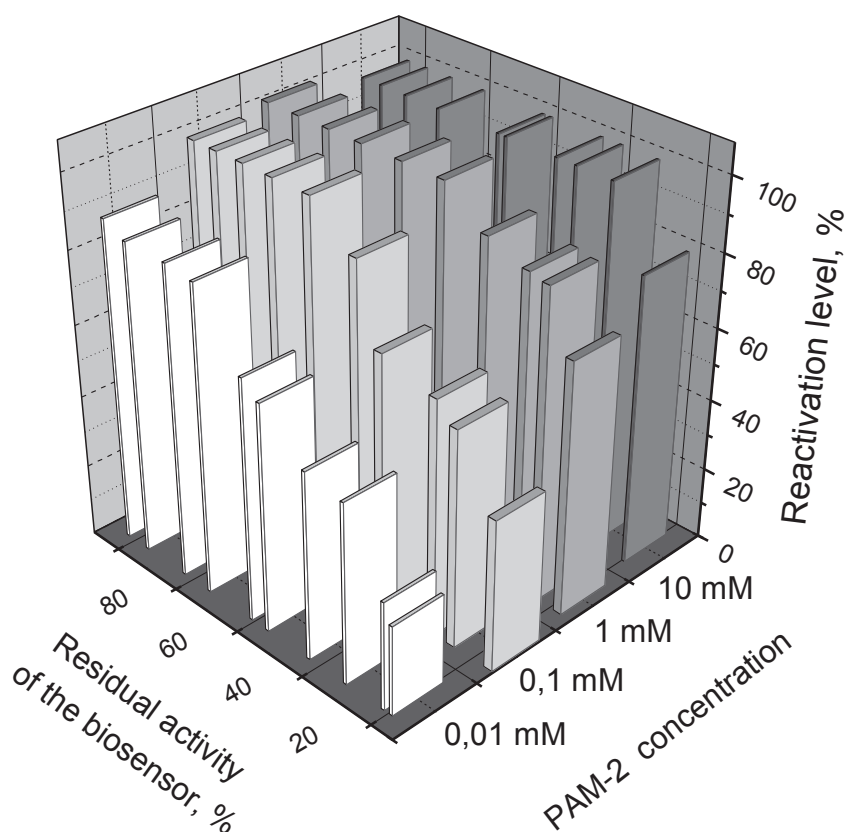


Fig. 5.4. Influence of PAM-2 concentration on the biosensor reactivation after inhibition with trichlorfon. Inhibition Time of inhibition in trichlorfon solutions of different concentrations : 20 min. Time of biosensor reactivation in PAM-2 solutions : 30 min. The measurements were performed in 5 mM phosphate buffer, pH 6.5.

As seen in the figure, 0.01 mM PAM-2 concentration was insufficient to restore the bioselective element activity even when the inhibition level was low. Using the 0.1 mM PAM-2 concentration enabled the restoration of biosensor activity only when the residual activity was higher than 50%. A 10 mM PAM-2 concentration was required for the

reactivation of strongly inhibited bioselective elements (inhibition level up to 85%). 10 mM PAM-2 was therefore chosen for subsequent experiments. 30 min was also experimentally determined as the optimal time of reactivation.

In the next step of the work, series of cyclic experiments involving inhibition-reactivation of the conductometric AChE-based biosensor were carried out. The aim was to demonstrate the possibility of repeated reactivation of the biosensor to show that it can be actually used several times.

First, we measured the signal to 1 mM AChCl and took its value for 100% (the first column in Fig. 5.5). Then, over one working day we received the responses to the same substrate concentration after inhibition in 100 mM trichlorfon solution (black bars in Fig. 5.5) and reactivation in 10 mM AMP-2 solution (grey bars in Fig. 5.5).

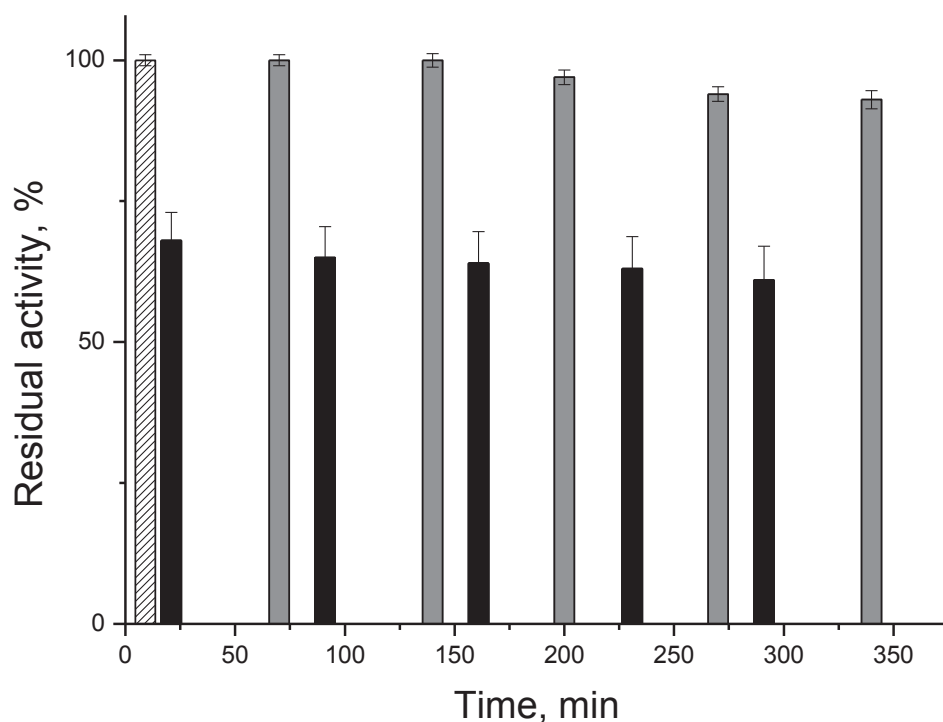


Fig. 5.5. Cyclic experiments on inhibition-reactivation of the conductometric AChE-based biosensor. signals after inhibition (black) and reactivation (grey). Time of biosensor inhibition in 100 μ M trichlorfon solution : 20 min, time of reactivation in PAM-2 solution : 30 min. Measurements were performed in 5 mM phosphate buffer, pH 6.5.

As seen in the figure, the biosensor signal obtained after 5 inhibition-reativation steps was less than 5% lower than the initial signal, indicating that the developed biosensor can be reused at least 5 times.

5.5. Conclusions

In this chapter, the development of a new conductometric AChE-biosensor was described and its applicability to the determination of several substances belonging to aflatoxins, organophosphorus pesticides, heavy metals, surfactants and glycoalkaloids families was evaluated. The biosensor response to 1mM AChE substrate, which corresponds to the optimal value, was very stable under repetitive analyses within one working day and decreased in presence of all the substances tested. The biosensor sensitivity to the toxic compounds decreased in the order α -solanine>trichlorfon>Cu²⁺>AFB1>benzalkonium ions and the wider linear range was achieved for AFB1. The possibility to reactivate the bioselective element after its inactivation by the different toxic compounds was demonstrated. A 5 mM phosphate buffer solution, pH 6.5, was sufficient to restore the enzyme activity after reversible inhibitions, only 3-5 min incubation being required after inhibition by aflatoxins or glycoalkaloids, whereas 30-40 min were needed to reactivate AChE after its inhibition by surfactants. 10 mM pralidoxime solution (PAM-2) and 5 mM EDTA pH 6.5 were used for irreversible inhibitions due to pesticides and heavy metals, respectively.

CHAPTER 6: APPLICABILITY OF THE DEVELOPED BIOSENSORS TO REAL SAMPLES ANALYSIS

6.1. Introduction

The determination of toxic substances in real samples is an actual challenge for analysts. Sample preparation as well as assessment and possibly correction of the matrix effects, due to interfering compounds present in the samples, are the most difficult issues when analyzing real samples. In this work, we targeted the application of the developed biosensors to different types of food samples potentially contaminated by aflatoxins, i.e. cereals, peas and walnuts.

6.2. Development of the extraction procedure

Since target samples are solid, and the proposed biosensor enables the determination of liquid samples, an extraction procedure had first to be developed. Different extractants have been reported in the literature, among which acetonitrile-water mixtures are very commonly used for aflatoxins extraction from contaminated samples [183]. For the experiment, uncontaminated samples of sesame, walnut and peas were bought in a supermarket and prepared according to the protocol of aflatoxin B1 extraction by ACN-H₂O (80:20) described in chapter 2.

Then, after the potentiometric biosensor response was recorded for a 4 mM solution of AChE substrate, 100 μ l of the extract were added to the measuring cell and the level of inhibition was determined. The histogram presented in Fig.6.1 shows the levels of inhibition calculated from 3 replicate measurements for each sample and for 100 μ l of ACN-H₂O (80:20) as control. All extracts and acetonitrile induced the same level of inhibition (about 10%) of the biosensing element, indicating the absence of aflatoxins (or presence at concentrations under the limit of detection) and an inhibitory effect of the extractant. To evaluate matrix effect, the extracts were further spiked at three different AFB1 concentration levels and measured. Fig. 6.1. shows that both ACN and sample matrix affects biosensor responses to AFB1, significantly changing the slope of the curve for sesame and decreasing the inhibition level but keeping the response curve almost parallel to the ACN curve for the

other samples. Using the calibration curve built from AFB1 standard solutions in ACN and responses to AFB1 additions in the three extracts, it was possible to quantify AFB1 concentrations in the real samples and therefore calculate recoveries. As reported in Table 6.1, the recovery values were quite low, confirming the high impact of sample matrix on biosensor response, and indicating that standard addition method will be required for AFB1 quantification in real matrix.

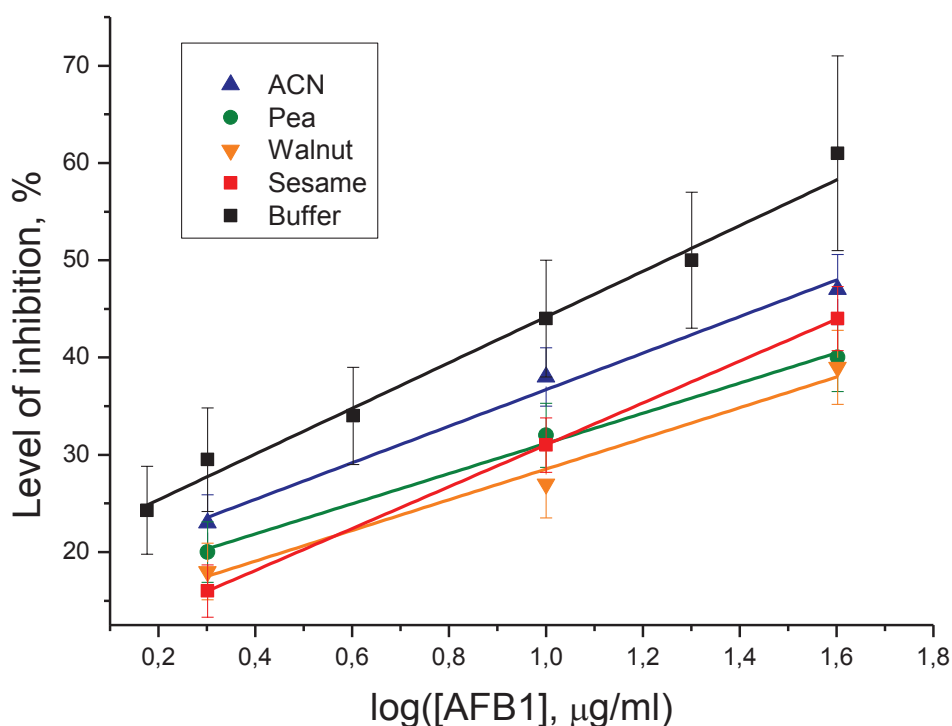


Fig. 6.1. Influence of extractant and sample matrix (sesame, walnut, peas) on the potentiometric biosensor sensitivity to AFB1 Measurements were conducted in 5 mM phosphate buffer, pH 6.5, AChCl concentration : 4 mM, addition of 100 μl of real sample in the measurement cell.

