SZENT ISTVÁN EGYETEM

ÁLLATORVOS-TUDOMÁNYI DOKTORI ISKOLA

Development of sensitive immundiagnostics for determination of toxic residues (mycotoxins, drugs) in biological fluids and animal feeds

PhD. Thesis

by

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Budapest
2002

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1. SUMMARY

Recent high publicity of the greatest food – scandals in some European countries (e.g. BSE, dioxin, mycotoxicosis, heavy metal contaminants, illegal hormone treatment or drug residues in meat) underline the importance of food and feed safety and has lead for the building up of a more rigorous food control system. This system focused mainly on the detection of harmful components, among them the control of toxic components e.g. ***mycotoxins*** **or** ***veterinary drugs*** *and antibiotics* are on the fist place.

In my dissertation the work was focused on the detection and screening of some of these toxic components. As head of the Diagnostic laboratory of Agricultural Biotechnology Center (Gödöllő) I conducted and coordinated these scientific activities.

A direct, competitive enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody has been developed for quantitative determination of **ochratoxin A** (**OA**) in different cereals. A dichloromethane/citric acid mixture was used for extraction of OA in cereals. This cleanup procedure proved to be as effective for ochratoxin A extraction as protocols using strong acids. The mean within-assay and interassay coefficients of variation for the standard curve was <10 %. The range of this test is 1-10 ng/g, with a detection limit of 0.5 ng/g OA. The toxin recovery from cereals contaminated with 0.5-100 ng/g OA varied between 90 and 130%. This test was commercialised in 1996 with branded name of *TOXIKLON* and has been used routinely for screening of samples in control laboratories. The test was modified and used for detection of OA content in human sera. The measuring range of this test (without sample dilution) was 0.2-2.0 ng/ml, and the detection limit was 0.2 ng/ml. The OA concentration of 355 sera samples varied from < 0.2 to 10 ng/ml. OA but 75% of the samples contained 0.2-1.0 ng/ml. In some cases (6.8%), more than 1.0 ng/ml OA was measured, which is probably a result of elevated intake of OA, which may even exceed the "virtually safe dose". Our data indicated that, like in many other countries, OA is present in food or feed products available in Hungary, and in order to save the health of consumers, their regular control is desirable. Another experiment was carried out to clear whether or not OA can be detected in seminal plasma after feeding of breeding boars with high amount of OA. The test was validated for semen and pig sera as well. Our findings suggested that OA might have an effect on the quality of semen. Another experiment was set up with broiler chickens after being fed with OA. The distribution of OA in different tissues (liver, kidney, thigh and breast muscles) was investigated. The test was validated for these organs as well. We could detect the highest amount of OA in liver and kidney, while the OA content in breast muscle was very low.

A direct, monoclonal antibody based, competitive ELISA for the quantitative determination of **fumonisin B_1** (**FB_1**) in cereals has also been developed. The measuring range of this test is 10-500 ng/g, with a detection limit of 7.6 ng/g FB_1. The toxin recovery from cereals contaminated with 50-200 ng/g of FB_1 varied between 61 and 84%. For practical application a test-kit, branded name of TOXIKLON has been developed. Our commercialised kit proved to be suitable for the rapid screening of food and feed samples for the presence of FBs.

For screening of drug residues (gentamicin and sulphonamides) in biological fluids, direct competitive ELISA tests have been developed. The **gentamicin**-ELISA test was validated for cattle milk and pig sera as well. The milk could be measured directly, the measuring range was 0.1-10 ng/ml, with detection limit of 0.03 ng/ml GE. The matrix effect caused by sera could be avoided with the dilution of sera (1:10) with 0.1%ANS/PBS/Tween 20 buffer, the measuring range of this test was 1.5-100 ng/ml, with detection limit of 1.2 ng/ml GE. For detection of **sulphadiazine** in milk and sera, a sensitive monoclonal antibody based ELISA test has been developed as well. The measuring range and the detection limit of this test meets well with the requirement of the EU (100 µg/kg).

Our laboratory has obtained an *ISO 9001:(2001)* certification for "production of specific monoclonal antibodies, development and manufacture of "ELISA" diagnostic tests for measurement of mycotoxins and antibiotics".

2. Introduction

As a result of the numerous, European food scandals that have occurred recently, such as hormones, PCBs and dioxins, BSE, consumers are becoming dissatisfied with the current production process and lay claim to such agricultural industry that is compatible with the production of high quality foodstuffs. The food and feed safety became immediately the first political matter in EU countries and Brussels planned to set up a Food-Control-Authority in the near future. The main task of this new Committee would be to make stronger legislation and to control the whole process of the food-chain, beginning from the farm - upto the table of consumers. This more reliable and efficient strategy of the feed and food control should contain not only the analysis of natural food components (carbohydrates, proteins, fats, vitamins, colorants, flavours) but also the detection of harmful compounds that may be dangerous for human and animal health and also for environment. The list of contaminants is quite long, including pesticide residues, antibiotics, drug residues, hormons and mycotoxins. As Hungary would like to join to EU in the near future, the quality control system of the food - and feed industry needs to be reorganised according to the new EU Directives.

For determination of the harmful residues in foods, acceptable, sensitive and reproducible methods are required such as enzyme- immunoassay (ELISA) based on antigen-antibody reaction.

As project leader of Diagnostic laboratory of ABC (Gödöllő), 12 years ago I started a long-term research program for development of a series of monoclonal antibody based ELISA tests along with assay technology for determination of mycotoxins and drug residues in foods and feeds. The mycotoxin research is one of the programs sponsored by the Hungarian Academy of Science and the Ministry of Agriculture and Landscape Development.

The first part of my dissertation is dealing with the development of direct-competitive ELISA test and reagent-kit for determination of **Fumonisin B1** mycotoxin contamination in cereals. Our other developed test, **Ochratoxin-A** was used for determination of ochratoxin-A contamination in human sera, seminal plasma of boars and in chicken tissues. Researches of both toxins are very important, because they are dangerous for human and animal health and their control in feeds and foods is necessary. With these series of tests named as **TOXIKLON**, at present there are five ELISA kits (T-2, zearalenone, ochratoxin-A, aflatoxin B1, fumonisin B1). Recently these kits are used in practice for quantitative rapid screening of the different mycotoxins in cereals.

Along with the mycotoxin project we have focused our interest on the development of ELISA tests for detection of veterinary drugs in different matrices. Residues of veterinary drugs, particularly of antibiotics, represent a potential hazard for human health. The main risk is the danger of increasing bacterial resistance, which can have dramatic consequences for public and animal health. Allergic reactions to antibiotics could also appear after consumption of food contaminated by antibiotic residues. Monitoring of the drug residues in food products (milk, meat) is an up-to-date program in EU because the drug residues in milk are harmful not only for milk consumption, but also for the processing of cheese and other dairy products. For this reason Maximum Residue Limits (MRLs) had been determined for these substances in EU, USA and Canada.

In the second part of the dissertation I summarised my R+D work for development of ELISA test for two veterinary drug residues, the **gentamicin** and **sulphadiazin.** The developed monoclonal antibody based ELISA tests can be used for determination of these components in milk, because this matrix is one of the most important foodstuff .The tests can be applied for other matrices, eg. sera as well.

I hope that these sensitive ELISA tests will be commercialised in the near future and can be used for screening of drug residues in milk or sera at an acceptable cost level.

3. Review of literature

3.1. Principles of Immunoassays

The term "Immunoassays" refers to a group of analytical techniques, which are used throughout by clinical laboratories. Among the immunoassays there is a growing need for such immunoassays using labelled antigen or antibody. The sensitivity of these methods is much higher than that of the traditional methods (immunoelectrophoresis, immunodiffusion). The first **radioimmunoassays (RIA**) were introduced by Yalow and Benson (1959) who were awarded the Noble Prize in Physiology and Medicine for this achievement in 1977. Since 1960, many RIA procedures have been developed for detecting of various materials occurring in body fluids and other test-materials at concentrations as low as 10^{-9} – 10^{-15} g/ml. Nevertheless, owing to the potential hazards of radioactive materials, the RIA techniques have been less widely employed than they expected. Soon after the principle of RIA had become known, research activities were focused on finding labels other than radioactive for visualisation of the highly specific and sensitive immunological reactions. Among others enzymes, fluorescent and luminescent molecules, stable free radicals and phages have been used as labels (**Table 1**.),(Braun, 1992).

The common property of the latter materials is that they have a certain multiplier effect, analogous to that of the scintillation multiplier owing to the radioactive decay in RIA test.

It has long been known that enzymes have a multiplier effect and are able to catalyse the turnover of many ten thousands of substrate molecules. It was obvious that the specific and sensitive immunological reactions should be combined with enzymatic reactions, which are measurable with high precision. The first enzyme-immunoassay (EIA) was published by Engvall and Perlmann in 1971. Ever since publications on EIA tend to increase in number year by year, indicating the great significance of the new methods.

Table 1. **Typology of Immunoassays** (Braun, 1992)

According to the reaction	Equilibrium Non equilibrium
According to the medium	Homogenous (separation –free) Heterogeneous (separation-required)
According to the labelling material	Chromogenic Radioactive Fluorescent Chemiluminescent Enzymatic Metal atom Stable free radical

From the beginning two main EIA procedures, known as the **homogenous and heterogeneous** techniques, have been developed.

The **homogenous EIA**, **EMIT** (Enzyme Multiplied Immunoassay Technique) detects haptens (eg. drugs), but cannot detect large molecular antigens. This method is sensitive down to nmol (10^{-9} mol) concentration, but insensitive to lower pmol (10^{-12} mol) and fmol (10^{-15} mol) concentrations of the materials to be assayed. The procedure is characterised by a measurable change in the activity of the labelling enzyme during the immunological reaction. It follows that separation of the bound and unbound fraction-which is necessary in RIA-is not required in the homogeneous EIA, therefore this technique is easily adaptable to automatic analysers. Disadvantage of homogeneous EIA is that certain disturbing components of the sample -which are eliminated from the heterogeneous EIA

system through the separation of bound and unbound fractions- may have an influence for the photometrical measurement.

The basic principle of an immunoassay is a reversible reaction between an antigen (Ag) and its antibody (Ab) to form a complex (AgAb):

$$Ag + Ag \underset{K_2}{\overset{K_1}{\rightleftharpoons}} Ag\text{-}Ab$$

K_1- is the association rate constant, or the forward reaction to form the complex

K_2- is the dissociation rate constant for the reverse reaction

The affinity or equilibrium constant, **K,** is the ratio of **K_1/ K_2.**

In the practice mainly the so called **heterogeneous EIA** technique is used, which is equally suitable for determination of large molecular weight antigens, small molecular weight haptens, and antibodies. If no precise measurement is required it is practicable to have a qualitative judgement based on visual determination (yes or no). The working principle of heterogeneous EIA is analogous to that of RIA, before the measuring the enzyme activity, it is necessary to separate the bound and unbound labelled fractions of the reaction partners. The antigen-antibody reaction takes place on a special solid phase, which can take many forms, including plastic (polystyrene, PVC, polyethylene) microplates, tubes, beads. These materials are able to adsorb the antigens or antibodies, which are chemically proteins, polysaccharides or lipids.

The heterogeneous EIA - by more popular term **ELISA** (Enzyme Linked ImmunoSorbent Assay) - has the disadvantage against RIA that the measurement of enzyme activity is less simple than scintillation counting, and requires therefore an additional working step. While the range of labels for RIA is limited (tritium, iodine, selenium or cobalt isotopes), many enzymes (eg. alkaline phosphatase, peroxidase, beta-galactosidase, glucose oxidase etc.) proved to be suitable for labelling in EIA.

3.1.1 The advantages of ELISA over RIA :

a./ very high sensitivity, detectability, and specificity are possible,
b./ no complicated and expensive equipment is necessary,
c./ assays may be very rapid and simple,
d./ reproducibility is high and evaluation is objective,
e./ no radiation hazards,
f./ reagents are relatively cheap and generally of long shelf-life.

It is indisputable that the prices of EIA kits and reagents are competitive. An enzyme with high effect multiplier requires an adequate substrate for the photometric measurement of the reaction, as far as possible in the visible light range. The most frequently used chromogens in ELISA are o-phenylenediamine (OPD), 2,2'-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and tetramethyl-benzidine (TMB), have high molar extinction coefficient at various wavelengths of the visible light range, thus enabling highly sensitive and precise measurement of the reaction.

As my research work focused on the detection of small molecules so-called "haptens", that molecular weight was less than 1000 Daltons, the selected immunoassay method was the competitive format.

3.1. 2 Characterisation of the heterogeneous enzyme-immune-analytical methods

a./ competitive immunoassays for haptens
b./ non competitive immunoassays for proteins
- indirect immunoassays for Ab detection,
- two site or sandwich immunoassays for Ag detection.

The competitive immunoassays are used mainly for haptens (eg. mycotoxins, hormones, anabolic, drugs, pesticide molecules etc.)

The competitive immunoassay can be written by the following formula:

$2Ab + Ag + Ag^{*} = AbAg + AbAg^{*}$,

where: **Ag** = free analyte (eg. mycotoxin, drug etc.),
Ag^{*} = labelled (eg. peroxidase enzyme) analyte,
Ab = specific antibody against the analyte

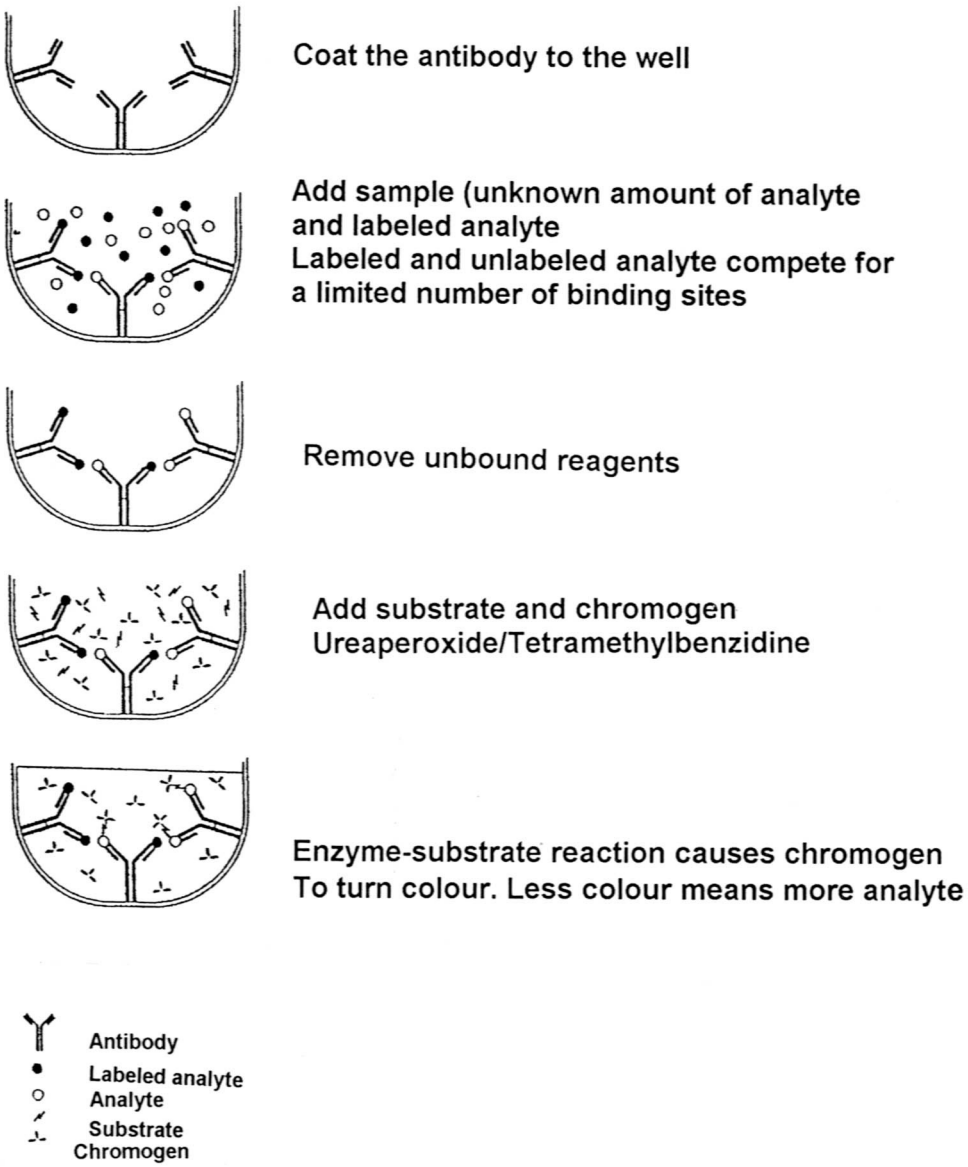
The **free Ag** and **labelled Ag** (Ag^{*}) are competing for the limited number of binding sites of the **Ab** at the same time. After a certain reaction time the unbound components will be removed and the enzyme activity of the bound component will be measured. Because of the competitive nature of the assay in which an excess of analyte will inhibit binding of the labelled analyte to the antibody, the analyte standard shows an inverse relationship. Depending on the type and specificity of the antibody, the detection limit of the analyte in sample can be ng/ml or pg/ml concentration.
(**Figure. 1**)

3.1. 3. Main steps of development an enzyme-immunoassay

- conjugation of hapten to macromolecular carriers (immunogen),
- immunization and raising antibodies,
- characterisation of antibodies,
- preparation of labelled hapten,
- designing the assay (study of assay conditions, optimalization of the test parameters),
- analysis of real samples,
- validation of the assay (reproducibility, specificity, sensitivity of the test)

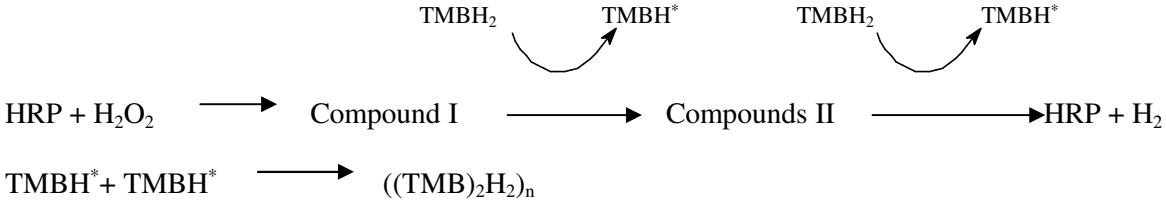
The development of a competitive ELISA test needs two main immunochemical reagents:

- a specific antibody, and
- an enzyme labelled hapten (eg. mycotoxin, drug molecule).



1. Figure. Flow chart of a competitive ELISA

General process of Tetramethylbenzidine (TMB) chromogen



$TMBH^{*} + TMBH^{*} \longrightarrow ((TMB)_2H_2)_n$

$((TMB)_2H_2)_n$ = coloured product of oxidation

HRP: Horseradish peroxidase enzyme

3.1. 4. Characteristics of enzyme-immunoassays

(Chu, 1990; Schuurs and Van Weemen 1977))

➨ **Sensitivity**

- Assay range and steepness of standard curve
- Maximum colour at zero concentration (Bo)
- Colour change per concentration unit (slope of the curve) $(B_2-B_1)/C_2-C_1)$
- 50% inhibition concentration (IC_{50})

➨ **Accuracy**

- Reproducibility within an assay (intra assay CV %)
- Reproducibility between assays (interassay CV %)
- Possible interference substances (Bs)
- Cross-reactivity with other related compounds
- Comparison of data with other methods

➨ **Simplicity**

- Easy to use and steps involved
- Time required for the tests

➨ **Stability of immunochemical reagents**

➨ **Cost**

An ELISA method should have the capability of providing reproducibility as well as sensitivity. Among the criteria the Sensitivity and Accuracy are the most important ones and necessary to evaluate very rigorously.

The following criteria should be examined for determination of the ***sensitivity***:

a./ Blank B reading in the absence of mycotoxin or drug (Bo),
b./ Blank reading for different sample matrices at appropriate dilutions in the absence of mycotoxin or drug (Bs).This value should not vary significantly from Bo,
c./ The change in B (B_2-B_1) per unit C (C_2-C_1) in the linear section of the curve should be defined (slope),
d./ The concentration of toxin or drug that cause 50% inhibition of binding ((IC_{50}),
e./ The concentration of toxin or drug analogs that cause 50% inhibition binding (specificity of the antibody).

A good competitive ELISA should have high Bo and $(B_2-B_1)/C_2-C_1)$ values, and a low IC_{50} value. If the standard curve is less steep, the slope of the curve will become smaller, a slight variation in B in the curve would result in a large change of concentration and consequently a large error. A larger IC_{50} means that the standard curve has shifted to the right and that the assay is less sensitive. The heterogeneity of the antibody-antigen interaction, low affinity antibody used in the assay and inadequate antibody or antigen concentration coated on the plate may give a standard curve less steep and shifting the curve to the right.

The ***accuracy*** **(**the extent to which the mean result of a large number of determinations coincides with the true value) of the immunoassay systems depends on:

(i) the immunological specificity or purity of the reagents,

(ii) the correct handling and assaying of the sample,
(iii) the use of an appropriate standard.

Accuracy tests of the complete system should include the determination of:

(i) Within-assay CV %- test one sample many times within an assay. This gives an over-optimistic estimation of precision,

(ii) Between-assay CV% (synonyms: inter-assay, day-to day, run-to-run CV %) is calculated from within-assay and total assay CV % values,

(iii) Recovery experiments or sample dilution experiments give useful information, particularly on possible effects (interference) of sample on the results ,

(iv) Checks on (immunological) specificity (specificity of the antibody, the degree of recognition between the labelled reactant and its immune counterpart (cross-reactivity with chemically similar compounds),

(v) Comparison of the data with other methods (e.g. HPLC, GC-Ms).

3. 2 Antibodies

3.2.1. Characterisation of antibodies.

The specificity of the ELISA test is influenced mainly by the acceptable quality (*affinity* and *avidity)* of antibody (polyclonal or monoclonal). *Specificity* denotes the degree of uniqueness of the antigens reacting with the binder, whereas the *affinity or equilibrium constant* K, is the ratio of K_1/ K_2 (see point 3.1., page 6) and shows the tightness of binding. (Gazzaz et al. 1992; Howanitz, 1988).

Antigens or immunogens are substances that can elicit an immune response. Antigens are usually of high molecular weight, greater than 1000 Da (a dalton is a unit of mass equal to that of a hydrogen atom = 1.67×10^{-24} g). A compound with a molecular weight less than 1000 Da is usually not immunogenic by itself, but can be made immunogenic through conjugation to a high molecular weight immunogenic carrier molecule, usually protein. These conjugated compounds are called haptens.

Immunity refers to all the mechanisms, involved in vertebrates to protect it against non-self environmental agents. The initial contact with a foreign agent (antigen immunization) triggers a chain of events that leads to the synthesis of antibodies specifically against that foreign agent. As antigens and haptens, usually foreign agents, they can evoke an immunological response in many animals. and result antibodies against the carrier protein and the hapten molecule as well.

Lymphocytes, plasma cells, and macrophages are three types of cells that play important roles in the immune system. These cells are derived from progenitor cells in the bone marrow and circulate in the blood, entering the tissues when required. *Lymphocytes* are small cells found in the blood, having the ability to recognise individual antigens through their specialised surface receptors. There are two major populations of lymphocytes, *B* and *T*. *B-lymphocytes* secrete humoral or serum antibodies and exhibit antigenic specificity. *T- lymphocytes* do not secrete antibody, but instead exhibit antigenic specificity, proliferate and differentiate in the presence of antigen-releasing, biologically important polypeptides called lymphokines. *T-lymphocytes* function is in helping *B-*

lymphocyte cells to synthesise antibodies by regulating the immune response. The third kinds of cells that play a major role in so called "acquired" immunity are the macrophages. *Macrophages* are large phagocyte cells that ingest particulate material and transfer the foreign matter to *T* cells. This step is important for *T*-cell activation and consequently for B-cell activation and antibody synthesis. Macrophages do not exhibit antigenic specificity and do not produce antibodies directly.

Antibody molecules or immunoglobulins (Ig) are complex biomolecules composed of heavy (H) and light (L) chain polypeptides. Immunoglobulins have many common structural features, including two heavy chains, molecular weight of 50.000 Da each, and two light chains, molecular weight of 25.000 Da each. The immunoglobulins have two, different types of light chain, λ and κ, with different amino acid sequence. The immunoglobulin molecule resembles a **Y**, or a partially opened zipper form. The H- and L-chains are held together by disulphide bridges. The H- and L-chains are organised into variable and constant regions, and the antigen-binding site, or combining site, is created by the association of the variable region portions (located at the amino end) of the H- and L-chains. The ability of an antibody molecule to specifically bind an antigen, or ligand molecule, is controlled by structural and chemical interactions that occur within the antibody combining site. The antigen-antibody interaction is a reversible interaction and does not involve formation of covalent bonds. Variety of interactions including hydrophobic, ionic, H-bonding, and van der Waals forces. Thus, the antigen-antibody interaction is analogous in many respects to the interactions that occur between an enzyme and its substrate, and it follows the same rules of physical chemistry. The antigen-antibody binding is a complex process involving a steric component (i.e., the analyte must fit properly into the combining site), as well as formation of specific chemical interactions between the antibody and antigen. It is generally thought that the greater the number of specific chemical interactions that occur between an analyte and the amino acid residues in the antibody combining site, the greater the binding energy (relative affinity of the antibody). Thus, the fundamental properties of an immunoassay, its specificity and sensitivity are controlled by the precise nature of the antigen-antibody binding process. (Beier et al.1996; Gazzaz et al.1992). The antigen-antibody interaction can be measured by the affinity constant (*K*), this value varied from 10^4 to 10^{12} l/mol. If the *K* value is between 10^4 –10^7 l/mol, it means a weak antigen-antibody binding, the antibody is unable to develop a good immunoassay, afterwards a *K* of 10^8-10^{12} l/mol refer to a good quality of antibody.

