ENZYME LINKED IMMUNOSORBENT ASSAY SYSTEMS AND FOOD ANALYSIS

Assoc. Prof. Dr Nihat TELLİ

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Enzyme Linked Immunosorbent Assay Systems and Food Analysis
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PREFACE

Quality and safe food production is of great importance for the protection of human health. The quality and safety of food depends primarily on the realization of hygienic conditions in the preparation, production, storage and distribution of food. In addition to establishing and maintaining hygienic conditions in food production, it is also very important to routinely evaluate the hygienic quality of the samples along the production line and the hygienic quality of the final product. Food with poor hygienic quality may contain biological, chemical and physical hazards that may pose a danger to human health. In order to prevent or minimize these hazards, it is necessary to determine the risks simply, quickly, accurately, reproducibly and economically.

Recent methods, such as electrical impedance and polymerase chain reaction, enable analyses at various stages from food production to consumption in less time and with reduced labor. Immunological methods, which are extensively utilized today, hold significant importance in food analysis due to their ease of application, rapidity, and practicality. The basic principle of immunological methods used to determine the safety and quality of foods is to reveal antigen antibody interactions. The methods employed for this purpose include enzyme linked immunosorbent assay (ELISA),

radioimmunoassay (RIA), affinity chromatography (AC), immunofluorescence (IF), precipitation and agglutination techniques.

This book explores the enzyme-linked immunosorbent assay (ELISA) and its diverse applications in food technology and analysis. By explaining the principles and practical uses of ELISA, it aims to enhance the understanding and implementation of this tool in ensuring food safety and quality. I believe this book will serve as a valuable resource for interested readers. I extend my gratitude to everyone who contributed to this work, whose efforts have made this publication possible.

May 2024

Assoc. Prof. Dr Nihat TELLİ

1. A BRIEF HISTORY OF ELISA

In 1987, during a period when the understanding of the nature and structure of living organisms, the German physician and scientist Paul Ehrlich proposed a theory aimed at explaining how cells interact with their external environment. He proposed that cells have specific side chains that bind to nutrients in order to sustain their vital activity. Subsequently, this theory was characterized as the theory of side chains, which is fundamental to the explanation of the interaction between antibodies and antigens. Ehrlich stated that antibodies produced by white blood cells act as side chains on the cell membrane. It was stated that side chains could bind to foreign bodies through specific chemical structures they have in the parts defined as binders. He characterized these chemical structures as receptors.

The binding between receptors and infectious agents was thought to be in a specific and organized harmony as in the key and lock example. However, it was also hypothesized that cellular structures exposed to microorganisms grow extra side chains to capture toxin elements. These additional side chains, designed to be transferred into the bloodstream, were identified as antibodies. According to the theory, there are numerous side chains on the surface of white blood cells that can form chemical bonds with different antigens. The idea was that for any antigen, there was at least one side chain with a definite binding side that could stimulate the cell to produce and release

more of the same type of antibody into the blood stream. These biomolecular entities were essentially antibodies, which Ehrlich first emphasized as "*magic bullets*" that targeted a particular type of toxin or pathogen without damaging the structural cells. Ehrlich, known as the *man with the magic bullet*, explained that the specificity of the receptor is defined before the antigen is encountered, so it is the antigen that selects the appropriate receptor. This is how the model of an antibody molecule, a branched structure consisting of multiple binding sites to capture foreign agents, was proposed. This model was similar to the lock and key theory first proposed by Emil Fischer for enzymes (Hosseini et al. 2018).

Enzyme Linked Immunosorbent Assay (ELISA) applications are the first systems in which the enzyme labelling technique is used for the first time in order to show antigen antibody reactions, which were introduced in the 1960s while searching for an alternative to Radio Immunoassay (RIA) methods. Compared to classical methods, they allow fast, economical and effective measurements with very high sensitivity in terms of confidence intervals and detection limits. Among the methods used for the detection of microbial metabolites (e.g. mycotoxins); gas liquid chromatography (GLC), high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) are very expensive methods that require specialized laboratories. In addition, cultural methods require

long periods of time, causing time-related disruptions in the production-consumption chain. ELISA systems are now widely used for economical and practical examination of foods with routine ELISA methods.

2. BASIC PRINCIPLE OF IMMUNOLOGICAL METHODS

Since the discovery of immune response events, serological tests that reveal specific antibodies against the causative agent of infectious diseases have been used for diagnostic purposes. These tests, which were only available in a few types until recently, have been further developed and are now widely used. Moreover, they are not limited to the search for specific antibodies, but are also used in the search for antigens. In addition to serum analyses, these tests can also be performed on body fluids or secretions. For this reason, the term serological test is insufficient and the term immunological test is more appropriate.

Immunological methods are used in the food industry, particularly in quality control within the meat industry, for the detection of toxic glycoalkaloids (e.g., solanine) in potatoes, and for the analysis of various substances such as toxins, hormones, drugs, pesticides, and antibiotic residues in different foods. In addition, it has been widely used in the detection of pathogenic microorganisms and microbial toxins in foods (Crowther 1995, Turantaş and Ünlütürk 1999, Alhabbab 2018, Hosseini et al. 2018).

Today, the basic principle of immunological methods used to investigate the safety and quality of foods is based on antigen antibody interactions (Candlish 1991, Alhabbab 2018).

2.1. Antibody

Antibodies are glycoprotein molecules formed by cells of the immune system, which have the ability to specifically recognize, bind and specifically combine antigens (Crowther 1995, Bonwick and Smith 2004, Alhabbab 2018, Hosseini et al. 2018).

Antibodies, also known as immunoglobulins, are glycoprotein molecules produced by the immune system in response to foreign organic substances that do not belong to the organism. These molecules are responsible for protecting the organism to which the immune system belongs from the possible damaging effects of foreign agents. Different types of immunoglobulins such as IgG, IgM, IgA, IgD and IgE can be mentioned (Table 1) (Crowther 1995, Hnasko 2015, Hosseini et al. 2018, Wikipedia 2024).

Table 1. Characteristics of immunoglobulin classes

Class	Serum (mg/mL)	Distribution	Function
IgA	1-4	Intravascular and secretions	Protect mucus membranes
IgD	0–0.4	Lymphocyte surface	Unknown
IgE	10–400 ng/mL	Basophils and mast cells in secretions	Protect against parasites
IgG	5–12	Intra- and extravascular	Secondary response
IgM	0.5–2	Intravascular	Primary response

As shown in Fig. 1, the antibody molecule has different binding sites and different chain-shaped structures in terms of molecular density (Candlish 1991, Crowther 1995, Alhabbab 2018).

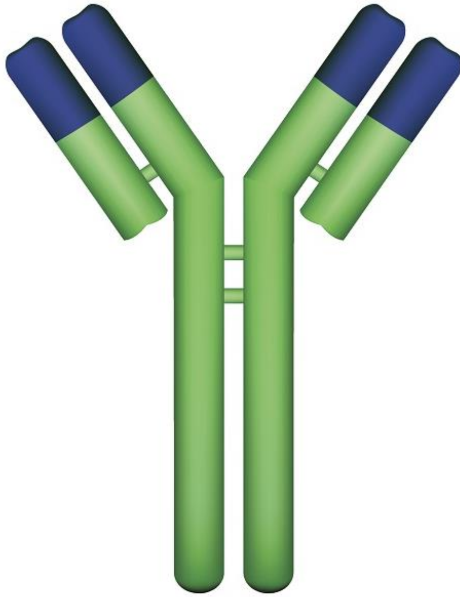

Figure 1. Structure of the antibody molecule

2.2. Antigen

Antigens are molecular structures that can specifically combine with antibodies (Fig. 2), which have the ability to recognize them specifically, leading to an immune system response (Candlish 1991, Crowther 1995, Hnasko 2015, Hosseini et al. 2018). Antigens, or in other words, immunogens, are molecules of foreign character which, when introduced into the organism, are recognized by the immune system and cause

antibody production. They typically exhibit a structure composed of proteins and polysaccharides. The most clinically important antigenic formations are found in the structure of bacteria. An antigen molecule consists of two basic parts, a protein substance and a hapten. The phenomenon of immunity or disease resistance can be defined as the ability to neutralize antigens included in the organism with antibodies (Crowther 1995, Hnasko 2015, Alhabbab 2018, Wikipedia 2024).