Table 6.1. Recovery study on spiked extracted samples

Added AFB1 ($\mu\text{g/mL}$)	Recovery (%)		
	walnut	sesame	pea
2	49,6	38,6	63,6
10	30,4	49,9	56,5
40	33,7	62,8	38,2

In order to determine whether the effect coming from the presence of acetonitrile in the extracts could be eliminated, extracts were evaporated to dryness and recovered either in ethanol, dimethyl sulfoxide (DMSO), or methanol. These solvents were chosen, since they are also known as aflatoxins solvents. The impact of the volume of these solvents injected in the measurement cell on the AChE-based biosensor responses was examined (Fig. 6.2).

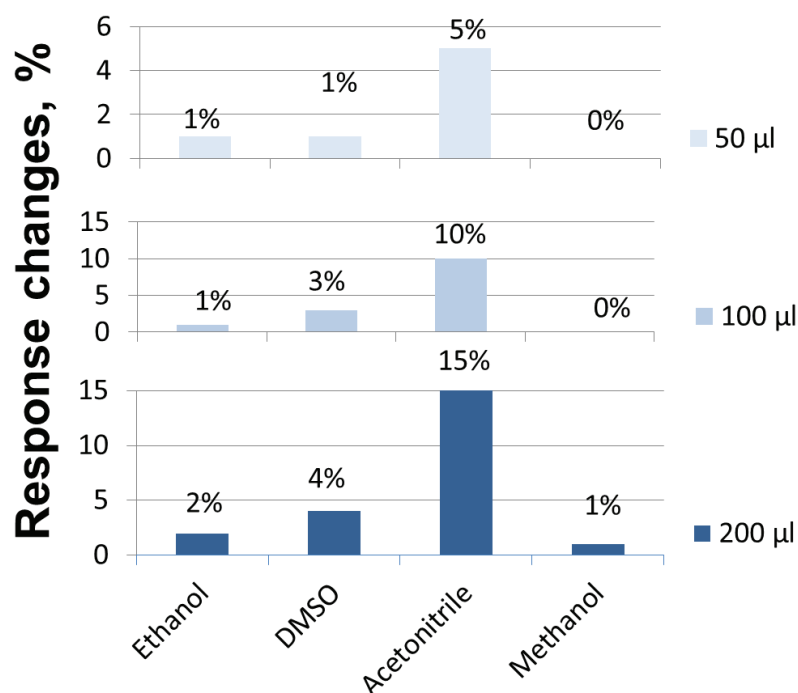


Fig. 6.2. Histograms representing the effect of different volumes (50 μl , 100 μl and 200 μl) of solvents (ethanol, DMSO, acetonitrile and methanol) on AChE-biosensor operation.

As shown in Fig. 6.2, ACN had the highest effect on AChE activity, while methanol had no effect for 50 or 100 μl volumes. As a consequence, further experiments were

performed using AC/H₂O as extractant and methanol as final recovery solvent. Standard addition method was used for quantification.

6.3. Aflatoxins analysis in samples infected with aflatoxins

In a further step, wheat samples infected with aflatoxins were prepared at the D.K. Zabolotny Institute of Microbiology and Virology, in Kiev, following the protocol of preparation described in detail in Materials and Methods. Shortly, *Aspergillus flavus* mold was grown on wheat in humidity and temperature conditions favoring the production of aflatoxins. The infected sample, as well as control wheat sample, were extracted and the level of inhibition was measured by the biosensor, while the same samples were also analyzed by HPLC. Different volumes (10 μ l to 100 μ l) of the non infected and infected samples were injected in the measurement cell after the response to 4 mM AChCl substrate was achieved. No significant inhibition of AChE was observed when the non infected sample was injected, whatever the volume. The injection of 10 and 25 μ l of the infected sample (250 and 100-fold dilutions) generated significant responses (14 and 27% of inhibition, respectively), which corresponds to 190 ± 3 and 200 ± 3 μ g/ml in the sample extract if AFB1 is used as standard for additions to the matrix. 3 replicates were performed for each volume.

The samples were also analyzed by HPLC using UV (200-400 nm), fluorescence (355 nm excitation and 465 nm emission) and mass spectrometric detections. Among the different toxins that *Aspergillus* may produce (Table 6.2.), only AFB2 could be detected and identified crossing the informations deduced from the different modes of detection used (retention time: 8 min in Fig.6.3 and corresponding mass spectrum in Fig.6.4) and from literature data. This result confirms the presence of AChE inhibitors in the sample already detected by the biosensor. Unfortunately, due to an important delay for AFB2 standard supplying, it was not possible to quantify AFB2 in the sample rapidly after sample production, and the sample degraded. We had not the time to generate new samples.

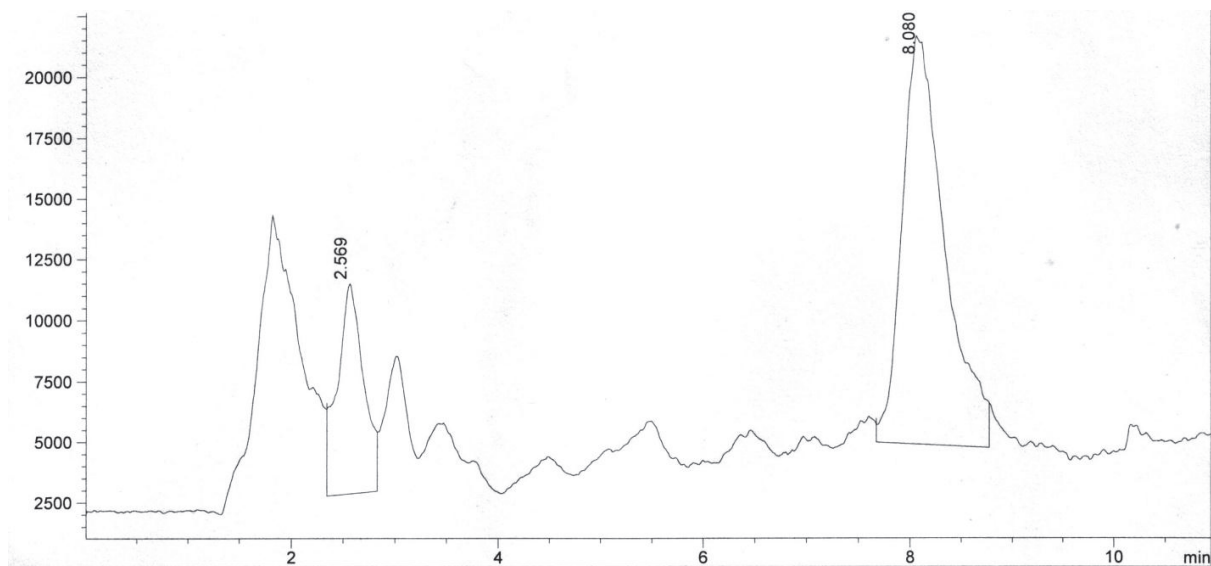


Fig. 6.3. Chromatogramme of the infected wheat sample extract. Fluorescence detection (excitation: 355 nm, emission: 465 nm). Separation conditions in chapter 2.

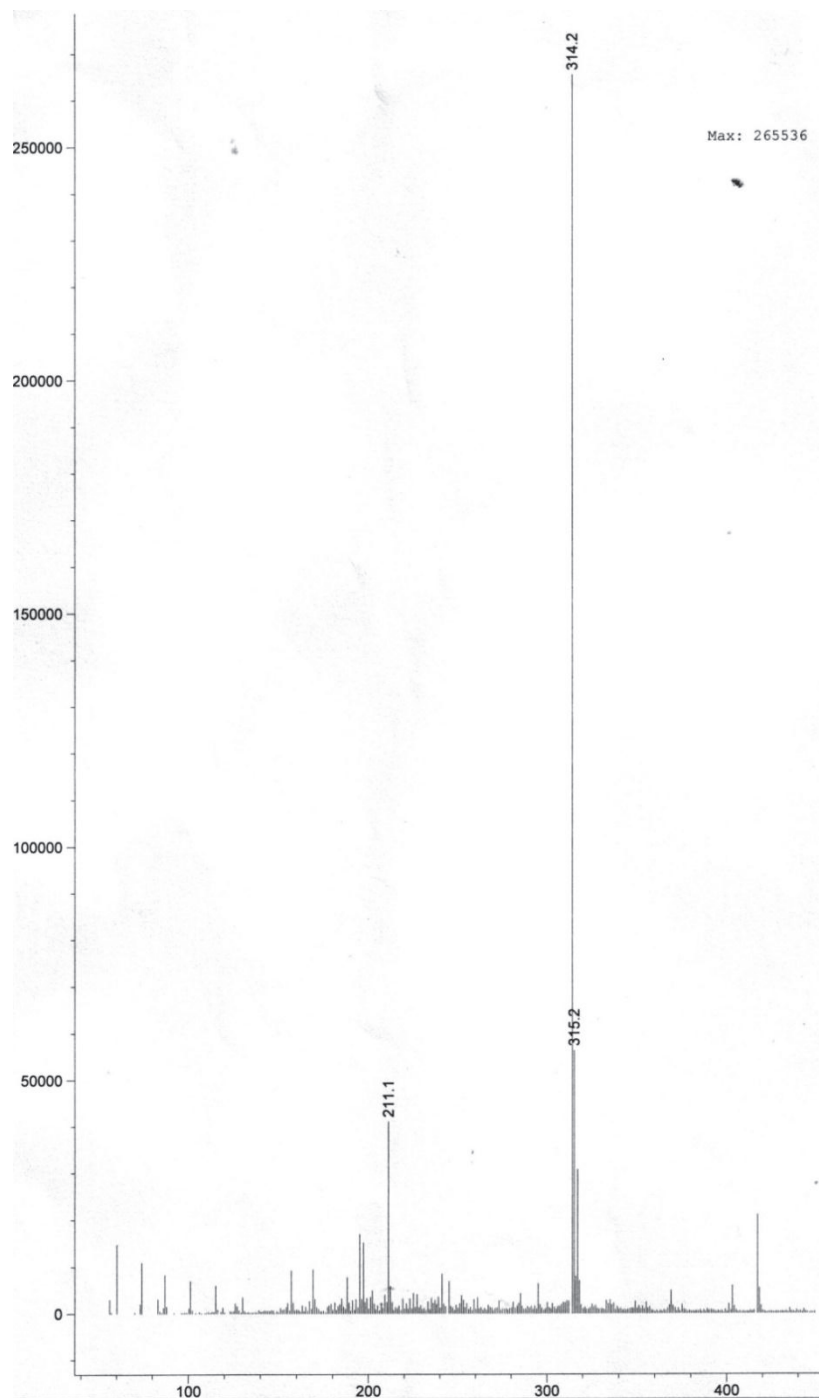


Fig. 6.4. Mass spectrum recorded at $Tr = 8$ min .

Table 6.2. Some toxins produced by Aspergillus with corresponding molecular mass and UV peaks of absorption.

