Immune-enzyme methods of food safety analysis

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Abstract. One of the topical issues of agricultural production is the quality and safety of raw materials and products. Food safety primarily concerns biological, more precisely microbiological objects, namely, viruses, pathogenic bacteria (salmonella, listeria, etc.) and fungi (mycotoxins), the analysis of which requires cheap, specific and fast methods. This article covers the essence of ELISA – enzyme-linked immunosorbent assay methods, as well as PSR - polymerase chain reactions, their varieties and advantages in comparison with traditional methods of analyzing microorganisms that pose a certain danger to human health (through food products) and animals (through feed). The article is relevant in the light of recent events, namely outbreaks of various epidemics (e.g., the Covid-19 coronavirus)

1. Introduction
Testing of enzymatic activity is the one of common methods, because it reflects the quality of food products. Since enzymes are labile proteins, they indicate the quality of the product and act as decomposition markers for the quality of the raw materials, for the process chain and for the safety of the final product. In this context, analysis involves measuring the rheological properties of the enzyme substrate either by physical measurement (viscosity) and analyzing the properties of the final product or by-products resulting from the enzyme activity. Common methods used include volumetric titration, gravimetric, colorimetric, polarimetric, flame photometry, and atomic adsorption spectrometry. In addition to them, methods that are fast, accurate and cheap (bioassays), replacing the classical approaches, have recently been discovered. One of the examples is identification through immunoassays using ELISA - Enzyme Linked Immune Sorbent Assay [1]. The purpose of this article is to discuss the essence of this method, its advantages and importance in the control of raw materials and products of the agrarian production chain.

2. Results and discussion
Immunoassays are a powerful analytical method for the analysis of food products. They are divided into the following groups:

- Based on primary responses, i.e. when the antibody-antigen response is directly measured (for example, radioimmunoassay and enzyme-labeled samples).
- Based on secondary responses, i.e. which measure the antibody-antigen response indirectly (eg, immunopresipitation). These terms - direct and indirect - refer to the fact that the antibody actually binds to the antigen, which will be measured or will measure some property of the antibody-antigen complex that is formed [2-4].
Recently, immunoassays, i.e. ELISA have begun to be used. Although ELISA itself is a relatively simple and promising method, the preparation of a food product sample for testing requires optimization. Sample preparation often requires extraction of the analyte and concentration from the complex matrix of PP, where there is difficulty in opening the analyte or the analyte becomes undetectable. There are various ELISA formats that could be used, but it is essential that they all follow the same principles and rely on the specificity of antibodies for the sensitivity of simple enzymatic assays. There are two main variations of this method: 1. Detection of the presence of antigens that the antibody is searching. 2. The antibody that the antigen is searching can be used to test. ELISA is a 5-step procedure: 1. Antigen coating of the micro-titer plate. 2. Blocking all unrelated sites to prevent false positive results. 3. Adding antibodies to the plate walls. 4. Addition of anti-serum IgG conjugated to the enzyme. 5. Reaction of the substrate with the enzyme to produce a colored product, indicating a positive reaction. There are many different types of ELISA, with different modifications; they depend on the presence of one component of the ligand pair fixed to a solid surface. The choice of which of the components binds and detects is carried out directly or indirectly, and whether there are two or more components in the immunoassay, will depend on the task of the study [5, 6].

The first step in preparing an immunoassay is to prepare the appropriate antibody. Animals are immunized with the antigen and then serum containing polyclonal antibodies is collected. Sample capabilities can often be improved by preparing monoclonal antibodies that exhibit high specificity for the target antigen. Spleen cells isolated from immunized animals are used to produce hybridoma cells that secrete monoclonal antibodies into the culture medium.

To obtain antibodies for a given antigen, several steps, such as extraction, concentration and purification of the target molecules are also required, and if the target molecule does not have antigenic rejection properties, it must be attached to the antigenic protein molecule to form a conjugate. This conjugate is then used to induce the formation of specific anti-conjugate antibodies that belong to one of the classes of immunoglobulins (e.g., G-class immunoglobulin (IgG); M-class immunoglobulin (IgM)).

It turned out that the ELISA method is applicable in various domains of analyzes related to food safety, for example, the detection of pathogenic microbes (mycotoxins) or their metabolites (bacterial toxins, mycotoxins A, etc.), hormones, antibiotics and growth promoters [7].