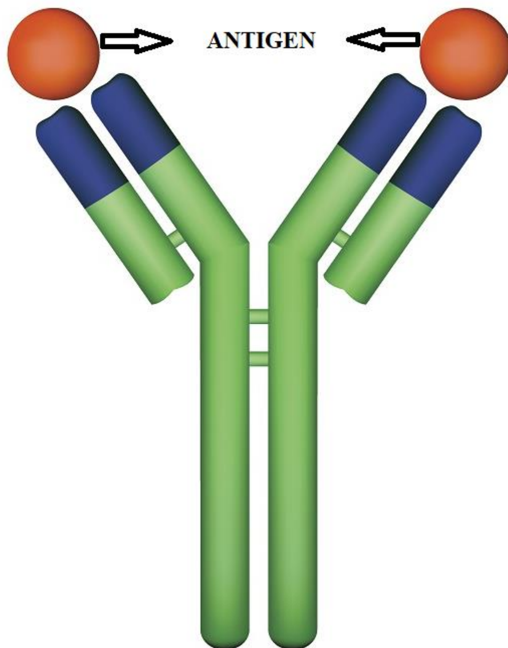

Figure 2. Structure of the antigen molecule

2.3. Immune Response and Antibody Formation

The immune system is composed of B and T cells, which are normally in a resting state but can respond to specific antigens when required. If an antigen is recognized or presented by the system, the lymphocytes proliferate clonally for a direct immune response. For an immune response to occur, antigens must interact with B and T lymphocytes. T cells can recognize small polypeptides of 8-20 amino acids bound to specific MHC (Major Histocompatibility Complex) molecules. Some antigens can cause B cell proliferation and initiate antibody formation without the help of the T cell (non-T cell-dependent). The first stage of the T cell-dependent antibody response is the activation of T cells by presenting the antigen processed by mononuclear phagocytes and dendritic cells to T cells. The presentation of antigen leads to the release of mediators (cytokines). This leads to maturation of T lymphocytes through interleukins. T cells form cellular immunity by separating in the direction of T helper I with IL-12 stimulation or humoral immunity by helping antibody production by separating in the direction of T helper II with IL-4 stimulation (Crowther 1995, Eroğlu 2001, Alhabbab 2018).

2.4. Features of Antigen Antibody Combination

The interaction between an antigen and an antibody is highly specific. This specificity makes it possible to investigate, detect and recognize the other by using it as a reagent when one of the two is available. Antigen molecules cause an antibody response. Antibodies bind to a hydrophilic region on the surface of antigens, known as an epitope, which typically consists of a specific sequence of amino acids. The epitope is the structure that provides the specificity of the antigen. The region of the antibodies that binds to the antigen is referred to as the paratope (Hnasko 2015, Alhabbab 2018, Hosseini et al. 2018). Epitope and paratope parts are shown in Fig. 3.

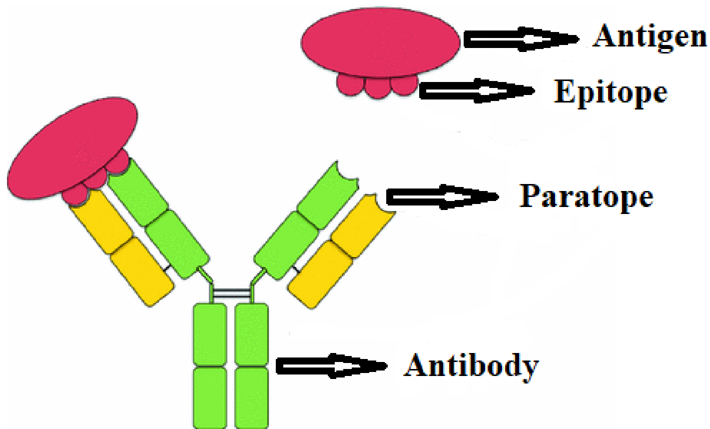

Figure 3. Epitope and paratope parts of antibody and antigen molecules

Antigen-antibody association is a chemical reaction. The interactions involved in this reaction include;

- *Electrostatic forces:* This is the combination of oppositely charged determinant groups in the amino acids of antibodies, which are attracted to each other by electrostatic attraction.

- *Hydrogen bonds:* These are irreversible bonds between hydrophilic groups that occur when antigen and antibody molecules approach each other.

- *Hydrophobic bonding:* These interactions involve the bonds formed by the hydrophobic effect, which occurs due to the repulsion of water molecules between two proteins that have glycine, alanine, leucine, and isoleucine residues on their surfaces. These bonds play the most important role in antigen antibody association.

- *Binding by Vander Walls forces:* These forces are related to the electron clouds surrounding the antigen and antibody molecule. It is realized when the distance between antigen and antibody decreases to 1 - 2 A°. Due to their strength, these bonds are difficult to reverse. (Crowther 1995, Hnasko 2015, Alhabbab 2018, Hosseini et al. 2018).

The antigen-antibody association reaction is a reversible reaction as shown in Fig. 4.

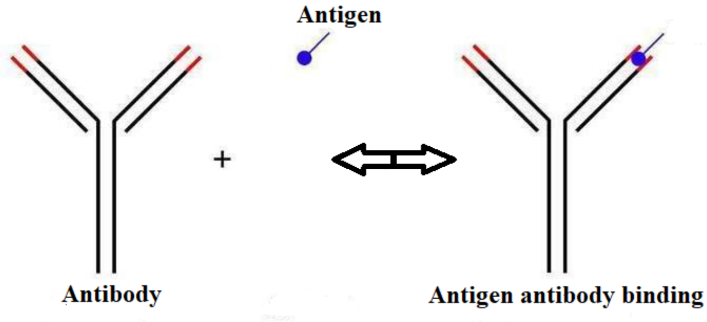

Figure 4. Antigen and antibody association

Antigen and antibody combine in appropriate proportions. The combination of antigen and antibody in appropriate proportions is called the equivalent zone (optimal zone). If there is an excess of antibody, this situation is called prezone and if there is an excess of antigen, it is called postzone event (Alhabbab 2018). Prezone, optimal zone and postzone events are shown in Fig. 5.

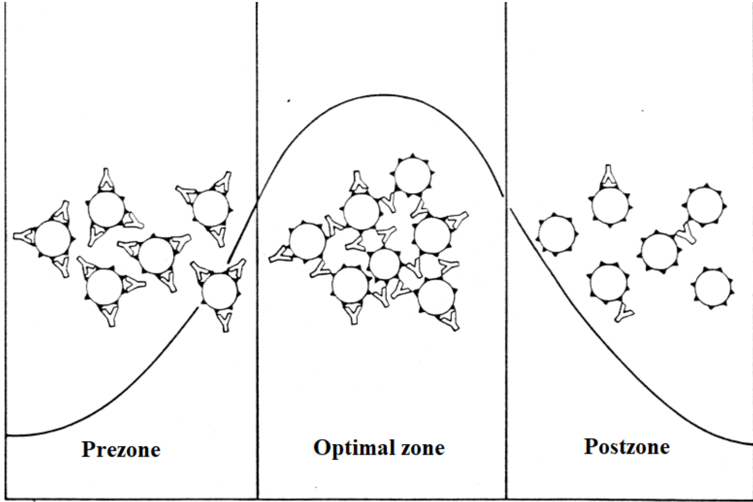

Figure 5. Optimal zone, prezone, postzone of antigen and antibody combination

Antigen-antibody association is a two-stage reaction. The first stage is the combination of specific antigen and antibody in a very short time. As a result of this reaction, 5 - 7 kcal energy is released. In the initial stage, the presence of electrolytes in the environment is not obligatory, and this phase is not perceptible to the naked eye. While the reaction in the second stage proceeds more slowly, a very small amount of heat energy is released. The presence of electrolytes in the environment is necessary and this phase can be seen visually. The finalization of this phase can take hours or even days (Alhabbab 2018).