Immunglobulin molecules are divided into five major classes or isotypes: IgG, IgM, IgA, IgE and IgD, based on the type of heavy chain (G, M, A, E and D) each possesses. Each one of these five immunoglobulins has a different and unique biological activity. Of the immunoglobulins, IgG (mol wt 150 000 Da) has been most widely studied. The IgM molecule or macroglobulin (mol wt. 900.000) consists of five IgG molecules joined together by several disulphide bonds between their fragment crystallizable (F_C) portions, and by one polypeptide chain called the J chain. IgM is multivalent and the functional affinity of IgM antibodies for antigens is high.

3.2.2. Production of polyclonal antibody

Immunization of animals (rabbit, mice, goat, sheep) with complex antigens generates antibodies of many different classes and specificities in the sera of animals. The antigens usually contain a number of distinct antigenic determinants so called epitopes. Each epitope may result in the production of antibody. Therefore, even a pure antigen is capable of eliciting production of a variety of specific antibodies directed at different epitopes, each of which is able to react with the parent antigen. Any given antiserum is therefore the sum of the immunoglobulin products of many different B-cell clones and considered **polyclonal.** The quality and quantity of polyclonal antibody varies from animal to animal, even at different times of bleeding from the same animal. Spite of this

fact high specificity and affinity polyclonal can be produced and applied for development immunoassays.

3.2.3. Production of monoclonal antibody

Monoclonal antibodies are derived from a clone of immunoglobulin-secreting hybridoma cells, which are created by fusing spleen cells from a mouse with a special type of tumor cell, often myelomas that can be experimentally induced in certain strains of mice. In this way, a single antibody with defined specificity can be continuously produced in vitro and unlimited amounts of standardised homogeneous antibodies can be made available for use. These antibodies have a single affinity and predictable specificity, but they are more difficult and more costly to prepare than polyclonal antibodies. (Howanitz, 1988; Gazzaz et al.1992)

Table 2. Advantages and disadvantages of monoclonal antibodies

Assay parameter	Advantages	Disadvantages
Assay configuration	Flexibility Easy to purify Superior for two-site immunoradiometric assay	Unable to cross-link Many antigens
Sensitivity	Appropriate affinity selectable	Many have low sensitivity
Specificity	Single epitope specificity Unpurified antigens used	Polyfunctional Too much specificity
Reproducibility	Unlimited quantity of identical antibody	-
Cost	Decreased quality control requirements	Expensive for identification of antibody required

Nearly everything that can be done with polyclonal antibodies can be done better with monoclonal antibodies.

The advantage of the use of monoclonal antibodies: it is not necessary pure antigen for immunization, the hybridoma cells can be continuously grown, producing exactly the same molecule for moths and even years. The stability of monoclonal antibodies has also proven to be excellent for a long time.

Some disadvantages have been found: the affinity of monoclonal antibodies is often low, assay precipitation is not always possible, costs are high, initial identification of the “right” monoclonal is labour-intensive, and in some cases, too much specificity may occur.

Despite of some disadvantages the monoclonal antibodies reached the 50% market share in diagnostics in 1990 and have today become the predominant immunoreagent. (Borrebaeck, 2000)

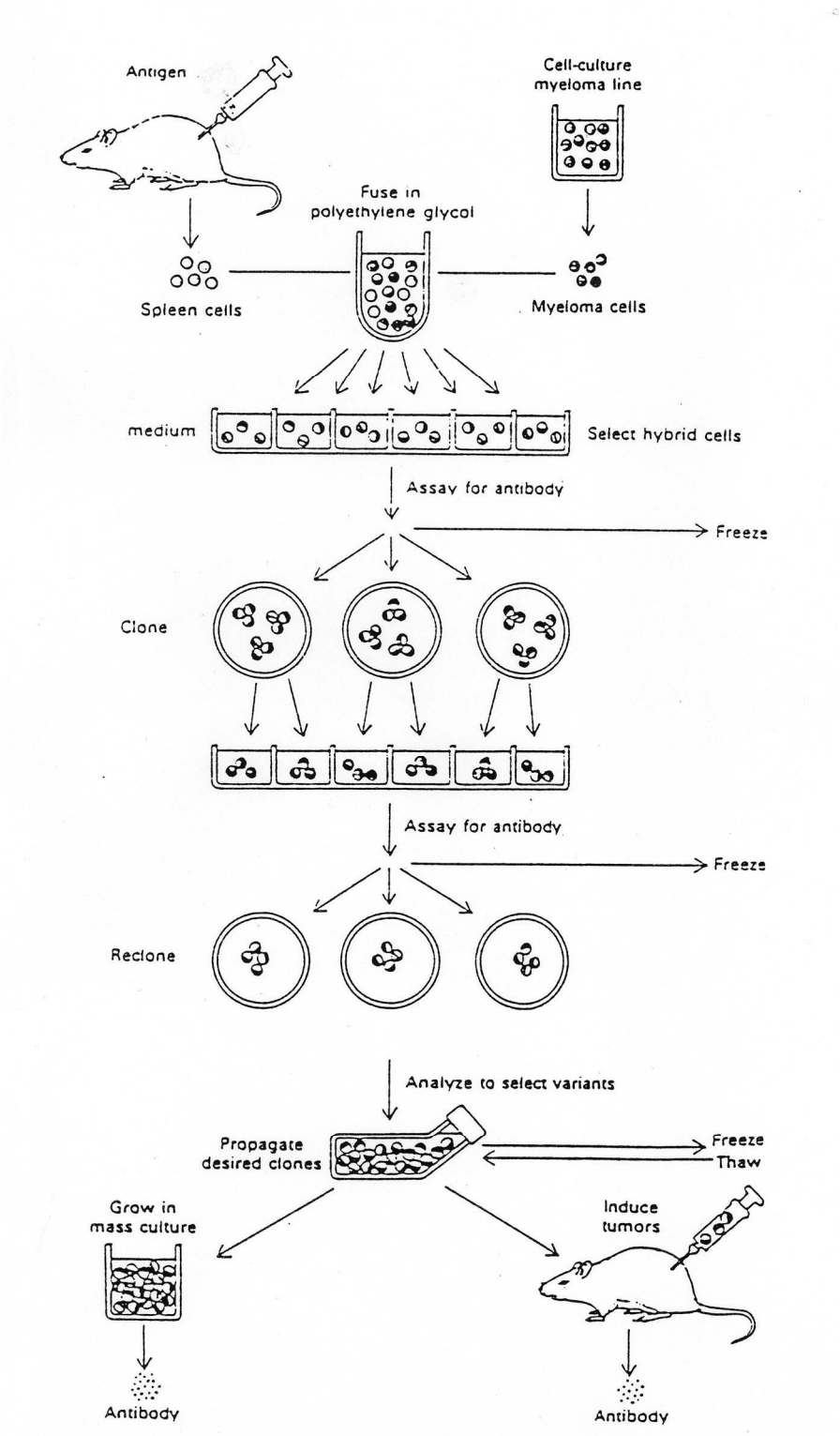


Figure 2. Production of monoclonal antibody

3.3. Preparation of hapten-protein and hapten-enzyme conjugates

For the development of a sensitive antibody needs an acceptable quality of antigen (immunogen).

In my dissertation the following haptens were selected: ochratoxin-A, Fumonisin B1, gentamycin and sulphadiazin. The reactive group of molecules and the conjugation methods for protein were summarized in **Table 3.**

These molecules were conjugated with large protein molecules such as bovine serum albumin or Keyhole-limpet hemocyanin (KLH) and were capable to raise antibodies in rabbit and mice.
The antigenic specificity, or antigenicity, is characterised by the capacity of the antigen to react with antibodies involving small reactive sites on the surface of the antigenic molecule. These sites are called antigenic determinants or epitopes.

The mammalian immune response can recognise many different structural and physicochemical features of complex compounds and can differentiate between the various structural features of a single immunogenic protein. An immunogenic protein may have one or more epitopes, each with a separate or distinct primary, secondary, tertiary, and/or quaternary structure as a recognition site.

Table 3. Conjugation of hapten to protein or peroxidase enzyme

Reactive group of analyte molecule	Reactive group of protein molecules	Conjugation method	References
Ochratoxin-A (-COOH)	BSA, KLH (-NH_2)	Active ester	Märtlbauer and Terplan (1988)
Ochratoxin-A (-COOH)	Peroxidase (-NH_2)	Mixed anhydride	Märtlbauer and Terplan (1988)
Fumonisin B_1 (-NH_2)	BSA, KLH (-NH_2)	aldehyde (OHC-R-CHO)	Avrameas and Ternynck(1969)
Fumonisin B_1 (-NH_2)	Peroxidase (-CHO)	Periodate	Wilson and Nakane (1978)
Gentamicin (-NH_2)	BSA, KLH (-NH_2)	aldehyde (OHC-R-CHO)	Avrameas and Ternynck(1969)
Gentamicin (-NH_2)	Peroxidase (-CHO)	Periodate	Wilson and Nakane (1978)
Sulphadiazine (-NH_2)	BSA, KLH (-NH_2)	diazo	Haasnoot et al.(2000)
Sulphadiazine (-NH_2)	Peroxidase (-CHO)	Periodate	Wilson and Nakane (1978)

Flow-charts of the conjugations methods are in Figure 3 – 7.

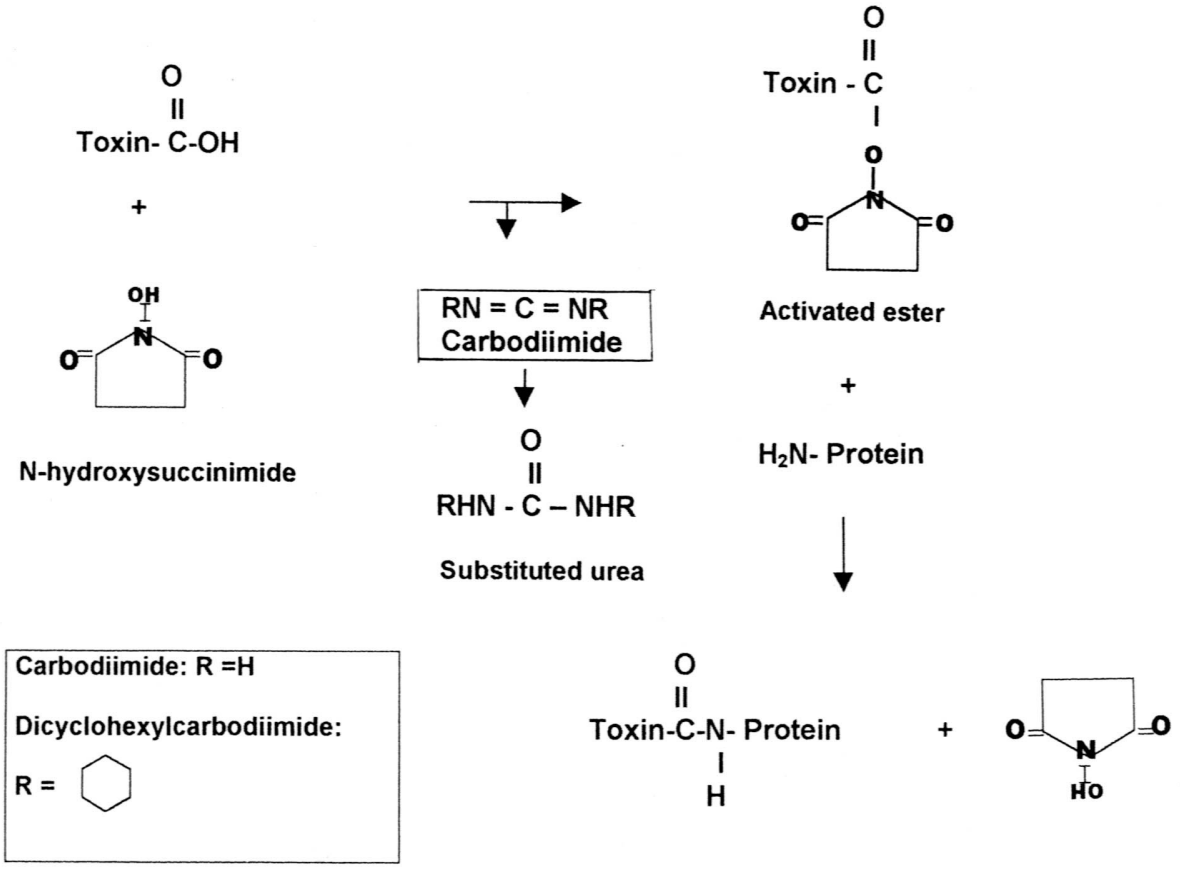


Figure 3. Hapten coupling to protein (or enzyme) by active ester method

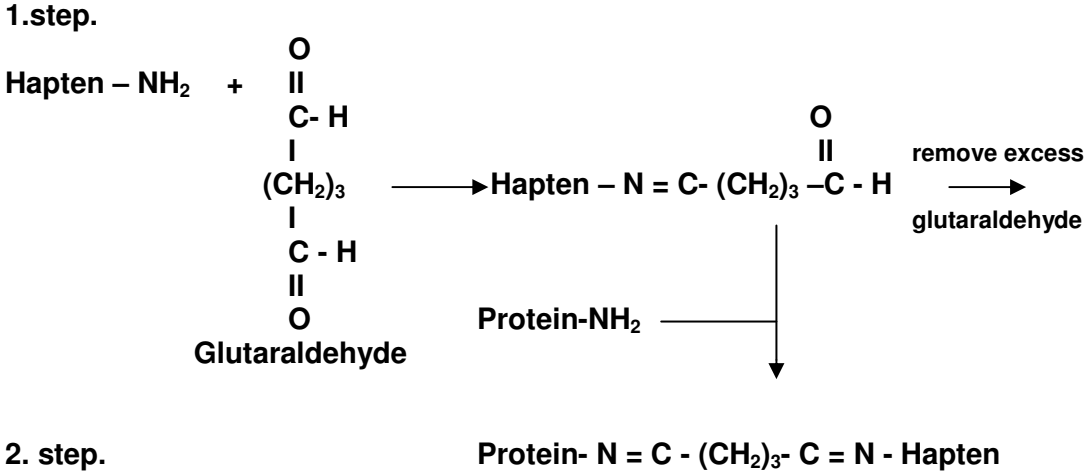


Figure 4. Coupling of hapten containing reactive amine to aldehyde

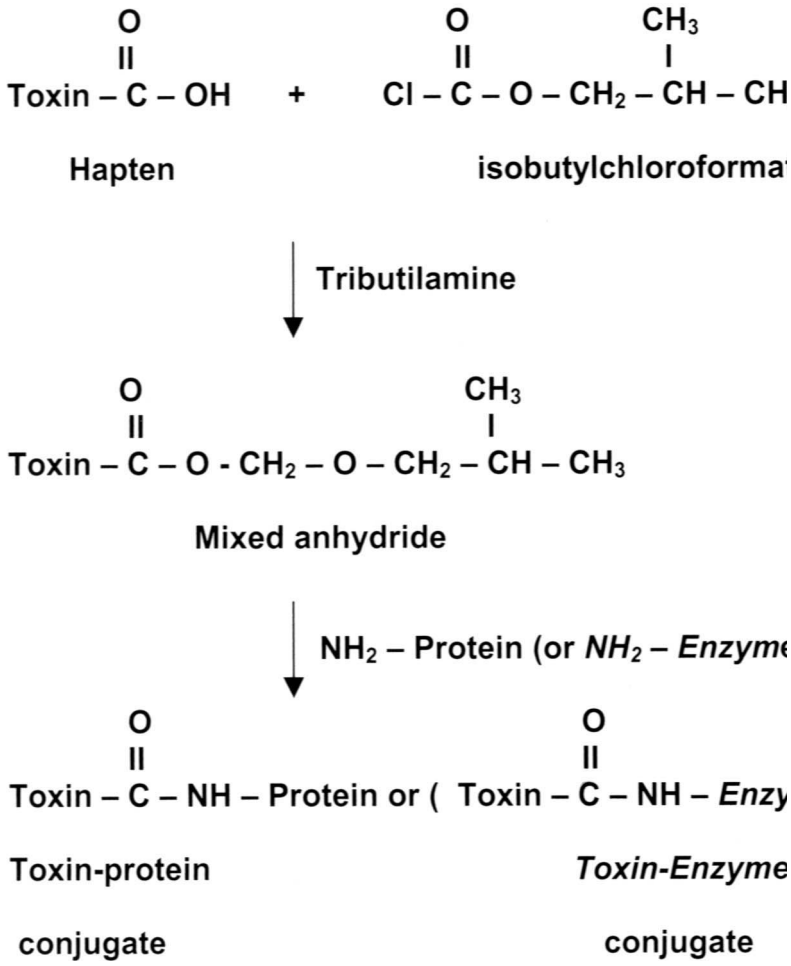


Figure 5. Hapten coupling to protein (or enzyme) by Mixed Anhydride method

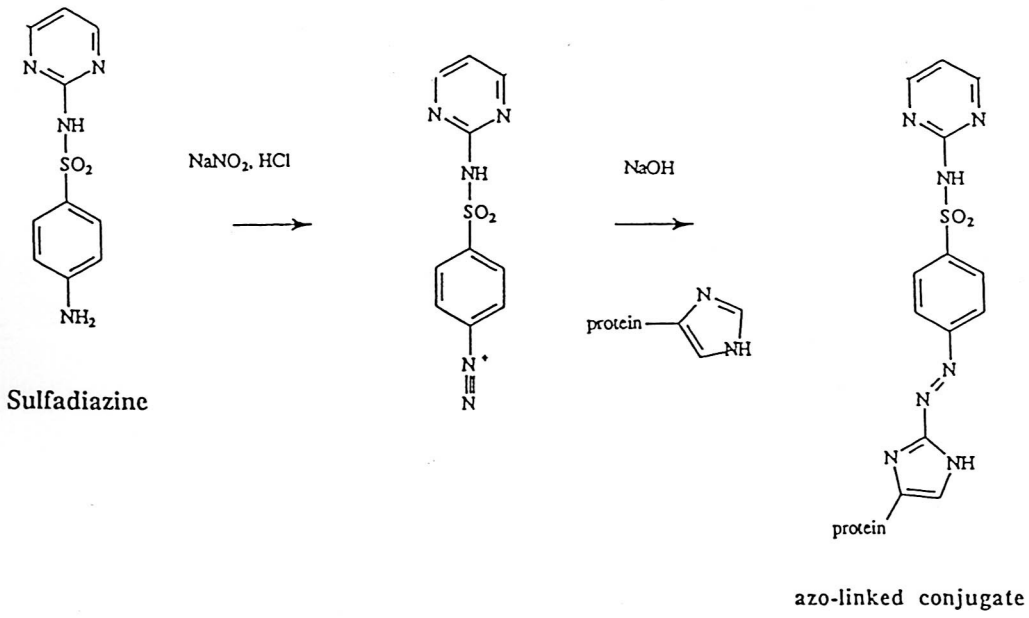


Figure 6. Hapten binding to protein by azo-coupling method.

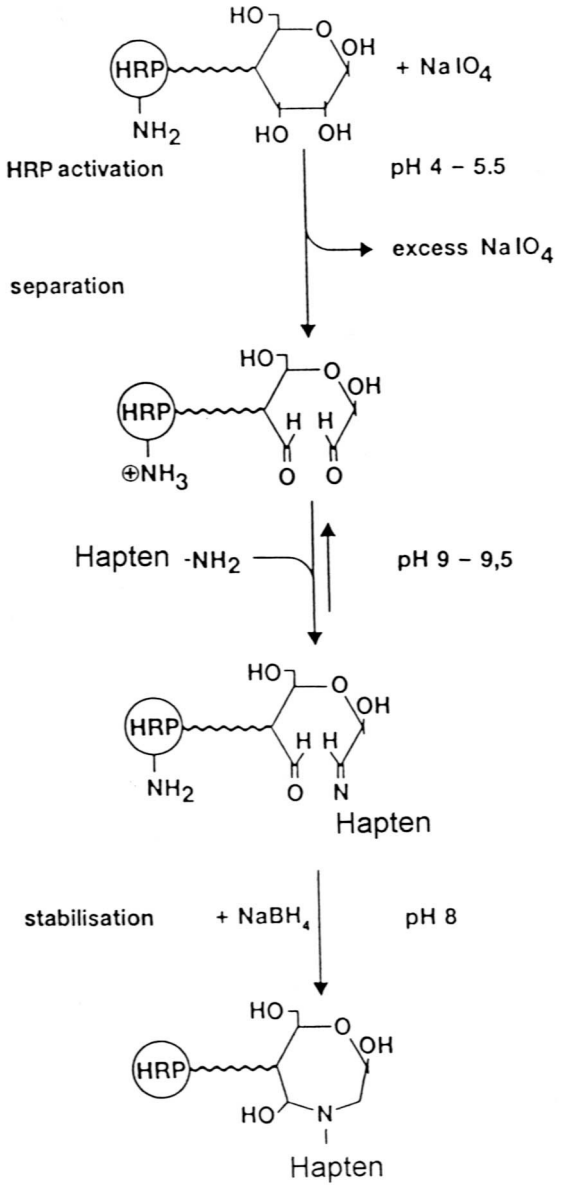


Figure 7. Hapten binding to enzyme by Periodate method

3.4. Mycotoxins

For the interest of public health of consumers, the main work of the complex food control is to assure the quality of foods and test all of the contaminants that can be dangerous for human health. This complex task underlines the account of diagnostic work. The list of contaminants is quite long, including pesticide residues, antibiotics, hormones, drug residues, toxins and mycotoxins. Among the contaminants the mycotoxins are remarkable.

Fungi are heterotrophic microorganisms that can be found throughout nature. Their propagation (reproduction) requires appropriate organic materials, carbohydrates, temperature and humidity. During life cycle, fungi produce different, relatively low molecular weight compounds, carbohydrates, citric acid, other acids and mycotoxines as well. **Mycotoxins** are defined as secondary, extra cellular metabolites of mould growth, which are generally believed to be produced in response to stress factors acting on the fungus. Individual moulds, fungi or mycotoxins rarely occur in isolation and two or more mycotoxins together may have a greater toxic effect than any one alone. (Lawlor and Lynch 2001). As of yet more than 300 mycotoxins have been identified to induce signs of toxicity in mammalian species. The most toxicant fungi are *Aspergillus, Fusarium, Penicillium Alternaria, Acremonium and Phomopsis* species. The deleterious effects are referred to as mycotoxicoses. The major toxigenic species of fungi and their mycotoxins are summarised in **Table 4.**

Mycotoxins have the potential to permeate the food chain (plant – animal – human) and are the common enemy of all food-related cereal, oilseed and animal industries.

According to numerous scientific papers about the mycotoxins and the latest "World Mycotoxin Forum" held in Noordwijk, the Netherlands in 2001, "Mycotoxins are likely to present a significantly greater and more widespread hazard to human health than was thought only a few years ago". This is common problem all over the world.

Some research groups in developed countries (USA, Germany, Japan) have carried out pioneering work in mycotoxin research (Chu et al. 1979; Kawamura et al. 1989; Pestka et al. 1981; Mārtlbauer et al. 1988). In their papers they drew attention to symptoms and diseases caused by mycotoxins and reported new analytical methods for their detection.

Mycotoxin research has been carried out by Ványi, Kovács and Kégl in Hungary (Kégl and Ványi 1991; Kovács and Ványi 1994). They have worked intensively with toxins that are frequent in the country and published reviews of the Hungarian mycotoxin situation.

According to the type of soil, the most common *plough-land fungi* belong to the Fusarium genus, which can infect cereals on the field. If the animal consumes them, they are likely to circulate in the blood, appear in milk and possible muscle tissue, and concentrate in liver and other organs. The Fusarium genus produces several mycotoxins with similar chemical structures; the most dangerous of them is the Trichotecene family (T-2, HT-2, DON, Nivalenol, Fusarenon X). The other carcinogenic mycotoxins, the zearalenons and fumonisins are the metabolites of Fusarium genus as well.

