**Charles University in Prague**
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# Disposable-miniaturized electrochemical immunosensor to determinate zearalenone in infant food

(diploma thesis)

In cooperation with
Universidad de Alcalá
Facultad de Farmacia
Departamento de Química Analítica e Ingeniería Química

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Alcalá de Henares, Hradec Králové 2008/2009 Tereza Čtvrtečková

**Univerzita Karlova v Praze**

**Farmaceutická fakulta v Hradec Králové**

**Katedra analytické chemie**

# Miniaturizovaný elektrochemický imunosensor ke stanovení zearalenonu v kojeneckých potravinách

(diplomová práce)

Ve spolupráci s

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I declare that I worked up this diploma thesis individually and all the literature and data are mentioned in the list of literature.

15th May 2009, in Hradec Králové ..........................................

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# 2. LIST OF ABBREVIATIONS

- **Ab**...................................Antibody
- **ACN**..................................Acetonitrile
- **Ag**....................................Antigen
- **AOAC**................................Association of Official Agricultural Chemists
- **BSA**....................................Bovine Serum Albumine
- **CRM**.................................Certified Reference Material
- **DPV**...................................Different Pulse Voltammetry
- **ELISA**................................Enzyme-Linked Immunosorbent Assay
- **FAO**...................................Food and Agriculture Organization
- **HPLC**................................High Performance Liquid Chromatography
- **HQN**..................................Hydroquinone
- **HRP**...................................Horseradish Peroxidase
- **IA**.......................................Immunoassay
- **LOD**................................. Limit of Detection
- **PBS**...................................Phosphate Buffered Saline
- **PBS-T**...............................Phosphate Buffered Saline - Tween
- **SPE**...................................Screen Printed Electrode
- **TLC**...................................Thin Layer Chromatography
- **ZEA**...................................Zearalenone
- **Z-HRP**...............................ZEA conjugated to Horseradish Peroxidase

# 3. INTRODUCTION AND AIM OF WORK

Unavoidably, natural contaminants in foods may have either chemical or biological origin. Mycotoxins-toxic secondary metabolites of fungi-are biological in origin. Despite efforts to control fungal contamination, toxigenic fungi are ubiquitous in nature and occur regularly in worldwide food supplies due to mold infestation of susceptible agricultural products, such as cereal grains, nuts, and fruits. Thousands of mycotoxins exist, but only a few present significant food safety challenges. The natural fungal flora associated with foods is dominated by three genera-*Aspergillus, Fusarium, and Penicillium.*

When present in foods in sufficiently high levels, these fungal metabolites can have toxic effects that range from acute (for example, liver or kidney deterioration), to chronic (for example, liver cancer), mutagenic, and teratogenic; and resulting symptoms range from skin irritation to immunosuppression, birth defects, neurotoxicity, and death.[1]

The importace of mycotoxin monitoring was first recognized in 1960s with the detection of the afalatoxins. Since then, analysis has evolved considerably also for the most predominant *Fusarium* toxins zearalenone (ZON), the fumonisins (FB´s), moniliformin (MON), and for the type-A and –B trichothecenes, particularly for deoxynivalenol (DON). Because most of these mycotoxins are a potential health risk to farm animals, several countries have regulated mycotoxins in feed at maximum tolerable levels. The need to comply with these regulations has increased the interest in the development of robust and reliable instrumental analytical methods and rapid test systems for in-field application.[2]

There is an increasing awareness of the hazards imposed on both human and animal health by mycotoxins present in food and feed. Legislation regarding the allowed levels of mycotoxins present in food and feed products and in raw materials is presented by the FAO in 1995 (FAO, 1995). Therefore, a need exists for a reliable, economical and easy to use assay for the measurement of the mycotoxin contents, especially in the raw materials for food and feed production. Common methods for mycotoxin determination include TLC, ELISA and HPLC. Several methods using one of these detection techniques have AOAC approval. Especially the HPLC method is a very sensitive method, which however is also the most costly and time consuming due to extraction and cleanup procedures necessary. [3]

runs different biological mechanisms, which act with goal to eliminate possible adverse products from presence of the mentioned molecules. [5]

Antibodies are heterogenous hyalomucoid molecules, which due to their electrophoresis mobility go under globulins, fraction β to γ, called immunogolobulins. Those can be polyclonal (formed with a couple of B-lymphocytes clones; with no-equivalent binding sites at all molecules) or monoclonal (with equivalent binding sites). Those are proteins with characteristic structure and specific binding sites for antigens. [6]

Typical structural attributes of an antibody molecule_are figured at Fig.1. The basic structure of antibodies consist of four polypeptide chains (150 kDa), united through disulfide bridges. [5]

Two heavy chains (H, 2x50 kDa) are connected with disulfide bridges. One light chain (L, 25kDa) is connected to every heavy chain one with cystine bridge. Heavy chains are composed of four, by some of five domains, which are structurally similar, formed with sequence of 110-120 amino-acids. Particular domains are joint with short sections of polypeptide chain. Light chains are compound of two immunoglobulin domains. Domains by the N-end of heavy as well as of light chain are "variable" and are called VH and VL. Definition of variable means that the structural details are individually different among molecules produced by varied clones of B-lymphocytes. Constant domains of light chains are named CL and domains of heavy chains CH1, CH2, CH3, eventually CH4, where numeration goes from N-end to C-end. Indexes L and H are replaced by marking with concrete types of chains. There are two types of light chains, namely κ and λ, which differ from each other by primary structure of constant domains and are coded by different genes situated on varied chromosomes. Constant sections of different tracers (classes) of heavy chains are coded by sections of genes organized on a single section of the chromosome. These heavy chains are called μ, δ, γ, α and ε. For the chains γ exist four subtypes γ1-γ4, for the chains α two subtypes α1 and α2. [7]

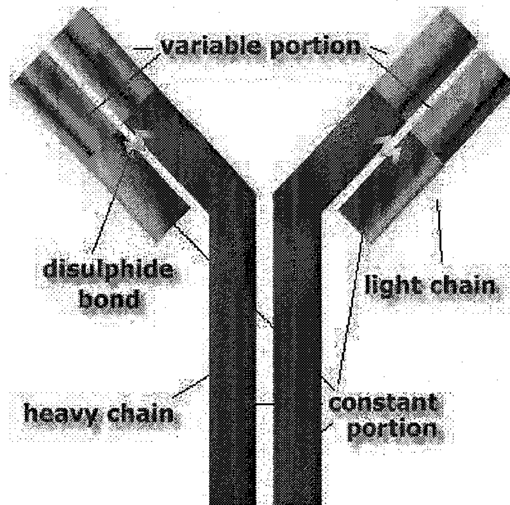



**Fig. 1**: Antibody structure [8]

Immunoglobulins formed by these different types of chains are called IgM, IgD, IgG (IgG1-IgG4), IgA (IgA1, IgA2) a IgE.[7] IgG immunoglobulins are the most used in immunoassay.[5] Chains $\gamma$ and $\alpha$ have three constant domains and other heavy chains four constant domains. Any type of H-chain can be coupled with any type of the light chain and form a full molecule of immunoglobulin. Binding sites for antigen are together created by variable domains of H and L-chains.

The molecule of immunoglobulin can be proteolytically dissociated into fragments, where we can get, in case of using papain-enzyme, two identical fragments called Fab (each of them includes one binding site for antigen, they are monovalent) and fragment Fc. In case of using pepsin enzyme, originate one bivalent fragment F(ab′)2 and the rest of molecule is dissociated by the enzyme into small pieces. The area where heavy chains are joint by cystine bridges is called hinge region and gives away definite flexibility to Fab fragments. Because of this, it is possible to change distance between binding sites promptly, depending on availability of the antigen. Heavy chains are considerably glycoside in section of Fc, that reflects that the immunoglobulins are, almost as all secreted proteins, hyalomucoids..