2.5. Antigens and Antibodies Used in Immunological Methods

Antigens and antibodies are required to have certain properties in order to be used in developed immunological methods. These are;

- They can be produced in sufficient quantities for practical and routine use,

- Being in a structure that does not deteriorate easily,

-They are reproducible and reusable (Candlish 1991, Crowther 1995).

3. CLASSIFICATION OF IMMUNOLOGICAL METHODS

Immunological methods can be divided into two main classes: primary reactions (such as radioimmunoassay, enzyme immunoassay, immunofluorescence) that involve the direct detection of antigen-antibody reactions, and secondary reactions (such as precipitation, agglutination, complement fixation test, virus neutralization test) that involve the indirect detection of antigen-antibody reactions (Bonwick and Smith 2004).

3.1. Secondary Immunological Methods

Secondary immunological methods are based on the principle that the binding between antigen and antibody becomes visible with the formation of lattice (matrix). In other words, antigen-antibody binding takes place first. In the second stage, spontaneously visible complexes are formed. This is called lattice formation. In this second stage, antibodies may precipitate soluble antigens (precipitation), agglutinate particulate antigens (agglutination) or cause complement to lyse indicator cells (hemolysis).

The primary advantages of secondary immunological methods lie in their ease of application and cost-effectiveness, while their principal drawbacks pertain to their limited sensitivity (Diker 1998).

3.1.1. Precipitation Method

The process involving the reaction of antigens dissolved in water with their specific antibodies in an electrolyte medium, initially resulting in turbidity and subsequently in the formation of fine granular precipitate, is referred to as precipitation (Fig. 6). On this basis, various methods have been developed and precipitation is widely utilized, particularly in microbiology (Bilgehan 1992, Alhabbab 2018).

The precipitation method is based on the binding and precipitation of non-particulate soluble antigens by specific antibodies. The antigens used in the precipitation test are obtained by chemical or physical lysis of microorganisms and cells. Tests based on precipitation can be applied in liquid and semi-solid media and the positive reaction is seen as a cloud-shaped precipitation (Diker 1998, Alhabbab 2018).

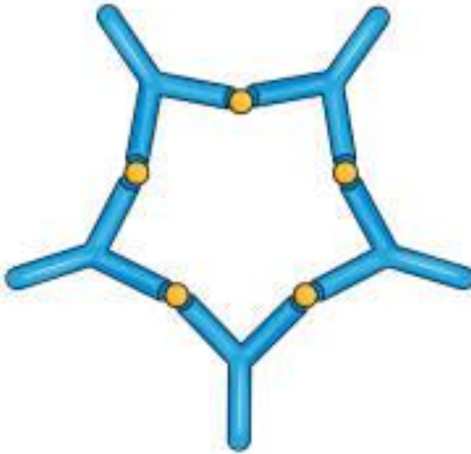

Figure 6. Precipitation of antigen and antibody complex

3.1.2. Agglutination Method

Antigen-carrying cells, including bacteria, erythrocytes, and leukocytes, as well as synthetic particles such as latex, bentonite, and polystyrene, are artificially coated with antigens. When suspended in an electrolyte medium and juxtaposed with sera containing antibodies against their respective antigens, these entities adhere to one another, culminating in the precipitation of particles visible to the naked eye. This

phenomenon is called agglutination (Fig. 7) (Bilgehan 1992, Alhabbab 2018).

Agglutination is used to investigate antibodies against microorganism antigens or to diagnose diseases by using erythrocytes and synthetic particles coated with known antibodies or to diagnose diseases by investigating antibodies formed against some antigens of erythrocytes and to investigate blood groups (Bilgehan 1992, Alhabbab 2018).

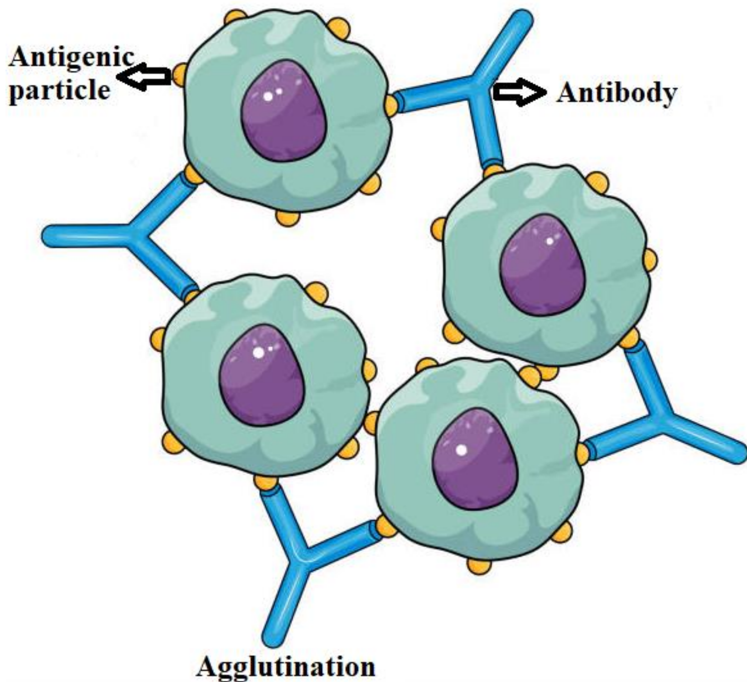

Figure 7. Agglutination of antigen and antibody complex

3.1.3. Complement Fixation Method

The core principle of the complement fixation test is that complement bound to the antigen-antibody complex lyses red blood cells. Before conducting the test, it is imperative to titrate the antigen, antibody, and complement (Diker 1998, Alhabbab 2018).

The complement system consists of 20 or more plasma proteins that interact with each other and with cell membranes. For the reaction to proceed, each protein constituent must interact sequentially under suitable conditions. Antigen-antibody mixtures are also among the activators and complement fixation test can be used to recognize one antigen or antibody when the other is unknown (Dündar et al. 1999, Alhabbab 2018).

The reaction consists of two steps. In the first step, antigen and antibody, one known and the other unknown (e.g. using a known antigen to determine whether antibodies to that antigen are present in the sample serum), are mixed together and a known amount of complement (usually guinea pig complement) is added. If the antigen and antibody are compatible, they combine and fix the complement. In the second step, a marker system of sensitized red blood cells (e.g. red blood cells + anti-red blood cell antibody) is added to the medium. If the antibody matches the antigen in the first step, complement has been

detected and there is little or no free complement left to bind to the sensitized red blood cells. As a result, hemolysis of the red blood cells does not occur, indicating a positive test result due to the presence of antibodies to the antigen in the serum sample. Conversely, if there is no match between the antibody and antigen in the initial step, the complement remains unbound and subsequently binds to and lyses susceptible red blood cells, resulting in a negative test outcome (Fig. 8) (Dündar et al. 1999, Alhabbab 2018).

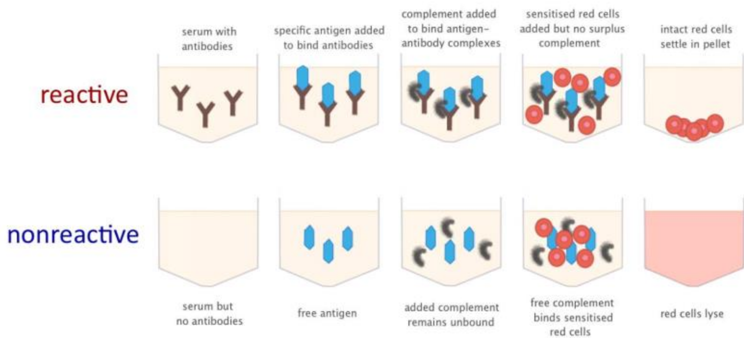

Figure 8. Complement fixation reaction

Complement must be carefully standardized and sample serum must be inactivated by heating at 56˚C for 30 minutes to avoid any human complement activity. The antigen must be quantified. The test result is reported as the highest serum

dilution giving a positive result. For the reliability of the results, controls are required to show whether the antigen or antibody alone induces complement fixation. If either the antigen or antibody alone is found to fix complement, it is indicative of anticomplementary activity. In this case, it is preferable not to use it for testing (Dündar et al. 1999, Alhabbab 2018).