Deleterious effects of the mycotoxins are summarised in **Table 5.**

Table. 4. The major toxigenic species of fungi and their principal mycotoxins
(Pittet, 1998; D'Mello and MacDonald, 1997; Varga et al. 1996; Téren et al. 1996))

Fungal species	**Mycotoxins**
A. flavus; A. parasiticus, A. nomius	Aflatoxins (B_1,B_2,G_1,G_2 and M_1
P.ochraceus; A.alliaceus; P. verrucosum;P. nordicum	Ochratoxin-A
P. expansum; A. clavatus; Byssochlamys nivea	Patulin
F. culmorum; F. graminearum; F. sporotrichioides, F. poae; F. acuminatum	Deoxynivalenol
F. sporotrichioides; F. poae	T-2 toxin
F. sporotrichioides; F. poae; F. graminearum	Diacetoxyscirpenol
F. culmorum; F. graminearum; F. sporotrichioides	Zearalenone
F. moniliforme; F. proliferatum; F. subglutinans	Fumonisins

A (Aspergillus) ; F (Fusarium); P (Penicillium)

Table 5. Deleterious effects of mycotoxins (*D'Mello and MacDonald, 1997)*

Mycotoxins	**Deleterious effects**
T-2, HT-2	Feed refusal, nervous system disturbances, diarrhoea, decreased milk production, acute toxicity, inhibition of protein synthesis, immunosuppressive
DON	Feed refusal, decreased weight gain and vomiting, teratogenic.
Zearalenone (F-2)	Infertility, reduced milk production and hyperoestrogenism in cows.
Ochratoxin-A	Teratogenic, carcinogenic, decreased foetal weight, immunosuppressive, strong inhibition of protein synthesis, nephrotoxicity, hepatotoxicity, strong acute toxicity
Aflatoxin B_1	Acut toxicity: LD_{50} values, 1.0-17.9 mg/kg BW (laboratory animals), 0.5 mg/kg BW (ducklings); hepatic lesions; teratogenic. Reduced feed efficiency, immune function and reproductive performance in ruminants. Carcinogenic in humans.
Aflatoxin M_1	Hepatotoxic and carcinogenic
Fumonisins	Hepatic lesions in pigs and cattle, Equine leukoencephalomalacia. Porcine pulmonary oedema. Implicated in oesophageal cancer in humans, hepato- and nephrotoxicity
Patulin	Acute toxicity, strong antibiotic effects, induces severe oedema

The other type of fungi, called *storage-fungi-group* grows and produces mycotoxins during inappropriate storage of cereals, dried fruits and spices. The *Aspergillus and Penicillium* genera belong to this group, their dangerous metabolite is ochratoxin-A (see **Table 5.).**

The aflatoxins are a group of hepatocarcinogenic bishydrofurano mycotoxins produced by certain strains of *Aspergillus* (see **Table 4.).** Aflatoxins are both acutely and chronically toxic to animals, including man, causing acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects. The four major naturally produced aflatoxins are known as aflatoxins B_1, B_2, G_1 and G_2. "B" and "G" refer to the blue and green fluorescent colours produced by these compounds under UV light on thin layer chromatography plates, while the subscript numbers 1 and 2 indicate major and minor compounds, respectively. When aflatoxin B_1 and B_2 are digested by lactating cows, a proportion (about 1.5%) is hydroxylated and excreted in the milk as aflatoxin M_1 and M_2, compounds of lower toxicity than the parent molecules, but significant because of the widespread consumption of cows' milk by infants. Because of their high toxicity, many countries have set low limits for aflatoxins in foods and feeds. (Pitt, 2000; Boutrif and Cane 1998). In 1977, the International Agency for Research on Cancer (IARC) published its first document of criteria for the evaluation of the carcinogenicity to experimental animals and humans. According to this document the aflatoxin B_1 in group 1 (agent carcinogenic to humans), the aflatoxin M_1 in group 2B (possibly carcinogenic to human) were classified. (Castegnaro and McGregor 1998).

Many countries have set legislative limits to the concentrations of a number of mycotoxins in foods. One of the major problems in setting legislative limits that will be acceptable to both the consumer and the producer understands the distribution of a mycotoxin in a bulk commodity especially if it is particulate. **Table 6.** shows the range of regulatory limits for mycotoxins in different countries.

Table 6. The range of regulatory limits for mycotoxins (Moss, 1996)

Mycotoxins	***Regulatory limit (μg/kg)***	***Number of countries***
Aflatoxins in food	0-50	53
Aflatoxin M1 in milk	0-0.5	15
DON in food	1000-4000	5
Ochratoxin-A in foods	1-300	6
Patulin in apple juice	20-50	10
T-2	100	2
Zearalenone	30-1000	4
Total Fumonisins	1000-10000	2

Table 7 shows the legitimate mycotoxin limits according to the Hungarian Mycotoxin Standard**.** These regulatory limits are in accordance with those of the European Union or even more strict. Among the mycotoxins my work concentrated on the detection of two very important mycotoxins, namely **ochratoxin-A and fumonisin B1** that causes health problems for man and animals all over the world.

Table 7. Acceptable limits for food - and feed products in Hungary

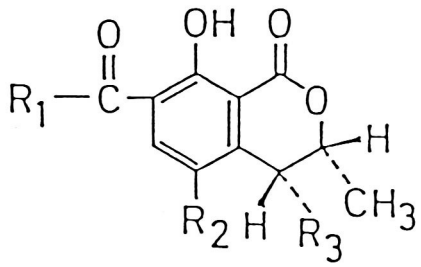
(*Collection of the Hungarian Mycotoxin Standard for animal feeds 90. IV/B and Hungarian Mycotoxin Collection for foods 17/1999 (VI. 16) and modified by the 40/2000, according to Ministry of Health)*

Mycotoxins	Food-products	Regulatory limit µg/kg	Feed-products	Regulatory limit µg/kg
Ochratoxin-A	raw coffee beans roasted coffee cereal products	**15** **10** **5**	laying hen, broilers, swine grown-up ruminants other feeds	**10** **100** **25**
Zearalenone (F-2)	flours, cereals, milling products, corn-based foods	**100**	breeding (cattle, swine, turkey) laying hen, broilers, swine grown-up ruminants raw materials	**80** **500** **2000** **10000**
T-2 /HT-2	flours, cereals, milling products, corn-based foods	**300**	grown-up ruminants laying hen, broilers, swine	**1000** **300**
Deoxynivalenol (DON)	bran for meal flours, milling products, corn-based foods	**1200** **1000**	grown-up ruminants laying hen, broilers, swine breeding (cattle, swine, turkey)	**2000** **2000** **400**
Aflatoxin B1	groundnut, hazelnut, almond, chestnut, dried fruits for direct human consumption or as an ingredient in foodstuffs	**2**	raw materials feeds for lactating animals laying hen, broilers, swine other feeds	**50** **5** **20** **10**
Total Aflatoxin (B1+B2+ G1+G2)		**4**		
Aflatoxin B1	groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	**8**	-	-
Total Aflatoxin (B1+B2+ G1+G2)		**15**		
Aflatoxin B1	nut, hazelnut, almond, chestnut, dried fruits and vegetables, spices to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	**5**	-	-
Total Aflatoxin (B1+B2+ G1+G2)		**10**		
Aflatoxin B1	cereals (including buckwheat) and processed products thereof intended for direct human consumption or as an ingredient in foodstuffs	**2**	-	-
Total Aflatoxin (B1+B2+ G1+G2)		**4**		
Aflatoxin B1	Confectionery products	**1**	-	-
Total Aflatoxin (B1+B2+ G1+G2)		**1**		
Aflatoxin M1	milk, milk powder	**0.05**	-	-
Patulin	fruit juice and vegetable products	**50**	-	-

3.4.1. Ochratoxins

The ochratoxins are metabolites of *Aspergillus* and *Penicillium* moulds (*P.ochraceus; A.alliaceus; P. verrucosum;P. nordicum*). The most important of these toxins is Ochratoxin-A (OA), which consists of a dihydro-isocoumarin moiety linked to the phenylalanine through an α-amide bond. Some ochratoxin derivatives have been isolated from laboratory cultures of these producing, moulds, only ochratoxin-A (OA) and very rarely ochratoxin-B (OB) and ochratoxin C (OC) have been found to occur naturally in mouldy plant products (**Figure 8.).** OA has been shown to be *nephrotoxic, carcinogenic, immunotoxic* and *teratogenic* to all animal species tested so far, and induces experimental liver and kidney tumours (Huff, 1991, Fink-Gremmels, 1995). Long term exposure to OA concentrations as low as 200 µg/kg may induce immuno-suppressive effect, the typical signs of nephrotoxicity in pigs were observed only at levels of OA 1400 µg/kg in feed. Since 1993, the International Agency for Research on Cancer (IARC) classified this toxin as a possible human ***carcinogen*** **(group 2B).**

The structure of OA as a derivative of L-phenylalanine makes it a *potent inhibitor* of phenylalanine-tRNA synthetase, which catalyses the phenylalanine-tRNA aminoacylation (Steyn and Stander 1999).



Ochratoxins	R_1	R_2	R_3
Ochratoxin A	$C_6H_5CH_2COOHNH$-	Cl	H
Ochratoxin B	$C_6H_5CH_2COOHNH$-	H	H
Ochratoxin C	$C_6H_5CH_2COO(C_2H_5)NH$-	Cl	H
(4R)-4-Hydroxy-ochratoxin A	$C_6H_5CH_2COOHNH$-	Cl	OH
Ochratoxin α	H	Cl	H

Figure 8. Structures of ochratoxins

OA contamination is widespread in cereals including corn, barley, wheat, rye, oats and rice. Beside cereals, OA has been found to occur in many other commodities such as coffee, cocoa beans, wine and grape juice, and dried vine fruits (Nakajima et al. 1997; Majerus and Otteneder 1996; Pittet, 1998; Blanc et al. 1998; Otteneder and Majerus 2001). Raw agricultural products, contaminated with OA and used as feed, can pass unchanged through the food chain and can be found in meat and meat products of non-ruminant animals such as poultry and pigs (Van Egmond and Spejiers 1994). As a preventive system in Scandinavian countries, contaminated meat is discharged to avoid human exposure to residues of OA (Fink-Gremmels 1999).

An additional concern is the human exposure, since OA has been detected in blood and breast milk (Miraglia et al. 1995). Recently a study was published about the presence of OA in human milk in relation to dietary intake (Skaug et al..2001). Twenty one % of the milk samples contained OA in the range 10-182 ng/l. The main source of OA contamination was liver paste, fruit and chocolate cakes, breakfast cereals, cheese and all kinds of juice.

Based on the *carcinogenicity study* with OA in rats, a "tolerable daily intake" (TDI) in humans was also estimated and ranged from 0.2 to 4.2 ng/kg body weight (BW). The FAO/WHO Joint Expert Committee on Food Additives established a provisional "tolerable weekly intake" (TWI) level of 112 ng/kg BW corresponding to 16 ng/kg BW daily, which was calculated on the lowest damaging level in the kidneys of pigs, which are the most sensitive species (Van Egmond, 1991). In view of its potential carcinogenicity, a daily OA intake in the order of 5 ng/kg BW may be a reasonable estimate for a "virtually safe dose" (VSD), (Zimmerli and Dick 1995).

The *toxicokinetics* of OA differ widely among the investigated animal species; the rhesus monkey showed the longest half-life value of 21 days (Breitholtz et al.1991). In human blood, these data vary between 20-50 days as OA binds strongly to serum albumin and recycling in the kidney contributes to its longer half-life. Due to this fact, OA concentration in blood may be higher than the daily toxin intake (Zimmerli and Dick 1995).

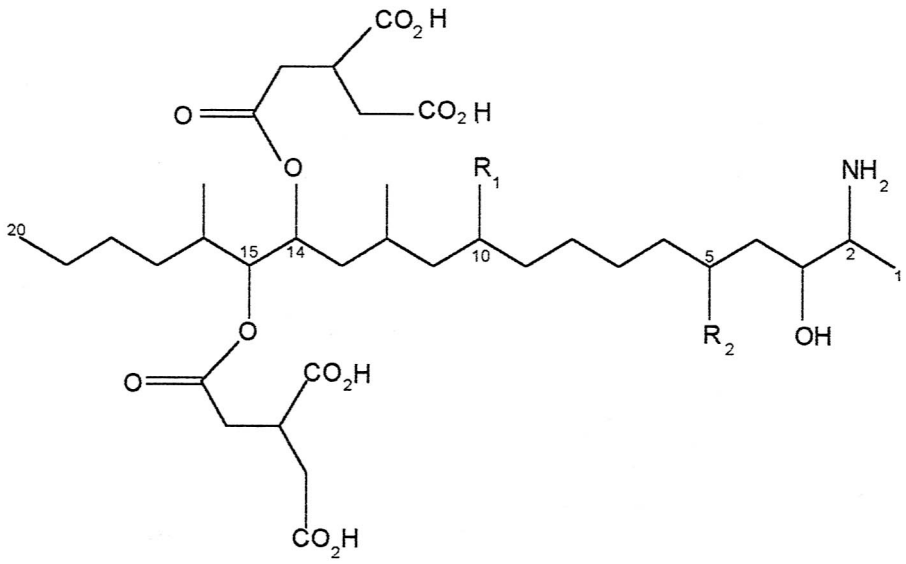
The OA may influence the sperm quality of boars and can cause fertility problems in pig breeding. In addition, OA has found to impair fertility in boars and to be teratogenic, but only at extremely high concentration (Marquardt and Frohlich 1992). This problem was studied in Hungary as well, OA was effected for the motility and longevity of boar sperm (Solti et al. 1999).

For determination of OA in cereals, body fluids (human and animal serum, seminal plasma) and animal tissue, different analytical methods are commonly used. Conventional analytical techniques (TLC, GC, HPLC) are officially accepted, they are expensive, time consuming and need appropriate instrumentation and/or trained personnel. Proposed legislation in some countries concerning "acceptable" limits of OA suggests that the assay must have high sensitivity.

Immunoassays such as ELISA fulfil these requirements and have several other advantages including simplicity, low cost, reliability, low requirements for technical skills and simple equipment. The sensitivity of these techniques enables the detection of OA at concentration of the below proposed regulatory values. Although many reports have been published about the development of ELISA using polyclonal (Clarke et al. 1993; Lee and Chu 1984; Märtlbauer and Terplan 1988; Pestka et al. 1981) or monoclonal (Ramakrishna et al. 1990; Candlish et al. 1988; Kawamura et al. 1989) antibodies against OA, only few of them have been commercialised (EZ-SCREEN Ochratoxin-A-(EDS-USA), RIDASCREEN Ochratoxin-A-(R-Biopharm-Germany), Ochratoxin-A assay kit, (BioKits-U.K.)).

As OA is present in Hungarian food and feed products as well, (remembering to the contaminated coffee scandal some years ago), the testing of this mycotoxin is essential. My research group decided to develop a monoclonal antibody based, very sensitive ELISA test for quantitative measurement of ochratoxin-A in different cereals. This work is part of the mycotoxin research program of ABC, started years ago. The developed OA test was first developed for screening of OA content of cereals and later has been modified, validated and applied for other matrices as well, e.g. for human and pig blood, seminal plasma of boars and tissues of chickens.

3.4.2. Fumonisins



Fumonisins	R_1	R_2
Fumonisin B_1	OH	OH
Fumonisin B_2	H	OH
Fumonisin B_3	OH	H
Fumonisin B_4	H	H

Figure 9. Structures of Fumonisins

Fumonisins are toxic metabolites produced by naturally occurring *Fusarium* fungi in corn, in particular *Fusarium moniliforme* and *Fusarium proliferatum* (**Table. 4)** *Fusarium moniliforme* as a mycotoxigenic species was recognised and published by Marasas (1976, 1988a). Fumonisins were first isolated from this fungus by Bezuidenhout in 1988.

Chemically, fumonisins are characterised by a 20-carbon backbone, two tricarballylic acid (TCA) groups, one to three hydroxyl groups and a single amino group. The TCA chains may be removed by alkaline hydrolysis resulting the partial (lacking one TCA) or full (lacking both TCA) hydrolysis products (HFB_1 and HFB_2) (Beier et al. 1996), (**Figure 9)**. There are at least four naturally occurring fumonisins known as FB_1, FB_2 FB_3 and FB_4.

The most investigated one is **FB_1** which can cause leukoencephalomalacia (LEM) in horses, pulmonary oedema in pigs, nephrotoxicity, liver cancer in rats and oesophageal cancer in human (Rheeder et al.1992; Kakuk, 1995; Fazekas et al. 1997). Butler gave an early description of LEM in 1902, which called the disease leucoencephalomalacia and produced the symptoms in a test animal with mouldy feed (Dutton, 1996).

The International Agency for Research on Cancer (IARC) classified the toxins from *Fusarium moniliforme* as possible **carcinogenic** to humans (**Group 2B**) (Peraica, 1999).

FBs can be found mainly in maize and different corn products but have been occasionally

detected in rice, wheat noodles, spices, beer and in raw milk (Dutton, 1996, Pittet, 1998; Maragos et al. 1997). The occurrence of FB_1 in maize in different countries was summarised by Dutton (1996), the FB1 level varied from 30 μg/kg to 33,4 mg/kg. FBs contaminate regularly maize in Hungary as well; case reports were published earlier (Szécsi and Vágújfalvi 1995; Fazekas et al. 1996, 1997).

With the widespread occurrence of fumonisins in maize and maize products it is important to understand how stable they are and what type of food processing can reduce the contamination. γ - Irradiation of maize is sufficient to microbiologically sterilise maize flour (15 kGy) but content of fumonisin decreased only by about 20 % and the mycotoxin was stable in this irradiated maize for at least 6 months at 25 ^{o}C. Fumonisin B_1 is relatively heat-stable and the half-life in dry maize was calculated to be 10, 38, 175 and 480 min at 150, 125, 100 and 75 ^{o}C, respectively. Fumonisins remained stable when maize was fermented for ethanol production, and most of the toxin was recovered from the distillers' grains. The fumonisins remained stable during heat treatment under alkaline conditions, only the tricarballyic acid residues were removed, but the molecule after this procedure remained toxic (Moss, 1998).

Studies on the FBs are still at a relatively early stage. Some studies have been established that the fumonisins specifically inhibited the conversion of sphinganine to dihydroceramides. Because FB1 has a close molecular resemblance to sphinganine, it interferes with ceramide biosynthesis. This can be an explanation of its carcinogenic properties (Dutton, 1996). It is difficult to make a risk assesment on the basis of the already established toxicological data, but Gelderblom et. al (1996) made estimates for the tolerable daily intake (TDI) which ranged from 31 to 160 ng/kg BW/day. These estimates are more than 1000 times those for aflatoxin B_1 but the fumonisins are often present at significantly higher concentrations than the aflatoxins (Moss, 1998).

As the presence of sometimes high content of fumonisins in cereals are a great problem all over the world, Switzerland proposed legislation for FB_1, the "acceptable" limit was determined in 1000 μg/kg (Boutrif and Canet 1998, Pittet, 1998). From the last year (2001) USA (FDA) has introduced regulations for FBs in food- and feed-products (**Table 8.).** As I know the Mycotoxin Committee in Hungary has prepared a similar "acceptable limit" for FBs but it has not been officially accepted yet.

Fumonisins are analysed typically by chromatographic methods (TLC, LC and LC-MS, GC-MS, and HPLC), which are expensive, time-consuming and need appropriate instrumentation and/or trained personnel (Dutton, 1996, Maragos et al. 1997). In the last few years some ELISA tests has been developed using polyclonal or monoclonal antibodies with different sensitivity and detection limits (Usleber et al.1994, Azcona-Olivera et al.1992a, Elissalde et al. 1995, Yu and Chu 1996).

Beside the OA test another research program was to develop a sensitive monoclonal antibody based direct, competitive ELISA test for screening fumonisin B_1 in different cereals. In this dissertation I summarized the steps of the developing procedure.

Table 8. Proposed limits for Fumonisins in food and feed-products in USA
(*Guidance for Industry, FDA (USA), November 2001)*

Human Foods	Total FBs (mg/kg)	Animal Feeds for	Total FBs (mg/kg)
Degermed dry milled corn products, fat content of <2,25%	**2**	Equins and rabbits	**5***
Whole or partially degermed dry milled corn products (e.g., flaking grits, corn grits, corn meal, corn flour with fat content of ≥2,25%, dry weight basis)	**4**	Swine and catfish	**20****
		Breeding ruminants, breeding poultry and breeding mink	**30****
Cleaned corn intended for popcorn	**3**	Ruminants ≥3 months old being raised for slaughter	**60****
Dry milled corn bran	**4**	Poultry being raised for slaughter	**100****
-	**-**	All other species or classes of livestock and pet animals	**10****

***** = no more than 20% of diet, dry weight basis
****** = no more than 50% of diet, dry weight basis

3.5. Antibiotics and veterinary drug residues

The recent food scandals involving European Agriculture drew the attention of European Agency for the Evaluation of Medicinal Products to change the quality control system of food products. The current European Legislation ought to be more rigorous; it needs more powerful analytical techniques to control the misused or illegally used antibiotics.

For residue control programs, the analysis of every sample for all possible residues is not a realistic way. Programs to monitor the meat supply for presence of drug residues must establish priorities for analysis. Among them the analysis of antibiotics in the tissue, sera and milk of animals would be important.

Antibiotics are often used in modern agriculture, as feed additives to increase weight gain and also as prophylactic treatment to avoid sickness. The substances authorised as veterinary drugs are listed in the annexes 1, 2, or 3 of the Council Regulation EEC No 2377/90 (Maghuin-Rogister, 2001). **Table 9.** Summarises the MRLs date of most important veterinary drugs. Despite this clear description of which antibiotics are authorised, there are still some substances with antimicrobial activity that are used illegally. Such misuse can lead to residue problems in food-products of animal origin.

Residues of veterinary drugs, particularly of antibiotics, represent a potential hazard for human health. The increasing *bacterial resistance* can have dramatic consequences for public and animal health all over the world.
Administration of antibiotics can lead to the selection of pathogenic *bacteria resistant* to antibiotics. Physicians attracted attention that resistance against the anti-microbial drugs is a great health problem in Europe, recently.
Allergic reactions to antibiotics could appear after consumption of food contaminated by antibiotic residues.
Finally, antibiotic residues in milk may cause technical problems, by *inhibiting the fermentation process* of cheese and yoghurt production.
The problem is the misuse of antibiotics on the black market and the administration of antibiotics

without veterinary prescription and not in the acceptable dose.

Table 9. MAXIMUM DRUG RESIDUE LEVEL IN BIOLOGICAL FLUIDS

(Council Regulation EEC No 2377/90)

Drug residues	Veterinary application	MRLs	Measure in biological fluids
Sulphonamides	**bacteriostatic compounds**	**100 µg/kg (EU) 10 µg/kg (US)**	**Milk, Meat, Sera**
Chloramphenicol	**Against gram-negative organism (E.coli,Salmonella), Against cattle, swine, poultry**	**0 µg/kg (FDA) 1µg/kg (EU)**	**Milk, Meat, Eggs**
Gentamicin	**Against of neonatal diarrhea of swine, swine dysentery, bovine mastitis therapy**	**100 µg/kg (EU) and 100 µg/l**	**Milk, Meat, Sera**
Tylosin	**Feed additives, treat of cattle, pig, poultry**	**50 µg/l (EU)**	**Meat, Milk**
Streptomycin	**Treatment of bovine mastitis**	**100 µg/l (FAO/WHO)**	**Milk**
Tetracyclines	**Feed additives**	**100 µg/l(EU) 2 µg/kg (FDA) 6 µg/kg (FDA)**	**Milk Meat Liver**
Kanamycin, Tobramycin	**Treatment of bovine mastitis**	**0.25 µg/kg (Japan)**	**Meat**
Beta-Lactam groups			
Penicillin G	**Treatment of bovine mastitis**	**5 µg/l(FDA) 50 µg/kg(EU)**	**Milk Meat, Kidney**
Amoxicillin, ampicillin	**antibacterials**	**50 µg/kg(EU) 10 µg/l (FDA)**	**Meat Milk**
Oxacillin, Cloxacillin	**antibacterials**	**300 µg/kg(EU)**	**Meat**
Centiofur	**Pneumonia, treat of cattle, pig, horse**	**0.5 µg/kg (FDA) 100 µg/l (EU)**	**Meat, Milk Milk**

Until a few years ago, in most countries the control of meat and milk for veterinary drug residues was based on microbiological methods (bacterial growth inhibition test). This method is not as sensitive as required by the MRL regulations.
Recently, several sensitive, accurate non-microbiological analytical methods have been introduced for determination of residues.

Proposed strategy for detecting antimicrobial residues in foodstuffs in EU:

1. Pre-screening at the level of the slaughterhouse using a simple microbiological test for the presence of bacterial growth inhibiting substances.
2. Selective screening of positives in Step 1. by means of immunoassays or other multianalyte methods, in order to identify the chemical nature of the antibiotics.
3. Detailed chemical identification using instrumental analytical methods e.g. GC, GC/MS, HPLC/UV, LC/MS for identification of the molecule.
4. Quantitative assay of the identified residue and evaluation with respect to MRLs (Council Regulation N^{o} 2377/90).

Usually the drug residues are screened by ELISA tests and the positive samples are confirmed by GC-MS/MS method.

Some enterprises produce and commercialize ELISA tests for antibiotics e.g. Ridascreen, (R-Biopharm, Germany), Euro-Diagnostica (The Netherlands), Biacore (Sweden), Idexx (USA) with different detection limits.

In Hungary the veterinary drugs are tested partly by microbiological method, by HPLC and by quick antibiotic (SNAP ® beta-lactam, tetracycline and sulphonamides) tests, produced by Idexx. These tests are not a quantitative test but give a quick- yes/no – answer for the user.

To our knowledge only the ABC group deals with the development of quantitative ELISA tests for veterinary drug residues in Hungary. This project was started some years ago, which resulted in two, monoclonal antibody based ELISA tests for quantitative detection of gentamicin and sulphadiazin in milk and sera. Parameters of both tests are in good agreement with the requirement of EU directives.

3.5.1. Gentamicin

Gentamicin, a broad-spectrum aminoglycoside antibiotic, is affective against most microorganisms associated with infections of the bovine reproductive tract. The aminoglycoside group contains streptomycin, dihydrostreptomycin, neomycin, kanamycin and gentamicin (VanEs et al. 2000). Gentamicin is commonly used to treat gram-negative bacterial diseases, especially Escherichia coli and Pasteurella spp, as well as gram-positive organisms, such as *C. pyogenes,* and streptococci spp that are frequently the causative microorganisms for endometritis, pyometra, and chronic metritis. Bacterial metritis is a leading cause of infertility in cows. Gentamicin (200 mg) injected intrauterinally is an effective single-dose therapy in the treatment of bovine metritis and/or cervicitis. After its absorption from the uterus, gentamicin can be detected in serum, urine, and milk and in endometrial tissue (Haddad et al. 1986).

Gentamicin residues in milk are dangerous not only for milk consumption, but they are blocking the processing of cheese and other dairy products as well.

Gentamicin is commonly used in human as well to treat gram-negative pneumonia, urinary tract infections and peritoneal, skeletal and soft tissue infections. Assay of gentamicin in serum may provide information whether the therapeutic concentration of the drug was used or not.
High level of aminoglycosides can cause severe kidney damage while a low level can lead to continued infection and may result in the development of bacterial resistance to these antibiotics.