<i>Toxin</i>	<i>Molecular mass</i>	<i>Wavelength of maximal absorption peaks</i>
B1	312	223;265;362
B2	314	220;265;363
G1	328	243;257;362
G2	330	217;245;365
M1	328	226;265;357
M2	330	221;264;357
P1	298	226;267;342;362;425
D1	286	227;324
Parasiticol (B3)	302	229;253;262;326
Q1	328	223;267;366
Aflatoxicol	314	254;261;332
Aflatoxicol B	314	254;261;325
B2a	330	228;256;363
G2a	346	223;242;262;365
Aflatoxicol ethyl-ester A	342	255;261;332
Aflatoxicol ethyl-ester B	342	255;261;331
Sterigmatocystin	324	208;235;249;329
Dihydro-sterigmatocystin	326	233;247;325
O-methyl-sterigmatocystin	338	236;310
Dihydro-O-methyl-sterigmatocystin	340	238;313
Aspertoxin	354	241;310

6.4. Algorithm for biosensor analysis of toxic substances of different classes in multicomponent solution

In the two previous sections, real samples analysis was performed on sample extracts spiked with one single and known toxin (AFB1) or on samples specially produced to contain a specific group, i.e., aflatoxins. However, in real food samples, many classes of toxic compounds may be present. In chapter 5, we have shown that aflatoxins, surfactants and glycoalkaloids are reversible inhibitors of AChE, and that organophosphorus pesticides and heavy metals are reversible inhibitors. AChE reactivation may be achieved using different solutions and different times of incubation. This results offer a way to selectively determine the toxic substances, even all present in the same aqueous sample, by using the AChE-based biosensors developed. The essence of this method can be represented as a block diagram (Fig. 6.5) demonstrating the analysis algorithm.

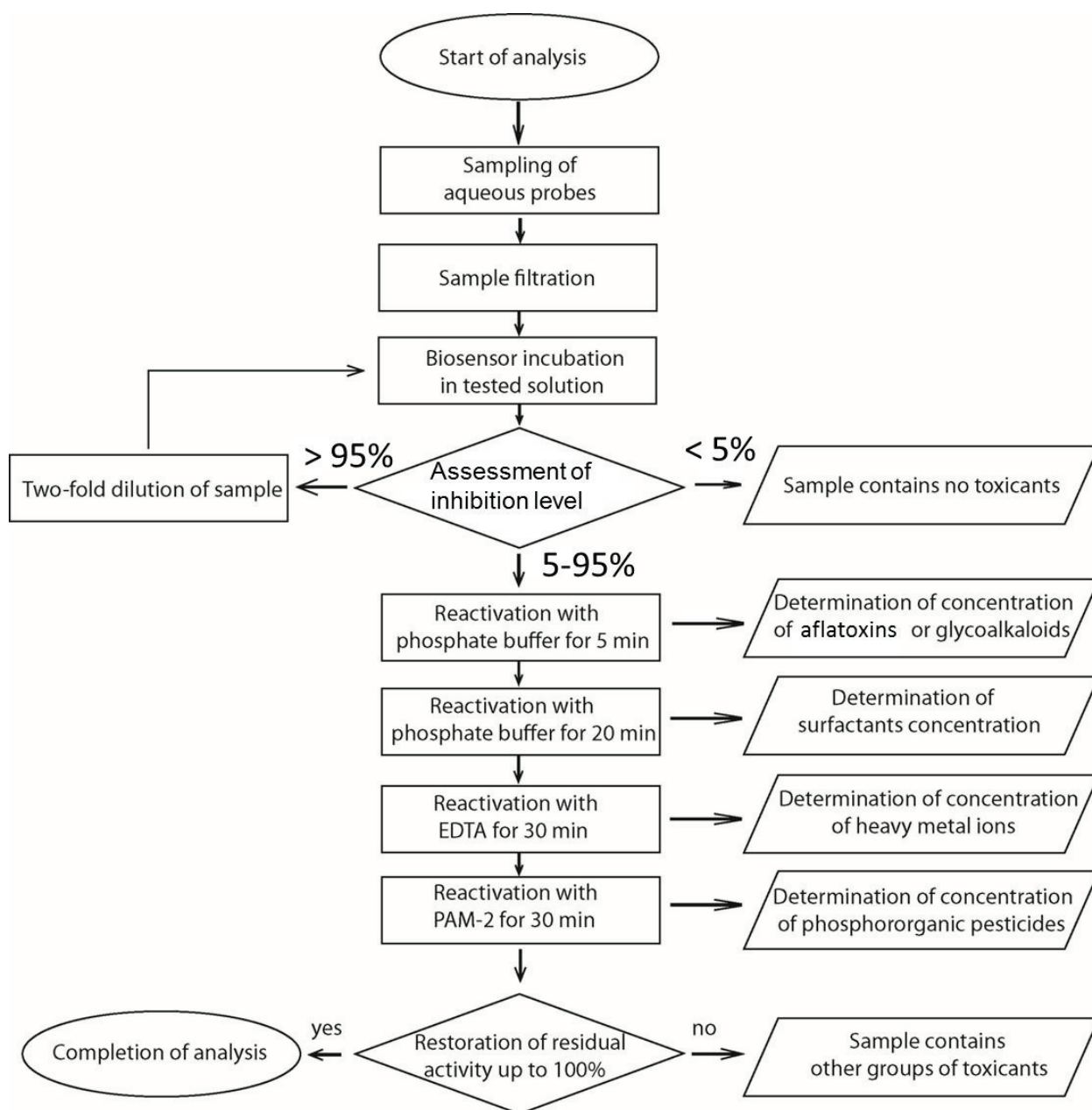


Fig. 6.5. Block diagram of inhibitory analysis of toxic substances by AChE biosensor using selective reactivation of bioselective element.

After direct sampling or extraction of the food solid sample, the aqueous probe is filtered to remove large particles which can damage the bioselective membrane mechanically. The membrane working activity is determined and taken as 100%. Next, the biosensor is incubated in the toxic solution and the inhibition level of the enzyme membrane is analyzed. If it is lower than 5%, it can be concluded that the concentration of toxic substances in the sample is too small to be registered by the biosensors. If the inhibition level is higher than 95%, the sample is diluted until the inhibition level ranges from 5 to 95%. To determine the

toxic composition, the biosensor is first washed with the working buffer. If a complete restoration of the membrane working activity is observed by washing for 5-10 min, it can be an evidence of the presence of reversible inhibitors in the sample, either glycoalkaloids or aflatoxins, depending on the sample origin. If the restoration requires more prolonged washing in the working buffer (20-30 min), it indicates the presence of surfactants in the sample. If after 30-40-min washing the biosensor response is not restored to 95%, then the presence of irreversible inhibitors in the tested solution can be stated.

To clarify the class of irreversible inhibitors, the selective reactivation is carried out. First, the biosensor is reactivated using EDTA to avoid the effect of heavy metal ions on the enzyme membrane. If this stage results in the restoration of biosensor activity, it may be assumed that heavy metal ions are present in the sample. If the activity is not restored to 100%, the biosensor is reactivated with PAM-2 to detect organophosphorus pesticides.

Thus, if AChE activity is totally restored after all phases of reactivation, it is possible to determine the toxic composition of the sample, analyzing the membrane activity at each stage of reactivation. If the activity is only partially restored, the sample likely contains toxic substances of some other classes than above mentioned. However, the total toxicity of the sample can be determined and if necessary, additional analyses may be used for further study.

In order to confirm the validity of the proposed algorithm, a series of experiments was performed using the potentiometric AChE-biosensor and a model solution containing a mixture of 75 μM trichlorfon and 12 μM AFB1. These two toxins were chosen because they can be naturally present in the same sample. First, the effect of each toxicant on the biosensor response was tested separately, and the relevant level of inhibition was calculated.

As shown in Fig.6.6., incubation of the biosensor in a solution of 12 μM AFB1 (4 $\mu\text{g/ml}$) for 30 min, the level of inhibition was 21%. Further 5-min incubation in the working buffer resulted in the complete restoration of biosensor activity. Incubing the biosensor in 75 μM trichlorfon solution for 30 min led to a level of inhibition of 15%. Further incubation in the working buffer did not restore the biosensor activity. After subsequent 30-min incubation in the 10 mM PAM-2 solution, the restoration of biosensor activity was observed.

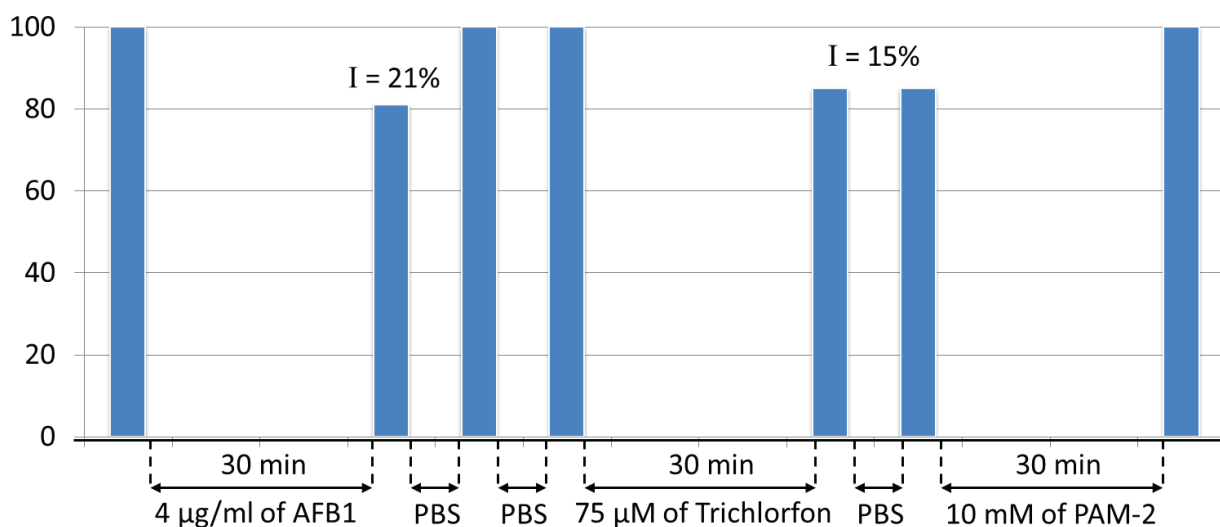


Fig. 6.6. Experimental scheme: separate inhibition of the potentiometric AChE biosensor by 4µg/ml AFB1 (12 µM) and 75µM trichlorfon; restoration of biosensor activity by short-time washing with working buffer and using special reactivator PAM-2.

The above experiment confirms the basic difference between the reversible inhibition by aflatoxin B1 and irreversible inhibition by trichlorfon. After inhibition by aflatoxin, the biosensor activity can be restored by rapid washing with the working buffer, while the special reactivator (PAM-2) should be used after inhibition by trichlorfon.

After restoration of the biosensor activity up to 100%, the transducer was incubated in the mixture of 12 µM AFB1 and 75 µM trichlorfon for 30 min. As shown in Fig. 6.7., the level of inhibition after incubation was 38%. Then the transducer was incubated in the working buffer for 5 min, which resulted in partial restoration of the biosensor activity, the level of inhibition was 14%. Further washing with the working buffer did not affect the biosensor response to the substrate. After 30-min incubation, the biosensor response was restored.