3.1.4. Virus Neutralization Method

These tests are mostly used to differentiate viruses or to measure serum antibody levels against them (Diker 1998). The virus neutralization test reveals antigen-antibody interactions and immunological properties and is increasingly used for the detection of virus infections (Buxton and Fraser 1977). Basically, antigen and antibody are first compared *in vitro* and then the mixture is administered to the test animal or tissue culture. If the antigen is bound by the antibody at the *in vitro* stage, it cannot exert its effect in the test animal or tissue culture. If binding has not occurred, antigen-induced effects are observed in the animal organism or cytopathy occurs in tissue culture. In these tests, the presence of suspected antigen can be detected if known antibody is used, and the presence of suspected antibody can be detected if known antigen is used. In addition, antigen and/or antibody level can be determined by diluting the antigen and/or serum utilized in the test (Diker 1998).

3.2. Primary Immunological Methods

Primary immunological methods are based on the binding of antigen and antibody. The reaction is not directly visible. Therefore, in order to evaluate the results, the reactions must be rendered visible or detectable. For this purpose, the antigen or antibody introduced into the test is labelled with a tool such as a radioisotope, fluorescent dye or enzyme that will make the reaction visible. After the test is completed, the presence or absence of antibody-antigen association is assessed by detecting this marking. According to these marking differences, primary immunological methods are divided into three main groups: radio immunoassay, enzyme immunoassay and immunofluorescence techniques. A common feature of these tests is that the reactions primarily occur on solid surfaces, with unbound antigens or antibodies subsequently removed through washing. The most important advantages of these tests are that they are highly sensitive and give results in a shorter time compared to secondary immunological methods. The most important disadvantages are that they are more expensive than secondary immunological methods and often require complicated equipment (Diker 1998).

Primary immunological methods have some different properties arising from the markers used. This situation is important for the usefulness and preference of these methods.

Table 2 shows the comparison of the markers used in primary immunological methods (Turantaş and Ünlütürk 1999).

Table 2. Comparison of markers used in immunological methods

Feature	Radioisotopes	Enzymes	Fluorescent molecules
Sensitivity	Good	Good	Low
Interaction with substances in the sample	None	Possible	Possible
Stability	Low	Good	Good
Marker cost	High	Low	Low
Equipment cost	High	Low	Low
Health risk	High	Low	Low

3.2.1. Radio Immunoassay (RIA)

Radioimmunoassay (RIA) was first introduced in 1960 by Solomon Berson and Rosalyn Yalow for the measurement of endogenous plasma. Rosalyn Yalow was awarded the Nobel Prize in Physiology Medicine in 1977 for her groundbreaking contributions to the development of immuno-radio-metric assays for peptide hormones. Immuno-radiometry was developed by Miles and Hales in 1968 and labeling of

antibodies with radioactive substances instead of antigens was applied to measure insulin in human plasma. Radio immunoassay includes techniques using antigens or antibodies labelled with radioisotopes. In cases where antigens are labelled with radioisotopes, the labelled immune complexes formed by antigens interacting with specific antibodies allow radioactive measurements. Radioisotopes such as C^{14} and I^{125} are used in RIA techniques. In practice, RIA techniques are mostly used to search for hormones, enzymes, drugs and other biological molecules in body fluids (Jay 1992, Diker 1998, Alhabbab 2018, Hosseini et al. 2018).

In the search for antibodies, plastic tubes or dimpled trays coated with appropriate antigen should be available. Serum suspected of containing antibodies is placed in these containers. During the incubation period, if the serum contains the appropriate antibody, it will adhere to the plastic surface. The excess unbound antibody is removed through washing. Following this step, a radioisotope labelled antibody is added. At the end of the incubation period, the unbound portions are removed by washing again. If the test is positive, i.e. if antibody matching the specific antigen is found, the plastic surface acquires radioactivity. The result is obtained by detecting the acquired radioactivity using a detector (Fig. 9) (Bilgehan 1992, Alhabbab 2018, Hosseini et al. 2018).

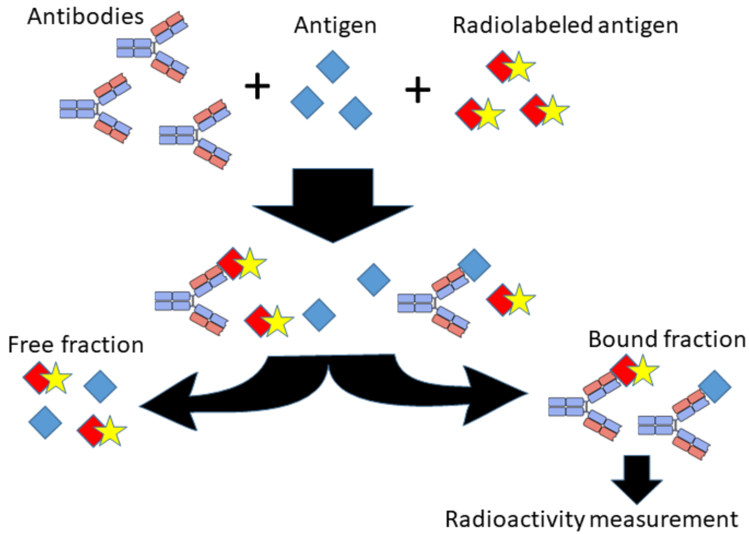

Figure 9. Radio immunoassay reaction

Suitable antibody-coated plastic surfaces are used for antigen detection. The liquid suspected to contain the antigen is placed on this surface. If the sample does contain antigen it will bind to the antibody on the surface. Following a washing step to remove any unbound material, a known antibody labeled with a radioisotope is introduced. After the known antibody labelled with radioisotope adheres to the surface and washed, the radioactivity is measured with the detector (Bilgehan 1992, Alhabbab 2018, Hosseini et al. 2018).

3.2.2. Immunofluorescence (IF)

Fluorescent dyes such as fluorescein isothiocyanate, rhodamine B isothiocyanate and lissamine rhodamine B can bind to antibodies by special methods. This binding does not affect the antibody's affinity for its specific antigen. Antibodies fluorescentized in this way become visible by fluorescence in fluorescence microscopy when they combine with their antigens in preparations on slides. Immunofluorescence techniques are based on this principle. The material to be examined can be cultures, tissue sections, disease materials containing bacteria, fungi, parasites and viruses or their antigens. The most important disadvantage of IF techniques is the need for fluorescence microscopy to see the reactions. This technique can be applied by direct or indirect methods (Bilgehan 1992, Alhabbab 2018).

The direct IF test is used to detect antigen in tissues or fluids. The tissue section or liquid is fixed on a slide. Fluorescein isothiocyanate labelled antibodies specific for the antigen sought are added to the slide. After washing, the slide is examined under a fluorescence microscope. Areas with green fluorescence indicate the presence and localization of the antigen. This technique has an important place in the direct diagnosis of pathogens that are difficult to isolate or found in small numbers (Fig. 10) (Bilgehan 1992, Alhabbab 2018).