The maximum residue/safe tolerance levels (MRLs/STLs) of gentamicin in milk and sera is 100 µg/l, as determined by EU, the accepted value of total aminoglycoside antibiotics in milk is 200 µg/l. The FDA have not set a STL for gentamicin in milk since the drug is not yet approved for use in lactating cows, although they state that any test method must be capable to measure the gentamicin at the 30 µg/l level (VanEs, 2001).

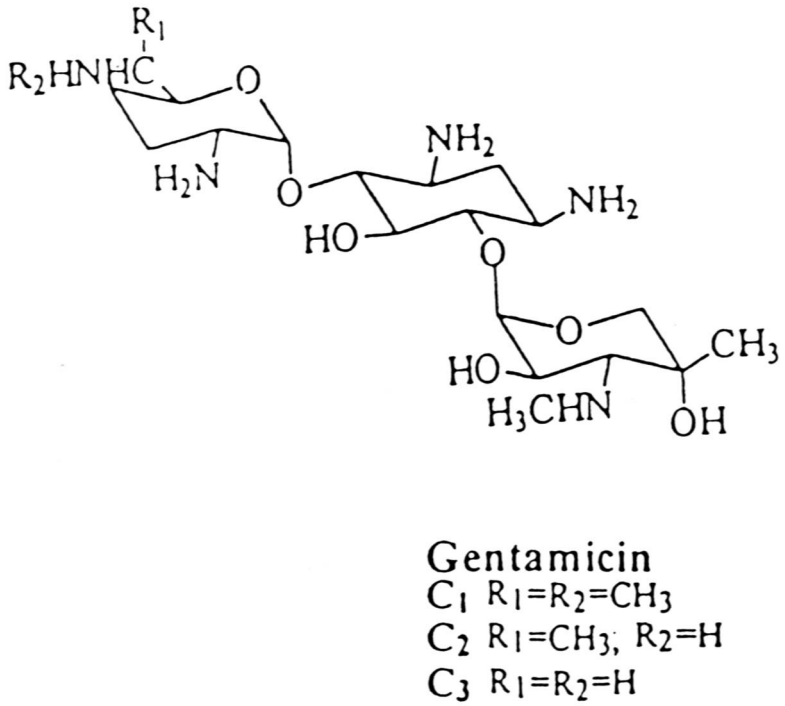


Figure 10. Structure of Gentamicin molecules

The gentamicin molecule is the C_3 molecule, where R1 and R2 are containing H atom, the others, the C_2 and C_1 are cross-reacting molecules.

A number of suitable physico-chemical and biological test methods have been reported for aminoglycosides in milk. As these compounds are thermolabile and hydrophilic molecules, their detection by LC-MS method is difficult. Drawbacks of the *physico-chemical* methods are the high cost, the need for complex instrumentation and trained personnel.

Biological test methods have traditionally been based on microbial inhibition assays. Commercial test kits are the Delvotest ® SP (DSM Food Specialties, The Netherlands), the BR-range (Laboratorium Enterotox, Krefeld, Germany), the Charm II test (Charm Sciences Inc. Malden, MA, USA) and the T101 test (Vario, Helsinki, Finland). Although these tests are simple to perform, they are non-specific and time-consuming.

The other type of assays is the *field-based* quantification of specific aminiglycosides would represent a useful screening tool for the farmer and dairy to select and eliminate the unacceptable milk. Schnappinger et al. (1993) have reported an enzyme-linked immunofiltration assay for detection of streptomycin and dihydrostreptomycin in milk with with visual detection limit of 2 and 5 μg/l. These tests required no sample preparation. Ara et al (1995) have reported a dipstick dot-ELISA for gentamicin in milk based on sandwich assay with detection limit of 100 μg/l. Although the device can be used on the field, it is not rapid, requires three 1 h incubation steps, and 5 min colour development. VanEs et al. (2001) developed an immunoassay coupled to flow-injection analysis and electrochemical detection with measuring range of 0-100 μg/l gentamicin in milk but it has not commercialised yet.

A new line in the immunoassays is the sensor chip, developed by Biacore firm (Sweden).

Based on this novel technology two Dutch groups (Wageningen, The Netherlands) developed a competitive and a non-competitive immunoassay for gentamicin. Although the assay was very quick (5 min/sample), the assay was not sensitive enough; the measuring range of gentamicin was 10-100 ng/ml only.

Although several papers have been published about detection of gentamicin, only some kits can be found on the market. According to my knowledge the following kits are available.

a./ "SIGNAL", manufacturer: Smithlkine Animal Health Products, West Chester, PA, USA,
b./ "Lactek", manufacturer: Idetek, San Bruno, CA, USA,
c/ Gentamicin EIA , manufacturer Eurodiagnostica, The Netherlands.

The aim of our present work was to develop a sensitive, monoclonal antibody ELISA test for quantitative determination of gentamicin in milk and sera. Our test suits the requirements of EU concerning the MRL value of 100 µg/l. The test is very easy, does not require complicated sample preparation before the assay. In this dissertation I summarize the most important steps of this work.

3.5.2. Sulphonamides

The potential danger of antibiotic and other veterinary drug misuse has been officially recognised by the European Union. Recently, four antibiotics that were widely used earlier in animal feed, have been banned due to their homology with human antibiotics.

The medical benefits of banning antibiotics are already clear. According to a Danish study, the incidence of antibiotic-resistant bacterial strains has decreased dramatically since the prohibition of using antibiotics in animal feed, introduced in 1995.

To protect consumers, regulatory authorities closely control the level of drug residues in food. In Europe a maximum residue limit (MRL) of 100 µg/kg in meat has been set for total sulphonamide content (the US has a limit of 10 µg/kg for each individual compounds used).

Sulphonamide group contains at least 18, chemically very similar molecules, among them the sulphadiazine, sulhamethazine, sulphathiazole and sulphamethoxypyridazine that are widely used for the treatment and prophylaxis of bacterial infections in the veterinary medicine.

Sulphonamides are derivates of sulphanilamide (p-aminobenzene sulphonic acid). They can be described as N1- or N4-substituted sulphanilamides depending on whether the substitution is on the amido or aromatic amino group, respectively. Substitution in the benzene ring of sulphonamides usually yields inactive compounds such as the main metabolites (N4-acetylated sulphonamides). Substitutions at the amido group with heterocyclic aromatic nuclei yields compounds with varying degrees of anti-microbial activity (Haasnoot et al. 2000) (**Figure 11.)**

Live animals, meat and meat products and milk can be screened for drug residues either on farms, at slaughter or prior to food processing. Although there is an extensive range of tests available, few techniques have satisfactory throughput, sensitivity or reliability to allow screening of large numbers of samples.

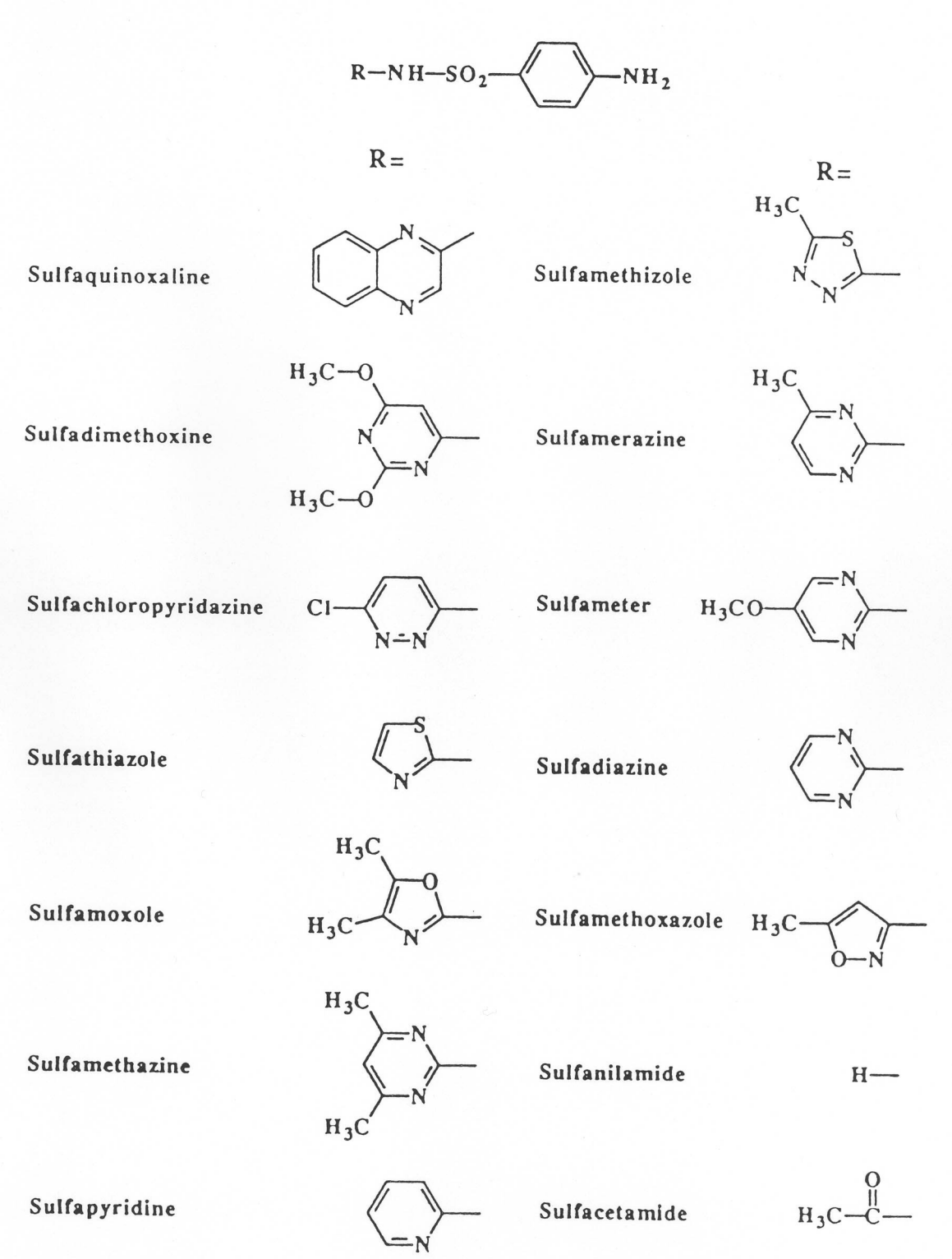


Figure 11. Structure of sulphonamides.

Microbial tests are often used for screening but they are usually too insensitive for sulphonamides. Instrumental techniques are accurate and sensitive but they are also costly, time-consuming, and therefore not particularly suitable for residue screening.

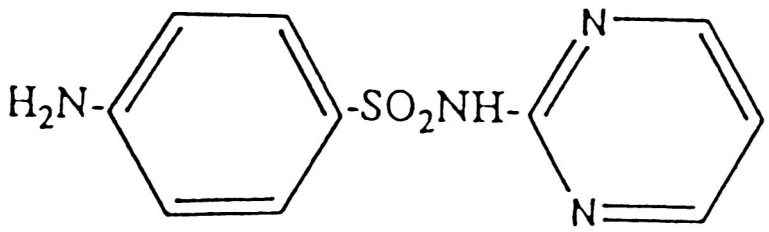


Figure 12. Structure of Sulphadiazine

Antibody-based methods, such as enzyme-immunoassays are suitable for screening sulphonamides, so many scientific papers have been published about the most important sulphonamides, such as sulphamethazine (Märtlbauer et al. 1992; Ostermaier et al. 1995; Bergström et al. 1999; Fránek et al. 1999), sulphathiazole (Thomson and Sporns 1995) and sulphadiazine (Elliott et al. 1999; Märtlbauer et al. 1992; Ostermaier et al..1995). Only individual sulphonamides can be determined, as no multi-residue ELISA kit is available for sulphonamides.
The selected positive samples shoud always be confirmed by GC-MS/MS method (Van Eeckhout et al.2000) according to the EU directives.

Some commercialised kits are available for sulphonamides by Riedel-de Haën (Germany), Biocore, (Sweden), Idexx (USA), Transia (France), Eurodiagnostica (The Netherlands), but there is no other research group in Hungary developing new ELISA tests for measuring antibiotics in food.

Among the several sulphonamides the sulphadiazin has first been selected and decided to develop an ELISA test for its detection in biological fluids, milk and sera.

4. Materials and Methods

4.1. Chemicals

Ochratoxin-A, Coumarin and L-beta-phenylalanine were purchased from Sigma–Aldrich (Germany). Ochratoxin-B (OB) and Ochratoxin alpha were generous gifts from M.E. Stack (U.S. Food and Drug Administration, Washington, DC); monoclonal anti-ochratoxin-A (used for control of enzyme labelled OA conjugate) was kindly supplied by A. A. G. Candlish (University of Strathclyde, Scotland) .
Tri-n-butylamin, and i-Butyl-chlorophormate were purchased from Fluka (Germany), N-hydroxisuccinimide, Dicyclohexylcarbodiimide (DCC) were obtained from Sigma-Aldrich, (Germany).
Fumonisin B_1 and Keyhole-limpet hemocyanin (KLH), Freund's Complet Adjuvant (FCA), Freund' Incomplete Adjuvant (FIA) were purchased from Sigma-Aldrich (Germany), Glutaraldehyde, Horseradish peroxidase (HRP) and Sodium borohydride were obtained from Reanal (Hungary), BSA was obtained from Serva (Germany), Sodium periodate was purchased from Fluka (Germany), FB_2 and HFB_1 were a gift from Jupiter Yeung , National Food Processors Association (NFPA,1401 New York Ave., N.W. Washington, D.C, 20005, USA), FB_3 was generously provided by Ronald D. Plattner (USDA-ARS-NCAUR, Peoria, IL, USA).
Gentamicin sulfate, Netilmicin, Sisomicin, **Sulphadiazine,** Sulphamethazine, Sulphathiazole, Thiamphenicol, Narasin, Tetracycline, Chloramphenycol, Streptomycin, Ammonium sulphamate, Potassium dichromate were purchased from Sigma-Aldrich (Germany), Sodium nitrite was obtained from Merck (Germany).
Spectrapore membrane tubing mw. 12.000-14.000 was puchased from Spectrum Medical Industry (USA).
Materials for monoclonal antibodies: Fetal calf serum (FCS), Hypoxantine/thymidine (HT; 50x), Aminopterin (50x), Dimethyl sulphoxide (DMSO), Polyethylene glycol 1300-1600, RPMI-1640 Medium Hybri-Max, mouse subclass specific antibodies in goat (anti-mouse IgG_1, anti-mouse IgG_{2a}, anti-mouse IgG_{2b}, anti-mouse IgG_3, anti-mouse IgA, anti-mouse κ) were obtained from Sigma-Aldrich (Germany).
Tissue culture plates (96- and 24 –wells, Culture flasks (50 ml-260 ml), Cryotubes, sterilized test tubes (50-100 ml) were purchased from Greiner (Germany).
Myeloma cell line. Sp2/0-Ag 14 was obtained from American Type Cell Collection CRL-1581.

Materials for ELISA testing: Microplate wells (Immunoplate F-8, Maxisorp) were obtained from NUNC (Denmark) and Immulon 4.HBX was purchased from Thermo Labsystems (USA), 3,3',5,5'-Tetramethylbenzidine (TMB) was obtained from Fluka (Germany), Urea-peroxid was obtained form Merck (Germany).
All other chemical used were analytical grade.

4.2. Preparation of immunogens.

4.2.1. Preparation of ochratoxin-A-BSA immunogen by active ester method (Märtlbauer and Terplan 1988)

Ochratoxin-A (OA) (3,2μmol) was dissolved with N-hydroxysuccinimide (15μmol) and Dicyclohexylcarbodiimide (30 μmol) in 150 μl of dry Dimethylformamide (DMF) and incubated at room temperature (RT) for 18 hours. This reaction mixture was added drop by drop to bovine albumin (BSA: 0.045 μmol), which was dissolved in 3 ml of Sodium hydrogen carbonát (0.13 mol/l) and was stirred for 3 hours at room temperature. The solution was dialysed in PBS (Phosphat buffer 0.01 mol/l with 0.15 mol/l NaCl, pH: 7.3) buffer for three days. The ochratoxin-A-BSA conjugate was stored at –20 ^{o}C until use. The estimated Mol $_{OA}$/ Mol $_{BSA}$ = 71. (Molecular weight (Mw) of OA: 403, Mw of BSA: 66000).

Content of PBS buffer: Phosphat buffer 0.01 mol/l with 0.15 mol/l NaCl, pH:7.3.
The conjugate was diluted to 1 mg/ml concentration for immunization of mice.

4.2.2. Preparation of Fumonisin B1 (FB_1) -KLH conjugate (Avrameas and Ternynck, 1969)

Conjugation of FB1 to KLH was performed by glutaraldehyde (GA) reaction with some modification. KLH (10 mg, 0.014 μmol) was dissolved in 12 ml of 0.8% NaCl and 1.3 ml of GA (2 %) was added and incubated for one day at RT. The excess GA was dialysed for 2 days, then centrifuged. FB_1 (1 mg, 1.39 μmol) was dissolved in 0.4 ml of methanol and drop-wise added to the activated protein, they incubated for 20 hours at RT. Finally the unreacted aldehyde groups were blocked by addition of 12 mg sodiumborohydride and the mixture was stirred for 4 hours. The conjugates were then dialysed in PBS for 3 days and stored at -20 ^{o}C. (Mw of FB1: 721.8, Mw of KLH is about 700 000, Mol_{FB1} / $Mol_{KLH} \cong 100$).
For immunization the conjugate was diluted to 1 mg/ml.

4.2.3. Preparation of Gentamicin-BSA conjugate (Avrameas and Ternynck, 1969)

The conjugation of GE was made by glutaraldehyde method with some modification.
BSA (20 mg, 0.3 μmol) was dissolved in 2 ml of PBS and dialysed at 4 ^{o}C for 2 days in PBS, then Gentamicin-sulphate (10.58 mg, 15.2 μmol) plus 30 μl, 1% glutaraldehyde (GA) was added and incubated for 3 hours at RT and 18 hours at 4 ^{o}C. The conjugate was dialysed against PBS for three days at 4 ^{o}C. (Mol_{GE} / $Mol_{BSA} = 50$, protein content was 10 mg/ml).
For immunization the conjugate was diluted to 1 mg/ml.

4.2.4. Preparation of sulphadiazine-BSA conjugate by diazo coupling (Haasnoot et al. 2000)

Sulphadiazine (SD), (10 mg, 40 μmol) was dissolved in 2 ml hydrochlorid acid (3.5 M) and 0.6 ml of a solution of sodium nitrite (10 mg/ml) was added dropwise to this mixture with stirring in the dark at 4 ^{o}C. After 30 min, the reaction was stopped by addition of 0.75 ml ammonium sulphamate (33 mg/ml). To a solution of BSA (20 mg, 0.3 μmol in 4 ml PBS) 1 ml of the diazo derivative solution was slowly added and the pH was adjusted to 9.5 ± 0.5 by the addition of a few drops of sodium hydroxide (1 M). The colour of the mixture turned to yellow. The reaction mixture was left at 4 ^{o}C for 6 h and the unreacted material was removed by dialysis against PBS. The dialysed solution was stored at –20 ^{o}C until used. Mol_{SD} / $Mol_{BSA} \cong 40$, Protein content: 6.3 mg/ml). Mw of SD is 250.3, Mw of BSA is 66 000.
For immunization the conjugate was diluted to 1 mg/ml.

4.3. Preparation of enzyme-conjugates

4.3.1. Preparation of ochratoxin-A-Peroxidase (HRP) conjugate by mixed anhydride method (Märtlbauer and Terplan, 1988 with some modification)

Ochratoxin-A (2.48 μmol) was dissolved in 100 μl of dry DMF and cooled to –15 ^{o}C. To this solution 1.5 μl Tri-n-butilamine was added and cooled again to –15 ^{o}C. After 15 min. incubation, 1 μl of i-butil-chlorophormate was added, stirred and cooled again to –15 ^{o}C and incubated for 15 min. HRP (RZ: 3.0, 0.05 μmol) was dissolved in 250 μl distilled water + 250 μl of DMF and cooled to –15 ^{o}C . The pH of the peroxidase solution was raised to 9.0 with some drops of 0.1 mol/l of $NaHCO_3$. The activated ochratoxin-A solution was added quickly, but drop to drop to HRP solution and stirred, the mixture was incubated for 1 hour at –15 ^{o}C, than 2 hours at + 4 ^{o}C. The pH of the mixture should be 9.0-10.0 during the incubation period. The solution was dialysed for 3 days in PBS buffer. The estimated Mol_{OA}/ $Mol_{HRP} = 50$. The working dilution of this conjugate was assessed in direct ELISA (Mw of HRP: 40000, Mw of OA 403).
The RZ (Reinheitszahl) shows the purity of the enzyme. (see Glossary).

4.3.2. Preparation of Fumonisin B_1 (FB_1) – HRP conjugate (Wilson and Nakane, 1978)

Eight mg of HRP (0.2 μmol) was dissolved in 2 ml distilled water, then 0.2 ml of 0.1 M sodium periodate was added and stirred for 20 min at RT, then dialysed overnight in 1 mM sodium acetate buffer (pH: 4.4). The pH of the activated HRP was adjusted to 9-10 with 0.1 M NaOH, mixed with 2 mg of FB_1 (2.77 μmol) dissolved in 0.5 ml distilled water and the mixture was stirred for 3 hours at RT. The reaction was stopped with 0.2 ml of a sodium borohydride solution (4 mg/ml) and incubated overnight at 4 ^oC. Finally the conjugate was dialysed against 0.1 M phosphate-buffered saline (PBS) and stored at - 20 ^oC.

(Mol_{FB1}/ $Mol_{HRP} \cong 14$)

4.3.3. Preparation of Gentamicin-Peroxidase conjugate (Wilson and Nakane, 1978)

The conjugation was prepared similarly to **point 4.5.,** but HRP was 10 mg (0.25 μmol) and Gentamicin-sulphate was 0.95 mg (1.4 μmol). (Mol_{GE} /Mol_{HRP} = 5.6).
(Mw_{GE}: 694)

4.3.4. Preparation of SD–HRP conjugate by periodate method (Wilson and Nakane, 1978)

The conjugation was prepared similarly to **point 4.5.,** but HRP was 4 mg (0.1 μmol) and SD was 10 mg (40 μmol). (Mol_{SD} /$Mol_{HRP} \cong 400$).

4.4. Monoclonal antibody production

4.4.1. General procedure for immunization of mice

Female Balb/c mice (2 months old) were immunized subcutaneously with 50 μg/ 50 μl immunogen (*OA-BSA or FB1-KLH or GE-BSA or SD-BSA*) emulsified with equal volumes of Freund's complete adjuvant (CFA) on the first day. After one month mice received a second injection intra-peritoneally with the same amounts of conjugates, emulsified with Freund's complete and incomplete adjuvants mixture (CFA : IFA = 1:3 , v/v). On day 50, the mice were bled and serum titers were assessed by dc-ELISA. The third immunization was performed on day 57 with the same dose of conjugate and adjuvant. Final immunizations were carried out four days prior to fusion (on day 70) intravenously.

4.4.2. General method of hybridoma production

Spleen cells ($1x10^8$) from a mouse producing antiserum for the given mycotoxins or antibiotics were fused with Sp2/0-Ag14 (from American Type Cell Collection CRL-1581) murine myeloma cell line using PEG 1600 according to Oi and Herzenberg (1980). Until 14 d after fusion, hybridomas were selectively grown in hypoxanthine, aminopterine, thymidine medium. At the half-monolayer state, the antibody-secreting hybridomas were detected by dc-ELISA. Positive hybridomas were grown in hypoxanthine, thymidine medium.
After cloning by the limiting dilution method, ascites was made with the cells that had produced supernatant fluids with the best sensitivity. Mature male Balb/c mice were injected intaperitoneally with $1x10^7$ hybridoma cells. After 10-14 d the ascites fluids were collected, centrifuged and stored at -20 ^oC until use. The protocol can be seen on **Figure 1.**

4.4.3. Measurement of the affinity of monoclonal antibody (Betty et al. 1987)

The affinity constant of the antibodies was determined by indirect ELISA method. Prior testing the

monoclonal antibody was purified from ascites and the protein content was determined by UV absorption and calculated in mg/ml concentration.

The immunogen of the appropriate monoclonal antibody was diluted in PBS buffer in concentration of 1.0- 25 µg/ml and 100 µl-100 µl were pipetted to microplate, incubated 18 h at RT. Excess immunogen was removed by washing the plate five times with distilled water. The monoclonal antibody was serially diluted in PBS buffer in concentration range of 500 µg/ml – 1 µg/ml and pipetted 100-100 µl from each antibody concentration to each immunogen concentration and incubated to 1 hour at RT. After washing, 100-100 µl of anti-mouse Ig-HRPO in 10.000 x dilution was added and incubated for 1 h again at RT. The plate was washed five times with distilled water and 150 µl of tetramethylbenzidine-H_2O_2 (TMB/H_2O_2) substrate per well added, then incubated for 15 min at RT. The colour reaction was stopped with 50 µl of 6N sulphuric acid and the OD_{450} was measured by an automated microplate reader (Labsystems Multiscan PLUS, Finland).

Plotting of curve

Antibody (Ab) concentrations (logarithm-scale) were plotted against optical densities (OD-linear scale) were plotted at different immunogen concentrations. The antibody concentrations were determined at OD-50 points (50% of the maximum OD value) of the sigmoid curves at constant Ag concentration.
Two pairs - Ab-Ag concentrations were selected and the affinity constant was calculated according to the following formulae.

$$K_{affinity} = \frac{n-1}{2\{n(A_b') - (A_b)\}}$$

Ab' concentration (µg/ml) at Ag1 concentration,
Ab concentration (µg/ml) at Ag2 concentration.