IgM and IgD as monomers can be found on the surface of B-lymphocytes and create BCR. Secreted IgM exists as pentamer (cca 900 kDa), where particular base units are put together by cystine bridges and by a structurally very different chain

called J. Pentamer IgM, molecule with ten binding sites for antigen. Chains μ are heavily glycoside. IgM is first isotope from antibodies, which is created after meeting with antigen. After are created another isotopes (IgG, IgA, IgE). Largest serum isotope is IgG (150 kDa), especially IgG1. IgA occurs in mucosal and serum form, where IgA consists of two monomers joint with J-chain and secretive component. IgE occurs in healthy persons in slight concentrations. It largely asserts itself against multicellular parasites on mucos and is a cause of atopic allergic reactions. [7]

### 4.1.2. Production of antibodies

Organism starts to product antibodies after a contact with the antigen, which is a result of the natural immunity, or proceeds after artificially implemented antigen by vaccination. Nowadays, animals are immunised for the preparation of polyclonal antibodies.[6] Polyclonal antibodies are complexes made of different types of antibodies. Monoclonal antibodies are identical molecules with the same specificity.[5] They are produced by artificial cellular cultures (hybridoms), which are obtained by immunisation with appropriate antigen and junction of immunity cells with tumor cells.[6]

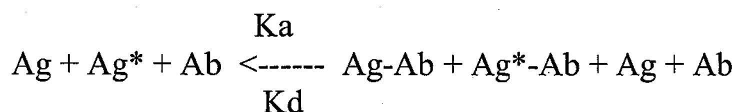
Not all molecules are capable of producing antibodies, this ability is related to the size of molecule. Immunogens are substances of heavy molecular weigh which are recognized by the organism as extraneous substances, starts in the same immunity response, with corresponding production of antibodies. On the contrary, haptens are substances of low molecular weigh, which therefore do not produce immunity response in organism.[5]

## 4.2. Classification of immunoanalytical methods

As described above, this method is determined by the interaction antigen-antibody. However, differing from others biological interactions such as enzyme catalysis, this reaction does not generate any chemical conversion. This highly complicates its detection and it also explains the existence of numerous strategies of transduction, which generate different criteria and classification of immunoanalytical methods. This way, although they are techniques able to measure the formation of complex antigen-antibody directly (mass changes or index of refraction), the main part of immunoanalytical methods represents the use of marker. The marker carries out measurable signal, which can be related to a grade of interactions between antigen and antibody. Nature of marker gives place to different methods. It can be radioactive molecule, fluorescence, electroactive molecule or enzyme.

Other fundamental criterion allows us to classify methods on the basis, if what is measured are the binding sites of antibody occupied or non-occupied by the analyte. In this way, it is possible to differ the competitive and non-competitive methods.

In the competitive assay is competing samples antigen (Ag) with the marked-antigen (Ag*) for limited number of binding sites of antibody (Ab), which is presented in limited concentration.[5] The marked antigen is presented in a slight excess. That means that qauntification provides the marked enzyme. Quantity of complex with marked enzyme is inversely proportional to quantity of determining non-marked antigen. The result is the higher concentration of sample, the lower intesity of measured signal in complex and the higher will be the concentration (signal) of free non-marked antigen.[6]




The occupied sites in the non-competitive assay are directly measured by the analyte, the signal therefore increases in a form directly proportional to the concentration of the analyte. It is used concentration of antibodies in excess.[5] In the

reaction participates only one antigen- analysing substance. The quantification depends on marked antibody, which reacts with antigen.[6]

Next criterion of classification allows us to differ homogenous methods from the heterogenous. Homogenous methods, after antigen-antibody interaction, do not require the separation of marker which stayed united or, more precisely, which can be found in solution (remaining). In this case is produced the alteration of signal, which produces the marker, when making part of the complex antigen-antibody. But in heterogenous methods, the signal is not modified after the formation of complex and it is necessary to introduce the step of separation to eliminate the remaining immunoreactive. The heterogenous methods present higher sensibility, because they are not exposed to the signal noise from the background, but require antibody or antigen to be immobilized by solid support.[5]

### 4.2.1. ELISA immunoassay

Enzyme-linked Immunosorbent Assay is the most used immunoassay. It is included in the group of so-called enzymoimmunoassays, which are characteristic for the usage of the enzyme as marker. Thanks to high catalytic constants of usually used enzymes (horseradish peroxidase, glucosa oxidase), it produces grand amplification of signal corresponding to each interaction antigen-antibody. Besides, thanks to possibility of using different substrates, the product of the reaction can be detected by different technics, both optical and electrochemical.

ELISA method is heterogenous method, which can be presented in competitive or non-competitive format. The most widely used methods are following:

#### 4.2.1.1. "Sandwich" method

In this non-competitive format, two types of different antibodies are used and each of them recognizes one antigenic determinant of the same antigen, which is polyvalent. In the more usual format, one of the antibodies is immobilized by solid support and the other is conjugated with enzyme. After sequential incubation, the antigen stays between the two antibodies. Antibodies should remain in excess. First, to assure complete adsorption of antigen and secondly, to assure complete occupation of immunocomplex. In this case, concentration of the analyte is directly proportional to the quantity of the enzyme united to the complex.

#### 4.2.1.2. Antibodies capture method

It can be used for detection and quantification of both antigens and antibodies. In this method, derivation of the antigen is immobilized by solid support. Solution of sample, containing unknown concentration of antigen, is added together with specific marked antibody. The samples antigen competes with the immobilized antigen for determined number of marked antibodies. Depending on the concentration of antigen, more or less free antibodies in the sample are left for their union to immobilized antigen. In this way, the quantification is determined by the quantity of marked antibody, which is left united to the immobilized antigen. The signal is, for this, inversely proportional to the concentration of the analyte (indirect competitive assay).

#### 4.2.1.3. Antigen capture method

This method is used for detection and quantification of antigens. It is based on competition between marked and non-marked antigens. In this method, the non-marked antibody is immobilized by solid support. The known quantity of marked-antigen, is then mixed with the analyzed sample and added to the immobilized

antibody. The antigen of determined solution competes with the marked-antigen for antibodies binding sites. If the concentration of the antigen in the sample is higher, quantity of the marked-antigen united to antibody will be low, as well as the signal obtained by the addiction of enzymatic substrate (direct competitive assay).

#### 4.2.1.4. Graphic interpretation

Calibration curves of different types of immunoassays present non-linear relation to the obtained response against concentration of analyte. The simple lineal analysis is applicable only in a stretch of interval of concentrations. Because of that, many mathematical methods were proposed in order to try to construct sufficient calibration curve. Logistic function about four parameters proposed by Healy is the most used of all the methods and it corresponds with the following equation.

$$y = (A-B)/ [1 + (x/C)^{D}] + B$$

Parameter A corresponds with the maximal signal, B represents the minimal signal, C stands for the concentration which corresponds to 50% of the minimal signal (IC50) and D depends on the point of curve inflection.

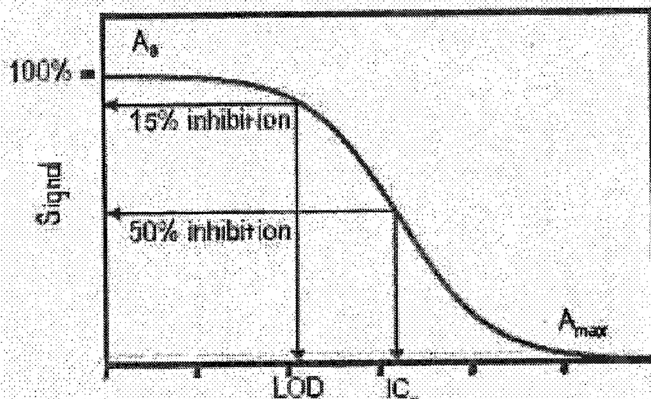



**Fig.2**: Typical curve of competitive assay. The LOD and IC50 correspond to 85% and to 50% of maximal signal in absence of analyte (Ao).

The value of IC50 presents the sensibility of assay and serves as a reference for comparison between different immunoassays. In this type of performance, the experimental error increases according to moving of the central point of the curve, that is why more specific measurements are obtained around this point.