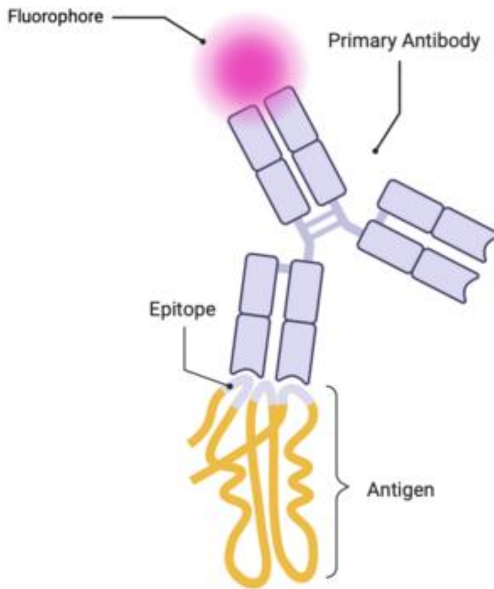

Figure 10. Immunofluorescence reaction

The indirect IF test can be used to detect antibodies or antigens. To detect antibodies, the sample serum is added to known antigen fixed on a slide. Fluorescein isothiocyanate labelled anti Ig is then applied. If the antibody is present in the sample serum, a green fluorescence will be visible under the fluorescence microscope. In order to detect antigens, antiserum or anti Ig specific to the target antigen is added to the slide-fixed sample material (Bilgehan 1992, Diker 1998, Alhabbab 2018).

3.2.3. Enzyme Immunoassay (EIA)

All techniques that use enzymes to demonstrate antigen-antibody reactions are generally called enzyme immunoassays (EIA). These tests based on enzyme-labelled immune reagents have a very important place in microbiology diagnostic laboratories. RIA techniques have been used earlier than EIA techniques. However, the use of short-lived isotopes such as I^{125} for labelling and the effects of radioactive substances harmful to public health and the environment have limited the use of RIA techniques in endocrinology laboratories. In the field of microbiology, IF techniques have found more widespread use than RIA techniques. However, the need for well-trained personnel and the subjective interpretation of the results prevent the widespread use of these techniques. The advantages over the other two techniques are that the reagents used in EIA techniques are long-lasting, have no hazards related to waste materials, and are simple and automated tests. However, they also have advantages such as the ability to work with a large number of samples and the short time of analyses (Crowther 1995, Yalınay Çırak 1998, Alhabbab 2018, Hosseini et al. 2018).

It is possible to classify the methods characterized as enzyme immunotest (Fig. 11) into two classes as homogeneous and heterogeneous. In homogeneous EIA method, free radicals, fluorescent dyes and enzymes can be used to mark antigens.

The test system includes three factors: antibody, labelled antigen and sample antigen. The homogeneous EIA reaction is a competitive reaction in which the sample antigen and the labelled antigen compete for binding with the specific antibody present in limited amounts in the medium. The enzyme used for labelling in the system is covalently bound to the antigen. When this complex binds to the antibody, the steric structure of the enzyme changes and loses its activity. As the amount of sample antigen in the medium increases, less and less labelled antigen will bind to the antibody. As a natural consequence of this, the signal given by the labelled antigen antibody complex will decrease. In this method, measuring enzyme activity, does not require separating the labelled antigen antibody complex; in other words, phase separation is not required. Although this method is easier and more practical than the heterogeneous EIA method, which requires phase separation, it is less sensitive and carries a higher risk of unwanted reactions with other substances in the environment. Homogeneous EIA methods are used in clinical studies to detect some substances with low molecular weight such as drugs and hormones (Bilgehan 1992, Crowther 1995, Turantaş and Ünlütürk 1999, Alhabbab 2018, Hosseini et al. 2018).

In heterogeneous EIA method, unlike homogeneous EIA method, there is no change in the enzyme activity used in labelling as a result of antigen and antibody reaction. Therefore,

in this method, it is necessary to separate free and bound antigens and antibodies from each other, in other words, phase separation is required. As a matter of fact, in the heterogeneous EIA method, the enzyme activity can be measured in both fractions after the enzyme-labelled antigen or antibody is separated from the enzyme-bound antigen-antibody complex (Crowther 1995, Turantaş and Ünlütürk 1999, Alhabbab 2018, Hosseini et al. 2018).

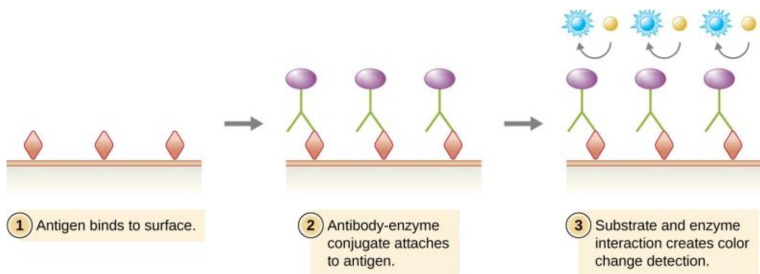

Figure 11. Enzyme immunoassay reaction

4. ELISA (Enzyme Linked Immunosorbent Assay) SYSTEMS

ELISA systems are a type of heterogeneous enzyme immunoassay (EIA) method. They were developed in the 1960s as an alternative to radioimmunoassay (RIA) techniques (Yalınay Çırak 1998, Alhabbab 2018). ELISA is a simple, sensitive, rapid, reliable and versatile analysis system for the measurement of antigens and antibodies (Moatsou and Anifantakis 2003). However, unlike RIA methods, ELISA systems, which is an immunological method, use enzymes instead of radioisotopes to mark either antigen or antibody (Jay 1992, Hosseini et al. 2018).

The basic principle of the method is to covalently bind an enzyme to a known antigen or antibody, then react the enzyme bound material with the sample and then measure the enzyme activity by adding a specific substrate to the enzyme (Dündar et al. 1999, Alhabbab 2018). ELISA systems are methods used to culture antibodies or antigens that can bind to a target protein enabling the detection of this protein both quantitatively and qualitatively. The main advantage of this approach is that antibodies or antigens can be produced specifically in response to the target protein, facilitating its recognition and quantification (Reid et al. 2006).

The advantages of the ELISA test can be generally discussed under the following headings.

1. *Simplicity*

-The reagents are added to the test in relatively small volumes.

-Removal of reagents is accomplished by simple washing operations.

-Passive adsorption of proteins is easy.

-The required equipment is easy to obtain.

2. *Reading*

-Colored end products can be read visually when evaluating test results.

-Statistical evaluation of the results is possible through multi-channel spectrophotometers.

3. *Rapidity*

-Results can be obtained within a few hours.

-The spectrophotometer stage is fast.

4. *Sensitivity*

-Detection levels of 0.01 to 1 μg/mL, considered sufficient for diagnosis, can be easily and consistently achieved in many testing procedures.

5. *Reagents*

-The commercial availability of the reagents provides usefulness in ELISA design and application of specific tests.

6. *Adaptability*

-Through the design of different configurations, it becomes possible to examine different methods and solve problems.

7. *Cost*

-Initial requirement and reagent costs are low.

8. *Acceptability*

-ELISA methods, standardized in laboratories, are considered the gold standard in terms of numerous testing procedures.

9. *Safety*

-Compared to the RIA method, waste disposal is not a problem.

-There are safe reagents that do not have mutagenic properties.

10. *Availability*

It can be utilized in a wide range of settings, including laboratories that lack the most advanced technology.

11. *Kits*

-ELISA kits are common, convenient and effective.

12. *Standardization*

-It allows statistics and standardization thanks to its feature of quantifying data (Crowther 2001).

Although ELISA systems are not very complex techniques, there are many variables to be controlled. Solid phase, washing processes, selection and efficiency of the enzymes and substrates used, management of ELISA device sections, termination time of reactions are the main variables to be controlled (Yalınay Çırak 1998, Hosseini et al. 2018).

4.1. Enzyme

Enzymes are protein-structured, colloidal biocatalysts that are complex in nature and widely used due to their high catalytic power. They react much faster than chemical catalysts. Enzymes can be simply aggregated proteins or they can have a complex protein structure containing more than one subunit. They regulate the rate and specificity of thousands of reactions occurring within the cell. Remarkably, enzymes retain their activity even outside the cell environment. Characterized by their large and intricate structures, enzymes serve as catalysts, accelerating chemical reactions and facilitating the conversion of one molecule into another. A very small quantity of enzyme

can effectively catalyze a reaction without undergoing any change of it’s structure. There is a special enzyme for each reaction. All of the enzymes are in protein structure or contain a protein component. Enzymes are named by adding the suffix "-ase" to the end of the names of the molecules they dissolve and break down, or according to the type of reaction they catalyze. For example, the enzyme that hydrolyses cellulose is called cellulase and the enzyme that hydrolyses xylan is called xylase (Karademir et al. 2002).