Ag2 /Ag1 = n

Ab concentration is calculated in Mol concentration (Mw of Ab is 160 000 g).

4.5. Competitive ELISA tests

4.5.1. Direct competitive ELISA (dc-ELISA) for detection of hybridoma supernatants

The hybridoma supernatants were screened in direct competitive ELISA. Briefly: Before the assay the supernatants were diluted with PBS (1:2). From each supernatant 100 -100 µl was added to two microplate wells previously coated with 150 µl of rabbit Ig anti-mouse Ig (IgM + IgG + IgA, developed in our laboratory, 15 µg/ml) and incubated for 1 h at RT. After washing, 50 µl PBS buffer (zero analyte concentration, B_0) was pipetted to one well, and 50 µl of 500-1000 ng/ml analyte (*OA or FB1 or GE or SD* in PBS to the other. Immediately thereafter, 50 - 50 µl of analyte-HRP conjugate in working dilution was added to each well and incubated for 1 h at RT. Plates were washed five times with distilled water and 150 µl of tetramethylbenzidine-H_2O_2 (TMB/H_2O_2) substrate per well added and incubated for 15 min at RT. The colour reaction was stopped with 50 µl of 6N sulphuric acid and the OD_{450} was measured by an automated microplate reader (Labsystems Multiscan PLUS, Finland).
Those antibodies were selected where OD_{450} at zero analyte concentration (B_0) was > 0.5 and the percent of inhibition was > 90 %.

4.5.2. Direct competitive ELISA (dc-ELISA) for detection of OA in cereals, sera, seminal plasma, animal tissues and FB_1 in cereals

The direct competitive ELISA was modified for measuring OA or FB_1 in different matrices. Briefly: Microplate wells (Immunoplate F-8, Maxisorp, Nunc, Denmark) were coated with anti-mouse Ig rabbit IgG globulin as previously, then 120 µl of diluted anti-OA ascites fluid (working dilution of 1:500) or anti-FB_1 ascites fluid (working dilution of 1: 100 000) was pipetted to each well and incubated for 18 hours at RT. After washing with distilled water, plates were dried and stored in a foil bag for up to several months at 4 ^{o}C. Fifty-microliter OA or FB_1 standard solution or extracted samples were coincubated in wells with 50 µl of diluted OA-HRP or FB_1-HRP conjugate in PBS-Tween 20 (0.1 %) for 1 h at RT. After four washing steps, the peroxidase activity was measured as before.

4.5.3. Direct competitive ELISA (dc-ELISA) for detection of GE or SD in biological fluids

Microplate wells (Immunoplate F-8, Maxisorp, Nunc, Denmark) were coated with 120 µl of diluted anti-GE ascites fluid (working dilution of 1:30000) or anti-SD ascites fluid (working dilution of 1: 4000) and incubated for 48 hours at RT or 18 hours at 37 ^{o}C. After washing with distilled water, plates were dried and stored in a foil bag for up to several months at 4 ^{o} C. Twenty µl of GE or SD standard solution or samples were coincubated in wells with 100 µl of diluted GE-HRP or SD-HRP conjugate in PBS-Tween 20 (0.1 %) for 1 h at RT. After four washing steps, the peroxidase activity was measured as before.

Plotting the standard curves:

The standard curve of OA, FB_1, GE or SD was obtained by plotting $\log_{10}$ concentration (*x-axis)* against B/B_o (*y-axis)*: B/B_o = (OD of standard or sample)/(OD of blank [no analyte added]), where optical density (OD) is the mean value.

4.6. Feeding trial with ochratoxin-A contaminated feed

4.6.1. Feeding of boars with ochratoxin-A

Toxin stock solution was prepared by dissolving 25 mg OA in 5 ml of 96% ethanol and stored at –20 ^{0}C. In this experiment eight clinically healthy, sexually matured breeding boars, with body weight of 250 kg were involved. Twenty micrograms of OA (low concentration of toxin, L) was given daily to two of the animals. Two boars were treated with 40 µg (higher toxin dose, H). Control boars (four animals) received no toxin. The experimental period was 5 weeks. Blood and semen samples were collected from the animals. Direct ELISA tested OA concentration of them.

4.6.2. Feeding of chicken with ochratoxin-A

The concentration of OA stock solution was 1.25 mg/ml in absolute ethanol in this experiment.
Twenty 28-day-old Ross broiler chicks (average body weight 839,19 ± 30.4 g) were involved into this experiment. Sixteen chicken were treated with 0.5 mg OA per animal weekly for 4 weeks. This corresponded to an average toxin dose of 0.354 mg/kg (calculated as an average of the actual weekly doses). The weekly toxin amount was divided into four portions and each bird was given 0.125 mg OA orally on the same regular days using a human baby nasogastric tube. Four chickens were fed with toxin-free diet (control group). On day 28, the four control chickens were sacrificed simultaneously with the last four experimental birds. Blood, liver, kidney and muscle from all treated and control birds were sampled and stored frozen at –70 ^{0}C until assayed by HPLC and ELISA.

4.7. Sample preparations, extractions, cleanup and measurement

4.7.1. Preparation of cereal samples for OA determination

Cereal samples (maize, barley, soya, mixed concentrates etc.) were triturated in a grinder and 2 g were transferred into a 50-ml Erlenmeyer flask. Thereafter 10 ml dichloromethane and 5 ml 1 M citric acid were added to the flask and the sample was vortex mixed for 5 minutes, sealed with parafilm and agitated for 2 hours at RT (about 22 ^{O}C) on a horizontal shaker. The whole suspension was then transferred to a centrifuge tube and centrifuged for 30 min at 3000 x g. Three phases, aqueous (upper), - sample cake (middle) and dichloromethane (lower) were obtained after centrifugation. The upper aqueous phase was discarded, the sample cake was cut through and 2 ml of the dichloromethane phase was transferred to another conical tube. Two milliliters of 1% sodium bicarbonate buffer was added to this, sealed with parafilm and shaken for 30 min during which OA was transferred into the aqueous solution. The mixture was centrifuged (20 min at 3000 x g) in order to obtain a clear buffer solution. Four hundred and ninety microliters of the upper buffer solution was pipetted into a test tube and 10 µl of 1 N HCl added. The sample was mixed thoroughly and 50 µl of this solution was used directly in the ELISA. If the expected OA concentration was higher than 10 ng/g, the final sample solution was diluted 1:10 with 1% sodiumbicarbonate buffer (pH: 7.5-8.0).

Evaluation:

The standard curve of OA was obtained by plotting log 10 concentration (x axis) against B/B_O (y axis).

B/B_O= (OD of standard or sample) / (OD of blank [no toxin added]),

(Where optical density /OD/ is the mean A 450 nm), (/) is label equation.

The concentration of OA in sample extracts were calculated using the calibration curve and were expressed in nanograms per gram (ng/g). The ng/ml value was multiplied either by 5 (where sample extract was used directly) or by 50 (at 1:10 sample dilution). The slope of the standard curve was the colour change per of unit concentration.

4.7.2. Recovery of OA toxin from artificially contaminated cereals

Pure OA (0.5-100 ng/g) was added to 2 g finely ground cereals and the mixture was homogenised one day prior to extraction. Samples were extracted and assayed as above.

4.7.3. HPLC analysis of OA toxin from artificially contaminated cereal.

HPLC analysis were carried out using a Hewlett-Packard HP 1090 Series II liquid chromatograph equipped with a binary solvent delivery system, an autoinjector, an autosampler, a temperature controlled column compartment and a fluorescence detector (HP 1046 A). The samples were analyzed on a BST (Bio Separation Technic Co., Budapest, Hungary), Rutin C 18 BD column (250 x 4 mm, 10 µm) (basic desactivated) according to Nesheim et al.(1992).

4.7.4. Preparation of human, pig, chicken serum and seminal plasma of boars for OA determination

Two milliliter of serum or seminal plasma, citric acid (2.5 ml 1 M) and 4 ml dichloromethane were added to centrifuge tube, vortexed for 5 minutes, sealed with parafilm and agitated for 1 hours at RT on a horizontal shaker, and finally centrifuged for 20 min at 3000 x g. Three phases, aqueous (upper), white ring (middle) and dichloromethane (lower) were obtained after centrifugation. Two milliliters from the lower (dichloromethane phase) was transferred to another conical tube, and 2 ml of 1% sodium bicarbonate buffer (pH= 8.8) was added, sealed with parafilm and shaken for 30 min

during which OA was transferred into the aqueous solution. The mixture was centrifuged (20 min at 3000 x g) in order to obtain a clear buffer solution. An aliquot (490 ml) of the upper buffer solution was pipetted into a test tube and 10 µl of 1 N HCl was added. The sample was mixed thoroughly and 50 µl of this solution was used directly in the ELISA. If the expected OA concentration was higher than 3.5 ng/ml, the final sample solution was further diluted with 1% sodiumbicarbonate buffer (pH: 7.5-8.0).

Evaluation:

The concentration of OA in serum or seminal plasma extracts were calculated using the calibration curve and were expressed in nanograms- per - milliliter (ng/ml). The ng/ml value was multiplied either by 2 (when sample extract was used directly), which gave a measuring range of 0.2-4 ng/ml. If the OA value was more than 3.5 ng/ml, the extracts had to be further diluted and the concentration calculated using the appropriate multiplication factor.

4.7.5. Recovery of OA from artificially contaminated human sera and seminal plasma

To 2 ml of low (0.26 ng/ml) concentration *human serum pool*, 0.1, 0.25, 0.5, and 1.0 ng/ml OA were added, mixed, extracted, and assayed as shown previously.

To 2 ml of low (0.1 ng/ml) concentration of *seminal plasma pool*, 4.0, 2.0, 1.0 ng/ml OA were added, mixed, extracted, and assayed as before.

4.7.6. Preparation of tissue sample for OA determination

Tissue samples (muscle, kidney, liver) of chickens were homogenised in a grinder and 5 g of each sample was transferred to a centrifuge tube. Seven milliliters of citric acid and 10 ml dichloromethane were added, vortexed for 5 min, agitated on a horizontal shaker for 1 h at room temperature and centrifuged for 20 min at 3000 x g. Two milliliters from the lower dichloromethane phase was transferred into a conical tube and further clean-up steps were identical to that of serum samples (see point 4.7.4.).

4.7. 7. Recovery of OA from artificially contaminated chicken sera, kidney, liver and muscle

Chicken sera, kidney, liver and muscle samples containing no OA were pooled as controls.

To 2 ml of *chicken pooled sera,* 1.0, 2.0, 3.0 and 5 .0 ng/ml OA were added, mixed, extracted and assayed as point 4.7.4.

To 5 g of *tissue samples*, 10, 20, 40 ng/ml OA were added, mixed, extracted and assayed as point 4.7.4.

4.7. 8. Preparation of cereal samples for FB_1 determination

To 5 g of finely ground cereals (wheat, maize, rye) 20 ml extraction solvent (composed of 50 parts acetonitrile, 39 parts water, 10 parts 0.5% KCl, 1 part 6% H_2SO_4) were added and shaken for 2 hours at RT. The extracts were centrifuged for 30 min at 4500 rpm/min and the supernatants diluted 1:5 with PBS-Tween 20 (0.1 %), shaked well then centrifuged again. From the clear supernatant 50 µl was used directly in the ELISA.

The FB_1 concentration in sample extracts were assessed by using the calibration curve and are expressed in nanograms per gram by multiplying the nanogram-per-milliliter value by 20.

4.7. 9. Recovery of FB1 from artificially contaminated cereals

To 5 g of finely ground cereals (wheat, maize, rye) 50-200 ng per gram of pure FB1 was added 1 day prior to extraction. Thereafter, the samples were shaken for 2 hours at RT with 20 ml of extraction solvent; all details are as described earlier.

4.7. 10. Determination of FB1 in naturally contaminated samples by HPLC

For this experiment, 11 maize samples were collected from one area (Heves county) of Hungary in 1998. The samples were homogenized and stored at -20 ^{o}C until use. HPLC analysis of FB1 and FB2 was done by method of Shephard (1990) and Sydenham (1992) as follows. Finely ground maize samples were extracted with MeOH/water (3:1 v/v), the crude extracts were cleaned using solid-phase strong anion exchange (SAX) cartridges (Varian, Harbor City, CA, USA). The fumonisins were separated from SAX cartridge with 1% acetic acid in methanol. Derivatization of fumonisins was performed with *ortho*-phthaldialdehyde (OPA) and mercaptoethanol. OPA-derivatives of fumonisins were separated on LiChrospher RP 18, 150 x 4 mm, 5 µm analytical column; mobile phase was acetonitrile-water-acetic acid (50/50/1). The contents were estimated with a fluorometric detector with excitation at 335 nm and emission at 450 nm. For quantitative determination a calibration curve was prepared. Validity of our method: Recovery rate was 75% at 200 ng/g FB_1 and FB_2 concentration and 71% at 1000 ng/g FB_1 and FB_2. Detection limit was 20 ng/g for FB_1 and 50 ng/g for FB_2. The signal to noise ratio was 10:1 for FB_1, 5:1 for FB_2 and the same ratio was for FB_3 as well.

4.7.11. Preparation of swine serum for GE and SD determination by ELISA

Serum samples of pig were diluted 1:10 with dilution buffer of PBS/Tween 20, containing 0.1 % 8-Anilinonaphtalene-1-sulfonic acid and 20 µl was tested further according to 4.5.3.

Using the calibration curve assessed the GE or SD concentrations of sera and 10 multiplied the ng/ml values.

4.7.12. Preparation of cattle milk samples for GE and SD determination by ELISA

Raw milk samples were de-fatted by centrifugation (3000 x g) and 20 µl of samples were tested directly by ELISA.

4.7.13. Recovery of GE from cattle milk

Potassium dichromate was added to de-fatted milk to the final concentration of 0.05% and stirred well. This material prevents the coagulation of milk. To this milk samples GE solution was added to final concentration of 10, 25, 50, 100 and 500 ng/ml and stirred thoroughly. Milk samples containing 10 ng/ml or more GE had to be diluted with PBS-Tween 20 before testing.

Gentamicin content of the milk samples were determined by the calibration curve and the values multiplied with the appropriate dilution factor.

4.7.14. Recovery of GE from swine sera

To swine serum GE solution was added to final concentration of 1.0, 5.0; 10.0; 25.0, 50.0; 100.0 ng/ml, stirred thoroughly than diluted 1:10 with dilution buffer (4.7.11.) and tested as before (4.5.3.)

4.7.15. Recovery of SD from cattle milk

Potassium dichromate was added to raw de-fatted milk to the final concentration of 0.05% and stirred well. To this milk samples SD solution was added to final concentration of 6.25; 12.5; 25; 50 and 100 ng/ml and stirred thoroughly.

Sulphadiazine content of the milk samples were determined from the calibration curve.

4.7.16. Recovery of SD from swine sera

SD solution was added to swine serum to final concentrations of 62.5; 125; 250, 500; 1000 ng/ml, stirred thoroughly than diluted 1:10 with dilution buffer (4.7.11) and tested as before (see 4.5.3.).

5. Results

5.1 Development of ELISA test for OA determination in cereals

All immunoassays are based on the reaction of analyte with its specific antibody. The quality of this antibody (affinity, avidity, cross-reactivity) is one of the most important factors for the development of sensitive assays. Using the appropriate immunogen, immunization protocol and screening procedures can obtain high quality antibody. In general, indirect or indirect-competitive ELISA is used for screening monoclonal antibodies against mycotoxins (Kawamura et al. 1989). Although highly sensitive monoclonal antibodies have been raised by our group using this selection method and applied in the direct competitive ELISA we could not produce the appropriate quality monoclonal antibodies against OA. In this study the direct ELISA was therefore used for antibody selection since the objective of the study was to develop a direct competitive ELISA for OA. This procedure avoided the selection of non-specific antibodies against the carrier proteins (BSA, HSA, KLH) as only the OA-specific monoclonal antibodies react. In addition to a specific antibody, a very good quality OA-perxidase conjugate was necessary as well. The selected monoclonal antibodies were ranked according to following criteria: assay-range, the slope of calibration curves and 50% displacement values of B/B_O (IC_{50}). Using this selection strategy, several monoclonal antibodies were obtained, the IC_{50} values varied between 10 and 0.4 ng/ml OA. Monoclonal antibody (hybridoma cell line-5/9G4A4H) with an IC_{50} value of 0.4 ng/ml OA, was used for the further studies.

The first step in developing a direct, competitive ELISA is to determine the optimal dilution of antibody and mycotoxin-peroxidase. In our experiment the monoclonal antibody was coated indirectly onto the microplate using rabbit anti-mouse Ig as the capture antibody, thereby the slope of standard curve was near to the optimal and the B_O value was higher compared to test systems where the specific antibody was coated directly onto the microplate. **Figure 13.** shows the dose-response curve of OA.

The characterisation of the monoclonal anti-OA antibody and that of the optimised OA test are summarised in **Table 10.** and **Table 11.**

Table 10. <u>Characterization of monoclonal anti-OA antibody:</u>

Clone number: 5/9G4A4H
Subclass of the antibody: IgG_1, κ
Affinity constant of the antibody: $7{,}8 \times 10^9$ l/ M
Cross-reactivity with some related toxins: ochratoxin-A 100 %
ochratoxin-B 9.3 %
(No cross reaction with ochratoxin-α, coumarin, 4-hydroxy-coumarin, D,L-phenylalanine)

The **Table 12.** shows the reproducibility data of the OA test**.**

Table 11. Characterisation of the optimised ELISA test for OA determination in cereals

Standard concentration of OA in PBS buffer: 0.1-2 ng/ml
IC_{50} value: 0.45 ng/ml
Slope of the standard curve: 0.95
Correlation coefficient of the standard curve: 0.991
Detection limit in buffer (0 ± 2SD.): 0.042 ng/ml
Detection limit for cereals: 0.5 ng/g
Measuring range: 0.5-10 ng/g (without sample dilution), 10-100 ng/g (with sample dilution)

Table 12. Reproducibility of the OA test

Within-assay and inter-assay of the ochratoxin-A (OA) standard were determined. The mean values of the coefficients of variation (CV) were less then 10 %.

Concentration of OA toxin standard (ng/ml)	Within-assay (CV %)	Inter-assay (CV %)
0	**5.6**	**6.91**
0.1	**7.3**	**6.86**
0.25	**8.5**	**8.86**
0.5	**8.3**	**7.96**
1.0	**8.4**	**7.4**
2.0	**7.5**	**6.9**

Number of samples: 12

Table 13. Recovery of ochratoxin-A from artificially contaminated cereals samples

Added OA (ng/g)	Barley detected (ng/g)	Goose-feed detected (ng/g)	Piglet-feed detected (ng/g)	Cole-seed detected (ng/g)
5	**4.81 (96%)[b]**	**4.5 (90.4%)[b]**	**6.5 (129%)[b]**	**6.3 (125%)[b]**
10	**9.8 (98%)**	**10.3 (103%)**	**10.1 (101%)**	**11.7(117%)**
20	**21 (105%)**	**21 (105%)**	**26 (130%)**	**22.8(114%)**
50	**64 (128%)**	**57 (114%)**	**69 (138%)**	**64 (128%)**
100	**127 (127%)**	**124 (124%)**	**108 (108%)**	**115 (115%)**

[a] Each sample was spiked in three parallel experiments and then extracted and assayed in three replicates.
[b] The recovery rate was calculated in per cent as well according to the formula:
Detected OA (ng/g) /added OA (ng/g) x 100.

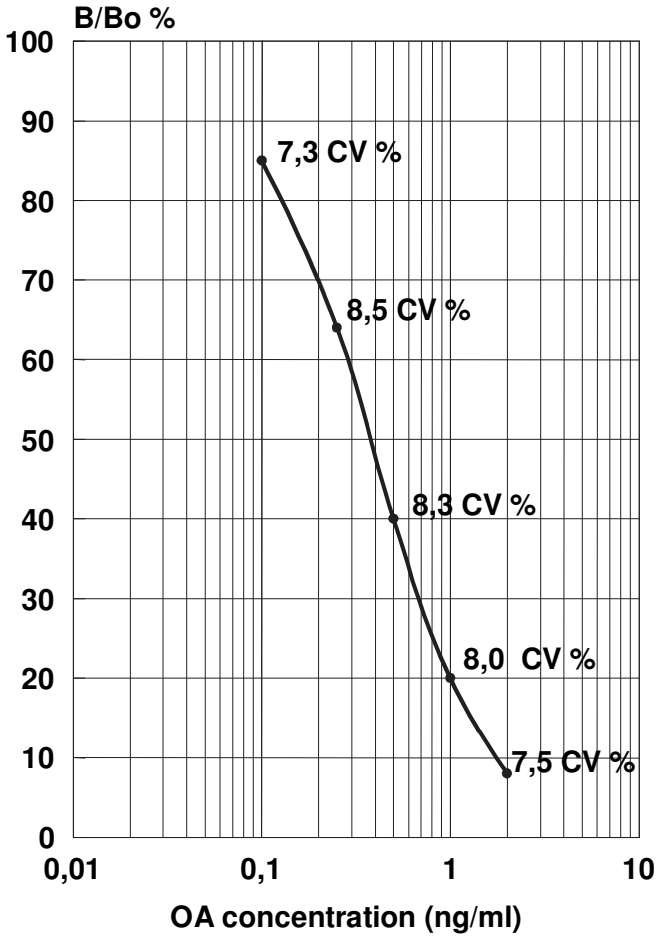


Figure 13. Dose-response curve of OA

Recovery values of OA from artificially infected cereals averaged 97% as summarised in **Table 13.** The extraction of maize was troublesome in some cases, as the sample cake remained diffuse after centrifugation. As a consequence the organic extract (lower phase) could not be aspirated properly resulting in false positive value.

The OA value of some samples measured by ELISA and HPLC was compared (**Table 14.**). The recoveries of OA at ELISA proved the effectiveness of the extraction procedure.

Table 14. Recovery of ochratoxin-A from artificially contaminated cereals as determined by ELISA and HPLC

Added OA (ng/g)	By ELISA		By HPLC	
	OA (ng/g)	detected (%)	OA(ng/g)	detected (%)
Barley 20	18,9	94,5	15,7	78,7
50	50,3	100,6	41,8	83,5
Corn 20	22,3	111,5	16,8	84,0
50	51,0	102	43,0	85,9

Each sample analysed by ELISA and HPLC was done in triplicate.

For easier application of the ELISA test in practice, reagent kit-package has been set up. The branded name of this commercialised kit is **TOXIKLON Ochratoxin-A.** With this kit 40 cereal samples can be determined in duplicate (Barna-Vetró et al. 1996).

5.1.1. Detection of OA in human sera

The sensitive TOXIKLON ochratoxin-A test could be used not only for cereals, but for other matrices as well, e.g. for determination of OA content in human sera.

First we had to modify the protocol of the sample preparation (see Materials and Methods **4.7.4.),** using almost half volume of dichloromethane and citric acid. The OA test was validated with a pool of negative (0.2-0.3 ng/ml of OA) and a pool of positive (2-3 ng/ml of OA) human sera.

Reliability of the ELISA test:

Stability. Because the shape of the calibration curves varied slightly throughout the experimental period, a daily calibration was used to ensure the reliability of the assay.

Reproducibility. The intra-assay and interassay errors were determined on the basis of eight separate extractions of the pooled negative and positive sera run on different days (**Table 15.).** The standard deviations in all cases were below 15%.

Precision. Recovery rates of OA from human sera were determined by OA artificially contaminated human serum. The **Table 16** summarizes the results with recovery rates between 53 and 91%.

Table 15. Intra-and Interassay variations of human serum pools

Pooled human serum OA (ng/ml)	Intra-assay CV (%)	Interassay CV (%)
0.26	9,53	14.8
2.73	7,87	10.2

Table 16. Recovery of ochratoxin A added to human sera*

OA added (ng/ml)	Expected OA (ng/ml)	Detected OA (ng/ml)**	CV (%)	Recovery (%)
1.0	**1.26**	**0.84 ± 0.1**	**12.5**	**67**
0.5	**0.76**	**0.4 ± 0.0.2**	**8.5**	**53**
0.25	**0.51**	**0.39 ± 0.06**	**18**	**75**
0.1	**0.36**	**0.33 ± 0.06**	**18**	**91**

* OA content of pooled human serum = 0.26 ng/ml.
** Mean ± S.D. (n=12).