The limit of detection is defined as quantity of the analyte, which corresponds with 85% of the maximal signal (or quantity of analyte, which determine the inhibition of 15%).[5]

## 4.3. Mycotoxins

Mycotoxins are toxic fungal metabolites that can occur in primary food products such as nuts, cereals and fruits as a result of mould growth. Some mycotoxins have been proved strong carcinogenic agents like aflatoxin B1. Others are under suspicion to have carcinogenic effects. Currently a few hundred mycotoxins are known

which are often produced by the genera *Aspergillus, Penicillium* and *Fusarium*. The most prominent toxins are aflatoxins, deoxynivalenol, zearalenone, ochratoxin, fumonisin and patulin.[3]

Mycotoxins are potent toxins which cause food-borne and feed-borne intoxication, and many are cytotoxic, carcinogenic, mutagenic, or immunosuppresive. Because of the potential health risk to humans and animals, national and regional food authorities have addressed the mycotoxin problem by adopting regulatory limits and are continuously amending existing regulations.[9]

The toxic effect of mycotoxins on animal and human health is referred as mycotoxicosis, the severity of which depends on the toxicity of the mycotoxin, the extent of exposure, age and nutritional status of the individual and possible synergistic effects of other chemicals to which the individual is exposed. The chemical structures vary considerably, but they are all relatively low molecular mass organic compounds.[10]

There is an increasing awareness of the hazards imposed on both human and animal health by mycotoxins present in food and feed. Legislation regarding the allowed levels of mycotoxins present in food and feed products and in raw materials is presented by the FAO in 1995 (FAO, 1995). Therefore, a need exists for a reliable, economical and easy to use assay for the measurement of the mycotoxin contents, especially in the raw materials for food and feed production.[3]

### 4.3.1. Zearalenone

Zearelone (ZEA) is a non-steroidal oestrogenic mycotoxin produced by various *Fusarium* fungi, such as F. *graminearum, F. culmorum, F. equisetti, F.cerealis, F. crookwellence, and F.semiectum*. It is found worldwide in a number of cereal crops (corn, barley, oats, wheat, rice, and sorghum) as a consequence of prolonged storage at high temperature or high humidity. ZEA has a relatively low acute toxicity (oral LD50>20,000mg.kg$^{-1}$ body weight) after oral administration in animals. In humans,

ZEA has been associated with early puberty, hyperplasic and neoplastic endometrium, and human cervical cancer.[4]

A myco-estrogen, zearalenone, has attracted recent attention due to concerns that environmental estrogens have the potential to disrupt sex steroid hormone functions. Occasional outbreaks of zearalenone mycotoxicosis in livestock are known to cause infertility. Alternatively, derivates of zearalenone are used in some livestock feeds for growth promotion (for example, Ralgo® in beef cattle), as alternatives to the more potent and controversial synthetic oestrogen, diethylstilbestrol.

This toxin is found almost entirely in grains and in highly variable amounts ranging from a few nanograms per gram to thousands of nanograms per gram. The average human intake of zearalenone was estimated to be approximately 0.02μg/kg on the basis of limited data obtained in Canada, United States, and Scandinavian countries, but it is likely that intakes are greater in countries from the regions of the world having less well- controlled grain storage systems.[1]

The fungal metabolite ZEA (C18H22O5, molecular weight 318.36) is a β-resorcyclic acid lactone with the systematic chemical name 3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3methyl-[S-(E)]-1H-2-benzoxacyclotetradecin-1,7(8H)-dione. ZEA is a white, odorless, crystalline substance, which sometimes has a faint yellow cast. Its meeting range lies between 161 and 164°C. ZEA is practically insoluble in pure water and tetrachloromethane ($CCl_4$) and soluble in diethyl ether, chloroform ($CHCl_3$), dichloromethane ($CH_2Cl$), ethyl acetate, alcohols and aqueous alkali. Of numerous ZEA derivates that can be produced by *Fusarium* spp., only trans-α-zearalenol has been found to occur naturally in grains. ZEA standards are usually prepared in ACN because of stability problems in MeOH.[2]

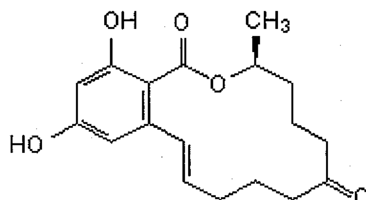



**Fig.3**: Chemical structure of zearalenone [11]

Nowadays, although the regulations for ZEA have been established in 16 countries (Food and Agriculture Organization (FAO) 2004), the maximum tolerable levels digger greatly between countries (20-1000μg/kg). The current maximum levels set by the European Commission (2005) are 20, 75, and 100μg/kg for children and baby food, cereal flour, and unprocessed cereals, respectively. If no specific level is fixed before 1 July 2007, the maximum limit of 200μg/kg will apply thereafter to unprocessed maize, maize flour, maize meal, and refined maize oil. However, no international harmonized maximum limit has been set for ZEA in foodstuffs.[4]

### 4.3.2. Detection methods of ZEA

The importance of mycotoxin monitoring was first recognized in the 1960s with the detection of the aflatoxins.[2]

Not only the chemical diversity of the mycotoxins but also the wide range of agricultural commodities and foods pose a challenge to method development. Concentrations in food and mixed feed may also vary substantially. Most of the developed analytical tools target single classes and specific substrates and comprise extraction and clean-up steps to reduce or eliminate unwanted matrix components. Besides validated official analytical methods based on chromatographic principles, rapid screening tests and several new techniques, for example, use of biosensors, are rapidly emerging.[9]

To minimize the risk for ZEA to humans and animals, extensive research has been conducted to develop sensitive and specific methods for the detection of ZEA in food and feed samples. ZEA is usually analyzed by chromatographic methods, such as thin-layer chromatography (TLC), gas chromatography-mass spectrometry (GC-MS), or high-performance liquid chromatography (HPLC) with fluorescence or mass spectrometry detection. Although these methods are widely accepted as official methods for ZEA determination, they are ether laborious or time consuming. Besides,

they require expensive equipment, complicated clean-up procedures, and pre-concentration steps, so they are treated as unsuitable methods for screening large numbers of samples.

Over the last 20 years the importance and application of immunochemical method have grown significantly. The immunochemical techniques, such as enzyme-linked immunosorbent assay (ELISA), fluorescence polarization immunoassay (FPIA), dipstick immunoassay, and immunochromatographic assay have been developed for the rapid detection of mycotoxin. Recently, an automatic flow-though immunosensor for the rapid and sensitive detection of ZEA in cereal and swine feed samples has been developed. Among these methods, ELISA has been widely used for ZEA determination because of the sensitivity, specifity, rapidity, adaptability, simplicity, selectivity, and cost-effectiveness of the assay. Thus, various commercial kit-based ELISAs have been developed and widely used for the determination of ZEA in food and agricultural products during the past two decades.[4]

#### 4.3.2.1 ELISA method

Immunoassays are analytical tests that use antibodies as specific recognition elements. The IA has proven to be one of the most productive technology contributions to medicine and fundamentals life science research in the twentieth century for both qualitative and quantitative analysis. A great number of research papers have appeared over the last years describing the development of novel IA strategy for detecting trace amounts of chemicals in environmental and food samples.[12]

Enzyme-linked immunosorbent assays (ELISAs) have become one of the most useful tools for rapid monitoring of mycotoxins, especially for the screening of raw materials. Despite high matrix dependence and possible overestimation, the great advantages of microtitre plate ELISAs are speed, ease of operation, sensitivity, and

high sample throughput. In recent years, test kits based mostly on direct competitive assays for field use have emerged.[9]

The technology is based on the ability of a specific antibody to distinguish the three-dimensional structure of a specific mycotoxin. The direct competitive ELISA is commonly used in mycotoxin analysis. A conventional microtiter plate ELISA requires equilibrium of the antibody-antigen reaction that would require an incubation time of approximately 1-2 h. Currently, most of the commercially available ELISA test kits for mycotoxins are working in the kinetics phase of antibody-antigen binding, which reduces the incubation time to minutes. Although reduction of incubation time may lead to some loss of assay sensitivity, the test kit can provide accurate and reproducible results.

A typical principle of direct competitive ELISA is shown in Fig.4.

After a mycotoxin is extracted from a ground sample with solvent, a portion of the sample extract and a conjugate of an enzyme-coupled mycotoxin are mixed and then added to the antibody-coated microtiter wells. Any mycotoxin in the sample extract or control standards is allowed to compete with the enzyme-conjugated mycotoxin for the antibody binding sites.