Enzymes used in almost every field of industry are generally obtained from microorganisms. This is because microorganism-derived enzymes have advantages over plant- or animal-derived enzymes, such as having higher catalytic activity, not producing undesirable by-products, being more stable and cheaper, and being available in large quantities. To date, more than 2000 enzymes have been identified and about 100 of them have been found to be suitable for commercial use. However, only 18 of them are produced for industrial purposes today (Zeman and McCrea 1985).

Proteases constitute 59% of commercially used enzymes, carbohydrases for 28%, lipases for 3% and other enzymes for 10%. The production of α-amylase, which belongs to the group of carbohydrase’s, holds significant importance, comprising 13% of enzyme production (Wiseman 1987). Due to the gradual development of enzyme technology, the diversity of the usage

areas of the products and the high economic value of the products, various research in the field of biotechnology related to industrial enzymes are becoming more important. Especially in recent years, enzyme production by utilizing recombinant DNA technology, which is considered as a strategic field, has reached large dimensions and its use has become increasingly widespread (Gessesse 1998).

4.1.1. Factors Affecting Enzymes

It is generally acknowledged that a larger surface area of the reactants typically leads to a faster reaction rate. As the particle size decreases, the total surface area and reaction rate increases, resulting more reactant molecules to participate in the reaction. Biological reactions typically occur in solution-like environments, where reaction rates are directly influenced by the concentration of the reactants. Therefore, reaction rates will be directly proportional to the concentration of the reactant. It is possible to list the factors affecting the functioning of enzymes as temperature, pH, enzyme density, substrate density, substrate surface, water, activator substance and inhibitor substance (Punekar 2018).

4.1.1.1. Temperature

It is generally accepted that higher the ambient temperatures correspond to increased reaction rates. Typically, for every 10 degree Celcius rise in temperature, the reaction rate

approximately doubles. Enzymes are notably affected by temperature since they are in protein structure (Fig. 12). Enzymes exhibit optimally activity between 30-36˚C. As temperatures decrease towards 0˚C, the enzyme activity slows down and stop working at 0˚C and below, yet resumes once temperatures rise above 0˚C, as their structures remain intact. At high temperatures, their structures deteriorate and they can no longer work even if the temperature is at normal values (Ersoy et al. 1979, Punekar 2018).

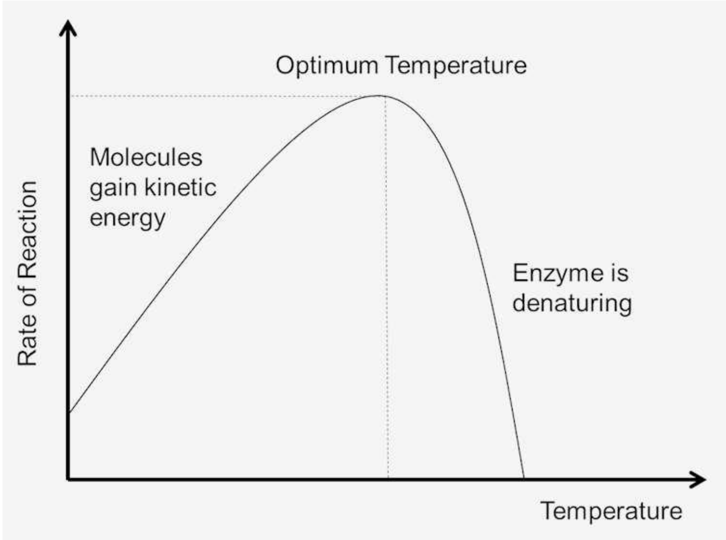

Figure 12. Enzyme-temperature reaction rate graph

4.1.1.2. pH

Enzyme activity is directly affected by pH. Enzyme-catalyzed reactions involve ionizable groups on the enzyme and/or substrate. This feature, which is almost always observed, also increases the importance of proton transfers. Therefore, it is important to maintain a well-defined pH (H+ concentration) in an enzyme-responsible reaction. As shown in Fig.13, the pH at which each enzyme can work optimally is different. The pH value at which the enzyme can work optimally is affected by temperature and substrate concentration (Punekar 2018).

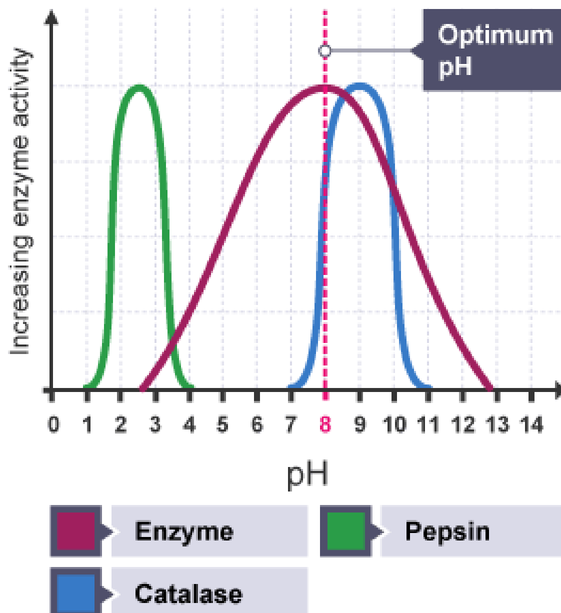

Figure 13. Enzyme-pH reaction rate graph

Table 3 shows the sources and optimum pH values of some sample enzymes (Ersoy et al. 1979).

Table 3. Enzymes and optimum pH values

Enzymes	Sources	Substrate	Optimum pH
Pepsin	Stomach	Proteins	2.0
Phosphatase	*Aspergillus*	Glycero-phosphoric acid	3.0
Amylase	Malt	Starch	4.5
Saccharase	Maya	Sucrose	5.0
Lipase	Melon tree	Olive oil	6.0
Catalase	Blood	Hydrogen peroxide	7.0
Amylase	Pancreas	Starch	7.0
Trypsin	Pancreas	Proteins	9.5

4.1.1.3. Enzyme density

In the presence of sample substrate in the medium, the reaction rate escalates with increasing enzyme concentration.

4.1.1.4. Substrate density

The reaction rate formed by the enzyme is related to the substrate concentration. When a certain amount of enzyme is

present in the medium, the reaction rate first increases as the amount of substrate increases and then continues at a constant rate. The reason for this tendency of the reaction rate can be explained as saturation of the enzyme with substrate (Ersoy et al. 1979).

4.1.1.5. Substrate surface

Enzymes act on the substrate from their outer surface. As the surface increases, the reaction rate increases. Fragmenting the substrate into smaller pieces enhances enzyme activity by increasing the total surface area available for interaction. Consequently, enzymes exhibit faster effects on small, fragmented molecules.

4.1.1.6. Water

Double distilled or deionized water are always used in dilutions and preparation of solutions for enzyme assays. Almost all of the assay components are easily soluble in water. Appropriate controls need to be taken to check whether water as an assay component affects the enzyme activity or the assay method. Apart from these features in terms of the reaction element, water is also an important issue in enzyme activity. The water content of the environment in which the enzymes exhibit their activity must be within certain limits. If the amount of water is below 15%, the enzymes stop working. Considering the moisture content levels of foods, it can be inferred that technological processes applied to some foods limit or eliminate enzyme activities. In particular, preservation practices aimed at reducing the water activity values of foods restrict both

microorganism and food-derived enzyme activities. As a matter of fact, this feature plays a significant role in the preservation of dry foods for extended periods (Ersoy et al. 1979, Punekar 2018).

4.1.1.7. Activator substance

Activator substances are mainly consist of metal ions and the presence of trace amounts of these ions is necessary for the activity of certain enzymes. Metal ions interact with enzymes to form complexes. Although the precise mechanism of action of metal ions remains incompletely understood, it is presumed that they probably facilitate substrate fixation on the enzyme. Some ions and the enzymes they influence are given in Table 4 (Ersoy et al. 1979).