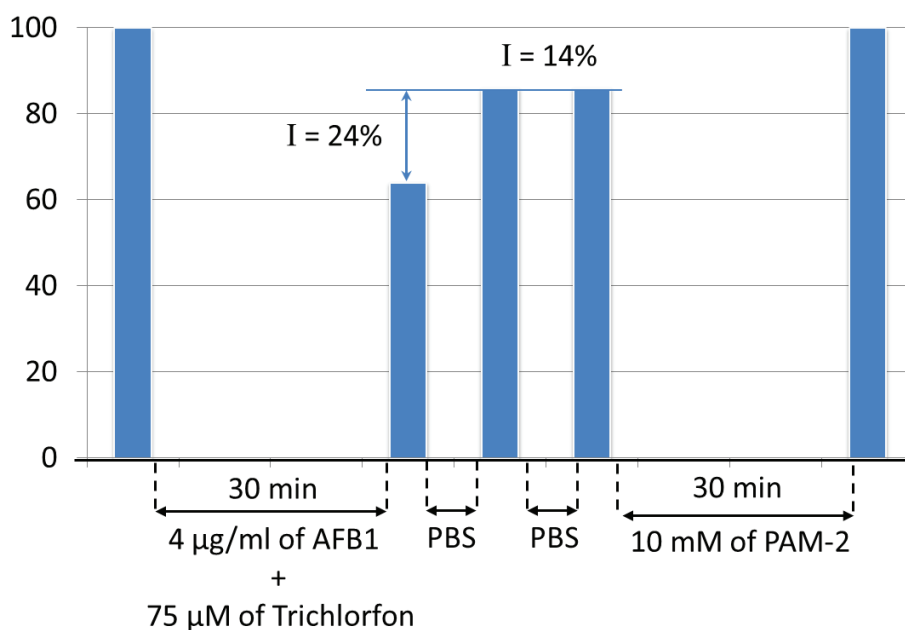


Fig. 6.7. Scheme of experiment: inhibition of potentiometric AChE biosensor by mixture of 4 µg/ml AFB1 and 75 µM trichlorfon; restoration of biosensor activity by washing with working buffer and using special reactivator PAM-2.

A conclusion can be made that 24% restoration of the activity after washing with the buffer corresponded to the inhibition by aflatoxin B1 whereas 14% of unrestored activity - to irreversible inhibition by trichlorfon. It is seen that when using the proposed algorithm, the levels of inhibition by the fixed concentration of toxins, either separate or in mixture, coincided within the margin of error of the experiment.

6.5. Conclusions

In this last chapter, different analytical steps required for further application of the developed biosensors to real food samples analysis were investigated. First, we showed that ACN, used as extractant for different types of samples (sesame, pea, walnut) slightly inhibits AChE and that ACN as well as extracted matrix components generate matrix effects, imposing the standard addition method as quantification method. By evaporation of the ACN extractant and recovery in methanol, it is possible to eliminate the inhibitory effect of ACN but not from the extracted matrix.

Wheat sample, infected by *Aspergillus flavus* mold, was able to generate AFB2 toxin, detected by HPLC, in agreement with the increase in inhibition level detected by the potentiometric biosensor.

A new approach was finally proposed for the selective detection of different toxic compounds by a single AChE-based biosensor. This approach consists in using successive stages of bioselective membrane reactivation using adequate solutions and times of incubation for aflatoxins, surfactants, glycoalkaloids (reversible inhibitors), and organophosphorus pesticides, heavy metals (irreversible inhibitors). This approach was successfully applied for the selective determination of trichlorfon and AFB1 in a model mixture.

GENERAL CONCLUSION AND PERSPECTIVES

In the present manuscript, we reported the successful development of two electrochemical biosensors based on AChE inhibition for the determination of different classes of toxic compounds. In both cases, AChE enzyme was immobilized at the surface of the transducers by cross-linking with glutaraldehyde vapour in presence of BSA.

First, a new potentiometric biosensor, using pH Sensitive Field-Effect Transistors (pH-FETs) as transducers, was investigated for aflatoxins determination and different elaboration and working parameters were optimized. Optimal concentrations of AChE and BSA in the biomembrane were found to be 1% (w/w) and 1% (w/w), respectively. 4 mM acetylcholine was chosen as AChE substrate concentration and inhibitory experiments were performed in 5 mM phosphate buffer, pH 6.5 using aflatoxin B1 (AFB1) as inhibitor. Under these conditions, the proposed biosensor was characterized by high operational stability and reproducibility of the signal during the work as well as during the storage. The biosensor response to AFB1, when plotted as level of inhibition (in%) vs $\log[\text{AFB1}]$ (in $\mu\text{g/ml}$) was linear in the 0,4 to 40 $\mu\text{g/ml}$ with a sensitivity of 26.642 % of inhibition per log of AFB1 concentration (in $\mu\text{g/ml}$). The biosensor sensitivity to AFG1 was similar to that to AFB1 and additive effects between the two aflatoxins were demonstrated, showing the possibility to measure the whole toxicity coming from aflatoxins in the samples by the biosensor developed. A mathematical simulation of the potentiometric biosensor response to aflatoxin B1 was proposed for the first time and validated. The applicability of the proposed biosensor to real food samples analysis was further investigated. First, we showed that ACN, used as extractant for different types of samples (sesame, pea, walnut) slightly inhibits AChE and that ACN as well as extracted matrix components generate matrix effects, imposing the standard addition method as quantification method. By evaporation of the ACN extractant and recovery in methanol, it is possible to eliminate the inhibitory effect of ACN but not of the extracted matrix. A contaminated wheat sample, produced by infection with AF producing *Aspergillus flavus* mold, was analyzed by the biosensor and HPLC. Only one aflatoxin (AFB2) could be identified by HPLC, consistently with the increase in inhibition level detected by the potentiometric biosensor.

Additionally, the mathematical model of operation of AChE-based biosensor for aflatoxins analysis was developed and the good agreement between simulation and experimental data is demonstrated. Such mathematical method of kinetic equations could be applied to different biosensors systems for their optimization in silico.

In a second step, a new conductometric biosensor using interdigitated gold microelectrodes was developed. The sensitivity of the biosensor to aflatoxins and other classes of toxic substances, such as organophosphorus pesticides, heavy metals ions, glycoalkaloids, and surfactants, was determined. The biosensor response to 1mM AChE substrate, which corresponds to the optimal value, was very stable under repetitive analyses within one working day and decreased in presence of all the substances tested. The biosensor sensitivity to the toxic compounds decreased in the order α -solanine>trichlorfon>Cu²⁺>AFB1>benzalkonium ions and the wider linear range was achieved for AFB1. The very low responses and sensitivity achieved for benzalkonium salts (maximum 15-20 % level of inhibition) render the biosensor hardly applicable to the reliable determination of surfactants. It is found that AChE-based biosensors can be used to identify different groups of toxins, but preferably to determine aflatoxin or to detect total toxicity of the sample. The linear range and the sensitivity of the conductometric biosensor to AFB1 are respectively wider and higher than the analytical characteristics obtained for the potentiometric biosensor but should be improved for quantitative determination in samples with low aflatoxins concentrations. In the same time the conductometric biosensor can not be selective enough for real samples work due to the complexity of the matrix of the target samples. In order to improve the selectivity using of pH-FET is more promising approach, because this type of transducer is selective only to protons.

The possibility to reactivate the bioselective element after its inactivation by the different toxic compounds was demonstrated. A 5 mM phosphate buffer solution, pH 6.5, was sufficient to restore the enzyme activity after reversible inhibitions, only 3-5 min incubation being required after inhibition by aflatoxins or glycoalkaloids, whereas 30-40 min were needed to reactivate AChE after its inhibition by surfactants. 10 mM pralidoxime solution (PAM-2) and 5 mM EDTA pH 6.5 were used for irreversible inhibitions due to pesticides and heavy metals, respectively. These differences were hardnessed to propose a new and original approach for the multidetection of toxic compounds belonging to different groups by a single AChE-based biosensor. This approach consists in using successive stages of bioselective membrane reactivation using adequate solutions and times of incubation for aflatoxins, surfactants, glycoalkaloids (reversible inhibitors), and organophosphorus pesticides, heavy metals (irreversible inhibitors). This approach was successfully applied to the selective determination of trichlorfon and AFB1 in a model mixture.

As a conclusion, we have proposed a new method usable for the rapid and easy assessment of the presence of compounds of different chemical groups in food samples. The

first validation results obtained in this work are really promising but they have to be confirmed and completed. Indeed, the sensitivity of the proposed biosensors still has to be improved in order that the method can be applied to the analysis of slightly contaminated samples. Moreover, the algorithm has to be further validated, first on model samples containing more than two compounds (on the same group and on different groups), by varying the ratio between the selected compounds, and then on real samples spiked with the same mixtures. Finally, a complete validation of the method should be performed using a reference method, e.g. HPLC.

BIBLIOGRAPHY

- [1] S. Marin, A.J. Ramos, G. Cano-Sancho, V. Sanchis, "Mycotoxins: occurrence, toxicology, and exposure assessment", *Food Chem. Toxicol.* vol. 60, 218–237, 2013.
- [2] M. E. Zain, "Impact of mycotoxins on humans and animals", *J. Saudi Chem. Soc.*, vol. 15, pp. 129–144, 2011.
- [3] F. Wu, J. D. Groopman, J. J. Pestka, "Public Health Impacts of Foodborne Mycotoxins", *Annu. Rev. Food Sci. Technol.*, vol. 5, 351–372, 2014.
- [4] S. D. Stoev, "Foodborne mycotoxicoses, risk assessment and underestimated hazard of masked mycotoxins and joint mycotoxin effects or interaction", *Environmental toxicology and pharmacology* 39 (2015) 794–809
- [5] A.G. Marroquín-Cardona, N.M. Johnson, T.D. Phillips, A.W. Hayes, "Mycotoxins in a changing global environment – A review", *Food Chem. Toxicol.*, vol. 69, pp.220–230, 2014.
- [6] H. P. Van Egmond, R. C. Schothorst, M. A. Jonker, "Regulations relating to mycotoxins in food - Perspectives in a global and European context", *Anal. Bioanal. Chem.* vol. 389, pp 47-157, 2007.
- [7] Food and Agriculture Organization (2004) Worldwide regulations for mycotoxins in food and feed in 2003. FAO Food and Nutrition Paper 81. Food and Agriculture Organization of the United Nations, Rome, Italy
- [8] European Commission, 2014. COMMISSION REGULATION (EC) No1881/2006 on setting maximum levels for certain contaminants in foodstuffs. Amended values. Retrieved from <http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02006R1881-20140701&from=FR>
- [9] W. P. Blount, "Turkey X Disease," *Journal of British Turkey Federation*, Vol. 9, No. 52, 1961, pp. 52-61.[9] K. Sargent, A. Sheridan, J. O'Kelly, and R. B. A. Carnaghan, "Toxicity associated with Certain Samples of Groundnuts," *Nature*, vol. 192, pp. 1096–1097, 1961.
- [10] B. F. Nesbitt, O. J., K. Sargeant, and A. Sheridan, "Toxic Metabolites of *Aspergillus flavus*," *Nature*, vol. 195, no. 4846, pp. 1062–1063, 1962.
- [11] T. W. Kensler, B. D. Roebuck, G. N. Wogan, and J. D. Groopman, "Aflatoxin: A 50-year Odyssey of mechanistic and translational toxicology," *Toxicol. Sci.*, vol. 120, no. SUPPL.1, pp. 28–48, 2011.
- [12] S.Z. Iqbal, S. Jinap, A.A. Pirouz, A.R. Ahmad Faizal, "Aflatoxin M1 in milk and dairy products, occurrence and recent challenges: A review", *Trends Food Sci. Technol.*, vol. 46, pp. 110-119, 2015.