ELISA test kits are favoured as a high through-put assays with low sample volume requirements and often less sample extract clean-up procedures compared to conventional methods such as TLC and HPLC. The methods can be fully quantitative. They are rapid, simple, specific, sensitive and portable for use in the field for the detection of mycotoxins in food and feeds. Although the antibodies have the advantage of high specifity and sensitivity, because the target compounds are mycotoxins but not the antigens, compounds with similar chemical groups can also interact with the antibodies. This so-called matrix effect or matrix interference commonly occurs in ELISA methods resulting in underestimates or overestimates in mycotoxin concentrations in commodity samples.[13]

When available, the trueness of the method should be confirmed using certified reference materials. Purely proportional systematic errors can be compensated by use of matrix calibration of standard addition.[9]

#### 4.3.2.2.Immunosensor

Applications of immunoelectrochemical sensors for the determination of different biologically active compounds have increased significantly during the last 15 years.[14] Electrochemical sensors have revolutionised modern analysis because of their technical simplicity, low cost, mass production and the possibility of decentralised in field analysis.[15]

These devices combine high specifity of traditional immunochemical methods with low detection limits of modern electrochemical systems. Other advantages of the electrochemical immunosensors are their simplicity and possibility to carry out continuous, fully automated assays. Immunoenzyme electrodes constitute a significant portion of the elaborated immunosensor. Operation of these instruments is based on the formation of enzyme-labelled immune complexes on the sensitive electrode surface and the subsequent detection of the immobilised enzyme. This approach allows applying standard ELISA formats and reactants for the immunosensor. The immunoenzyme electrodes retain the main advantages of solid-phase immunoassays, namely high sensitivity and specifity, and furthermore, simplify and accelerate the analytical procedure.

#### 4.3.2.3. Solid support

For the design of an immunosensor, the crucial step is the immobilisation of immuno-reagents onto the electrode surface. The immobilisation method will determine the sensitivity and reproducibility of the immunosensor. General strategies for the immobilisation of immuno-reagents on solid surface include physical adsorption, entrapment in polymer matrix and covalent attachment. Proteins have an amphiphilic nature and they therefore have a tendency to adsorb to a solid surface. So the direct adsorption of antibodies on the electrode surface seems to be a promising approach for immunosensor construction. It does not require the use of chemical

linkers simplifying the fabrication procedure of the immunosensor. Other alternative for oriented immobilisation of antibodies is through protein A or protein G.[14]

The use of the electrode surface as solid phase as well as electrochemical transducer presents some problems: a shielding of the surface by biospecifically bound antibody molecules can cause hindrance of the electron transfer, resulting in a reduced electrochemical signal. An interesting approach to increase the sensitivity involves the use of electrodes for the transduction step, whereas the affinity is performed using a different support, as, for example, magnetic beads.

The use of magnetic beads greatly improves the performance of the immunological reaction, due to an increase in the surface area, as well as the faster assay kinetics achieved because the beads are in suspension and the analytical target does not have to migrate very far.[15] Moreover, the matrix effect is minimized due to surface area.

Additionally, the magnetic beads can be easy magnetically manipulated by using permanent magnets or electromagnets. Therefore, the analysis of samples performed on magnetic beads can be easily achieved without any pre-enrichment, purification, or pre-treatment steps, which are normally necessary for standard methods.[12]

Therefore immobilisation of biological molecules on solid surface is a key step in the development of biosensor.[16]

In this work were used magnetic particles covered with protein G. Protein G is monomeric protein (30000-35000 Daltons), isolated from bacterial cellular wall (*Streptococcus).*[5]

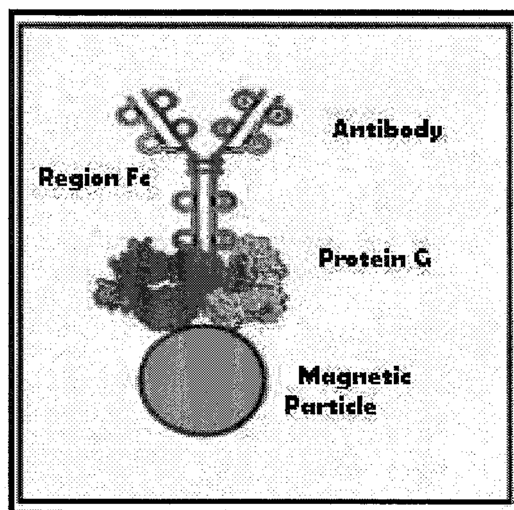



**Fig.4**: Immobilization of antibody by solid support – magnetic particle covered with protein G. [5]

### 4.3.2.4. Electrodes

A wide variety of electrodes have been used as support to fabricate immunosensor device including carbon paste electrodes, glassy carbon electrodes or gold electrodes. Recently, several immunosensor devices have been developed on screen-printed electrodes. The screen-printing microfabrication technology is nowadays well established for the production of thick-film electrochemical transducers. This technology allows the mass production of reproducible yet inexpensive and mechanically robust strip solid electrodes. Other important features that these electrodes exhibit are related to the miniaturization of the corresponding device along with their ease of handling and manipulation in a disposable manner.[15]

In this experiment I have worked with disposable Screen-printed Carbon Electrodes (Fig.6), which exhibit a high electrochemical activity. They are ideal for working with microvolumes, for decentralized assay or to develop specific sensor.

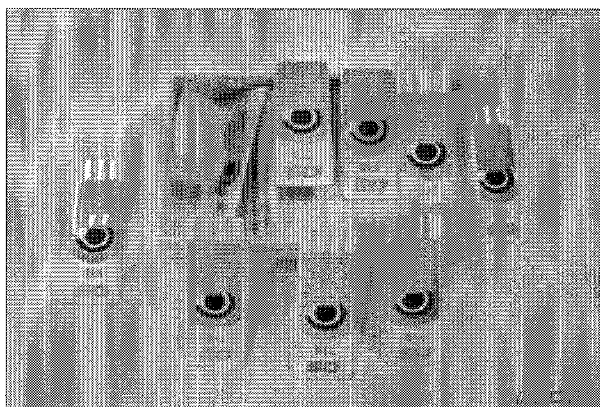


**Fig.5**: Screen-printed carbon electrodes, DropSens

Electric contacts: Silver

The electrochemical cell consists of: Working electrode: Carbon (4mm diameter)

Counter electrode: Carbon

Reference electrode: Silver. [17]

# 5. EXPERIMENTAL PART

- Cereal milkshake, Puleva, (containing a mixture of wheat, rice, corn, barley, oats, and rye), (purchased in local supermarket).

## 5.4. Magnetic particles based immunoassay (ELISA) with electrochemical detection

For preparation of this immunosensor, there is a need to implement enzymatic immunoassay in ELISA microplate wells, using magnetic particles as a support for the immunoreactants. Assay includes these particular steps:

1) Immobilization of known and fixed amount of specific antibody for zearalenone on the surface of magnetic particles (Dynabeads).
2) Competition between zearalenone (represented by sample or standard solution) and its enzymatic conjugate, for the binding sites on the specific antibody.
3) Washing steps to eliminate non-bounded reactants.
4) Introduction of enzymatic substrate.

### 5.4.1. Preparation of solvents

- Washing buffer: citrate phosphate buffer, pH 5.0
  -Dissolving these quantities in 1l of disionted water: 4.7g citric acid + 9.2g $Na_2HPO_4$
- Phosphate saline buffer (PBS), pH 7.2
  - Dissolving in 1l of disionted water of these quantities:
  1.44g $Na_2HPO_4$ + 0.2g $K_2HPO4$ + 0.2g KCl + 8g NaCl
- PBS-T 0.05% (v/v)
  Dissolving 0.5 ml of Tween-20 in litre of PBS
- Dilution buffer PBS-Tween + 0.1% BSA (bovine serum albumin)

- The peroxidase substrate contains: hydrogen peroxide + hydroquinone (which is necessary to optimize concentrations of each). This substrate is prepared *de novo* for every single use.

## 5.4.2. Analytical procedure

### 5.4.2.1. Conditioning of magnetic beads

1) Resuspension of magnetic particles until we receive a homogenic suspension.

2) Transfer of desired volume (2µl) of Dynabeads Protein G magnetic particles into the microplate well where the immunoassay was performed, at room temperature.

3) Magnetic beads are separated from the conservation supernatant by placing the microplate wells in a magnetic rack for 1min until magnetic beads migrate to the side walls and the liquid is clear to remove it with pipete Pasteur.