Table 4. Ions and the enzymes they affect

Ions	Enzymes Affected
Ca^{++}	Rennet and promthrombase
Fe^{++}	Xanthine-dehydrase
Mg^{++}	Decarboxylases and phosphatases
Mn^{++}	Arginase and amino-polypeptidase
Cu^{++}	Polyphenol-oxidases

4.1.1.8. Inhibitory substance

There are two types of inhibitory substance effects: irreversible inhibition, which results in the breakdown of the enzyme structure, or reversible inhibition, in which the enzyme activity can reappear when the inhibitor stops working. Some inhibitory substances and the enzymes they affect are given in Table 5 (Ersoy et al. 1979, Punekar 2018).

Table 5. Inhibitory substances and the enzymes

Inhibitory Substances	Enzymes Affected
Malonic acid	Succino-dehydrogenase
Pantoyl-taurine	Acetylase
Isoniazid	Transaminase
Ca^{++}	Adenosine-triphosphate
Cu^{++}, Ag^{+}, Hg^{++}, Pb^{++}	Urease
Cyanide	Cytochrome oxidase
Fluorides	Glycero-mutase, phosphatase

4.1.2. Enzymes in ELISA Systems

The enzymes to be used in ELISA systems should be well defined in terms of their kinetics and degree of binding with proteins. Many enzymes such as alkaline phosphatase, horseradish peroxidase, glucose oxidase and β-d-galactosidase are used in ELISA systems (Voller et al. 1978). These enzymes have the ability to convert substrates into coloured products (Yalınay Çırak 1998). Enzymes to be used in ELISA systems should have the following properties;

- Their structure should remain stable at the temperatures encountered during the test,

-They should maintain their integrity for at least 6 months without degradation.

-They must be commercially available.

-They should be capable of binding with proteins effectively.

-They need to be cost-effective.

-Their activities should be readily measurable.

-They should be available in a highly purified form.

4.2. Substrate

The substance that specifically changes its form under the action of an enzyme is defined as the substrate of the enzyme in question and the substance obtained is defined as the product.

Certain enzymes can bind more than one substrate. Substrate binding can change the three-dimensional structure of the enzyme (Ersoy et al. 1979, Punekar 2018).

4.2.1. Substrates Used in ELISA Systems

Substrates employed in ELISA systems are usually used to obtain a colored product following enzymatic degradation. An insoluble or partially soluble substrate product (e.g., 5-amino-salicylic acid or diaminobenzidine for peroxidase compounds) is needed to visually read ELISA systems. For quantitative ELISA systems, however, the substrate must be soluble. Orthophenylene-diamine is suitable for use with peroxidase compounds, although it is known for its photosensitivity and mutagenic properties. Para-nitrophenyl phosphate is an excellent substrate for alkaline phosphatase compounds. Para-nitrophenyl phosphate is a very safe substrate, available in tablet form and yielding a yellow stable product. Although fluorogenic methyl-umbelliferin substrates are highly sensitive, they are impractical for routine use (Voller et al. 1978).

4.2.2. Enzyme Substrate Interactions

Enzymes act on the substrate at their outer surface where specific regions called active sites facilitate binding. The relationship between enzyme and substrate is likened to key-lock compatibility as shown in Fig. 14.

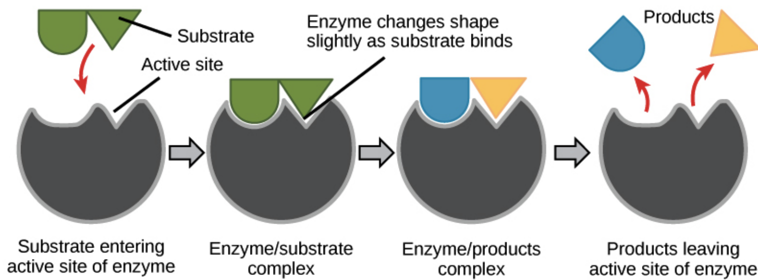

Figure 14. Enzyme-substrate relationship

4.3. Solid Phase

The procedure of any diagnostic test determines whether the method has any practical value. In recent years a number of solid phase variations of ELISA systems have been used. Substances such as cellulose, agarose and polyacrylamide have been used, which are composed of discrete particles and have the ability to bind equivalently with the antigen or antibody. The antigen is passively adsorbed onto carriers such as polyvinyl, polypropylene, polycarbonate, glass or silicone (Voller et al. 1978, Crowther 1995).

For large-scale experiments, plastics are commonly used as the solid phase, primarily in the form of disposable microtiter plates. In addition to polystyrene microtiter plates, other materials such as plastic beads, ferrous beads, and nitrocellulose membranes can also be used as solid phases. The use of these

phases simplifies the processing procedures (Yalınay Çırak 1998, Crowther 1995). Although various substances other than those mentioned above can be used, these variables can affect the test result. Therefore, each new species should be thoroughly tested before determining the amount and reproducibility of antigen or antibody retention (Voller et al. 1978, Crowther 1995).

4.3.1. Microplates

In ELISA systems, there are two different types of microplates containing 50 and 96 wells. Microplates with 96 wells shown in Fig. 15 are frequently preferred because they allow working with more samples.

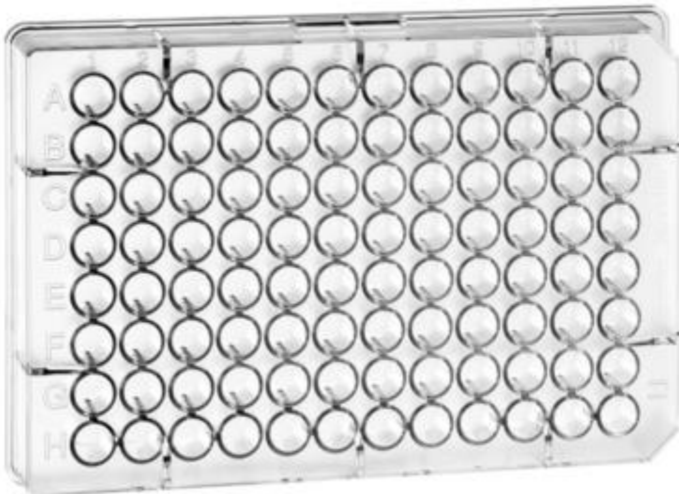

Figure 15. 96-well microplate

The total filling capacity of each well of the microplate is 300 µl. Microplate wells can be in different geometrical shapes. Fig. 16 shows different microplate shapes.

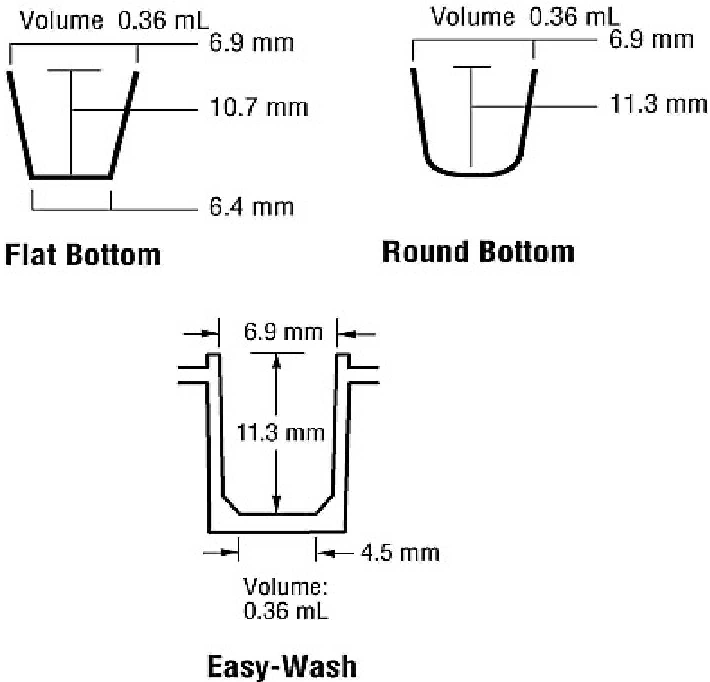

Figure 16. Microplate shapes with different geometric properties

4.3.2. Preparation of ELISA Plates

Preparation of ELISA plates involves binding an antigen-specific antibody to the solid phase if the target is an antigen. Conversely, if the target is an antibody, an antibody-specific antigen should be bound to the solid phase. The coating of the plates is carried out by washing after two hours at 37˚C or overnight at room temperature or + 4˚C (Jitsukawa et al. 1989, Yalınay Çırak 1998).