- [13] M. M. Moake, O. I. Padilla-Zakour, and R.W. Worobo, "Comprehensive review of patulin control methods in foods", *Comprehensive Reviews in Food Science and Food Safety*, vol. 1, pp 8-21, 2005.
- [14] O. Puel, P. Galtier I.P. Oswald, "Biosynthesis and Toxicological Effects of Patulin", *Toxins*, vol.2, pp.613-631, 2010.
- [15] N. Glaser and H. Stopper, "Patulin: Mechanism of genotoxicity," *Food Chem. Toxicol.*, vol. 50, no. 5, pp. 1796–1801, 2012.
- [16] A. Lupescu, K. Jilani, M. Zbidah, F.Lang "Patulin-induced suicidal erythrocyte death", *Cell Physiol Biochem.*, vol. 32(2), pp. 291-299, 2013. doi: 10.1159/000354437.
- [17] N. Hartmann, M. Erbs, H.-R.Forrer, S. Vogelgsang, F.E. Wettstein, R.P. Schwarzenbach and T. D. Bucheli, "Occurrence of Zearalenone on *Fusarium graminearum* Infected Wheat and Maize Fields in Crop Organs, Soil, and Drainage Water", *Environ. Sci. Technol.*, vol. 42 (15), pp 5455–5460, 2008.
- [18] L. Escriva, G. Font, L. Manyes, "In vivo toxicity studies of fusarium mycotoxins in the last decade: A review", *Food Chem. Toxicol.*, vol. 78, pp. 185–206, 2015.
- [19] P. Bayman and J. L. Baker, "Ochratoxins: A global perspective," *Mycopathologia*, vol. 162, pp. 215–223, 2006.
- [20] A. el Khoury, A. Atoui, "Ochratoxin a: general overview and actual molecular status", *Toxins*, vol. 2, pp. 461–493, 2010.
- [21] E. Petzinger and A. Weidenbach, "Mycotoxins in the food chain: the role of ochratoxins," *Livest. Prod. Sci.*, vol. 76, pp. 245–250, 2002.
- [22] L. Anfossi, C. Giovannoli, C. Baggiani, "Mycotoxin detection", *Curr. Opin. Biotechnol.*, vol. 37, pp.120–126, 2016.
- [23] N. W. Turner, H. Bramhmbhatt, M. Szabo-Vezse, A. Poma, R. Coker, S. A. Piletsky, "Analytical methods for determination of mycotoxins: An update (2009-2014)", *Anal. Chim. Acta*, vol. 901 pp. 12-33, 2015.
- [24] J. N. Selvaraj, L. Zhou , Y. Wang, Y.-j. Zhao , F.-g. Xing, X.-f. Dai , Y. Liu, "Mycotoxin detection- Recent trends at global level", *J. Integr. Agr.*, vol. 14, pp. 2265–2281, 2015.
- [25] V.L. Pereira, J.O. Fernandes , S.C. Cunha, ,"Mycotoxins in cereals and related foodstuffs: A review on occurrence and recent methods of analysis", *Tr. Food Sci. Technol.*, vol. 36, pp. 96-136, 2014.
- [26] J.P. Meneely, F. Ricci, H.P. van Egmond, C.T. Elliott, "Current methods of analysis for the determination of trichothecene mycotoxins in food", *Trends Anal. Chem.*, vol. 30, pp. 192-203, 2011.
- [27] H. Yao, Z. Hruska, J. Diana Di Mavungu, "Developments in detection and determination of aflatoxins", *World Mycotoxin Journal*, vol. 8, pp.181-191, 2015.

- [28] G. S. Shephard, "Aflatoxin analysis at the beginning of the twenty-first century", *Anal. Bioanal. Chem.*, vol.395, pp. 1215–1224, 2009.
- [29] P. Li, Z. Zhang, X. Hu, Q. Zhang, "Advanced hyphenated chromatographic-mass spectrometry in mycotoxin determination: current status and prospects", *Mass Spectrom. Rev.*, vol. 32, pp. 420–452, 2013.
- [30] A. Garrido Frenich, R. Romero-Gonzalez, M. M. Aguilera-Luiz, "Comprehensive analysis of toxics (pesticides, veterinary drugs and mycotoxins) in food by UHPLC-MS", *Trends Anal. Chem.*, vol. 63, pp. 158–169, 2014.
- [31] J. O'Mahonya, L. Clarke, M. Whelan, R. O'Kennedy, S. J. Lehotay, M. Danaher, "The use of ultra-high pressure liquid chromatography with tandem mass spectrometric detection in the analysis of agrochemical residues and mycotoxins in food – Challenges and applications", *J. Chrom. A*, vol. 1292, pp. 83–95, 2013.
- [32] M. Solfrizzo, A. De Girolamo, V. M. T. Lattanzio, A. Visconti, J. Stroka, A. Aldrick, and H. P. van Egmond, "Results of a proficiency test for multi-mycotoxin determination in maize by using methods based on LC-MS/(MS)," *Qual. Assur. Saf. Crop. Foods*, vol. 5, no. 1, pp. 15–48, 2013.
- [33] S. Hickert, J. Gerding, E. Ncube, F. Hübner, B. Flett, B. Cramer, H.-U. Humpf, "A new approach using micro HPLC-MS/MS for multi-mycotoxin analysis in maize samples", *Mycotoxin Res.*, vol.31, 109-115, 2015.
- [34] G. Martínez-Domínguez, R. Romero-González, A. Garrido Frenich, "Multi-class methodology to determine pesticides and mycotoxins in green tea and royal jelly supplements by liquid chromatography coupled to Orbitrap high resolution mass spectrometry", *Food Chem.*, vol. 197, pp. 907–915, 2016.
- [35] C.-D. Liao, J. W. Wong, K. Zhang, P. Yang, J. B. Wittenberg, M. W. Trucksess, D. G. Hayward, N. S. Lee, J. S. Chang, "Multi-mycotoxin Analysis of Finished Grain and Nut Products Using Ultrahigh-Performance Liquid Chromatography and Positive Electrospray Ionization–Quadrupole Orbital Ion Trap High-Resolution Mass Spectrometry", *J. Agric. Food Chem.*, vol. 63, pp. 8314–8332, 2015.
- [36] C. McElhinney, P. O'Kiely, C. Elliott, M. Danaher, "Development and validation of an UHPLC-MS/MS method for the determination of mycotoxins in grass silages", *Food Additives & Contaminants: Part A*, vol. 32, p.2101–2112, 2015.
- [38] A. Breidbach, F. Ulberth, "Two-dimensional heart-cut LC-LC improves accuracy of exact-matching double isotope dilution mass spectrometry measurements of aflatoxin B1 in cereal-based baby food, maize, and maize-based feed", *Anal. Bioanal. Chem.*, vol. 407, pp. 3159–3167, 2015.
- [38] A. Garrido Frenich, R. Romero-Gonzalez, M. M. Aguilera-Luiz, "Comprehensive analysis of toxics (pesticides, veterinary drugs and mycotoxins) in food by UHPLC-MS", *Trends Anal. Chem.*, vol. 63, pp. 158–169, 2014.

- [39] J. O'Mahonya, L. Clarke, M. Whelan, R. O'Kennedy, S. J. Lehotay, M. Danaher, "The use of ultra-high pressure liquid chromatography with tandem mass spectrometric detection in the analysis of agrochemical residues and mycotoxins in food – Challenges and applications", *J. Chrom. A*, vol. 1292, pp. 83–95, 2013.
- [40] T. P. Lee, R. Sakai, N. A. Manaf, A. M. Rodhi, and B. Saad, "High performance liquid chromatography method for the determination of patulin and 5-hydroxymethylfurfural in fruit juices marketed in Malaysia," *Food Control*, vol. 38, no. 1, pp. 142–149, 2014.
- [41] M. Vaclavikova, Z. Dzuman, O. Lacina, M. Fenclova, Z. Veprikova, M. Zachariasova, and J. Hajslova, "Monitoring survey of patulin in a variety of fruit-based products using a sensitive UHPLC–MS/MS analytical procedure," *Food Control*, vol. 47, pp. 577–584, 2015.
- [42] E. Beltrán, M. Ibáñez, J. V. Sancho, and F. Hernández, "Determination of patulin in apple and derived products by uhplc-ms/ms. Study of matrix effects with atmospheric pressure ionisation sources," *Food Chem.*, vol. 142, pp. 400–407, 2014.
- [43] S. C. Cunha, M. a. Faria, V. L. Pereira, T. M. Oliveira, a. C. Lima, and E. Pinto, "Patulin assessment and fungi identification in organic and conventional fruits and derived products," *Food Control*, vol. 44, pp. 185–190, 2014.
- [44] Y. Rodríguez-Carrasco, J. C. Moltó, H. Berrada, and J. Mañes, "A survey of trichothecenes, zearalenone and patulin in milled grain-based products using GC-MS/MS," *Food Chem.*, vol. 146, pp. 212–219, 2014.
- [45] Z. Dzuman, M. Vaclavikova, I. Polisenska, Z. Veprikova, M. Fenclova, M. Zachariasova, and J. Hajslova, "Enzyme-linked immunosorbent assay in analysis of deoxynivalenol: investigation of the impact of sample matrix on results accuracy.," *Anal. Bioanal. Chem.*, vol. 406, no. 2, pp. 505–14, 2014.
- [46] H. Du, J. Liu, Y. Xun, J. Liang, S. Li, and G. Chen, "Determination of Deoxynivalenol, Zearalenone, Aflatoxin B1, and Ochratoxin by an Enzyme-Linked Immunosorbent Assay," *Anal. Lett.*, vol. 47, no. 11, pp. 1912–1920, 2014.
- [47] B. H. Liu, Y. T. Hsu, C. C. Lu, and F. Y. Yu, "Detecting aflatoxin B1 in foods and feeds by using sensitive rapid enzyme-linked immunosorbent assay and gold nanoparticle immunochromatographic strip," *Food Control*, vol. 30, no. 1, pp. 184–189, 2013.
- [48] W. Jiang, Z. Wang, G. Nölke, J. Zhang, L. Niu, and J. Shen, "Simultaneous Determination of Aflatoxin B1 and Aflatoxin M1 in Food Matrices by Enzyme-Linked Immunosorbent Assay," *Food Anal. Methods*, vol. 6, no. 3, pp. 767–774, 2013.
- [49] M. Kazemi, A. M. Tahmasbi, R. Valizadeh, A. A. Naserian, and A. Soni, "Organophosphate pesticides: A general review," *Agric. Sci. Res. Journals*, vol. 2, no. 9, pp. 512–522, 2012.
- [50] S. Suratman, J. W. Edwards, and K. Babina, "Organophosphate pesticides exposure among farmworkers: Pathways and risk of adverse health effects," *Rev. Environ. Health*, vol. 30, no. 1, pp. 65–79, 2015.