4) When the supernatant is removed, magnetic particles Dynabeads® Protein G must be washed with citrate-phosphate buffer pH5.0, accordingly to the manufacture protocol, in order to eliminate storage buffer and conditioning them for the IgG capture procedure. 0.2 ml of this buffer is added.

5) Repetition of steps 3, 4 and 3 (again).

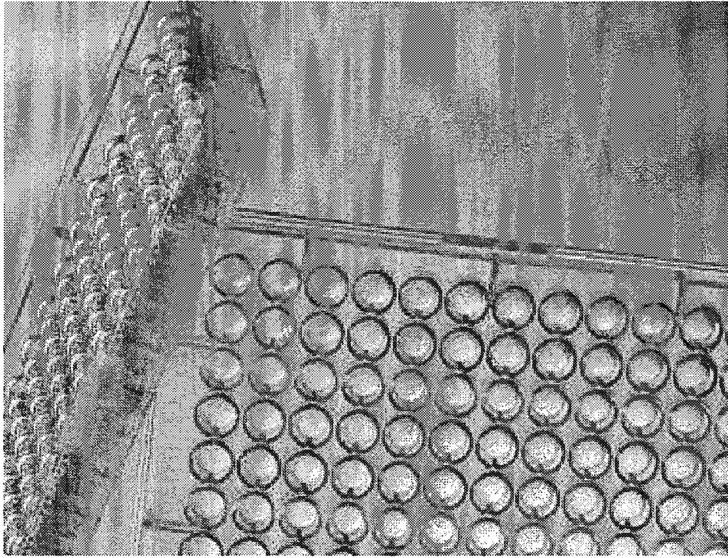


**Fig.6**: ELISA microplate wells [18]

#### 5.4.2.2. Immobilization of specific monoclonal antibody for ZEA

1) Introduction of 50μl of specific antibody (0.005mg/ml) prepared in citrate-phosphate buffer pH 5.0 to previously conditioned particles.

2) The solution is to be stirred gently for 30min at room temperature to obtain antimycotoxin-Dynabeads protein G derivatized beads.

3) To eliminate antibody not bound to protein G, it is to be washed with PBS-T solution (200μl) – five times in the following way:

a) Microplate well is placed on a magnetic rack for 1min until it is possible to remove the supernatant by a pipete Pasteur,

b) After adding 200μl of PBS-T and stirring for a short time to eliminate the remains which were not removed before.

c) Replacement of the microplate well on a magnetic rack for 1min and removal of the supernatant again.

d) The steps 1, 2 and 3 are repeated four times to ensure that all the immobilized antibody is eliminated.

#### 5.4.2.3. Competition between free antigen and antigen conjugated with peroxidase

Addition of antigen (Z, standard or sample) and conjugation with peroxidase enzyme (Z-HRP) is gradual. It helps to prior binding of zearalenone to the binding sites of the antibody.

1) 50µl of antigen solution (standard or sample) is added into the microplate well where particles with antibody are modified.

2) After sufficient incubation time (10min) for preferred union of antigen, necessary volume (0.25µl) of conjugate (Z-HRP) solution is added to obtain the final determined concentration.

3) Competitive immunological reaction – when non-occupied binding sites of antibody are completed – is allowed to proceed with gently stirring for the next 20min at room temperature.

5) Similarly as before, the magnetic particles are washed (five times) again with 200µl of PBS-T to eliminate the not bound species.

### 5.4.2.4. Preparation of the enzymatic substrate

Ag-AB beads complex is removed from the supernatant (using magnetic rack) and one drop (10μl) of this molecular complex is added on the surface of the screen printed electrode (configuration of 3 electrodes) for its detection. We intended to concentrate all the particles in the middle of the 3 electrodes (working electrode). Its immobilization is enabled because of the magnet, which is situated under the electrode and allows us to capture all the magnetic particles. Use of this method results in reduce of the total time of the analysis, because the enzymatical substrate is put directly on the surface of the immunosensor.
The enzymatic substrate is always prepared before every single use.

20μl of PBS pH 7.4 solution is introduced after, to cover all the electrodes surface and finally drop (10μl) of solution containing the mediator (hydroquinone) and the enzymatic substrate (hydrogen peroxide) is added. Thus the total volume of drop put on SPE electrode is 40μM. Afterwards, the enzymatic reaction is allowed to proceed within 5min.
The detection can begin. Electrochemical measurement is realized: different pulse voltammetry (DPV).

The response is determined by polarizing the carbon SPE working electrode at -0.250V where reduction of the enzymatically oxidized mediator hydroquinone takes place.

### 5.4.2.5. Preparation of samples

Different cereal samples, in particular solid matrix (cereal baby food powder / CRM-corn) and liquid (cereal milkshake) have been analyzed by the developed immunoanalytical method. As the first object of validation of accuracy of this developed method, analysis of CRM-certified reference material of ZEA in corn was

carried out. Subsequently, commercially accessible samples from infant foodstuff were analyzed.

In the case of solid matrixes (cereal baby food powder and CRM-corn) the procedure of extraction is needed first.

#### 5.4.2.6. Extraction of ZEA in CRM

For a higher degree of accuracy validation studies were analyzed in the corn samples as representative of certified reference material (83±9 μg/kg) CRM was extracted accordingly to this procedure:
Samples of 1g were weight and transferred to the centrifugal tube and implied with 4ml of acetonitrile:water solution (75:25, v/v) for 90 min at room temperature in ultra sonic bath (150 rpm). The sample was then centrifuged at 4000 rpm for 10min, then it was necessary to extract the supernatant and dilute in PBS-T-BSA one hundredfold. The diluted extracts were immediately assayed.

#### 5.4.2.7. Solid samples – baby food powder

Subsamples of 1g were weight and transferred to a centrifugal tube and directly spiked with a stock solution of zearalenone in acetonitrile (0.5ml) to get the concentration of 80 μg/kg. The samples were left to equilibrate at least for 30min before the extraction itself.

The procedure of extraction: Every centrifugal tube containing 1g of baby food powder spiked with ZEA was extracted with 4mL of acetonitrile:water (75:25, v/v) for 90mins at room temperature in ultra sonic bath. It was then centrifugated at 4000 rpm for 10min and the supernatant (extract) was diluted one hundredfold with PBS-T-BSA

to minimize the solvents influence. In this way, the final concentration of 0.2μg/kg was achieved.

#### 5.4.2.8. Liquid samples - cereal milkshake

Matrixes of cereal milkshake (1ml) are directly spiked with zearalenone of concentration 0.2μg/kg and undergo all the steps of immunoassay described above

All of the experiments were analyzed triplicate.

# 6. RESULTS AND DISCUSSION

## 6.1. Immunooassay optimization

Optimization of the immunosensor in a drop involves previous studies of parameters, such as concentration of enzymatic substrate ($H_2O_2$) and electrochemical mediator (HQN), which significantly affects the acquired signal as well as the incubation time of the enzymatic substrate and mediator with the immunoreactants.

A set of assays was realized, where increasing concentrations of electrochemical mediator were used, while the concentration of the enzymatic substrate was stable.

The concentrations of assayed electrochemical mediator were: 1, 5, 10, 20, 40 and 80μM.

Measurements of the maximal signal were carried out ([Zea] = 0μg/kg) in this study.

Fig.7 shows dependence between concentration of electrochemical mediator and developed signal.

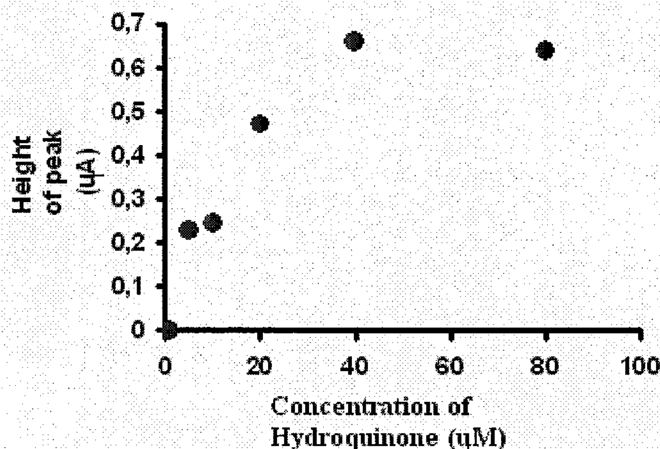



**Fig.7**: Optimization of the concentration of electrochemical mediator.

Fig.7 clearly displays that the signal of electrochemical mediator is increasing until the concentration of 40μM, where it becomes stable as well.