In a three-month period (from March to July 1995), a total of 355 serum samples from random internal medicine patients were collected at the Szent János Hospital. The relative frequency of OA contamination of these human sera is shown on **Figure 14.**

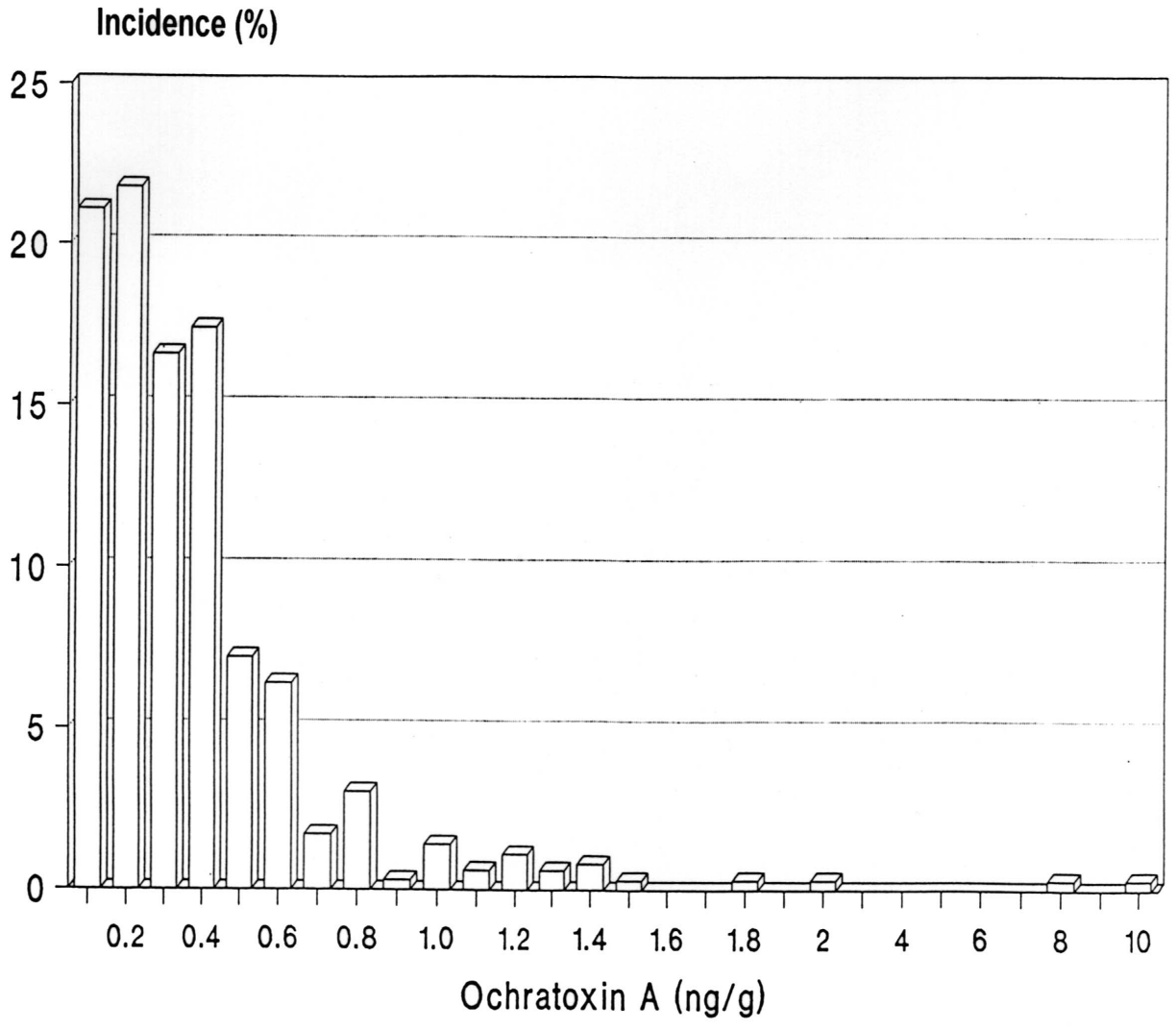


Figure 14. Relative frequency of OA contamination in random human serum samples

The OA content of the samples varied between < 0.2 and 10 ng/ml. According to their OA values, the sera were assigned into three groups (**Table 17).** The OA content of the first group (<0.2 ng/ml) was below the detection limit of our assay; that of the second group was on the linear part of the calibration curve (0.2-1.0 ng/ml), whereas the remaining sera samples were above 1.0 ng/ml. Low ((<0.2 ng/ml) OA values considered to be negative were found in 65 of the 355 analysed serum samples (18.2 %). Most of the samples (75%) fell in the range of 0.2-1.0 ng/g, whereas 24 samples (6.8 %) contained 1.0 ng/ml OA or more. However, only two samples contained OA in very high concentrations (8.8 and 10 ng/ml, respectively) (Solti et al. 1997).

Table 17. OA concentration in human sera

Number of samples	Range (ng/ml)	Relative Frequency of Occurrence (%)		
		< 0.2 ng/ml	0.2 –1.0 ng/ml	> 1.0 ng/ml
355	< 0.2 –10.0	18.2	75	6.8

5.1. 2. Detection of OA in serum and seminal plasma

The aim of the experiment was to investigate whether or not OA can be detected in seminal plasma after feeding the toxin in five and 10 times of the human tolerable daily intake (TDI) with breeding boars. The estimated human TDI value is 16 ng/ kg/day.

As collaborating partner in this work, the earlier optimized OA test was used to measure the OA concentration. Before this, the OA test had to validated for seminal plasma and swine sera as well. The extraction of OA from swine serum and seminal plasma samples were the same as for human sera (Materials and Methods **4.7.4.** page 39**).**

The test was validated with a *pool of negative* (0.2-0.3 ng/ml of OA) and a *pool of positive* (2-3 ng/ml of OA) seminal plasma. The positive seminal pool was prepared using negative seminal plasma pools artificially contaminated with 3 ng/ml OA. The OA content of the positive seminal plasma was determined by five, parallel extractions.

Reproducibility: The Intra- and Interassay variations of the negative and the positive seminal plasma pools are shown in **Table 18.**

Table 18. Intra-and Interassay variations of seminal plasma pools

Pooled seminal plasma OA (ng/ml)	Intra-assay CV (%)	Interassay CV (%)
0.24	**10.9**	**11.2**
2.7	**3.5**	**5.2**

Number of samples: 48
The positive and negative seminal pools were used as internal controls for OA determination of seminal plasma samples in the ELISA.

Precision. Recovery rates of OA from seminal plasma were determined by OA artificially contaminated with negative seminal plasma pools (**Table 19).**

Table 19. Recovery of ochratoxin-A added to seminal plasma

OA added (ng/ml)	Intra-assay		Interassay	
	*Detected OA (ng/ml)	CV (%)	Detected OA (ng/ml)	CV (%)
1.0	1.11 ± 0.14	10.3	1.2 ± 0.11	15
2.0	2.32 ± 0.22	9.73	2.3 ± 0.2	8.75
4.0	3.4 ± 0.17	10.6	3.28 ± 0.24	15.5

***** Values are means ± SD. Number of samples were 12.
OA content of the negative seminal plasma pool was: 0.1 ng/ml

After oral treatment the animals with low and high dose of OA, the blood and seminal plasma samples were tested with the validated OA test, using either the OA standards and plus two, internal controls (+ and – seminal plasma).

Average OA content of serum in experimental animals presented a well-defined, sharp OA concentration curve. The sera and seminal plasma of the animals were tested individually.

In low-dose boars the serum OA value of one of the animals reached a maximum of 2.06 ng/ml and a second peak of 0.919 ng/ml. In high-dose boars the serum OA of one of the animals reached a higher value (2.56 ng/ml) and the second peak was around 2.0 ng/ml.

The OA concentration of the seminal plasma at low-dose boars fluctuated from 0.45 ng/ml to 0.579 ng/ml. In high dose animals a peak was reached on day 28, with a value of 0.709 ng/ml (**Figure 15).** (Solti et al. 1999).

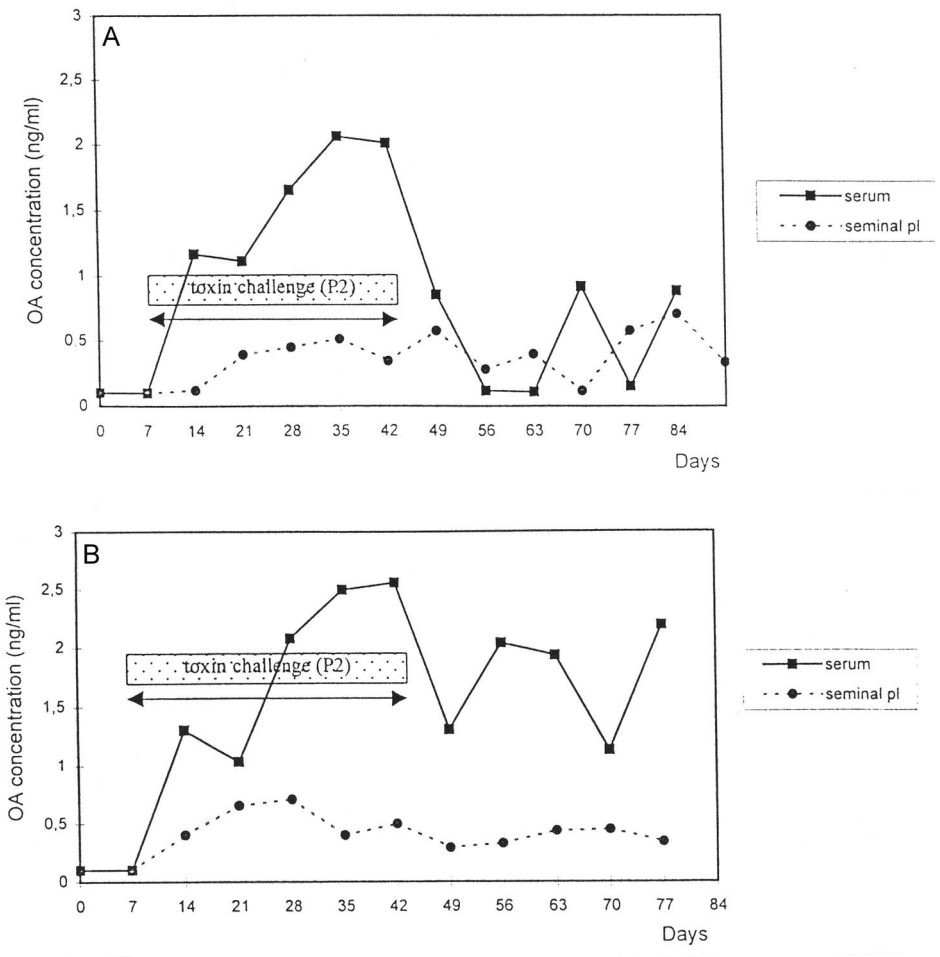


Figure 15. OA concentration in serum and seminal plasma of boars
(A.) in low dose animals; (B) in high-dose animas

Due to the low number of animals that were available for the experiment, no significant differences were found in sperm motility between the high-dose and low-dose toxin treated animals.

We have found at one of the animals, that the initial sperm motility reduced strongly by 26% of the control (Solti et al. 1999).

5.1. 3. Detection of OA in tissue of chickens

As collaborating partner in this experiment, we wanted to investigate the OA content in poultry tissues and organs after long-term administration of OA doses approximating the range of natural contamination in feed.

For this experiment the TOXIKLON ochratoxin-A test was used for measuring the OA content in different tissue samples. To test the OA in tissue, the sample preparation and clean-up had to be modified and first the test had to be validated for the different tissue samples (Materials and Methods 4.7.6. and 4.7.7., page 40**).**
The OA test was validated for different animal tissues, the detailed data are summarized in **Table 20.**

Table 20. Recovery of ochratoxin-A added to sera, muscle, liver and kidney of chicken

Chicken sera

OA added (ng/ml)	*Detected OA (ng/ml)*	Recovery (%)
5	3.56 ± 11.0	71.2
3	1.96 ± 13.7	65.3
2	1.41 ± 6.2	70.5
1	0.55 ± 8.3	54.8
OA added (ng/g)	***Detected OA (ng/g)***	**Recovery (%)**
	Chicken tissue	
40	24.2 ± 10.8	60.5
20	12.5 ± 7.0	62.5
10	5.18 ± 8.1	51.8
	Chicken kidney	
40	21.1 ± 10.5	53.0
20	11. 04 ± 5.9	55.2
10	4.94 ± 4.7	49.4
	Chicken liver	
40	20.0 ± 5.6	50.1
20	11.48 ± 4.5	57.4
10	5.64 ± 5.7	56.4

Each sample was assayed in five replicates.

The average body weight of experimental animals did not differ significantly from that of the controls.

The OA *serum* concentration-time curve was established based on ELISA and HPLC. From the second week, ELISA values were constantly higher than those obtained by HPLC. Maximum values for HPLC (1.16 ng/ml) and ELISA (2.12 ng/ml) were reached simultaneously on day 14. The toxin concentration declined to 0.64 ng/ml on day 28, measured by ELISA (**Figure 16.)**

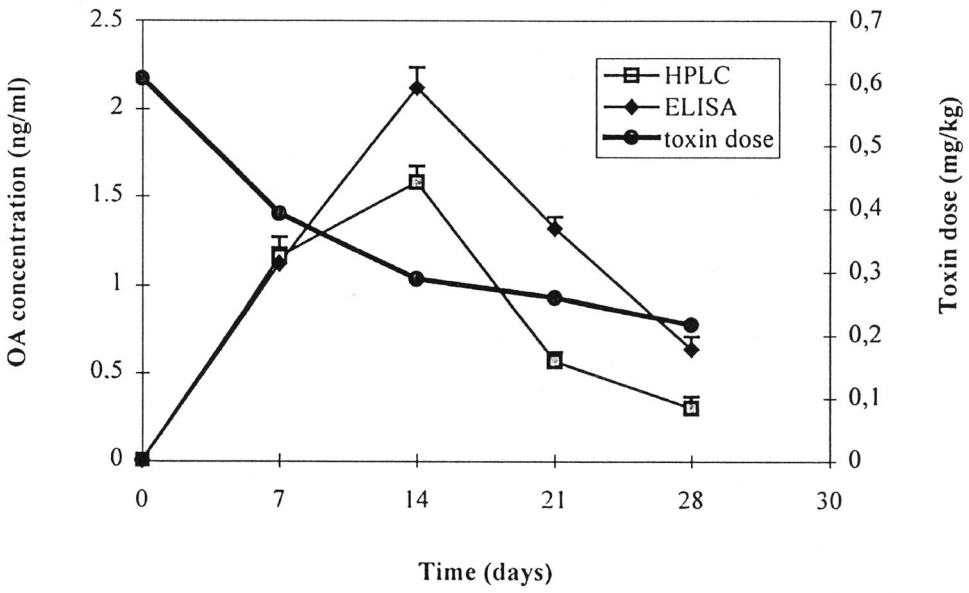


Figure 16. Ochratoxin-A plasma concentration-time curve

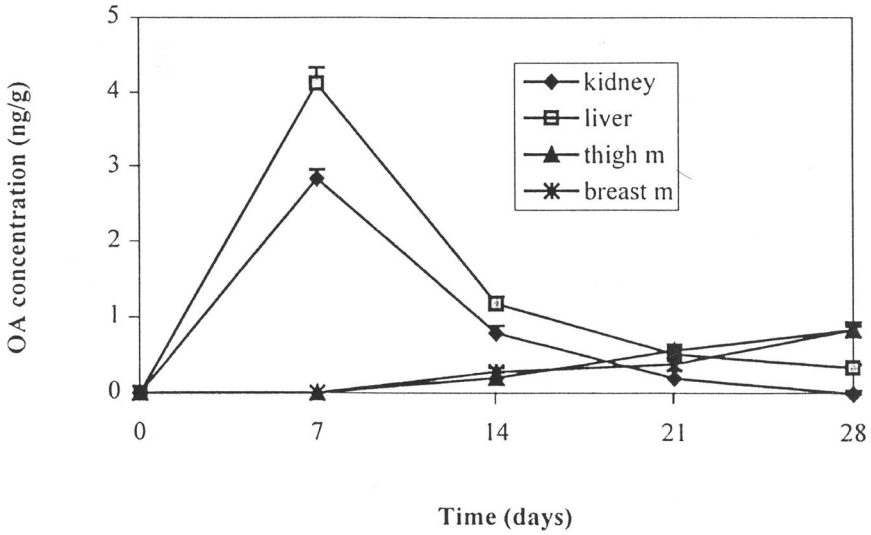


Figure 17. Ochratoxin-A residues in tissues of broilers detected by ELISA

The OA *tissue* concentration-time curve can be seen on **Figure 17.** The highest toxin concentration was found in the liver on day 7, the OA value was 4 ng/g detected by ELISA. The OA concentration markedly dropped to 1.12 ng/g in the second week and this toxin was still detectable even on day 28. Compared with the residual toxin amount in the liver, a considerably lower concentration of OA (2.84 ng/g) was detected in the kidney with ELISA. On the second week the OA concentration decreased markedly (0.7 ng/g) in kidney.

In the thigh and breast muscle ELISA identified OA at a level around 0.2 to 0.28 ng/g on day 14, and a slight increasing tendency was seen (0.84 ng/g) on day 28. OA residues were not detected in any organs or tissues of control animals.
According to this experiment, residues of OA were found in serum and in all tissues (kidney, liver and muscle), indicating wide tissue distribution (Biró et al. 2002).

5.2. Development of ELISA test for determination of Fumonisin B_1 in cereals

For preparation of monoclonal FB_1 antibodies the following strategy was used: immunization with FB_1-KLH immunogen using shorter cross-linker reagent as glutaraldehyde (GA) and using another cross-linker reagent for preparation of immunogen within the next experiment if FB_1-GA-KLH resulted in antibodies with poor affinity.

Using FB_1-GA-KLH immunogen, all treated animals (n=6) produced specific FB_1 antibodies with approx. 50 % inhibition in presence of free FB_1 (1µg/ml). Spleen cells from hyperimmunized mouse were fused with Sp2/0-Ag14 myeloma cells and the resulting hybridoma supernatants were screened by dc-ELISA. The use of dc-ELISA avoided the selection of nonspecific antibodies against the carrier protein as only the FB_1-specific monoclonal antibody reacted. With this selection strategy, sixteen hybridomas were obtained that all recognised the unconjugated FB_1. From these active hybridomas four cells were cloned and recloned by limiting dilution method and retested again by dc-ELISA. The resulted antibodies were very sensitive, the inhibitions at 500 ng/ml of free FB_1 toxin level (it means 25 ng/well FB_1) were more than 90%. Ascites fluid containing the monoclonal antibody (1D6F11E3) was used in all subsequent experiments. The affinity constant of the antibody was determined by the method of Beatty et al. (1987) .The specificity of the antibody toward fumonisins and other mycotoxins was checked by dc-ELISA. The cross-reactivity values were calculated as (IC_{50} of FB_1 /IC_{50} of compound) x 100. (IC_{50} is defined, as the toxin concentration required inhibiting the colour development by 50 %). **Table 21 and 22** summarize the most important data of the monoclonal antibody and the optimised ELISA test. **Figure 18** shows the dose-response curve of FB_1.

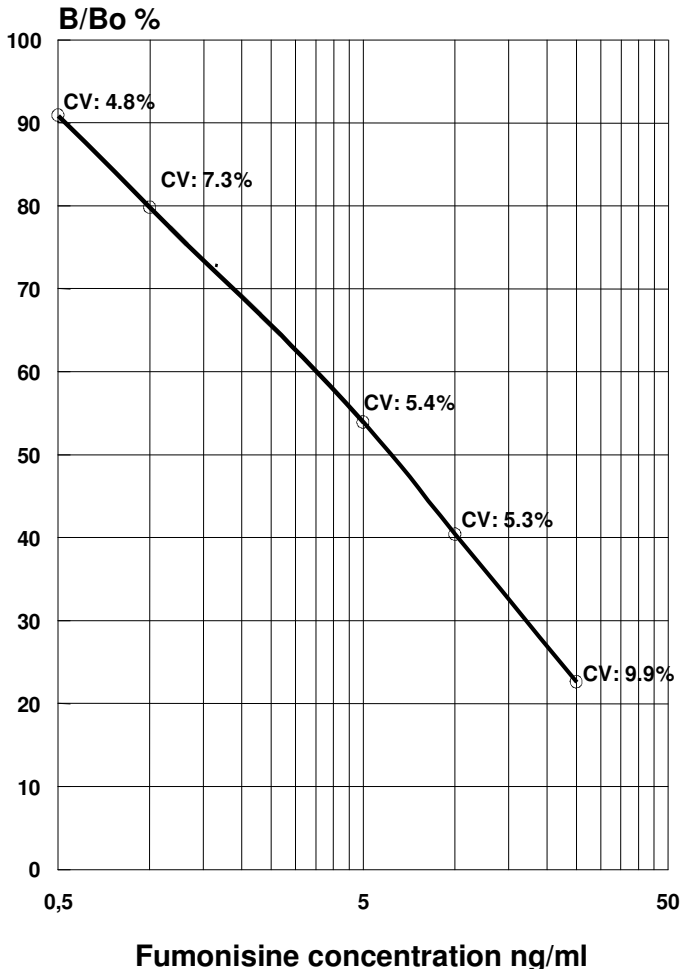


Figure 18. Dose-response curve of FB_1

Table 21. Characterization of monoclonal anti-FB_1 antibody:

Clone number: **1D6F11E3**
Subclass of the antibody: **IgG_1, κ**
Affinity constant of the antibody: **1.3×10^{10} l/ M**
Cross-reactivity with some related toxins: **FB_1 100 %**
FB_2 91.8 %
FB_3 209 %
HFB_1 0 %

Our anti-fumonisin antibody did not recognise the zearalenone (F-2), T-2 and deoxynivalenol mycotoxins that are commonly found in grains.

Table 22. Characterisation of the optimised ELISA test for FB_1 determination in cereals

Working dilution of monoclonal antibody:	**100 000**
Working dilution of FB_1-HRPO conjugate:	**140 000**
Standard concentration of FB_1 in PBS buffer:	**0.5-25 ng/ml**
IC_{50} value:	**5.4 ng/ml**
Slope of the standard curve:	**1.19**
Correlation coefficient of the standard curve:	**0.91**
Detection limit in buffer (0 ± 2SD.):	**0.38 ng/ml**
Detection limit for cereals:	**7.6 ng/g**
Measuring range in cereals:	**10-500 ng/g**

The within-assay (n=10) and interassay (n=30) coefficients of variation for standard concentration of FB_1 (0.5-25 ng/ml) were < 10 % (**Table 23).**

Table 23. Reproducibility of the FB_1 test

Concentration of FB_1 toxin standard (ng/ml)	Within-assay CV %	Interassay CV %
0	**4.03**	**5.65**
0.5	**3.1**	**4.82**
1.0	**5.9**	**7.34**
5.0	**2.67**	**5.39**
10.0	**2.0**	**5.29**
25.0	**5.8**	**10.5**

A very important part of the validation is the detection of matrix effect, which should be minimized

by the sample preparation procedure. To extract mycotoxins from different cereals, methanol- or acetonitrile-based organic solvents have been used in different concentrations (50 -75%) combined with different cleanup steps (Azcona-Olivera et al. 1992a, Yeung et al., 1996). The aim of our work was to simplify the procedure by eliminating the sample cleanup. However, although the acetonitrile-based solvent was compatible with the ELISA, we were unable to simplify the procedure because the centrifugation step could not be eliminated.

Content of the extraction solvent: 50 part acetonitril/ 39 part distilled water/ 10 part 0.5 % KCl / 1 part 6 % sulphuric acid).

Recoveries of FB_1 from artificially infected cereals are summarised in **Table 24,** ranging from 61 to 84%. The mesauring range of our test was 10-500 ng/g with detection limit of 7.6 ng/g.

Table 24. Recovery of FB_1 mycotoxin from artificially contaminated cereals[a]

FB_1 added (ng/g)	Rye-flour FB1 detected (ng/g)	Maize FB1 detected (ng/g)	Maize-flour FB_1 detected (ng/g)	Wheat FB_1 detected (ng/g)
200	**156 ± 21 (78%)**	**123 ± 21 (62%)**	**151 ± 10 (76%)**	**145 ± 27 (72%)**
100	**83 ± 15.9 (83%)**	**63 ± 11 (63%)**	**63 ± 7.0 (63%)**	**78 ± 14 (78%)**
50	**42 ± 9.9 (84%)**	**31 ± 7.1 (61%)**	**42 ± 11 (84%)**	**35 ± 8.7 (70%)**

[a] Different amount of FB_1 toxin was added to each sample in three parallel experimental series, then extracted and each extract was assayed in triplicates (n= 9).
Recovery % is detected FB_1 (ng/g)/added FB_1 (ng/g) x 100.

To compare the immunoassay method with an established procedure, 11 naturally infected maize samples were extracted and their fumonisin content were analysed by HPLC and dc-ELISA as summarised in **Table 25.** The negative toxin content of the samples was confirmed by both methods. In some samples our ELISA showed a higher value for FB_1 than did HPLC. We believe that the higher ELISA values at these samples are partly due to greater sensitivity of ELISA and partly to the high reactivity of our monoclonal antibody to other fumonisins. This cross-reactivity is particularly advantageous since it enables simultaneous detection of all three fumonisins in doubtful or suspicious samples. In our case we measure total fumonisins rather than FB_1.

Table 25. Comparison of FB concentrations obtained by ELISA and HPLC

Samples	HPLC		ELISA
	FB_1 (ng/g)	FB_2 (ng/g)	FB_1 equivalents (ng/g)
1	< 20	< 50	< 10
2	< 20	< 50	< 10
3	< 20	< 50	< 10
4	147	< 50	222
5	28	< 50	86
6	105	< 50	183
7	< 20	< 50	< 10
8	< 20	< 50	< 10
9	< 20	< 50	< 10
10	2444	926	> 500
11	< 20	< 50	25

For the sake of economy and stability, in our commercialised ELISA kit, the FB_1-specific antibody was pre-coated on the surface of polystyrene strips, the enzyme-labelled FB_1 was supplied in a stabilised buffer and all other reagents (washing buffer, substrate/chromogen and stopping solution) were kept in concentrated form. The brand name of our kit is **TOXIKLON Fumonisin B_1**. The stability of these reagents was 6 months at 4 ^oC. On one microplate FB_1 content of 40 cereal samples can be determined in duplicate (Barna-Vetró et al. 2000).

5.3. Development of ELISA test for determination of gentamicin in biological fluids

The aim of our work was to develop a sensitive ELISA for detection of gentamicin in porcine sera and cattle milk according to the EU directives. This simple test must not require complicated sample preparation. At first we had to prepare the main immunological reagents, i.e.the specific antibody and the enzyme labelled conjugate.