- [51] F. Sanchez-Santed, M. T. Colomina, E. Herrero Hernandez, "Organophosphate pesticide exposure and neurodegeneration", *Cortex*, vol. 74 pp. 417-426, 2016.
- [52] M. Bayrami, T. Hashemi, A. A. Malekirad, H. Ashayeri, F. Faraji, and M. Abdollahi, "Electroencephalogram, cognitive state, psychological disorders, clinical symptom, and oxidative stress in horticulture farmers exposed to organophosphate pesticides.," *Toxicol. Ind. Health*, vol. 28, no. 1, pp. 90–96, 2012.
- [53] M. T. Muñoz-Quezada, B. A. Lucero, D. B. Barr, K. Steenland, K. Levy, P. B. Ryan, V. Iglesias, S. Alvarado, C. Concha, E. Rojas, and C. Vega, "Neurodevelopmental effects in children associated with exposure to organophosphate pesticides: a systematic review.," *Neurotoxicology*, vol. 39, pp. 158–68, 2013.
- [54] V. Andreu and Y. Picó, "Determination of currently used pesticides in biota", *Anal. Bioanal. Chem.*, vol.404, pp. 2659–2681, 2012.
- [55] D. Sharma, A. Nagpal, Y. B. Pakade, J. K. Katnoria, "Analytical methods for estimation of organophosphorus pesticide residues in fruits and vegetables: A review", *Talanta*, vol. 82, pp. 1077–1089, 2010.
- [56] G. Martínez-Domínguez, P. Plaza-Bolaños, R. Romero-González, A. Garrido-Frenich, "Analytical approaches for the determination of pesticide residues in nutraceutical products and related matrices by chromatographic techniques coupled to mass spectrometry", *Talanta*, vol. 118, pp. 277–291, 2014.
- [57] R. Raina-Fulton, "New Trends in Pesticide Residue Analysis in Food, Dietary Supplements, and Highly Processed Consumer Products", *J. AOAC Int.*, vol. 98, pp. 1163-1170, 2015
- [58] K. Sharafi, N. Fattahi, A. H. Mahvi, M. Pirsaeheb, N. Azizzadeh, and M. Noori, "Trace analysis of some organophosphorus pesticides in rice samples using ultrasound-assisted dispersive liquid-liquid microextraction and high-performance liquid chromatography," *J. Sep. Sci.*, vol. 38, no. 6, pp. 1010–1016, 2015.
- [59] Q. Wang, X. Zhang, Z. Xu, and H. Gao, "Simultaneous Determination of Three Trace Organophosphorus Pesticide Residues in Vegetables Using Molecularly Imprinted Solid-Phase Extraction Coupled with High-Performance Liquid Chromatography," *Food Anal. Methods*, 2015.
- [60] X. Miao, D. Liu, Y. Wang, Y. Yang, X. Yang, and H. Gong, "Modified QuEChERS in Combination with Dispersive Liquid–Liquid Microextraction Based on Solidification of the Floating Organic Droplet Method for the Determination of Organophosphorus Pesticides in Milk Samples," *J. Chromatogr. Sci.*, no. 2, p. bmv089, 2015.
- [61] J. H. Xin, X. G. Qiao, Z. X. Xu, and J. Zhou, "Molecularly Imprinted Polymer as Sorbent for Solid-Phase Extraction Coupling to Gas Chromatography for the Simultaneous Determination of Trichlorfon and Monocrotophos Residues in Vegetables," *Food Anal. Methods*, vol. 6, no. 1, pp. 274–281, 2013.
- [62] Q. Tang, X. Wang, F. Yu, X. Qiao, and Z. Xu, "Simultaneous determination of ten organophosphate pesticide residues in fruits by gas chromatography coupled with magnetic separation.," *J. Sep. Sci.*, vol. 37, no. 7, pp. 820–7, 2014.

- [63] L. Wu, Y. Song, M. Hu, H. Zhang, A. Yu, C. Yu, Q. Ma, and Z. Wang, "Application of magnetic solvent bar liquid-phase microextraction for determination of organophosphorus pesticides in fruit juice samples by gas chromatography mass spectrometry," *Food Chem.*, vol. 176, pp. 197–204, 2015.
- [64] T. Zhou, X. Xiao, G. Li, "Microwave Accelerated Selective Soxhlet Extraction for the Determination of Organophosphorus and Carbamate Pesticides in Ginseng with Gas Chromatography/Mass Spectrometry", *Anal. Chem.*, vol.84, pp. 5816–5822, 2012.
- [65] J.-W. Li, Y.-L. Wang, S. Yan, X.-J. Li, S.-Y. Pan, "Molecularly imprinted calixarene fiber for solid-phase microextraction of four organophosphorous pesticides in fruits", *Food Chem.*, 192 pp. 260–267, 2016.
- [66] D. Ferreira da Silva, F. E. Paiva Silva, F. G. S Silva, G. S. Nunes, M. Badea, "Direct determination of methyl parathion insecticide in rice samples by headspace solid-phase microextraction–gas chromatography–mass spectrometry", *Pest. Manag. Sci.*, vol.71, pp. 1497–1502, 2015.
- [67] J.-C. Wei, J. Hu, J.-L. Cao, J.-B. Wan, C.-W. He, Y.-J. Hu, H. Hu, P. Li, "Sensitive Detection of Organophosphorus Pesticides in Medicinal Plants Using Ultrasound-Assisted Dispersive Liquid–Liquid Microextraction Combined with Sweeping Micellar Electrokinetic Chromatography", *J. Agric. Food Chem.*, vol. 64, pp. 932–940, 2016.
- [68] M. Ishibashi, Y. Izumi, M. Sakai, T. Ando, E. Fukusaki, T. Bamba, "High-Throughput Simultaneous Analysis of Pesticides by Supercritical Fluid Chromatography Coupled with High-Resolution Mass Spectrometry", *J. Agric. Food Chem.*, vol. 63, pp. 4457–4463, 2015.
- [69] A. T. Jan, M. Azam, K. Siddiqui, A. Ali, I. Choi, Q. M. R. Haq, "Heavy Metals and Human Health: Mechanistic Insight into Toxicity and Counter Defense System of Antioxidants", *Int. J. Mol. Sci.*, vol. 16, pp. 29592–29630, 2015.
- [70] H. S. Kim, Y. J. Kim, Y. R. Seo, "An Overview of Carcinogenic Heavy Metal: Molecular Toxicity Mechanism and Prevention", *J. Cancer Prev.*, vol. 20, pp. 232-240, 2015.
- [71] X. Yuan, R. L. Chapman, Zhiqian Wu, "Analytical Methods for Heavy Metals in Herbal Medicines", *Phytochem. Anal.*, vol. 22, pp. 189–198, 2011.
- [72] M. Zaib, M. M. Athar, A. Saeed, U. Farooq, "Electrochemical determination of inorganic mercury and arsenic—A review", *Biosens. Bioelectron.*, vol. 74, pp. 895–908, 2015.
- [73] G. March, T. D. Nguyen, B. Piro, "Modified Electrodes Used for Electrochemical Detection of Metal Ions in Environmental Analysis", *Biosensors*, vol.5, pp. 241-275, 2015.
- [74] Y. I. Korpan, E. A. Nazarenko, I. V. Skryshevskaya, C. Martelet, N. Jaffrezic-Renault, A. V. El'skaya, "Potato glycoalkaloids: true safety or false sense of security?", *Trends in Biotechnology*, Vol. 22(3), pp147–151, 2004.

- [75] T.T. Mensinga, A.J. Sips, C.J. Rompelberg, K. Van Twillert, J. Meulenbelt, H.J. Van den Top, H.P. Van Egmond, "Potato glycoalkaloids and adverse effects in humans: an ascending dose study", *Regul Toxicol Pharmacol.*, vol. 41(1), pp 66-72, 2005.
- [76] I. Ginzberg, J. G. Tokuhisa, R. E. Veilleux, "Potato Steroidal Glycoalkaloids: Biosynthesis and Genetic Manipulation", *Potato Research*, vol. 52, pp 1–15, 2009. DOI 10.1007/s11540-008-9103-4
- [77] F. Matsuda, K. Morino, H. Miyazawa, M. Miyashita, H. Miyagawa, "Determination of potato glycoalkaloids using high-pressure liquid chromatography-electrospray ionisation/mass spectrometry", *Phytochem Anal.*, vol. 15(2), pp 121-4, 2004.
- [78] P. Abreua, A. Relvaa, S. Matthewa, Z. Gomesb, Z. Moraisd, "High-performance liquid chromatographic determination of glycoalkaloids in potatoes from conventional, integrated, and organic crop systems", *Food Control*, vol. 18(1), pp 40–44, 2007.
- [79] G. CaprioliI, M. G. CahillIII, K. J. JamesII, S. VittoriI, "Liquid chromatography-orbitrap mass spectrometry method for the determination of toxic glycoalkaloids and their aglycons in potato upper soil", *J. Braz. Chem. Soc.*, vol.25 (9), pp 1681-1687, 2014.
- [80] E. Olkowska, Ż. Polkowska, J. Namieśnik, "Analytical procedures for the determination of surfactants in environmental samples", *Talanta*, vol. 88, pp 1-13, 2012.
- [81] E. Jurado, M. Fernandez-Serrano, J. Nunez-Olea, G. Luzon, M. Lechuga, "Simplified spectrophotometric method using methylene blue for determining anionic surfactants: Applications to the study of primary biodegradation in aerobic screening tests", *Chemosphere*, vol. 65, pp 278–285, 2006.
- [82] Y. Yokoyama, E. Tai, H.Sato, "Spectrometric determination of anionic surfactants in environmental waters based on anisole extraction of their bis[2-(5-chloro-2-pyridylazo)-5-diethylaminophenolato]cobalt(III) ion pairs.", *Anal Sci.*, vol. 27(8), pp 845-9, 2011.
- [83] M. Beneito-Cambra, J. M. Herrero-Martinez and G. Ramis-Ramos, "Analytical methods for the characterization and determination of nonionic surfactants in cosmetics and environmental matrices", *Anal. Methods*, vol. 5, pp 341-354, 2013.
- [84] M. Zahrobsky, D. Camporese, O. Rist, P.H. Carlsen, "HPLC analysis of aliphatic sulfonate surfactants using ion-pair detection.", *Molecules.*, vol. 10(9), pp 1179-1189, 2005.
- [85] V. Scognamiglio, F. Arduini, G. Palleschi, G. Rea, "Biosensing technology for sustainable food safety", *Trends Anal. Chem.*, vol. 62, pp. 1-10, 2014.
- [86] T.F. McGrath, C.T. Elliott, T.L. Fodey, "Biosensors for the analysis of microbiological and chemical contaminants in food", *Anal. Bioanal. Chem.*, vol. 403, pp. 75–92, 2012
- [87] V. Perumal, U. Hashim, "Advances in biosensors: Principle, architecture and applications", *J. Appl. Biomed.*, vol 12, pp.1-15, 2014.
- [88] Ronkainen, N.J., Halsall, H.B., Heineman, W.R., 2010. Electrochemical biosensors. *Chem. Soc. Rev.* 39, 1747-1763.

- [89] Hayat, A., Catanante, G., Marty, J.L., 2014. Current trends in nanomaterial-based amperometric biosensors. *Sensors* 14, 23439-23461.
- [90] A. Bratov, N. Abramova, A. Ipatov, "Recent trends in potentiometric sensor arrays-A review", *Anal. Chim. Acta*, vol.678, pp.149-159, 2010.
- [91] N. Jaffrezic-Renault and S.V. Dzyadevych, "Conductometric microbiosensors for environmental monitoring", *Sensors*, vol. 8, pp.2569-2588, 2008.
- [92] E. P. Randviir and C. E. Banks, "Electrochemical impedance spectroscopy: an overview of bioanalytical applications", *Anal. Methods*, vol.5, pp. 1098-1115, 2013.
- [93] K. Narsaiah, S. N. Jha, R. Bhardwaj, R. Sharma, R. Kumar, "Optical biosensors for food quality and safety assurance—a review", *J. Food Sci. Technol.*, vol. 49, pp.383–406, 2012.
- [94] Y. Zhang, S. Tadigadapa, "Calorimetric biosensors with integrated microfluidic channels", *Biosensors and Bioelectronics*, vol. 19, pp 1733–1743, 2004.
- [95] K. Länge, B.E. Rapp, M. Rapp, "Surface acoustic wave biosensors: a review", *Anal Bioanal Chem* (2008) 391:1509–1519
- [96] V. Scognamiglio, "Nanotechnology in glucose monitoring: Advances and challenges in the last 10 years", *Biosens. Bioelectron.*, vol. 47, pp.12–25. 2013
- [97] B. Perez-Lopez, A. Merkoci, "Nanomaterials based biosensors for food analysis applications", *Trends Food Sci. Technol.*, vol. 22, pp. 625-639, 2011.
- [98] V. K. Nigam, P. Shukla, "Enzyme Based Biosensors for Detection of Environmental Pollutants. A Review", *J. Microbiol. Biotechnol.*, vol. 25, pp. 1773–1781, 2015.
- [99] R. Monosik, M. Stredansky, J. Tkac, E. Sturdik, "Application of enzyme biosensors in analysis of food and beverages", *Food Anal. Methods*, vol. 5, pp. 40–53, 2012.
- [100] S. Dzyadevych, N. Jaffrezic-Renault, "6 – Conductometric biosensors", *Biological Identification of DNA Amplification and Sequencing, Optical Sensing, Lab-On-chip and Portable Systems*, pp. 153–193, 2014
- [101] N. J. Ronkainen, H. B. Halsall and W. R. Heineman, "Electrochemical biosensors", *Chem. Soc. Rev.*, vol. 39, pp. 1747–1763, 2010.
- [102] S. V. Dzyadevych, V.N. Arkhypova, A. P. Soldatkin, A. V. El'skaya, C. Martelet, N. Jaffrezic-Renault, "Conductometric Enzyme Biosensors", Part Four. *Transducer Technologies for Biosensors, Handbook of Biosensors and Biochips*. Edited by Robert S. Marks, David C. Cullen, Isao Karube, Christopher R. Lowe and Howard H. Weetall., 2008 DOI: 10.1002/9780470061565.hbb037