Reviewing these results, concentration of the electrochemical mediator at 40μM was chosen, because it provides considerable signal.

After that, signal using stable concentration of electrochemical mediator at 40μM and varied concentrations of hydrogen peroxide from 10 to 160μM, maintaining the rest of the conditions identical, was studied.
The maximum signal was observed at the concentration value of 80μM of hydrogen peroxide, indicating the maximum speed of the enzymatic reaction.

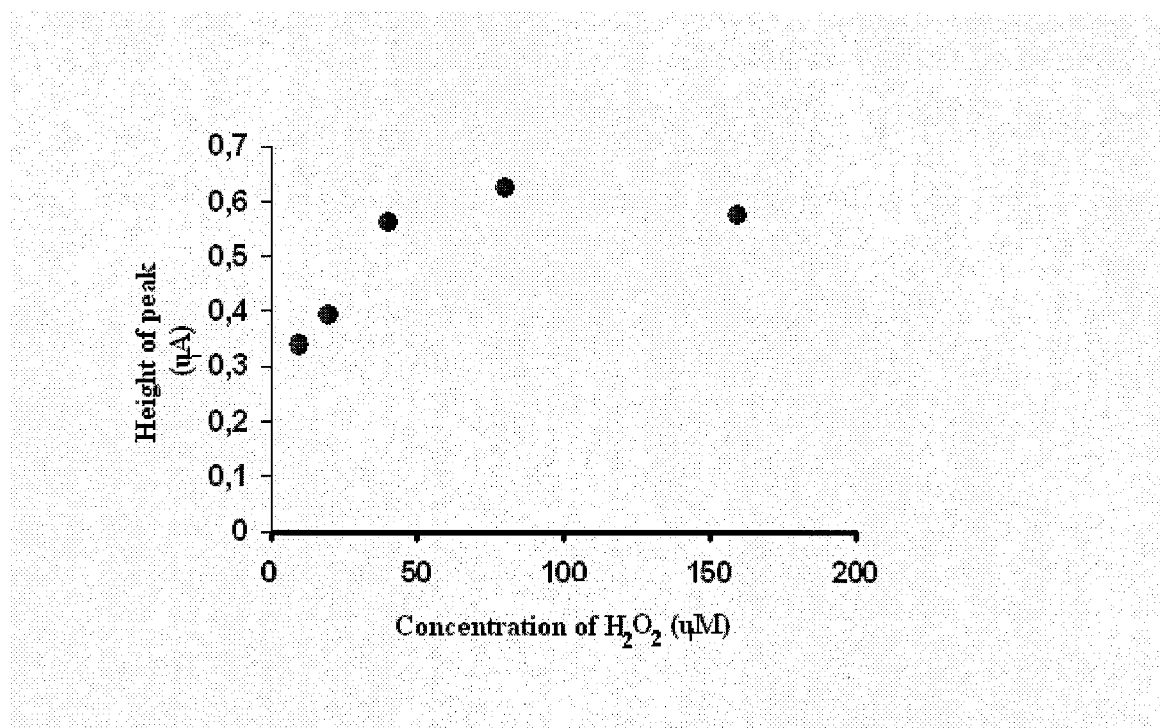



**Fig.8**: Optimization of the concentration of the enzymatic substrate.

The concentrations of 80μM for $H_20_2$ and 40μM for HQN were chosen as the most suitable for our assay.

## 6.2. Evaluation of immunosensors capacity for the determination of ZEA in cereal samples

### 6.2.1. Calibration curve

The evaluation of immunosensors capacity is verified by evaluation of the detection limit (LOD) and accuracy of developed method. The selectivity is operated by the antibody.

When then optimization of conditions was done, calibration curve was realized. The competitive calibration curves were performed by varying the amount of zearalenone from 0 to 1000μg/kg in PBS solution. Dose response curves for zearalenone were analysed with four parameter logistic equation using the software Graph Pad Prism 5.

The competition curve for zearalenone is shown at Fig.9. The signal is reported as binding percentage *Bx/Bo* (%), versus logarithm of the zearalenone concentration, where *Bo* is the maximum signal obtained without competition with the tracer, and *Bx* is the signal obtained during the competition process.

The curve exhibited the typical sigmoidal shape of a competitive immunoassay. The limit of detection (LOD), obtained as 85% of the top value (the maximal voltammetric signal) is 7ng/kg.

EC50 is the antigen concentration necessary for half of the current signal. The EC50 value that corresponds to the analysed concentration necessary to displace 50% of the enzyme tracer was assessed to be 0.0878μg/kg.

The detection limit value is very low comparing to the actual demands in the matter of zearalenone´s regulation in foodstuff, because the most restrictive limit found for maximum permit amount of zearalenone in foodstuff is 20μg/kg.

This fact allows us to apply this developed immunosensor for analysing the mycotoxin in real samples of foodstuff.

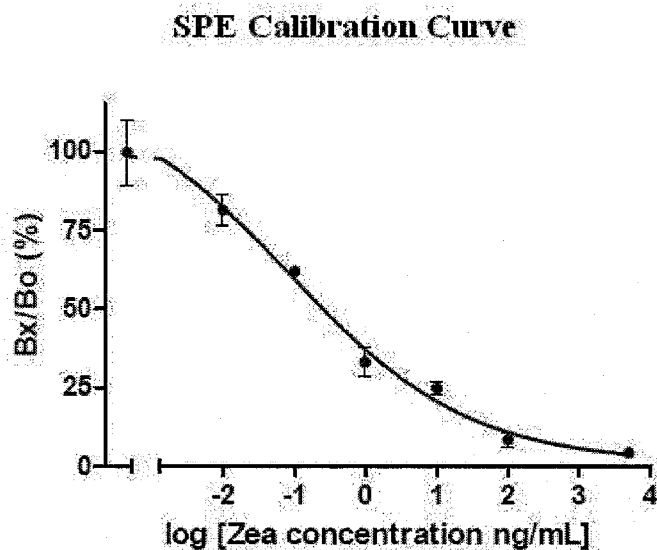



**Fig.9:** SPE calibration curve

## 6.3. Analysis of certified reference material (CRM)

After verification of detection limit, which is, according to actual needs in the matter of regulations, very low, the evaluation of the accuracy developed method - analysis of CRM (zearalenone in corn, 83±9 µg/kg) – was carried out.

Due to the solid character of the sample, there is a need of previous treatment, with proposing mycotoxins extraction and its analysis.

The specific procedure of extraction implies the use of ACN:$H_2$0 (75:25, v/v) as extractive solution (4ml/1g of sample) and subsequent stirring for 90min. After the

phase of centrifugation, the supernatant is diluted in PBS-T-BSA buffer one hundredfold, to avoid denaturalization of immunoreactants caused by solvent. The final concentration of zearalenone after the extraction procedure is 0.21μg/kg. Used extraction was assayed at 3 independent 1g - samples of CRM. Content of zearalenone in each assay was, after application of corresponding ELISA method, determined through the use of calibration curve, realized the same day.