Biosensors. The term sensor means a system (electronic, signaling) that is responsible for a specific physical or chemical property that can be identified or quantified. Biosensor is an analytical tool containing a biologically sensitive element, connected or integrated with a transducer, which translates a biological phenomenon into what should happen. The biologically sensitive element can be either catalytic (enzyme, microorganism), or non-catalytic, such as an affinity sensor (antibody receptor). Biosensor technology is widely used [8, 9], but there is still no understanding of the requirements and specific conditions for the use of biosensors in food analysis. The biosensor contains two elements: 1) a searching biological element (antibody, enzyme, cell, receptor) and a signal-translating element (optical, amperometric, acoustic, electrochemical) connected with the data of the search and processing system [10]. The use of various chemiluminescent biosensors for the detection of various food analyses has been reported recently [11]. Immunosensors, originally developed for medical purposes, have tremendous potential for the analysis of food products, since has recently been emphasized for the analysis of B vitamins in fortified foods using an automatic flow system based on the surface plasma resonance (SPR) method (table 1)

**Table 1.** Various food components and contaminants measured by biosensors [1].

| No. | Food components | Type of biosensor | Determination mechanism | Type of sample |
|-----|-----------------|-------------------|------------------------|----------------|
| 1   | glucose, sucrose| enzymatic, glucose oxidase | membrane, electric potential | vegetables, grains, dairy products |
### 2.1. Molecularly printed polymer based methods (MIPs)

An alternative to immuno-sensors and immunoaffinity methods is the method of molecularly printed polymers based on synthetic polymers created with specific “sites” as (bio) sensors for special target (print) molecules (PM) (figure 1).

**Figure 1.** Mechanism of formation of molecularly printed polymers (MIPs) and their application as a method for the identification and measurement of various food organic contaminants.

The procedure involves preparing a “mold” by surrounding functional monomers (Mo) around the PM (antibiotics, steroids, toxins, etc.). The print of the molecule (PM) and the resulting complex is copolymerized with crosslinking groups (for example, ethylene glycol, dimethyl acrylate) in rigid polymer.

The PM is then extracted, making search sites free. Using this method, “platinum antibodies” are created as special MIPs “mold” into affinity sites to capture specific PMs, which then becomes capable of acting as biological antibodies.

In practice, the MIP method includes 4 stages.

1. The printed molecule dissolves in the pores together with the monomers and forms a non-covalently soluble complex.
2. A polymerization initiator is added to form a rigid polymer.
3. The polymer mass settles and envelops the solvents, forming particles of 20-25 mm (called MIP), which act as supports for SPE (Solid Phase Extraction), electrophoresis or applied to an HPLC column.
4. PM is extracted from MIP “molds”, which provides special (compartments) affinity sites (biosensors) for “catching” other different printouts of the molecule (poisons, herbicides, sugars, nucleotides, amino acids, proteins) from the mixture.

Some examples of MIPs used for the analysis of contaminants are: identification and quantification of herbicides triazine and chloro triacin, penicillin, chloramphenicol using MIP-based SPE, fluorometry and HPLC, at 0.2-1000 ppm content [6]. MIP is also used in combination with transducer elements (optical fiber, electrochemistry, piezoelectric or acoustic sensors) for rapid “biometric sensor” detection and quantification, as well as for contaminants (e.g. O-xylene, RAN) at 0.01-1 ppm content.

The application of MIPs has mainly been aimed at contaminants soluble in organic solvents, but now this method is widely used both in the agrarian industry and for environmental analysis. With new approaches like surface printing, MIP can also be applied in aqueous media for water-soluble analytes, extending it to proteins, DNA, carbohydrates and microorganisms. Recently, new synthetic materials that can recognize proteins have been found. Protein-binding nanoparticles were prepared on a carbohydrate-like surface using thin films produced by RF plasma. Protein-specific nanometer-sized plates have been used as a substrate to separate specific proteins from a mixture like albumin, immunoglobulins, fibrinogen, and lysozyme.