As gentamicin molecule (GE) has many NH_2 groups, for preparation of immunogen glutaraldehyde (GA) was used as cross-linker reagent similar to the fumonisin immunogen but the carrier protein was BSA instead of KLH.
The immunogenity of the GE-GA-BSA conjugate proved to be very good, all treated animals (n=5) produced specific antibodies. The fusion, hybridoma selection, cloning and recloning procedures were the same as for production of fumonisin antibody (**Results 5.2** page 53). During this procedure we could get 8 stabile clones with similar sensitivity. Ascites fluid of the best clone (3A11F9/C5) was selected, characterised and used in all subsequent experiments. **Table 26-27** summarize the most important data of this antibody.

Table 26. Characterization of monoclonal anti-GE antibody:

Clone number: **3A11F9/C5**
Subclass of the antibody: **IgG_1, κ**
Affinity constant of the antibody: **4.2×10^{10} l/M**

Table 27. Specificity of monoclonal anti-GE antibody

Substance	IC_{50} (ng/ml)	Cross-reaction (%)*
Gentamicin sulphate	**0.7**	**100**
Sisomicin	**20**	**3.5**
Netilmicin	**6.4**	**10.9**

No cross-reaction was found with sulphonamides, thiamphenicol, tobramycin, narasin, tetracycline, chloramphenicol, streptomycin.

* The cross-reactivity values were calculated as (IC_{50} of GE /IC_{50} of substance) x 100. (IC_{50} is defined, as the concentrations of GE required inhibiting colour development by 50 %).

Using the periodate method a good quality peroxidase-GE conjugate was prepared with working dilution of 1:200 000.

With the selected monoclonal antibody and enzyme conjugate a direct, competitive ELISA test (dc-ELISA) was set up. The monoclonal antibody was coated directly to the microplate in working dilution of 1:30000. In the drug tests we changed the rate of the volume (standards or samples)/ volume of conjugate to 1:5, this rate was 1:1 in the mycotoxin tests.

The reproducibility of the GE standards in PBS buffer is summarized in **Table 28.**

We wanted to use the optimised GE for determination of GE in *sera and milk* so we had to validate this test for milk and sera as well. Before the determination of within- and interassay data, the gentamicin standards had to prepare either in milk or in pig serum.

***Preparation of GE standards in milk*:** Potassium dichromate was added to de-fatted raw milk in a final concentration of 0.05% and stirred well. This treatment prevented the coagulation of milk for about one week.. The stock solution of GE (1 mg/ml) was diluted with milk in concentration range of 0.1-10 ng/ml, than tested in dc ELISA. Potassium dichromate did not disturb at all the enzyme reaction, the optical density was the same as without this stabiliser material.

***Preparation of GE standards in porcine serum**:* Porcine serum caused several problems. One of them was a very high non-specific binding to the antibody coated to the microplate. The measured optical density (Bo) of a normal, GE free sera (serum-blank) was very low compared to the buffer control. This problem could be solved by the use of a special salt (ANS) in the dilution buffer and with the dilution of sera to 1:10 in this buffer. The optimal concentration of 8-Anilino-1-Naphthalenesulfonic Acid (ANS) was determined. The final concentration of ANS was 0.01% in the diluted sera (**Figure 19).**

The stock solution of GE (1mg/ml) was diluted in this prepared pig serum in concentration range of 100-1 ng/ml. The GE serum-standards were diluted further to 1:10 in 0.1% ANS/ PBS/Tween 20 buffers prior to the test.

Table 29 shows the reproducibility of GE standards in milk and Table 30 shows that of in pig sera**.**

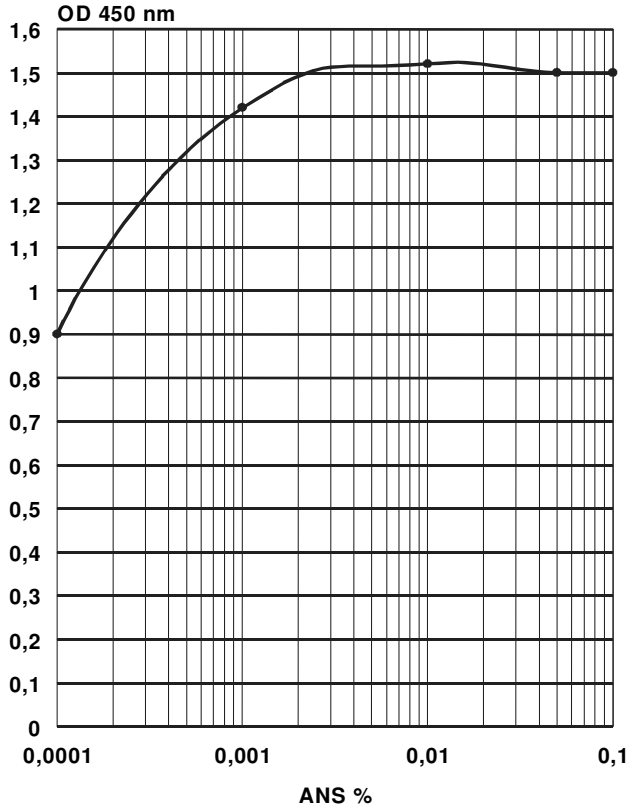


Figure 19. Dose-response curve of ANS in swine sera

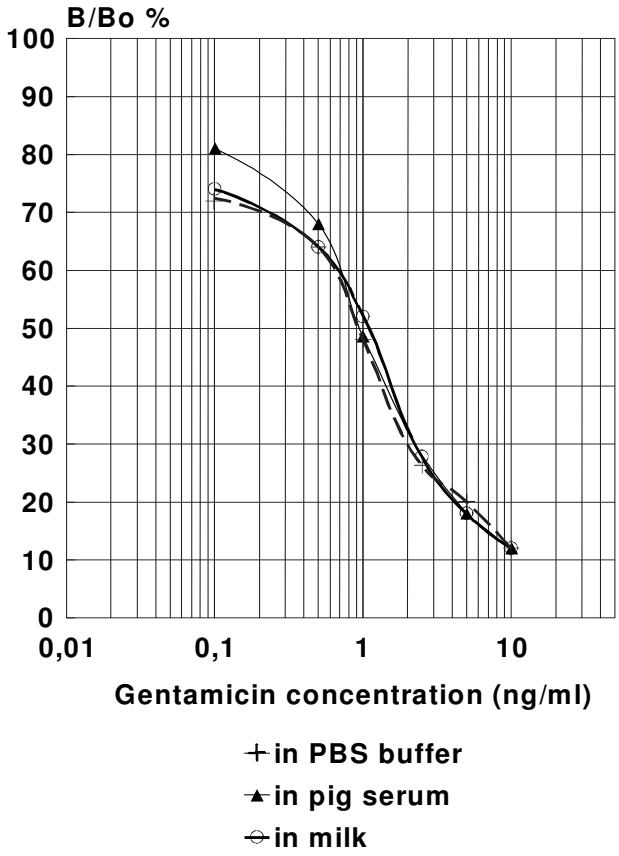


Figure 20. Dose-response curve of Gentamicin in different matrices

Table 28. Reproducibility of GE standards in PBS buffer

Gentamicin std. (ng/ml)	Within assay (CV%) 1. day	2. day	3. day	Interassay (CV%)
0	0.63	2.47	1.56	4.5
0.1	0.43	1.3	2.69	2.4
0.5	0.50	1.7	1.15	6.6
1.0	4.76	1.17	3.15	4.8
2.5	0.9	1.13	7.07	6.4
5	1.3	1.23	2.01	5.0
10	4.2	9.9	4.6	10.4

Number of samples:12

Table 29. Reproducibility of GE standards in milk

Gentamicin std. in milk (ng/ml)	Within assay (CV%) 1. day	2. day	3. day	Interassay (CV%)
0	2.4	2.2	1.6	6.3
0.1	3.3	3.3	3.4	1.4
0.5	1.4	1.4	0.93	1.3
1.0	1.8	2.1	2.0	2.4
2.5	4.0	3.5	4.6	2.1
5	5.0	5.8	7.1	5.0
10	6.0	5.2	8.1	7.2

Number of samples:12

Table 30. Reproducibility of GE standards in swine serum

Gentamicin std. in swine serum (ng/ml)	Within assay (CV%) 1. day	2. day	3. day	Interassay (CV%)
0	3.2	3.0	2.8	6.2
0.1	6.4	7.3	5.5	7.0
0.5	5.4	8.6	12	4.9
1.0	3.7	5.6	3.4	3.8
5	4.8	6.9	12	10.4
10	4.3	3.5	5.2	9.1

Number of samples:12

According to the reproducibility data of **Table 28-30**., the CV values of the within- and interassay are < 15%.

The precision of the GE test in milk and sera was determined by the recovery data.

Milk was infected artificially with gentamicin in a concentration range of 10 – 500 ng/ml. The milk samples were diluted before the test. **Table 31** shows the recovery of GE from milk.

Table 31. Recovery of GE from artificially infected milk

Added GE (ng/ml)	Dilution rate	[1]Detected GE mean (ng/ml)	CV (%)	Recovery (%)	[2]Detected GE mean (ng/ml)	CV (%)	Recovery (%)
500	**200**	**513**	**17**	**102**	**478**	**6.0**	**96**
100	**40**	**105**	**18**	**105**	**96**	**5.0**	**96**
50	**20**	**52**	**18**	**104**	**48**	**3.4**	**96**
25	**10**	**26**	**17**	**104**	**23**	**6.7**	**91**
10	**4**	**10**	**17**	**100**	**9.0**	**3.0**	**90**

1.Detected milk GE samples according to the GE standards in buffer
2.Detected milk GE samples according to the milk-GE standards

To determine the *precision* of GE test in pig sera**,** the samples were prepared similarly to the milk.

GE was added to pig sera in concentration of 1-100 ng/ml, then the sera was diluted 1:10 in PBS/Tween 20/ANS buffer prior to testing. The results are summarised in **Table 32.**

Table 32. Recovery of GE from artificially infected swine serum

Added GE (ng/ml)	Dilution rate	[1]Detected GE mean (ng/ml)	CV (%)	Recovery (%)	[2]Detected GE mean (ng/ml)	CV (%)	Recovery (%)
100	**10**	**97**	**1.1**	**97**	**99**	**1.0**	**99**
50	**10**	**50**	**4.8**	**100**	**47**	**4.0**	**94**
25	**10**	**26**	**8.0**	**104**	**24.9**	**4.4**	**99.6**
10	**10**	**9.8**	**1.4**	**98**	**10**	**2.9**	**100**
5	**10**	**4.8**	**5.0**	**96**	**4.98**	**3.2**	**99.6**
1	**10**	**1.2**	**10.3**	**120**	**1.2**	**14.0**	**120**

1.GE standards in buffer. Detected serum-GE samples were multiplied with the dilution factor.
2.GE standards in pig serum. Detected serum-GE samples were multiplied with the dilution factor.

As seen on **Figure 21,** the recovery of GE from milk and sera was very good; plotting the values of the added GE (*x-scale*) versus the measured GE (*y-scale*) resulted in a close correlation (correlation coefficient, r).
The linear equations are the followings:
(y_{milk}: 1.025 x + 0.76, r: 0.999),
(y_{sera}: 0.97 x + 0.56, r : 0.999).

The most important data of the validated GE-ELISA tests are summarized in **Table 33.**

Table 33. Characterisation of the GE-ELISA tests in biological fluids

Gentamicin ELISA test	*In PBS buffer*	*In swine serum*	*In milk*
Working dilution of anti-GE ab.	**1:30000**	**1:30000**	**1:30000**
Working dilution of GE-HRPO conjugate	**1:200 000**	**1:200 000**	**1:200 000**
Volume of std./sample (µl)	**20**	**20**	**20**
Volume of GE-HRPO conjugate (µl)	**100**	**100**	**100**
Dilution of samples prior to the test **Measuring range (ng/ml)**	**no** **0.1-10**	**10** **1.5-100**	**no** **0.1-10**
Dilution of samples prior to the test	**no**	**10 –1000**	**1-1000**
Measuring range (ng/ml)	**0.1-10**	**1.5 -10000**	**0.1-10000**
Concentration of GE (ng/ml) at IC_{50}	**0.7**	**9**	**0.9**
Slope of the standard curves	**0.78**	**1.07**	**0.84**
Correlation coefficient (r)	**0.837**	**0.89**	**0.86**
Detection limit (ng/ml)	**0.03**	**1.2**	**0.03**

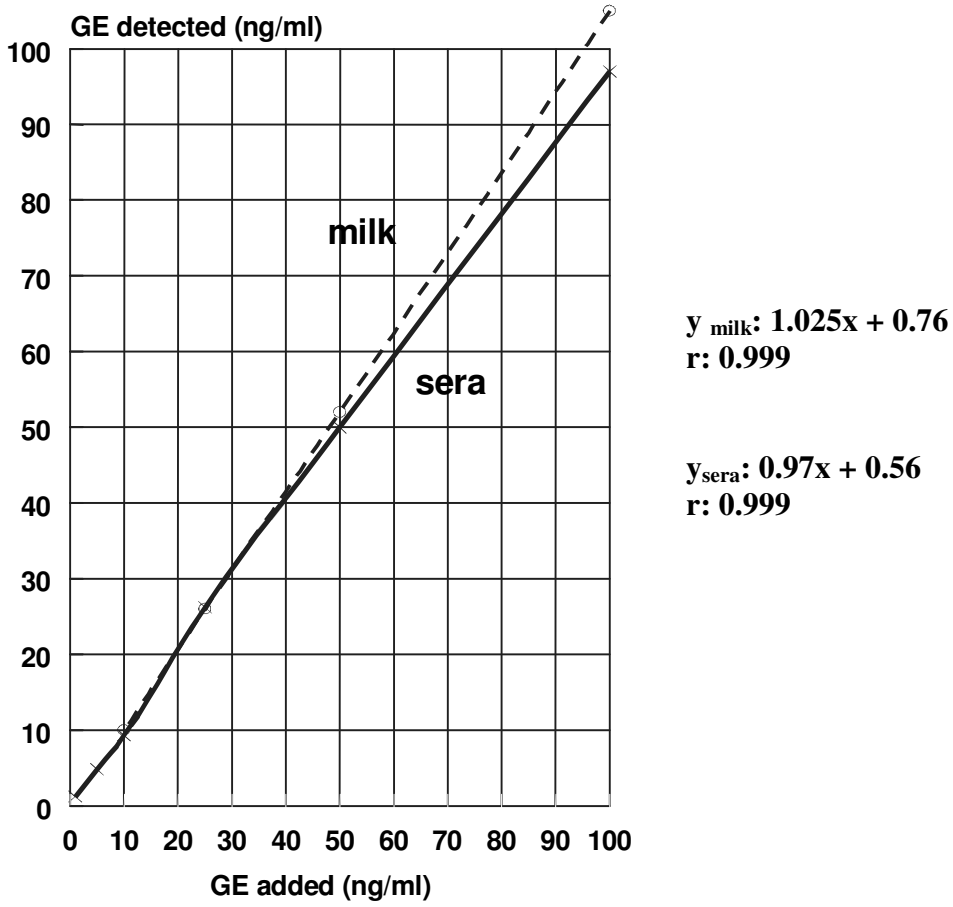


Figure 21. Recovery of GE in milk and porcine sera.

5.4. Development of ELISA test for determination of sulphadiazine in biological fluids

Immunological methods have been used both for environmental and food testing for a long time. Their use enabled rapid and effective screening of sulphonamides either on-site or in a fixed laboratory.

Continuing our drug-test program, the next selected drug-group was the sulphonamides.

Among the most important sulphonamides, the first we started the work was the sulphadiazine (SD). Our plan was to develop a sensitive ELISA for detection of SD in swine sera and cattle milk according to the EU directives.

Similarly to GE, we wanted to develop a simple test, which does not require complicated sample preparation.

First the main immunological reagents, the specific antibody and the enzyme labelled conjugate had to prepared.

As sulphadiazine molecule has NH_2 groups, for preparation of immunogen the same, glutaraldehyde (GA)-coupling method was tried as for fumonisin and gentamicin..
However, the immunogenity of the SD-glutaraldehyde-BSA complex was not so good as the former immunogens. The immun-response of the immunized mice were poor and the titer of sera remained low in spite of several booster injections.

According to many papers, the azo-coupling method for preparation of SD-BSA immunogen resulted in high titer antibodies therefore we tried this method too.

The synthesis of SD to BSA protein was made according to Haasnoot et al. 2000,)(Materials and Methods. 4.2.4.,page 35).

All treated animals (n=6) produced specific antibodies and the dilution of sera was quite high. One mouse with appropriate serum titer was selected for fusion. The fusion, hybridoma selection, cloning and recloning procedures were the same as for production of fumonisin antibody (**Results 5.2**). During this procedure 6 stabile clones were raised with similar sensitivity. Ascites fluid of the best clone (**1F3G8H5G8**) was selected, characterised and used in all subsequent experiments. **Table 34-35** summarize the most important data of this antibody.

Table 34. <u>Characterization of monoclonal anti-SD antibody:</u>

Clone number:	**1F3G8H5G8**
Subclass of the antibody:	**IgG_1, λ**
Affinity constant of the antibody:	**2.51×10^{10} l/ M**

Table 35. Specificity of monoclonal anti-SD antibody

Substance	IC_{50} (ng/ml)	Cross-reaction (%)*
Sulphadiazine	10	100
Sulphamerazine	80	12.5
Sulphathiazole	460	2.1
Sulphamethazine	2270	0.4
4-acethyl-sulphamethazine	4000	0.25
Sulphanilamide	10 000	< 0.1
Sulphadimethoxine	10 000	< 0.1
Sulphamethoxypyridazine	10 000	< 0.1

* Cross-reaction (%):IC_{50} (ng/ml) SD per IC_{50} (ng/ml) of Substance x 100

No cross-reaction with: thiamphenicol, tobramycin, narasin, tetracycline, chloramphenicol, streptomycin, gentamicin, sisomicin, netilmicin

The SD-HRPO conjugate was prepared either with the diazo-coupling method or the periodate method of Wilson and Nakane (1978). The conjugate prepared with diazo-coupling resulted in low working dilution of conjugate and a high background. Using the periodate method a good quality peroxidase-SD conjugate could be prepared with working dilution of 1:25 000.

With the selected monoclonal antibody and enzyme conjugate a direct, competitive ELISA test (dc-ELISA) was set up. The monoclonal antibody was coated directly to the microplate in working dilution of 1:4000. In the SD tests the rate of volume (standards or samples) per volume of conjugate was 1:5, the same as in GE test.

We wanted to use the optimised test for determination of SD in ***porcine sera and cattle milk*** therefore the test should be validated for milk and sera as well.

Before determination of intra- and interassay CV of the standards, SD standards had to be prepared either in milk or in pig serum, similar to the GE test.

Preparation of SD standards in cattle milk: de-fatted raw milk was stabilised by 0.05 % Potassium dichromate. The stock solution of SD (1 mg/ml) was diluted with milk in concentration range of 50-2.5 ng/ml, than tested in dc ELISA. Potassium dichromate did not disturb at all the enzyme reaction, the optical density was as same as without this stabiliser material.

Preparation of SD standards in porcine sera*:* The stock solution of SD (1 mg/ml) was diluted in pig sera in concentration range of 1000 – 25 ng/ml. Before the test, the standards were diluted 1:10 in PBS/Tween 20 buffer, which contained 0.1% of 8-Anilino-1-Naphthalenesulfonic Acid (ANS).
The reproducibility data of the SD standards in PBS buffer, in milk and in pig sera are summarized in **Table 36-38.**

Table 36. Reproducibility of SD standards in PBS buffer

SD std. (ng/ml)	Within assay (CV%) 1. day	2. day	3. day	Interassay (CV%)
0	2.0	2.2	1.8	1.1
50	5.4	8.9	5.5	2.7
25	5.4	6.3	6.7	3.6
10	5.8	4.2	7.0	3.0
5	5.8	4.6	2.9	6.1
2.5	3.2	2.6	3.7	7.1

Number of samples:12

Table 37. Reproducibility of SD standards in cattle milk

SD std. in milk (ng/ml)	Within assay (CV%) 1. day	2. day	3. day	Interassay (CV%)
0	2.4	3.9	3.1	6.4
50	8.2	7.0	9.5	7.3
25	5.2	9.4	6.2	2.7
10	6.0	4.5	6.7	3.2
5	3.8	5.3	2.6	1.7
2.5	1.3	1.3	1.0	6.1

Number of samples:12

Table 38. Reproducibility of SD standards in swine serum

SD std. in swine serum (ng/ml)	Within assay (CV%) 1. day	2. day	3. day	Interassay (CV%)
0	9.1	6.8	5.1	1.4
500	5.5	2.2	3.3	1.0
250	7.6	3.0	6.2	1.7
100	1.8	1.2	4.5	2.9
50	4.0	1.7	7.0	2.6
25	1.1	1.6	7.7	1.6

Number of samples:12
Serum standards were diluted 1:10 before the test.

According to the reproducibility data of **Table 36-38**., the CV values of the within- and interassay are < 15%.

Precision of the SD test in cattle milk and swine sera was determined by the recovery data.

De-fatted cattle raw milk was artificially contaminated with SD in concentration range of 6.25- 100 ng/ml. These milk samples were tested directly. **Table 39** shows the recovery of SD from milk.

To determine the precision of SD test in swine sera, the samples were prepared similarly to the milk.

SD was added to swine sera in concentration of 62.5-1000 ng/ml, then the sera were diluted 1:10 in PBS/Tween 20/ANS buffer before testing. The results are summarized in **Table 40.**

Table 39. Recovery of SD from cattle milk

Added SD (ng/ml)	[1]Detected SD mean (ng/ml)	CV (%)	Recovery (%)	[2]Detected SD mean (ng/ml)	CV (%)	Recovery (%)
100	**96**	**3.1**	**96**	**92.9**	**2.5**	**92.9**
50	**48.1**	**2.7**	**96**	**49.6**	**3.1**	**99.2**
25	**25.6**	**1.9**	**102**	**32**	**2.9**	**128**
12,5	**11.4**	**2.4**	**88**	**12.3**	**4.2**	**98.4**
6.25	**5.8**	**2.1**	**92.8**	**6.25**	**5.0**	**100**

1.Detected milk SD samples according to the SD standards in buffer
2.Detected milk SD samples according to the milk-SD standards

Table 40. Recovery of SD from swine serum

Added SD (ng/ml)	[1]Detected SD mean (ng/ml)	CV (%)	Recovery (%)	[2]Detected SD mean (ng/ml)	CV (%)	Recovery (%)
100	**95**	**3.5**	**95**	**94**	**4.2**	**94.3**
50	**47**	**1.2**	**94**	**49**	**5.1**	**97.6**
25	**25.5**	**2.9**	**102**	**24.9**	**3.2**	**99.6**
12.5	**11.3**	**2.1**	**90.4**	**12.3**	**3.5**	**98**
6.25	**7.0**	**6.6**	**112**	**6.8**	**5.9**	**108**

1.SD standards in buffer. Detected serum-SD samples according to the SD standards in buffer
2.SD standards in swine serum. Detected serum-SD samples according to the serum-SD standards

The SD standards in serum and serum samples were diluted 1:10 before the test.
Table 40 contains the SD values without multiplication.

Figure 22. Dose-response curves of SD in different matrices.