**Table 1.** Results of the data obtained for CRM analysis

Calibration curve – 3 points (linear interval)

| [Zea] μg/kg | log [Zea] | Height 1 (μA) | Height 2 (μA) | Height average |
|---|---|---|---|---|
| 1 | 0 | 0.350 | 0.420 | 0.385 |
| 0.1 | -1 | 0.727 | 0.520 | 0.6235 |
| 0.01 | -2 | 1.098 | 1.400 | 1.249 |

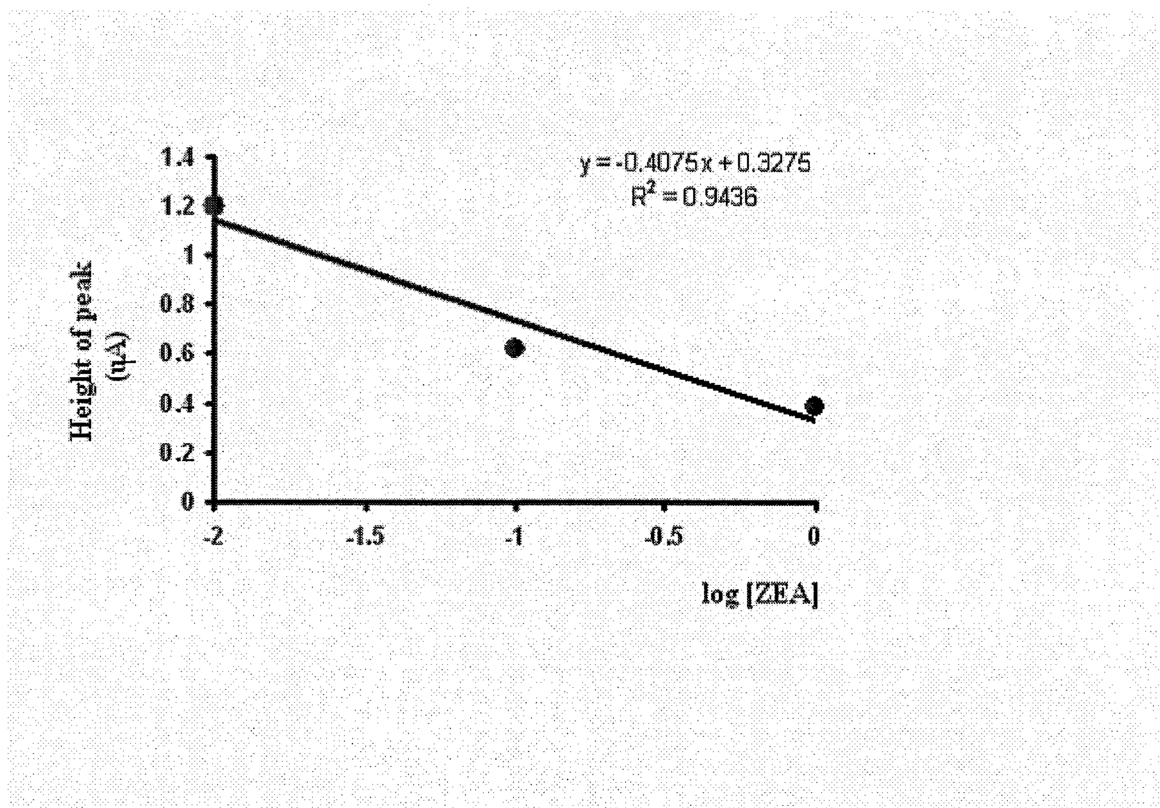



**Fig.10**: Calibration curve, realized the same day as CRM analysis

**Table 2.** – Summary of obtained results

| CRM | Height average (μA) | [Zea] interpolated (μg/kg) | [Zea] real (μg/kg) | Recovery (%) |
|---|---|---|---|---|
| 1 | 0.595 ± 0.042 | 0.22 | 88 | 106.0 |
| 2 | 0.590 ± 0.046 | 0.23 | 92 | 110.8 |
| 3 | 0.603 ± 0.01 | 0.21 | 84 | 101.2 |

Table 2. shows an excellent agreement from the obtained results within the uncertainty of the certified reference material.

## 6.4. Analysis of real samples

When the accuracy of the method was proved, determination of zearalenone in real samples was carried out.

Two matrixes with different complexity, representing infant food, were chosen. The objects of analysis are corresponding with a liquid representative – cereal milkshake, and solid representative –infant preparation, baby food powder.

Obtained data for each matrix are presented. Similarly as with the procedures before, there is a need to undergo developed ELISA method.

### 6.4.1. Analysis of liquid samples represented by cereal milkshake

One of the great advantages of the developed immunological method is wide application possibility on real samples having liquid character, thanks to its selectivity and sensibility. In this case, determination of zearalenone was realized directly in the cereal milkshake, without previous extraction and dilution. Recovery analysis in the matrix, previously found to contain undetectable level of the mycotoxin, was realized. The matrix was spiked with zearalenone of concentration 0.2μg/kg (point close to the IC50).

Data presented in Table 3. correspond with recovery mentioned above.

**Table 3.** - Calibartion curve – 3 points (linear interval)

| [Zea] μg/kg | log [Zea] | Height 1 (μA) | Height 2 (μA) | Height average |
|---|---|---|---|---|
| 1 | 0 | 0.379 | 0.455 | 0.417 |
| 0.1 | -1 | 0.964 | 1.067 | 1.015 |
| 0.01 | -2 | 1.378 | 1.571 | 1.474 |

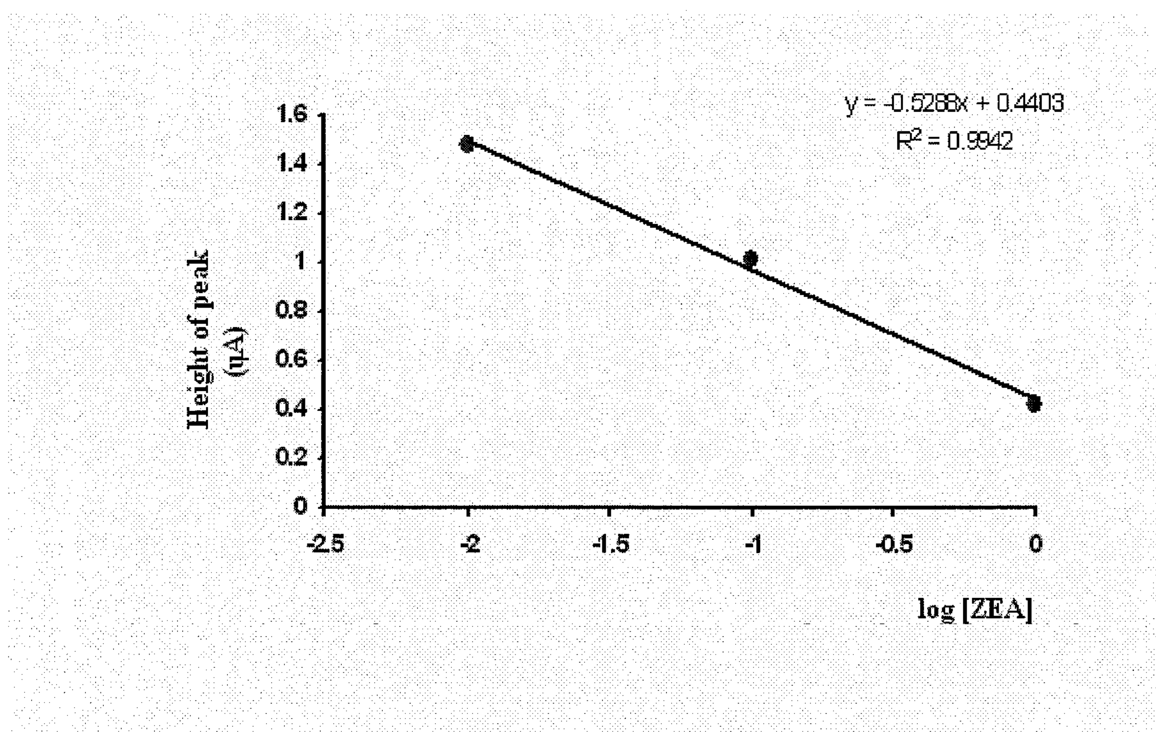



**Fig.11:** Calibration curve of 3 points, realized the same day as spiked milkshake

**Table 4.** – Summary of obtained data

| Spiked Milkshake 0.2 µg/kg | High average (µA) | [Zea] interpolated (µg/kg) | Recovery (%) |
|---|---|---|---|
| 1 | 0.813 ± 0.06 | 0.197 | 98.5 |
| 2 | 0.820 ± 0.03 | 0.193 | 96.5 |
| 3 | 0.813 ± 0.05 | 0.197 | 98.5 |

.Accuracy of the electrochemical immunoassay to determine the concentration of mycotoxin directly in the raw liquid milkshake sample was studied at 3 spiked samples. Recovery values presented in the Table 4., shows as the obtained results are very close to the spiked values.

These data demonstrate suitability of the developed method for detection of zearalenone in real samples of this character.

### 6.4.2. Analysis of solid samples represented by infant preparation

In order to demonstrate the possibility of application of the developed method for analysis of zearalenone in different types of real samples, determination was realized in a sample of grand interest, infant food preparation combined from different cereals in form of baby food powder.

Due to the solid character, it was necessary to carry out the phase of extraction previously, in the same way like in the CRM case.