ELISA (Enzyme linked immunosorbent assay) is becoming one of the most widely used serological tests for the detection of antigens [12, 13]. This method uses enzymes as a label for antibody molecules used for antigen detection. By adding a chromogenic substrate to the antibody-enzyme-conjugate-antigen complex, an enzymatic reaction is induced, resulting in a colored product that is analyzed with a colorimeter. Typical enzymes used are horseradish peroxidase, alkaline phosphatase and β-galactosidase. There are basically two flavors of ELISA method used: 1) direct (sandwich) and 2) indirect (assay). The first method is used to detect antigens, and the second to detect antibodies. In the first sandwich sample (1st specific “capture”), the antibody is immobilized on a hard surface, usually a plastic (polystyrene) microtiter plate, tube, nitrocellulose membrane, or magnetic tape. Then a sample of antigen is introduced, which reacts with the fixed antibody. After washing out unbound antigens, the 2nd sample with an enzyme-conjugated reporter antibody is added. Unbound antibodies are removed again by elution. A three-layer complex that looks like a sandwich is formed. The reporter antibody is detected by the addition of a substrate specific to the enzyme. If an enzyme bound antibody is present, an enzymatic reaction occurs which results in a colored product. The intensity of the color that appears indicates the proportionality of the amount of antibody present.

In an indirect ELISA assay, a “sensitive” antigen is adsorbed onto a solid surface (mainly microtubes or latex tapes) and then the free antigens are removed by washing. An antiserum test is then added and if antigen-specific antibody is present, a (linked) antibody-antigen complex is formed. Free antibodies are then removed by washing, after which enzyme-linked antibodies are added. The conjugate is combined with the antibody sample, and then free conjugate is removed by washing, the attacked ligands are shown by a stained substrate. Available antibodies are quantified in the same manner as antigens in the double sandwich method.

Although the ELISA method gives short-term results (usually from 1 to 3 hours), to increase the number of cells to the level necessary for real determination (about 10^5 cells per ml), preliminary enrichment and simple enrichment steps are required. The duration of traditional methods is two or more days. A number of approaches have been used to further increase the accuracy and speed of detection, for example, a system that captures Salmonella on a probe. The probe is inserted into the pre-enriched wort, where Salmonella cells are captured. The stick is then transferred to the growth medium, allowing an increase in the number of cells before the ELISA assay. This is useful for a selective enrichment stage, which will save extra days [14].

2.2. Radioimmunoassays (RIA)
In principle, these tests resemble the ELISA method, only they differ in that instead of enzymes, radioisotopes are used as a label for antibodies. The original RIA method uses a radiolabeled antigen that is present in an unlabeled antibody or antigen in a test sample. The antigen radioactivity is then measured using a radioisotope analyzer. If the analyzed sample contains a large number of antigens, then they are captured with a radiolabeled antibody at the site of the antigen-binding antibody and the radioactivity is greatly reduced. Isotope $^{125}$I is commonly used. The use of isotopes has some disadvantages such as isotope instability, health risks and environmental pollution problems. For these reasons, labeled isotopes are not used very often, and other alternative “cold” labeling systems, such as fluorescence, have been developed and put into practice [15].

2.3. Automatic immunoassays
Automatic immunoassays are becoming more and more popular in food microbiology laboratories. They are designed primarily for testing large quantities of samples, but currently only small batches of samples are available for testing. In addition to ELISA, fluorescence immunoassays (FIA) and chemimiminescent immunoassays (CLIA) are also automated. The instrument performs all stages of testing (adding reagents, washing, detecting and calculating) automatically. In addition, some commercial immunoassay systems are fully automated and include the Specimen ID barcode. For food microbial antigens, the VIDAS (bio Merilux), Oris (Tesla) and EIAFoss (Foss Electric) systems are examples of automated immunoassays [16].

2.4. PCR samples
Recently, the PCR method - polymerase chain reaction (Polimerase Chain Reaction) was developed and it had a revolutionary significance in the field of molecular biology, among which was molecular diagnostics. Many of the molecular biology techniques prior to PCR were expensive, time consuming and required a high level of technical expertise. Moreover, the work with trace amounts of DNA made it difficult to use it as the main method, for example, hybridization of NAs.