- [103] I. Žuraa, D. Babićb, M. D. Steinbergc, I. Murković Steinberg, “Low-cost conductometric transducers for use in thin polymer film chemical sensors”, *Sensors and Actuators B: Chemical*, vol. 193, pp. 128–135, 2014.
- [104] D. W. Kimmel, G. LeBlanc, M. E. Meschievitz, and D. E. Cliffel, “Electrochemical Sensors and Biosensors”, *Anal. Chem.*, vol. 84, pp. 685–707, 2012
- [105] J. X. Zhang and K. Hoshino, “Molecular Sensors and Nanodevices, Principles, Designs and Applications in Biomedical Engineering”, Chapter 4 - Electrical Transducers: Electrochemical Sensors and Semiconductor Molecular Sensors, pp. 169–232, 2014
- [106] A. Sassolas, L.J. Blum, B.D. Leca-Bouvier, "Immobilization strategies to develop enzymatic biosensors", *Biotechnol. Adv.*, vol.30, pp. 489–511, 2012.
- [107] D. Baratella, M. Magro, G. Sinigaglia, R. Zboril, G. Salviulo, and F. Vianello, “A glucose biosensor based on surface active maghemite nanoparticles,” *Biosens. Bioelectron.*, vol. 45, no. 1, pp. 13–18, 2013.
- [108] H. D. Jang, S. K. Kim, H. Chang, K. M. Roh, J. W. Choi, and J. Huang, “A glucose biosensor based on TiO₂-Graphene composite,” *Biosens. Bioelectron.*, vol. 38, no. 1, pp. 184–188, 2012.
- [109] B. Unnikrishnan, S. Palanisamy, and S. M. Chen, “A simple electrochemical approach to fabricate a glucose biosensor based on graphene-glucose oxidase biocomposite,” *Biosens. Bioelectron.*, vol. 39, no. 1, pp. 70–75, 2013.
- [110] M. Tyagi, M. Tomar, and V. Gupta, “NiO nanoparticle-based urea biosensor,” *Biosens. Bioelectron.*, vol. 41, no. 1, pp. 110–115, 2013.
- [111] S. V. Marchenko, I. S. Kucherenko, O. O. Soldatkin, and A. P. Soldatkin, “Potentiometric biosensor system based on recombinant urease and creatinine deiminase for urea and creatinine determination in blood dialysate and serum,” *Electroanalysis*, vol. 27, pp. 1–9, 2015.
- [112] V. Serafín, P. Hernández, L. Agüí, P. Yáñez-Sedeno, and J. M. Pingarrón, “Electrochemical biosensor for creatinine based on the immobilization of creatininase, creatinase and sarcosine oxidase onto a ferrocene/horseradish peroxidase/gold nanoparticles/multi-walled carbon nanotubes/Teflon composite electrode,” *Electrochim. Acta*, vol. 97, pp. 175–183, 2013.
- [113] C. S. Pundir, S. Yadav, and A. Kumar, “Creatinine sensors,” *TrAC - Trends Anal. Chem.*, vol. 50, pp. 42–52, 2013.
- [114] I. Isildak, O. Cubuk, M. Altikatoglu, M. Yolcu, V. Erci, and N. Tinkilic, “A novel conductometric creatinine biosensor based on solid-state contact ammonium sensitive PVC-NH₂ membrane,” *Biochem. Eng. J.*, vol. 62, pp. 34–38, 2012.
- [115] X. Shang, L. Luo, K. Ren, X. Wei, Y. Feng, X. Li, and X. Xu, “Synthesis and cytotoxicity of azo nano-materials as new biosensors for L-Arginine determination,” *Mater. Sci. Eng. C*, vol. 51, pp. 279–286, 2015.

- [116] M. Sheliakina, V. Arkhypova, O. Soldatkin, O. Saiapina, B. Akata, and S. Dzyadevych, "Urease-based ISFET biosensor for arginine determination," *Talanta*, vol. 121, pp. 18–23, 2014.
- [117] S. Hou, Z. Ou, Q. Chen, and B. Wu, "Amperometric acetylcholine biosensor based on self-assembly of gold nanoparticles and acetylcholinesterase on the sol-gel/multi-walled carbon nanotubes/choline oxidase composite-modified platinum electrode," *Biosens. Bioelectron.*, vol. 33, no. 1, pp. 44–49, 2012.
- [118] A. I. Journal, A. T. Tunç, E. A. Koyuncu, and F. Arslan, "Development of an acetylcholinesterase – choline oxidase based biosensor for acetylcholine determination", *Artif. Cells, Nanomedicine, Biotechnol.*, vol. 1401, no. October, 2015.
- [119] Vidal, J.C., Bonel, L., Ezquerro, A., Hernández, S., Bertolín, J.R., Cubel, C., Castillo, J.R., 2013. Electrochemical affinity biosensors for detection of mycotoxins: A review, *Biosens. Bioelectron.* 49, 146–158.
- [120] L. Reverte, B. Prieto-Simon, M. Campas, " New advances in electrochemical biosensors for the detection of toxins: Nanomaterials, magnetic beads and microfluidics systems. A review", *Analytica Chim. Acta*, vol. 908, pp. 8-21, 2016.
- [121] F. Dridi, M. Marrakchi, M. Gargouri, A. Garcia-Cruz, S. Dzyadevych, F. Vocanson, J. Saulnier, N. Jaffrezic-Renault, F. Lagarde, "Thermolysin entrapped in a gold nanoparticles/polymer composite for direct and sensitive conductometric biosensing of ochratoxin A in olive oil", *Sens. Actuators B*, vol. 221, pp. 480-490, 2015.
- [122] M.A. Alonso-Lomillo, O. Domínguez-Renedo, L.D.T. Román, M.J. Arcos-Martínez, "Horseradish peroxidase-screen printed biosensors for determination of Ochratoxin A", *Anal. Chim. Acta*, vol. 688, pp. 49–53, 2011.
- [123] M.A. Alonso-Lomillo, O. Domínguez-Renedo, L. Ferreira-Gonçalves, M.J. Arcos-Martínez, "Sensitive enzyme-biosensor based on screen-printed electrodes for Ochratoxin A", *Biosens. Bioelectron.*, vol. 25, pp. 1333–1337, 2010.
- [124] S.C. Li, J.H. Chen, H. Cao, D.S. Yao, D.L. Liu, "Amperometric biosensor for aflatoxin B-1 based on aflatoxin-oxidase immobilized on multiwalled carbon nanotubes", *Food Control*, vol. 22, pp. 43-49, 2011.
- [125] W. Zhang, A.M. Asiri, D. Liu, D. Du, Y. Lin, "Nanomaterial-based biosensors for environmental and biological monitoring of organophosphorus pesticides and nerve agents", *Trends Anal. Chem.*, vol. 54, pp.1–10, 2014.
- [126] P. Kumar, K.-H. Kim, A. Deep, "Recent advancements in sensing techniques based on functional materials for organophosphate pesticides", *Biosens. Bioelectron.*, vol. 70, pp. 469–481, 2015.
- [127] A. Sassolas, B. Prieto-Simón, J.-L. Marty, "Biosensors for Pesticide Detection: New Trends", *Am. J. Anal. Chem.*, vol. 3, pp. 210-232, 2012.
- [128] J. Mehta, S. K. Bhardwaj, N. Bhardwaj, A.K. Paul, P. Kumar, K.-H. Kim, A. Deep, "Progress in the biosensing techniques for trace-level heavy metals", *Biotechnol. Adv.*,

vol. 34, pp. 47–60, 2016.

- [129] M. B. Gumpu, S. Sethuraman, U. M. Krishnan, J. B. B Rayappan, "A review on detection of heavy metal ions in water – An electrochemical approach", *Sens. Actuators B*, vol. 213 pp. 515–533, 2015.
- [130] M. Gopal, A. Bhaskaran, W. I Khalife, and A. Barbagelata, "Heart Disease in Patients with HIV/AIDS-An Emerging Clinical Problem", *Curr Cardiol Rev.*, vol. 5(2), pp 149–154, 2009. doi: 10.2174/157340309788166705
- [131] K. Bullard Dunn, M. Heffler, and V. Golubovskaya, "Evolving Therapies and FAK Inhibitors for the Treatment of Cancer", *Anticancer Agents Med Chem.* vol. 10(10), pp 722–734, 2010.
- [132] E. B Friedrich and M. Böhm, "Management of end stage heart failure", *Heart.* vol. 93(5), pp 626–631, 2007. doi: 10.1136/hrt.2006.098814
- [133] A.V. Herrera-Herrera, M. Asensio-Ramos, J. Hernández-Borges, M.Á. Rodríguez-Delgado, "Pesticides and Herbicides: Types, Uses, and Determination of Herbicides", *Encyclopedia of Food and Health*, pp 326–332, 2016. doi:10.1016/B978-0-12-384947-2.00536-5
- [134] A. Ata, S. Naz and E. M. Elias, "Naturally occurring enzyme inhibitors and their pharmaceutical applications", *Pure Appl. Chem.* vol. 83(9), pp 1741–1749, 2011. doi:10.1351/PAC-CON-10-11-16
- [135] Wu KK, "Aspirin and other cyclooxygenase inhibitors: new therapeutic insights", *Semin Vasc Med.* vol. 3(2), pp 107-12, 2003.
- [136] A. Amine, F. Arduini, D. Moscone, and G. Palleschi, "Recent Advances in Biosensors Based on Enzyme Inhibition," *Biosens. Bioelectron.*, vol.76, pp.180-194, 2016.
- [137] C. P. L. Guilbault G.G., Kramer D.N., "Electrochemical Determination of Organophosphorous Compounds," *Anal. Chem.*, vol. 34, no. 11, pp. 1437–1439, 1962.
- [138] L. S. B. Upadhyay and N. Verma, "Enzyme Inhibition Based Biosensors: A Review," *Anal. Lett.*, vol. 46, p. 225-241, 2013.
- [139] A. L. Alvarado-Gámez, M. A. Alonso-Lomillo, O. Domínguez-Renedo, and M. J. Arcos-Martínez, "Vanadium determination in water using alkaline phosphatase based screen-printed carbon electrodes modified with gold nanoparticles," *J. Electroanal. Chem.*, vol. 693, pp. 51–55, 2013.
- [140] A. Samphao, P. Suebsanoh, Y. Wongsa, B. Pecec, J. Jitchareon, K. Kalcher, "Alkaline Phosphatase Inhibition-Based Amperometric Biosensor for the Detection of Carbofuran", *Int. J. Electrochem. Sci.*, vol. 8, pp. 3254 - 3264, 2013.
- [141] L. S. B. Upadhyay, N. Verma, "Alkaline phosphatase inhibition based conductometric biosensor for phosphate estimation in biological fluids", *Biosens. Bioelectron.*, vol. 68, pp. 611–616, 2015.
- [142] E. Akyilmaz and M. Turemis, "An inhibition type alkaline phosphatase biosensor for