The food matrix previously found to contain undetectable level of zearalenone was spiked with a solution of zearalenone in acetonitrile, thus the concentration of ZEA in powder was 80 mg/kg. After the extraction procedure, the supernatant was diluted one hundredfold in PBS-T-BSA buffer, obtained theoretic final concentration is 0.2 mg/kg. Three matrixes of 1g were determined based on the same calibration curve obtained as average of two calibration curves realized the days before.

**Table 5.** – Calibration curve – 3 points (linear interval)

| [Zea] μg/kg | log [Zea] | Height 1 (μA) | Height 2 (μA) | Height average |
|---|---|---|---|---|
| 1 | 0 | 0.369 | 0.396 | 0.395 |
| 0.1 | -1 | 1.296 | 1.0512 | 1.404 |
| 0.01 | -2 | 2.191 | 2.168 | 2.179 |

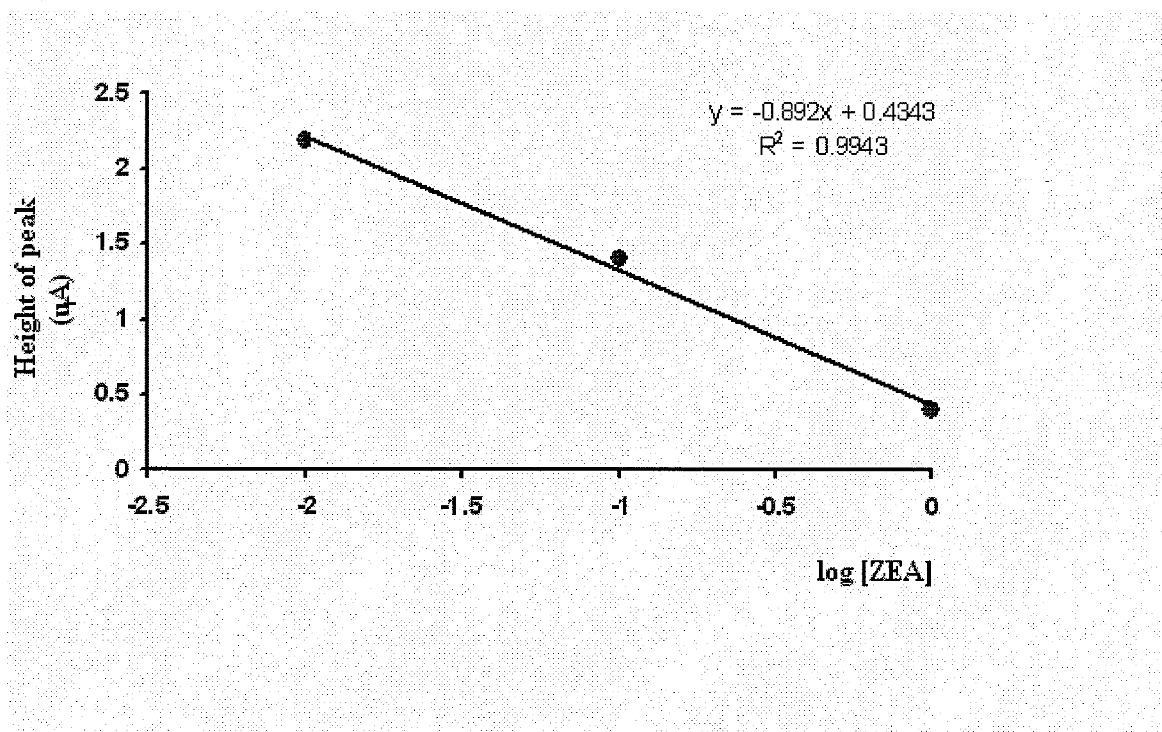



**Fig.12:** Calibration curve

**Table 6.** Summary of obtained results

| Spiked baby powder 0.2 μg/kg | Height average (μA) | [Zea] interpolated (μg/kg) | Recovery (%) |
|---|---|---|---|
| 1 | 1.045 ± 0.07 | 0.21 | 105 |
| 2 | 1.066 ± 0.035 | 0.196 | 98.0 |
| 3 | 1.058 ± 0.04 | 0.199 | 99.5 |

Table above shows the agreement between the spiked concentration and the obtained results demonstrating the suitability of the method even for extremely low concentrations.

# 7. CONCLUSION

The immunoanalytical method proposed for the determination of zearalenone in cereal samples based on magnetic particles and monitored voltammetrically on screen printed electrodes is a valid method and also highly competitive when compared to the already existing methods, thanks to the limits of the detection, which were found very low according to the valid legislation allowed levels, as well as the great accuracy of the method reflected on certified reference material.

Moreover, this method offers various advantages in comparison to other possibilities, for example easy usage, cheap equipment, rapidness of procedure and disposability.

These characteristics make the method perfectly suitable for controlling safety of the infant food.

# 8. ABSTRACT

The concentration of zearalenone in food matrixes was detected using enzymatic immunoassay (direct competitive ELISA using an enzyme tracer). Drop of total volume 40μl (10μl of anti-mycotoxin antibody complex + 20μl of PBS pH 7.4 +10μl of enzymatic substrate) was introduced on the surface of SPE electrode. Indirect measurement was realized after introduction of the enzymatic substrate ($H_20_2$ + HQN), which reacts with an enzyme producing a sample, giving us certain signal. The retrieved signal is inversely proportional to the analytes concentration in the sample. For the sensitive detection of an electrochemical mediator (HQN) was used different pulse voltammetry, directly related to the activity of the enzyme tracer, on the surface of SPE electrode.

The first part of work was the optimization of variables: $H_20_2$ and HQN as substrate and electrochemical mediator, proceed by detecting the concentrations producing the maximal signal ([ZEA] = 0). The concentrations of 80μM for $H_20_2$ and 40μM for HQN, were chosen.

Subsequently, the calibration curve for evaluation of immunosensors capacity was carried out-limit of detection: 7ng/kg and accuracy of the method, verified by using certified reference material with known concentration of ZEA. Recovery values in the range from 101.2 % to 110.8 % were reached.

In the end, the method was applied on real samples of infant food stuff, previously found to contain undetectable level of zearalenone, which were spiked with known quantity of zearalenone and assayed. Recovery values in the range from 96.5 % to 105 % were reached.

The method was found suitable for the detection of zearalenone in infant food.

# 9. SOUHRN

Koncentrace zearalenonu ve vzorcích kojenecké stravy byla detekována za využití enzymatické imunoeseje (přímé kompetitivní ELISA metody, užívající značení enzymem). Kapka o celkovém objemu 40μl (10μl imunokomplexu + 20μl PBS pH 7.4 + 10μl enzymatického substrátu) byla injektovaná na povrch SPE elektrody. Nepřímé měření bylo realizováno po přidání enzymatického substrátu ($H_2O_2$ + HQN), jež reaguje s enzymem za vzniku produktu, který nám poskytuje signál. Získaný signál je nepřímo úměrný analyzované koncentraci. Pro citlivou detekci elektrochemického mediátoru (hydrochinonu) byla použita diferenciálně pulsní voltametrie, přímo úměrná aktivitě enzymu na povrchu SPE elektrody.

První částí práce byla optimalizace proměnných: $H_20_2$ a HQN - substrátu a elektrochemického mediátoru, zjišťováním koncentrací poskytující maximální signál ([ZEA] = 0). Takto byly vybrány koncentrace [$H_20_2$]=80μM a [HQN]=40μM.

Poté byla sestrojena kalibrační křivka pro vyhodnocení parametrů výkonnosti imunosensoru - limit detekce: 7ng/kg a přesnost metody, ověřená pomocí certifikovaného referenčního materiálu o známé koncentraci ZEA. Využitím vyvinuté metody, zjištěné koncentrace odpovídaly 101.2% až 110.8% hodnoty koncentrace referenčního vzorku.

Na závěr byla metoda aplikována na reálné vzorky kojeneckých potravin (u kterých byl předem potvrzen nedetekovatelný obsah zearalenonu), kdy byl vzorek injektován známým množství zearalenonu. Zjištěné hodnoty nabývaly 96% až 105% injektované. koncentrace.

Tato metoda byla shledána vhodnou pro detekci zearalenonu v kojeneckých potravinách..

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