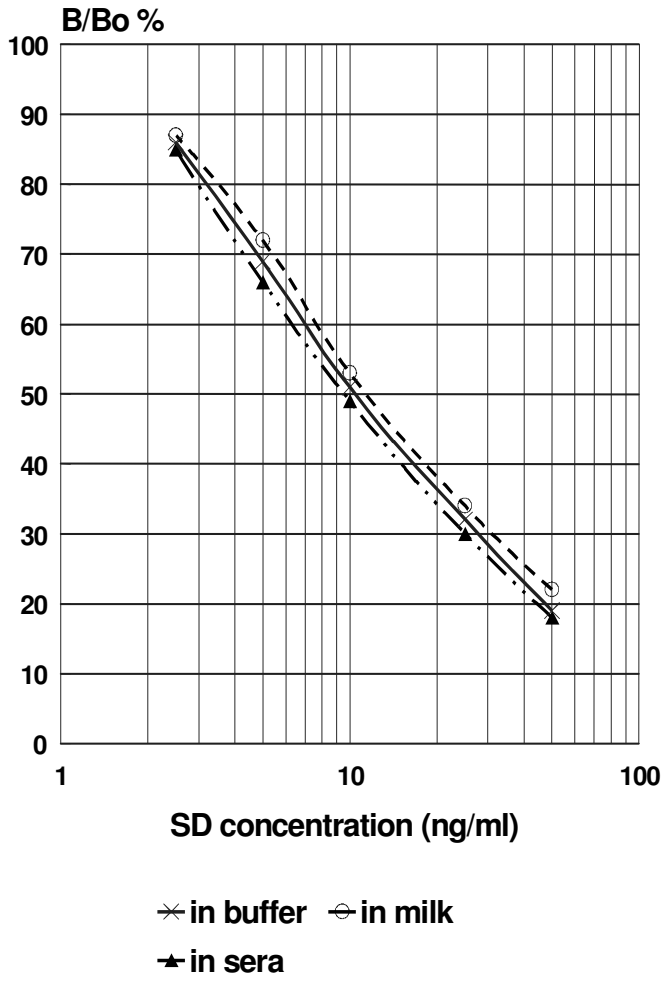


Figure 22. Dose-response curve of SD in different matrices

Table 41. Characterisation of the SD-ELISA tests in biological fluids

SD ELISA Test	*In PBS buffer*	*In swine serum*	*In milk*
Working dilution of anti-SD ab.	**1:4000**	**1:4000**	**1:4000**
Working dilution of SD-HRPO conjugate	**1:25 000**	**1:25 000**	**1:25 000**
Volume of std./sample (µl)	**20**	**20**	**20**
Volume of SD-HRPO conjugate (µl)	**100**	**100**	**100**
Dilution of samples prior to the test **Measuring range (ng/ml)**	**no** **50 – 2.5**	**10** **500 -25**	**no** **50-2.5**
Concentration of SD (ng/ml) at IC_{50}	**10**	**8.5**	**12**
Slope of the standard curves	**0.86**	**0.7**	**0.83**
Correlation coefficient (r)	**0.9**	**0,88**	**0.91**
Detection limit (ng/ml)	**1.2**	**10**	**1.5**

The most important data of the validated SD-ELISA tests are summarized **in Table 41.**

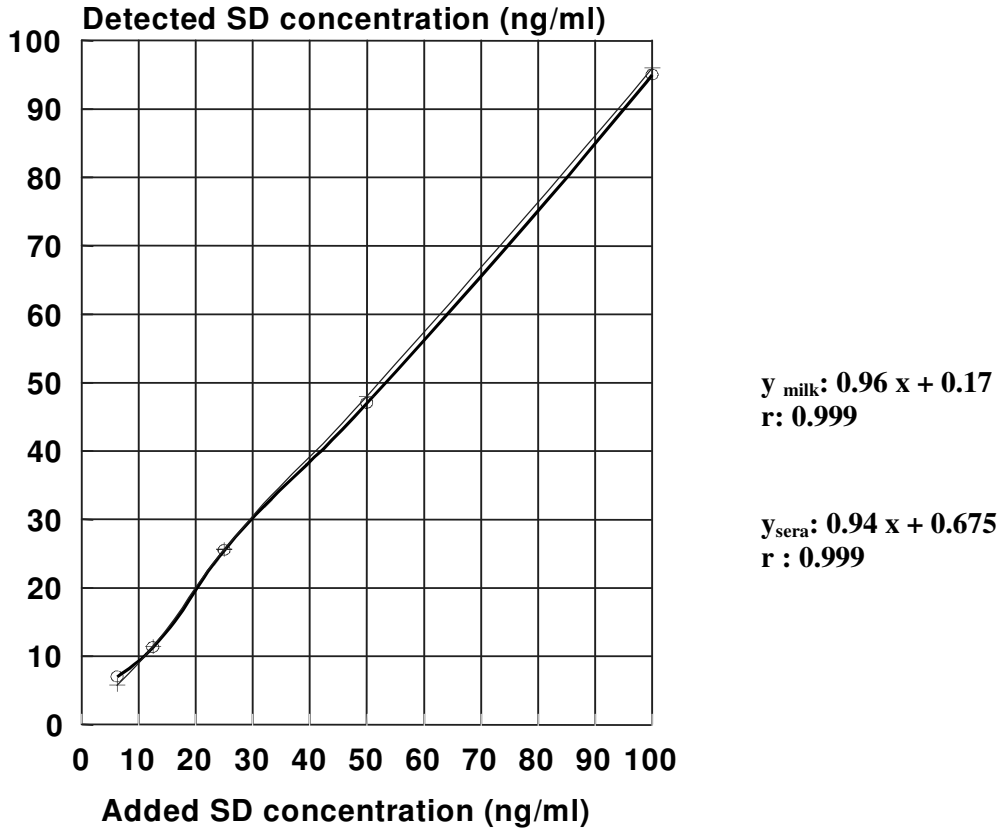


Figure 23. Recovery of SD from milk and swine sera
(+) in milk
(-o-) in serum

The recovery of SD from milk and sera were plotted. The added SD (*x-scale*) versus detected SD (*y-scale*) resulted a very good correlation **Figure 23.**

The linear equations are the followings:

(y_{milk}: 0.96 x + 0.17, r: 0.999),

(y_{sera}: 0.94 x + 0.675, r : 0.999).

6. Discussion

Our program was initiated to develop monoclonal antibody based ELISA tests for determination of harmful mycotoxins like OA and FB1 and drug residues (gentamicin, sulphadiazin) in different matrices. These tests were developed in the period of 1995-2002.

OCHRATOXIN-A

The aim of our work, concerning the **ochratoxin-A**, was the development of a sensitive OA ELISA test, which suits the international requirements for OA determination in **foods and feeds.** Instead of the generally used indirect ELISA for selection of monoclonal antibodies direct competitive ELISA has been applied which resulted in specific and sensitive monoclonal antibodies against OA.

A very important step of validation is the detection of matrix effects, which should be minimised by sample preparation procedure. Many reports have been published for extraction of OA from cereals using chloroform-, methanol-, or dichloromethane-based organic solvents supplemented with different acids (HCl, acetic acid, ascorbic acid, phosphoric acid) (Ramakrishna et al. 1990; Lee and Chu 1984; Märtlbauer et al. 1988; Kawamura et al. 1989; Seidel et al. 1993). Among the several solvents tried in our experiments, the dichloromethane based extraction method described by Märtlbauer et al. (1988) with modification was selected. Instead of HCl, a milder acid (citric acid) was used and proved to be as effective as HCl. This type of acid has not been previously used for the extraction of OA. In this procedure the centrifugation step is one of the most crucial steps as caution must be taken to avoid the non-specific reaction caused by incomplete separation of the aqueous buffer solution from the organic phase.

From practical and economic point of view a reagent kit was set up, branded name of **TOXIKLON**. This kit is available on the market from 1996 and used by several mycotoxin-control laboratories.

This test can be used for detection of OA content in human serum, porcine serum and seminal plasma and in different tissues.

As OA is known to be harmful, causing many serious disorders in the human, its level in human blood was investigated in several countries as summarised in a report by Zimmerli and Dick 1995). According to this paper, most of the human sera in European countries contained OA in a concentration of < 0.1 or 0.1-1.0 ng/ml. According to our result, 75% of the human sera contained 0.2-1.0 ng/ml of OA, which is in good agreement with the values found in Switzerland (Solti et al. 1997). In Bulgaria, a significantly greater proportion of human serum samples from patients with endemic nephropathy or urinary system tumours or both contained more than 2 ng/ml of OA in serum compared with samples from non-endemic areas. According to the paper of Kovács et al.(1995), 52 of 100 human blood samples (52%) and 38 of 92 human colostrum samples (41%) analysed by HPLC were reported to contain OA. In our experiment, the prevalence of serum samples containing detectable amount of OA was even higher (81.8%). However, only 6.8% of the 355 samples with OA concentration above 1 ng/ml were regarded as positive, which reflected an elevated toxin intake. Hungarian food law has set up new acceptable limits or modified the existing ones for aflatoxins, OA, DON, zearalenone, T-2 (HT-2), DAS and patulin in different food matrices. According to these regulations, the tolerance level of OA in rosted coffee is 10 ng/g and that of in cereal products 5 ng/g (**Table 7,** page 22.).

There is an empirical formula by which the daily intake of OA can be estimated (Zimmerli and Dick 1995; Breitholtz et al. 1991). According to this estimation, a daily OA intake of 0.7 ng/kg BW corresponds to a mean serum concentration of 0.25 ng/ml, whereas 5 ng/kg BW would result in a serum concentration of approximately 2 ng/ml. In our case, 24 blood samples (6.8%) contained over 1 ng/ml OA, and two of them contained considerably more than 2 ng/ml.

Although from our present survey, a direct conclusion regarding a later nephropathy or liver tumour cannot be drawn, but the data should call the attention of control organisations of each country to a thorough food and feed inspection, especially in connection with OA.

A great number of experiments have been conducted to elucidate **OA toxicity and pharmacokinetics** (Fink-Gremmels et al. 1995), but only very few data are available in the literature referring to mycotoxins **altering sperm quality of boars** in feeding trials. In our experiment, dietary OA was administered in microgram range and reached ppb values in serum and seminal plasma. Our OA test was sensitive enough to detect OA in serum and seminal plasma a few days after toxin challenge had started. Toxin profile of serum corresponded to that of semen in the experimental animals. Our findings that secondary distribution peaks of OA were detected both in plasma and semen seem to corfirm data given by Fuchs et al (1988) and Roth et al. (1988), that intestinal absorption of the toxin is supported by enterohepatic circulation/biliary excretion, which might be responsible for toxin accumulation and prolonged elimination from the body. It was reported that after a single oral exposure to OA, maximal serum levels of the toxin were found within 10-48 h in the pig. This experiment has suggested that OA might have the potential to affect sperm production and boar semen quality, but further investigation on more animals is needed to clear up the effect of OA to the quality of sperm.

Pharmacokinetic properties of **OA** differ widely between animal species. Absorption of the **toxin in chicken** was found to be faster than that of pigs, but pigs show much higher oral bioavailability (around 60%) compared with chickens (40%) (Galtier et al. 1981). High toxin concentrations were detected in the serum, liver and kidney during the first experimental week. Thereafter, only serum OA concentration increased until day 14. From the third week, a considerable decrease in plasma and tissue levels was detected. This can be explaned by the relative reduction in dose, ranging from 0.6 mg/body weight (kg) on day 1 to 0.22 mg/body weight (kg) in week 4 (**Figure 16,** page 52**).**
According to Galtier et al. (1981), the chicken can eliminate or metabolise the toxin very quickly, due to very high plasma clearance values. In the present study, no detectable amounts of residues were found in the kidneys after 28 days with ELISA. Therefore, other routes of toxin elimination such as biliary excretion should be considered in the poultry.

Our **TOXIKLON OA** test is versatile, it can be applied for large number of samples as well as for screening of OA in cereals, sera, seminal plasma and animal tissues. Our extraction method using the milder ***citric acid*** was as effective as other strong acids for purification of OA .
Our sensitive **TOXIKLON OA** test proved to be a useful tool for screening OA production among the black Aspergili as well (Varga et al. 1996; Téren et al. 1996).

FUMONISIN B_1

As the FB_1 molecule has one amino group on C_2 position its coupling to different carrier proteins (OVA, BSA, HSA, KLH or cholera toxin (CT) (Beier et al. 1996) is very easy. Different cross-linker reagents are available for coupling e.g. glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) (Fukuda et al. 1994), sulfo-SMPB (Kamps-Holtzapple et al. 1994), succinimidyl 6-(N-maleimido) hexanoate (MHS) (Szurdoki et al. 1996). According to Elissalde et al. (1995) the use of longer cross-linker (about 16 atoms) between the hapten and carrier can result in antibodies with higher affinity and assays with greater sensitivity than those obtained using shorter cross-linker (i.e. glutaraldehyde). An alternative method for coupling fumonisin B_1 to protein is one of its carboxylic acid groups by mixed anhydride method, which resulted in sensitive ELISA test for fumonisin B_1, while immunogen prepared through $-NH_2$ group produced only low titer of antibody (Yeung et al. 1996).

Although others (Beier et al. 1996 and Elissalde et al. 1995) have stated that linkage of FB_1 through its primary amine to a carrier protein is unlikely to produce high quality antibodies, we found that our immunogen (FB_1-GA-KLH) *elicited high-titre, high-affinity antibodies* that recognise free fumonisins. The working dilution of our monoclonal antibody was very high (1:100.000). In our experiment the monoclonal antibody was coated indirectly onto the microplate using rabbit anti-mouse Ig as the capture antibody.

The high cross-reactivity data indicated that the acidic side-chains of the intact molecule (at C-14 and C-15) might play part in the antigen-antibody interaction, confirming the statements of Beier et al (1996) and Elissalde et al (1995). Their other findings that the absence of hydroxyl group at C-5 in the case of FB_3 decreased the affinity of the Mabs by an average of threefold was not observed in our studies. Our antibody recognised FB_2 and FB_1 equally well and FB_3 much better, *similar antibody has not been published yet* in the literature.

As a result, our dc-ELISA may show higher value for FB_1 than other instrumental (e.g. HPLC) methods. The cross-reactivity of the monoclonal antibody is particularly advantageous since it enables simultaneous detection of all three fumonisins in doubtful or suspicious samples. In our case we measure *total fumonisins* rather than FB_1.

GENTAMICIN

Antibiotics cause a number of health risks that urge the detection of these contaminants in food products. In this respect, contamination of milk and milk products, sera and meat with antibiotics is being monitored in most European countries (**Table 9,** page 28).

Aminoglycosides are one of the major classes of antibiotics used in veterinary medicine. The present microbial, immunochemical and physiochemical methods of detection are either not sensitive enough or not suited for routine screening. To develop immunoassays that meet the required Maximum Residue Levels (MRL) as set up by the EU for gentamicin (100 $\mu g/kg^{-1}$), we decided to work on a monoclonal antibody based, simple and sensitive ELISA test for detection of GE in porcine sera and cattle milk.

By using a good immunogen GE-BSA), prepared by glutaraldehyde coupling and that of our selection method for hybridomas, some clones producing very sensitive monoclonal antibodies, could be selected.

A competitive ELISA was set up with the selected monoclonal antibody (**3A11F9/C5)** either for milk or for pig sera. The greatest difficulty with direct analysis of body fluids was the effect of the matrix on the assay. The aim of our study was to investigate if the development of a sensitive immunoassay could overcome the effect caused by the matrix.

The test-protocol of the validated GE test of milk became very simple, the samples (20 µl) could be used *directly*, it was not necessary a clean up or dilute the sample before the assay. In the drug tests, the volume of the conjugate per volume of sample (or standards) was **5,** compared to our mycotoxin tests, where this rate was **1**. With this kind of test-modification we could set up a very sensitive assay. The measuring range of GE, in the milk test, is **0.1-10 ng/ml**, with detection limit of **0.03 ng/ml.**

During the setting up the test for sera we had to face with many problems. When we tried to use directly a normal, gentamicin free serum as negative control (Bo sera), similar to the milk, lower optical density was measured, almost 50 % of the buffer control (Bo buffer). Probable the serum

protein caused this high, non-specific binding-effect in the test. By dilution of sera with PBS-Tween 20 buffer (1:10), that contained a special salt (**ANS**) in appropriate concentration this problem could be solved. This assay-buffer compensated the non-specific binding effect. The measuring range of the GE test in swine serum is 1.5-100 ng/ml, with detection limit of 1.2 ng/ml.

As seen on, the dose-response curves of GE (**Figure 20.** page 58) in PBS, sera and milk, that are in good agreement with each other, it is not necessary to prepare different GE-standards for testing of milk or sera samples. The application of different GE-standards would have resulted in a too complicated and expensive test. According to the test-protocol, we used simply only six GE standards in PBS buffer (0.1 – 10 ng/ml) and the GE concentration of milk samples can be read directly from the calibration curve (ng/ml). The GE concentration of diluted serum samples can be calculated from the same standard curve, using the appropriate multiplication factor.

According to Berkowitz et al. (1986), the gentamicin adsorbs to glass and partly to plastic surface. We did not find the feature of the gentamicin-solution during 6 months, storing the GE-standards in plastic and glass vessels.

Recently Van Es et al. (2001) published a paper about the measurement of GE in milk by a flow-injection assay using two controls, 0 and 100 ng/ml of GE. Although the procedure was very short, it is a qualitative test and not as sensitive as our quantitative GE-ELISA test.

In the VIth. International Conference on Agri-Food Antibodies, held in Prague in October 2001, Koets et al. (Agrotechnological Research Institute, Wageningen, The Netherlands) reported about a monoclonal and a recombinant antibody based ELISA tests for gentamicin and dihydrostreptomicin. According to this short paper, the IC_{50} value of GE was 135 ng/ml in PBS and 655 ng/ml in milk. Compared these data with our data in **Table 33.** (page 61), our tests are more sensitive than these ones.

We plan to validate our GE test for meat as well and to promote the practical application, our GE-ELISA tests will be commercialised in the near future.

SULPHADIAZINE

In order to prevent drug residue problems in the dairy and swine industries, proper medication practices on the farm and appropriate feeding protocol are required, complemented with reliable screening methods to identify if a residue problem exists or not. Antibody-based methods (e.g ELISA) are used both for environmental and food testing during the past decade, quickly becoming the screening method of choice for many compounds. Their use enabled rapid and effective screening of drug residues e.g. sulphonamides either on–site or in a laboratory.

Drug residues are detected mainly in porcine blood, milk, tissues, urine and bile. The greatest difficulty with direct analysis of body fluids is the effect of the matrix on the assay.

Among the sulphonamides, the most important compounds are the sulphadiazine, sulphamethazine, sulphathiazole, sulphamerazine and sulphamethoxypyridazine.
For control of these residues in foods, some commercialised kits are already available on the market.

Some years ago our group started a project with the aim to develop monoclonal antibody based ELISA tests for the most important sulphonamides. We already have monoclonal antibodies against sulphadiazine, sulphamethazine and sulphathiazole. Among these works I selected the sulphadiazine ELISA test, introduced and summarised the most important steps of the test-

development.

The first step in development of an immunoassay is the preparation of immunogen.

As sulphadiazine has one amino group, some methods are known for the coupling to carrier protein. One common cross-reacting compound is the glutaraldehyde with a glutaramyl spacer arm, (-NH-CO-CH_2-CH_2-CH_2-CO-NH-). First we tried the glutaraldehyde method to prepare SD-BSA complex, but in our case, we failed to raise good antibody against this immunogen.
Earlier with the glutaraldehyde-coupling we could produce good quality immunogens (FB_1-KLH, GE-BSA) and could raise very high titre of monoclonal antibodies.

Using glutaraldehyde Märtlbauer et al. (1992) prepared some sulphonamide-BSA immunogens and could get good titre of polyclonal antibodies against sulphamethazine and sulphadiazine with cross-reactivity of some other sulphonamides.

The other possible method, the azo-coupling (- N=N -) for preparation of immunogen resulted better immunogen resulted in raising antibodies with higher titer. Fránek.et al (1999) reported a similar result concerning the azo-coupling immunogen.

In general, the sensitivity of any immunoassay is based partly on the selected spacer in the production of enzyme conjugate. This approach consists of a chemical change of the spacer arm in the same position as used for attachment to the enzyme and the carrier protein (Fránek et al 1999).

Our observation concerning the preparation of *immunogen* and *enzyme conjugate* with different chemical methods using different spacers (*heterogeneous system*) and the effect of these reagents for the sensitivity of our assay was the same. The preparation of the SD-HRPO by azo-coupling (homologue system) resulted in a poor sensitivity with high background. Thus we selected another coupling method (periodate) and finally developed a sensitive test with no background.

We set up a direct, competitive ELISA with the measuring range of 2.5-50 ng/ml and with detection limit of 1.2 ng/ml in buffer. This test was applied for determination of SD in body fluids.

In general the greatest difficulty with direct analysis of body fluids is the effects of the matrix on the assay. The aim of this study was to investigate if the development of a highly sensitive immunoassay could overcome the matrix effect. One possibility for this problem is the dilution of the matrices (100-1000) prior to the test (Fránek et al 1999).

However, we followed another way, i.e. we modified the volume of standards (or sample) per volume of conjugate to 1:5, the volume of standard (or sample) was 20 µl.

By using this little volume the dilution of milk was avoided and according to the recovery data there was no matrix effect (**Table 39,** page 65).

In the case of sera we experienced matrix effect, when the sera was used directly in the test. But in these cases the dilution of sera to 1:10 with our special dilution buffer (0.1%ANS/PBS/Tween 20) made the matrix effect disappeared (**Table 40,** page 65).

Our monoclonal anti-SD antibody is specific mainly to sulphadiazine and partly recognises the sulphamerazine and sulphathiazole. It means that we measure mainly the sulphadiazine.

From other point of view it would be better a generic antibody (monoclonal or polyclonal), which recognise many sulphonamides, so the test would be versatile (Hassnoot et al. 2000). According to

Hassnoot, the synthesis of a sulphathiazole (TS) or sulphanilamide (PS) derivative could solve this problem, they could produce antibody with higher cross-reactivity to many sulphonamides.
Our plan is to raise a similar antibody with the preparation of TS immunogen.

The parameters of our validated SD-ELISA tests are applicable for screening milk and sera according to the EU directives. We would like to continue this work and validate the test for meat samples too.

Our work for development of ELISA tests was qualified by obtaining ISO 9001:2001 certifications for "production of specific monoclonal antibodies, development and manufacture of "ELISA" diagnostic tests for measurement of mycotoxins and antibiotics".

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9. Glossary

Affinity - a mathematical measure of the binding energy between an antigen or ligand and an antibody.

Analyte -substance to be detected; the target compound in an immunoassay.

Antigen - a large, chemically complex molecule or hapten-carrier conjugate that can induce an immune response, resulting in the formation of specific antibodies.

Antigen-antibody complex - the product that results from an antibody binding to an antigen.

Antibody-antigen reaction - the dynamic, reversible reaction between an antibody and antigen to form an antigen-antibody complex:

$$Ag + Ab \underset{K_2}{\overset{K_1}{\rightleftharpoons}} AgAb$$

Where Ab is free antibody, Ag is free antigen, AgAb is the resulting antigen-antibody complex, K_1 is the association rate constant, or the forward reaction to form the complex, and K_2 is the dissociation rate constant for the reverse reaction.

The reaction eventually reaches equilibrium, as shown in this equation:

$$Ag + Ab \overset{K_1}{=} AbAg$$
$$K_2$$

The affinity or equilibrium constant, K, is the ration of K_1/K_2

Antigenic determinant (or epitope) - part of an antigen that reacts with an antibody.

Antibody - a protein produced in response to an antigen that binds a specific antigen or hapten and form a complex.

Antibody-binding site s- areas of the antibody able to bind to corresponding antigenic determinants

Avidity - describes the stability of a complex formed by multivalent antibodies with an antigen.

Chromogen - a colour-generating reagent.

Clone - a group of homogeneous cells that are the progeny of a single cell.

Competitive binding assay - a type of ELISA in which the analyte in a sample (antigen) competes with a known amount of enzyme conjugate (labeled antigen) for a limited number of binding sites on a specific antibody. The amount of conjugate displaced from binding sites indicates how much analyte is in the sample.

Cross-reactivity - the binding of an antibody to an antigen or a hapten other than the target antigen or hapten. It is a measure of specificity.

EIA - Enzyme immunoassay. An immunoassay using a colour-changing enzyme-substrate system for reading results.

ELISA - Enzyme-linked immunosorbent assay. A heterogenous EIA using a solid phase for separation bound from free enzyme conjugate.

Enzyme conjugate- an antibody, antigen or hapten coupled with an enzyme in a way that allows each to retain its activity.

Epitope - an antigenic determinant.

Hapten - a small molecule that cannot induce antibody production unless it is covalently bound to a carrier

molecule. A hapten can react with the specific antibodies produced in response to the hapten-carrier conjugate.

Heterogeneous EIA - an EIA requiring incubation and separation steps, such as an ELISA.

Hybridoma - a hybrid cell produced by fusing an antibody-producing cell and myeloma (tumor) cell to produce an immortal cell line. Hybridoma cells can produce monoclonal antibodies indefinitely in culture.

Horseradish-peroxidase (HRP) -an enzyme frequently used in EIA. The purity of enzyme is shown by RZ value. The RZ (Reinheitszahl) is the absorbance ratio A_{403}/A_{280} determined at 0.5-1 mg/ml in deionized water. It is a measure of hemin content, not enzymic activity.

Immunoglobulin- one of a family of globular proteins capable of acting as antibodies. These include IgG, IgA, IgM, IgD and IgE.

Immunoassay - an analytical test to measure or detect a substance using antibody-antigen reactions.

Lymphocyte - cell that mediates immune-response specificity.

Macrophage - phagocytes found in tissue and blood.

Monoclonal antibody - a homogeneous antibody population derived from one specific antibody-producing cell.

Multivalent antigen - antigen with more than two binding sites.

Myeloma cell -a tumor cell used to produce a hybridoma.

Polyclonal antisera - antibodies obtained from sera, often a heterogeneous population of antibodies varying in specificity and affinity, resulting from many antibody-producing cells.

Qualitative - detecting a substance through a yes/no result.

Quantitative - mesuring how much of an analyte is in a sample.

Sensitivity-the ability of an antibody to detect very small levels of an antigen.

Specificity - the ability of an antibody to detect only the target analyte.

Standard - one of a group of reagents with known, graduated concentrations of analyte spanning the range of an assay (see standard curve).

Standard curve -a dose-response graph constructed using standards of graduated concentrations. The results of a qualitative immunoassay are plotted on this curve.

Substrate -the reagent which, when it reacts with the enzyme in an EIA, causes the chromogen to produce a measurable colour change.

Titre - the reciprocal of the dilution of an antiserum able to bind 50% of the labeled antigen. The geater the number, the greater the antibody concentration and affinity.

10. Acknowledgements

I wish to express my thanks to:

Professor **László Solti** (Szent-István University, Faculty of Veterinary Science, Budapest), who has been supervised my R+D work since 1989. As the director of the Institute of Animal Science of ABC (Gödöllő), he started and conducted the mycotoxin project. Although he left the institute, our scientific connection in different subjects remained unchanged and he is always helping me with valuable advices,

Professor **Ervin Balázs** (Director of Environmental Bio-Safety Research Institute of ABC, Gödöllő), who was the General director of ABC until 2000. He is our present director as my group belongs to the Bio-Safety Research Institute. My director always helped and stimulated our food safety program for developing of mycotoxin and antibiotic test-kit,.

Professor **Ferenc Nagy** (General Director of ABC), who supported me to continue my research project and summarize it in a Ph.D. work,

My research fellow, dr. **János Schrett** for the preparation of anti-sulphadiazine monoclonal antibody,

Special thanks to Mrs. **Erzsébet Szabó, Éva Tóth**, and Mr. **László Sziráki** for their excellent technical assistance in the developing of ELISA tests and to Mrs. **Antónia Szigeti** and **Gizella Dezső-Szabó** for their excellent technical assistance in the preparation of monoclonal antibodies,

I have to mention and thank to my former colleagues, Mrs. **Dr. Gyöngyösi, Ágnes Horváth** and Mrs. **Farkas, Márta Rajkort** for the preparation of monoclonal anti-OA antibody and **Anna Wölfling**, who took part in the preparation of OA-HRPO conjugate.