- amperometric determination of caffeine,” *Electrochim. Acta*, vol. 55, no. 18, pp. 5195–5199, 2010.
- [143] H. Chen, C. Mousty, L. Chen, and S. Cosnier, “A new approach for nitrite determination based on a HRP/catalase biosensor,” *Mater. Sci. Eng. C*, vol. 28, no. 5–6, pp. 726–730, 2008.
- [144] Biosensor- M. Cuccioloni, M. Mozzicafreddo, S. Barocci, F. Ciuti, I. Pecorelli, A. M. Eleuteri, M. Spina, E. Fioretti, M. Angeletti, "Biosensor-Based Screening Method for the Detection of Aflatoxins B1-G1, *Anal. Chem.*, vol. 80, pp. 9250–9256, 2008.
- [145] M. E. Ghica, R. C. Carvalho, A. Amine, and C. M. A. Brett, “Glucose oxidase enzyme inhibition sensors for heavy metals at carbon film electrodes modified with cobalt or copper hexacyanoferrate,” *Sens. Actuators B Chem.*, vol. 178, pp. 270–278, 2013.
- [146] J.G. Ayenimo and S. B.Adeloju, "Rapid amperometric detection of trace metals by inhibition of an ultrathinpolypyrrole-based glucose biosensor", *Talanta*, vol.148, pp. 502–510, 2016.
- [147] A. Attar, M. Emilia Ghica, A. Amine, and C. M. A. Brett, “Poly(neutral red) based hydrogen peroxide biosensor for chromium determination by inhibition measurements,” *J. Hazard. Mater.*, vol. 279, pp. 348–355, 2014.
- [148] D. Shan, Q. Li, H. Xue, and S. Cosnier, “A highly reversible and sensitive tyrosinase inhibition-based amperometric biosensor for benzoic acid monitoring,” *Sensors Actuators, B Chem.*, vol. 134, no. 2, pp. 1016–1021, 2008.
- [149] D. Shan, Q. Shi, D. Zhu, and H. Xue, “Inhibitive detection of benzoic acid using a novel phenols biosensor based on polyaniline-polyacrylonitrile composite matrix.,” *Talanta*, vol. 72, no. 5, pp. 1767–72, 2007.
- [150] X. Zeng, J. Liu, Z. Zhang, S. Kong, "Sensitive and Selective Detection of Mercury Ions by Potentiometric Biosensor Based on Urease Immobilized in Chitosan–Poly(Vinyl Alcohol) Hydrogel Film", *Int. J. Electrochem. Sci.*, vol. 10, p. 8344 - 8352, 2015.
- [151] Y.Braham, H. Barhoumi, Abderrazak Maaref, "Urease capacitive biosensors using functionalized magnetic nanoparticles for atrazine pesticide detection in environmental samples", *Anal. Methods*, vol. 5, pp. 4898-4904, 2013.
- [152] D. M. Quinn, “Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states,” *Chem. Rev.*, vol. 87, no. 5, pp. 955–979, 1987.
- [153] M. Pohanka, “Cholinesterases in Biorecognition and Biosensors Construction: A Review,” *Anal. Lett.*, vol. 46, no. 12, pp. 1849–1868, 2013.
- [154] M. B. Colovic, D. Z. Krstic, T. D. Lazarevic-Pasti, A. M. Bondzic, V.M. Vasic, "Acetylcholinesterase Inhibitors: Pharmacology and Toxicology", *Current Neuropharmacology*, vol. 11, pp. 315-335, 2013.
- [155] C. S. Pundir and N. Chauhan, “Acetylcholinesterase inhibition-based biosensors for pesticide determination: A review,” *Anal. Biochem.*, vol. 429, no. 1, pp. 19–31, 2012.

- [156] V. Dhull, A. Gahlaut, N. Dilbaghi, and V. Hooda, "Acetylcholinesterase biosensors for electrochemical detection of organophosphorus compounds: A review," *Biochem. Res. Int.*, vol. 2013, pp. 1–18, 2013.
- [157] W. Zhang, A. M. Asiri, D. Liu, D. Du, Y. Lin, "Nanomaterial-based biosensors for environmental and biological monitoring of organophosphorus pesticides and nerve agents", *Trends Anal. Chem.*, vol. 54, pp. 1–10, 2014.
- [158] N. Xia, Y. Gao, "Carbon Nanostructures for Development of Acetylcholinesterase Electrochemical Biosensors for Determination of Pesticides", *Int. J. Electrochem. Sci.*, vol. 10, pp. 713 - 724, 2015.
- [159] H. Zhao, X. Ji, B. Wang, N. Wang, X. Li, R. Ni, and J. Ren, "An ultra-sensitive acetylcholinesterase biosensor based on reduced graphene oxide-Au nanoparticles- β -cyclodextrin/Prussian blue-chitosan nanocomposites for organophosphorus pesticides detection", *Biosens. Bioelectron.*, vol. 65, pp. 23–30, 2015.
- [160] H. Guan, F. Zhang, J. Yu, and D. Chi, "The novel acetylcholinesterase biosensors based on liposome bioreactors–chitosan nanocomposite film for detection of organophosphates pesticides", *Food Res. Int.*, vol. 49, no. 1, pp. 15–21, 2012.
- [161] J. Gong, Z. Guan, and D. Song, "Biosensor based on acetylcholinesterase immobilized onto layered double hydroxides for flow injection/amperometric detection of organophosphate pesticides", *Biosens. Bioelectron.*, vol. 39, no. 1, pp. 320–323, 2013.
- [162] N. Ben Oujji, I. Bakas, G. Istamboulié, I. Ait-Ichou, E. Ait-Addi, R. Rouillon, and T. Noguier, "Sol-gel immobilization of acetylcholinesterase for the determination of organophosphate pesticides in olive oil with biosensors", *Food Control*, vol. 30, no. 2, pp. 657–661, 2013.
- [163] M. Wei, G. Zeng, and Q. Lu, "Determination of organophosphate pesticides using an acetylcholinesterase-based biosensor based on a boron-doped diamond electrode modified with gold nanoparticles and carbon spheres," *Microchim. Acta*, vol. 181, pp. 121–127, 2014.
- [164] J. Yan, H. Guan, J. Yu, and D. Chi, "Acetylcholinesterase biosensor based on assembly of multiwall carbon nanotubes onto liposome bioreactors for detection of organophosphates pesticides," *Pestic. Biochem. Physiol.*, vol. 105, no. 3, pp. 197–202, 2013.
- [165] M. Wei and J. Wang, "A novel acetylcholinesterase biosensor based on ionic liquids-AuNPs-porous carbon composite matrix for detection of organophosphate pesticides", *Sens. Actuators B*, vol. 211, pp. 290–296, 2015.
- [166] A. Amine, H. Mohammadi, I. Bourais, G. Palleschi, "Enzyme inhibition-based biosensors for food safety and environmental monitoring", *Biosens Bioelectron.*, vol. 15;21(8), pp 1405-23, 2006.
- [167] L. D. Mell and J. T. Maloy, "A model for the amperometric enzyme electrode obtained through digital simulation and applied to the immobilized glucose oxidase system," *Anal. Chem.*, vol. 47, no. 2, pp. 299–307, 1975.

- [168] N. Gajovic, A. Warsinke, T. Huang, T. Schulmeister, and F. W. Scheller, "Characterization and Mathematical Modeling of a Bienzyme Electrode for l -Malate with Cofactor Recycling," *Anal.Chem.*, vol. 71, no. 20, pp. 4657–4662, 1999.
- [169] M. R. Romero, A. M. Baruzzi, and F. Garay, "Mathematical modeling and experimental results of a sandwich-type amperometric biosensor," *Sensors Actuators, B Chem.*, vol. 162, no. 1, pp. 284–291, 2012.
- [170] S. Loghambal and L. Rajendran, "Mathematical modeling of diffusion and kinetics in amperometric immobilized enzyme electrodes," *Electrochim. Acta*, vol. 55, no. 18, pp. 5230–5238, 2010.
- [171] S. Loghambal and L. Rajendran, "Mathematical modeling in amperometric oxidase enzyme-membrane electrodes," *J. Memb. Sci.*, vol. 373, no. 1–2, pp. 20–28, 2011.
- [172] A. Meena and L. Rajendran, "Mathematical modeling of amperometric and potentiometric biosensors and system of non-linear equations - Homotopy perturbation approach," *J. Electroanal. Chem.*, vol. 644, no. 1, pp. 50–59, 2010.
- [173] V. Ašeris, E. Gaidamauskaitė, J. Kulys, and R. Baronas, "Modelling glucose dehydrogenase-based amperometric biosensor utilizing synergistic substrates conversion," *Electrochim. Acta*, vol. 146, pp. 752–758, 2014.
- [174] V. Ašeris, R. Baronas, and J. Kulys, "Modelling the biosensor utilising parallel substrates conversion," *J. Electroanal. Chem.*, vol. 685, pp. 63–71, 2012.
- [175] K. V. Stepurska, O. O. Soldatkin, I. S. Kucherenko, V. M. Arkhypova, S. V. Dzyadevych, and a. P. Soldatkin, "Feasibility of application of conductometric biosensor based on acetylcholinesterase for the inhibitory analysis of toxic compounds of different nature," *Anal. Chim. Acta*, vol. 854, pp. 161–168, 2015.
- [176] F. Achi, S. Bourouina-Bacha, M. Bourouina, and a. Amine, "Mathematical model and numerical simulation of inhibition based biosensor for the detection of Hg(II)," *Sensors Actuators B Chem.*, vol. 207, pp. 413–423, 2015.
- [177] G. Dimarco, L. Pareschi, "Numerical methods for kinetic equations", *Acta Numerica*, Cambridge University Press (CUP), pp.369-520, 2014.
- [178] B. Moser and J. Rayburn, "Evaluation of developmental toxicity of interaction between caffeine and pseudoephedrine using frog embryo teratogenesis assay-Xenopus (Fetax)", *BIOS*, 78(1), pp. 1-9, 2007.
- [179] A. Amine, L. El Harrad, F. Arduini, D. Moscone, and G. Palleschi, "Analytical aspects of enzyme reversible inhibition," *Talanta*, vol. 118, pp. 368–374, 2014.
- [180] G. A. Zhylyak, S. V. Dzyadevich, Y. I. Korpan, A. P. Soldatkin, and A. V. El'skaya, "Application of urease conductometric biosensor for heavy-metal ion determination," *Sensors Actuators B. Chem.*, vol. 24, no. 1–3, pp. 145–148, 1995.

- [181] O. O. Soldatkin, I. S. Kucherenko, V. M. Pyeshkova, A. L. Kukla, N. Jaffrezic-Renault, A. V. El'skaya, S. V. Dzyadevych, and A. P. Soldatkin, "Novel conductometric biosensor based on three-enzyme system for selective determination of heavy metal ions," *Bioelectrochemistry*, vol. 83, no. 1, pp. 25–30, 2012.
- [182] A. P. Soldatkin, V. N. Arkhypova, S. V. Dzyadevych, A. V. El'Skaya, J. M. Gravouelle, N. Jaffrezic-Renault, and C. Martelet, "Analysis of the potato glycoalkaloids by using of enzyme biosensor based on pH-ISFETs," *Talanta*, vol. 66, no. 1, pp. 28–33, 2005.
- [183] H. Akiyama, Y. Goda, T. Tanaka, and M. Toyoda, "Determination of aflatoxins B1, B2, G1 and G2 in spices using a multifunctional column clean-up," *J. Chromatogr. A*, vol. 932, no. 1–2, pp. 153–157, 2001.