2.5. Fundamentals of the PCR analysis
The purpose of PCR analysis is to obtain a relatively large number of special types of DNA (piece) from very small quantities in vitro. The technical analysis is carried out by monitoring the enzymatic application of a DNA seeding molecule containing the specific DNA sequence of interest. The primer (support) can be any DNA format, such as genome DNA, plasmid or bacteriophage DNA and other substances. In theory, only one copy of the backing DNA is needed to generate millions of copies of new DNA molecules. The ability to amplify an exact DNA sequence for further analysis or manipulation is the real strength of PCR analysis. Some of the main reasons why PCR is such a powerful tool is its simplicity and specificity. All that is needed for this reaction are relatively inexpensive reagents. The specificity of the PCR assay stems from its means to target and copy one specific segment of DNA (or gene) from the entire genome or isolated DNA.

PCR reactions consist of repeated cycles of the following three stages: denaturation of the primer DNA, annealing of the oligonucleotide (primers) and synthesis of complementary DNA strands.

The main stages of PCR analysis are explained below:

1. Denaturation of DNA - separation of two strands of primer DNA by thermal denaturation - usually about 95 °C.
2. Attachment of primers - annealing of primers to the substrate chain is carried out by cooling the system to 45-65 °C.
3. Extension. DNA polymerase extends primers - this usually goes through around 72°C. The primers are propagated along the target sequence using heat-resistant DNA polymerase. As a result, two new complementary DNA strands are synthesized.
By repeating the three-step process many times, an almost exponential increase in the number of final target DNAs is achieved. The products of each PCR cycle are complementary to the primers and capable of binding the primers, and therefore the amount of DNA exponentially doubles in each successful cycle. 20 cycles could theoretically form 1 million copies of the target sequence (2^{20}-10^6), but for practical purposes 30-40 cycles are usually used, forming at least 10^6 copies, increasing the concentration of the target DNA. The power of PCR is shown by the fact that if each cycle lasts about 5 minutes, 2^{12} copies can be obtained per hour.

The required reagents for PCR are:

1. Buffer solution containing Mg^{2+}.
2. DNA-substrate (primer).
3. 2 primers that should to amplify the DNA fragment.
4. Deoxyribonucleotides (dNTP, like dATP, dTTP, dCTP).
5. Thermostable DNA polymerase.

DNA polymerase used in PCR must be thermostable and thermosetting, i.e. should not be inactivated by repeated heat treatment of PCR in stage 1 (90-93 °C), and it should also be active when the primer extension (70 °C). DNA polymerase from thermophilic archaea satisfies these requirements. Cloning and subsequent modification of the gene of this polymerase leads to a reduction in cost and a much-targeted variant of the enzyme.

Subsequent sample preparation includes mixing in one microtube DNA substrate, oligonucleotide primers, thermostable polymerase, 4 deoxynucleotide triphosphates and a buffer solution. The tube is placed in the thermal cycler. A thermal cycler consists of a block that contains samples and can be rapidly heated and cooled over the entire temperature range. The heating and cooling rate of this thermal unit is referred to (determined) as “temperature cycling” or “thermal cycle”.

A simpler and more general method for the determination of PCR products is based on the observation of DNA groups of the expected size in an ethyl bromide-containing agar gel. For diagnostic purposes, another more sophisticated endpoint detection method has been developed, which can detect PCR products not only after gel separation, but also in solution. The most important detection systems are:

1. Agarose or polyacrylamide or (PAGE).
2. Labeled cofluorescent PCR products and laser-induced fluorescence detection.
3. Capture on the plate and sandwich assay of hybridization.

Not only DNA, but also RNA can be the target of the sequence for amplification. However, it must be converted to the original (initial) DNA. Enzymatic reverse transcription (RT) method is used to synthesize copies of complementary DNA strands (cDNA), which can then serve as a substrate for a standard PCR method. RT-PCR is useful for the detection of RNA viruses, and gene expression can be studied by this method through the detection of transcripts (copies) of t-RNA.

3. Conclusion
When considering food safety aspects, it is important to have an approach to the various potential hazards, and even more important to have knowledge of the factors that turns risks into hazards. Lowering limits for hazardous agent detection, increasing environmental exposure and product liability are just a few of the many factors that are forcing companies to deepen their sometimes-extreme food safety controls. Therefore, it is essential that decisions can be made on a qualitative basis and with an announcement of reasons, as can be seen from the materials of this article. Therefore, ideally, a food safety expert should have knowledge of not only possible hazards, but also how to analyze them, and should be familiar with the relevant sections, such as quality assurance systems,
food production systems, modeling, risk analysis. In this regard, ELISA and PSR methods, as shown in this article, are quite modern and demanded by experts in quality and food safety.

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