4.4. Components of the ELISA Test Device

There are basically two different parts in ELISA devices. As shown in Fig. 17, these are the parts that make up the device and provide the operation of the device. In other words, it consists of the preparation of the samples, placing them in the device and other subsequent processes and the software programs required for the interpretation of the results.

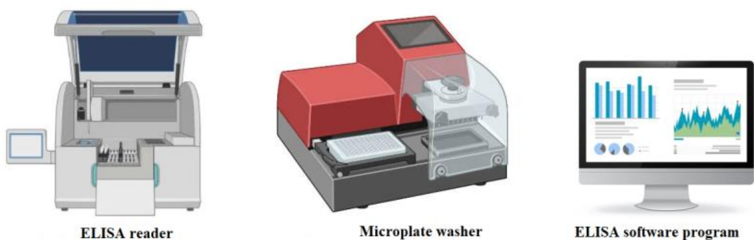

Figure 17. ELISA device systems

4.4.1. Main Device

After the samples are pipetted into microplates (Fig. 18), they are placed on the reciprocating arm of the ELISA device. The operation of the arm is controlled by optical monitors and/or mechanical sensors. In addition, a separate microprocessor controls the four driving motors (hydraulic interface, vacuum and delivery pumps, photometer interface, on-board sensors). Fig. 19 shows the main components of the ELISA device.

Figure 18. ELISA pipetting procedure

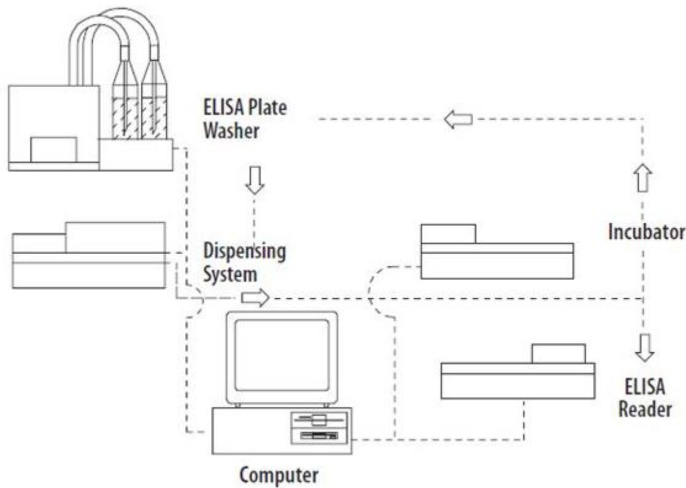

Figure 19. The components of the ELISA device

4.4.2. Incubators

In the incubation area, there are two independent control chambers that can maintain the temperature even when the device is not working. The microprocessor controlling the incubation ensures that the incubation chambers are maintained between a minimum of 5˚C and a maximum of 45˚C with an error margin of ± 1˚C.

4.4.3. Washing Rooms

There are 16 washing heads in the washing rooms. Among them, 8 heads distribute the washing solutions while the remaining 8 aspirate. Liquid levels are automatically controlled

by sensors. Variables such as liquid volume amounts (100-2000 ml), number of washing rounds (1-9), washing times (1 sec-2 min), liquid pressures (low, normal, high) can be programmed according to the requirements of the study. Fig. 20 shows the washing chamber sections of the ELISA device.

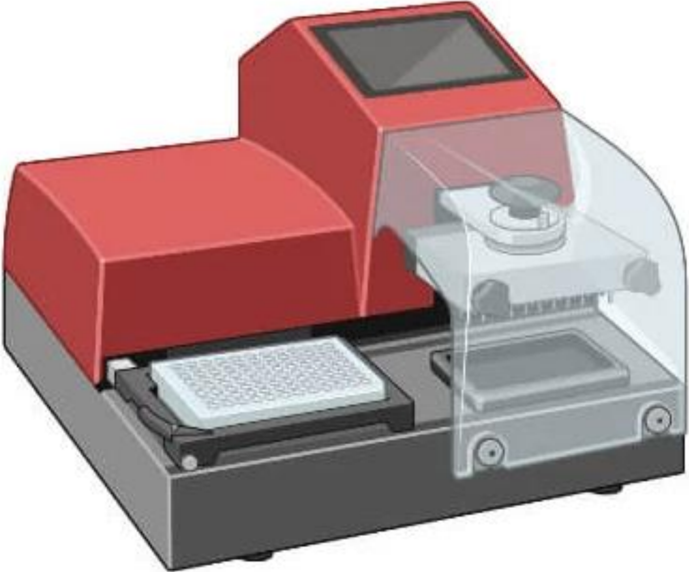

Figure 20. Washing rooms sections

4.4.4. Photometric Readers

The photometric readers (Fig. 21) read each of the micropillars individually in a 1 x 8, 2 x 8……., 12 x 8

arrangement in vertical rows in the 0-3 OD (optical density) ranges.

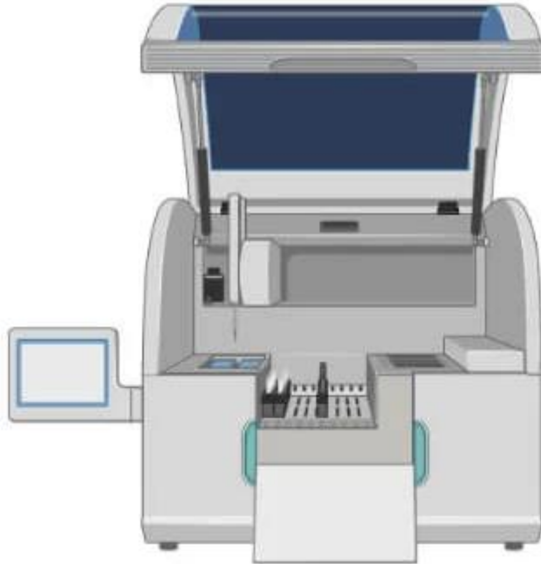

Figure 21. ELISA photometric reader

The photometric reading is performed with 16 illuminant channels and 8 mixing filters. In the photometric reader zone of the photometric readers, each absorbance value related to the sample concentration can be read directly vertically or indirectly depending on the test procedure. Microprocessors are used to control the movement of the illuminator channels, sensors, mixing filters and microplates in the reader zone.

4.5. Classification of ELISA Test Methods

ELISA systems have many different protocols. Table 6 shows the general classifications of ELISA methods, their usage areas and the required chemicals.

Table 6. Classification and protocols for ELISA methods

ELISA type	Uses	Chemicals	Comments
Indirect	Antibody search	Antigen pure or semi-pure; Antibody-containing test solution; Ig-binding enzyme conjugate in immunized samples.	The use of pre-existing specific antibodies is not required; a relatively large amount of antigen is required.
Direct competitive (competitor)	In antigen search; soluble in antigen detection	The antigen is pure or semi-pure; Test solution containing antigen; Enzyme-antibody conjugate for specific antigen.	Rapid test with only two steps; very useful for measuring antigenic cross-reaction
Antibody-sandwich method	In antigen search; soluble in antigen detection	Antibody capture (capture) (pure or semi-pure specific antibody); Test solution	The most sensitive antigen test; Requires relatively large amounts of pure or semi-pure

		containing antigen; Enzyme-antibody conjugate specific for antigen.	antibodies (antibody capture)
Double antibody-sandwich method	Antibody search	Antibody capture (capture) (specific for Ig in immunized samples); Test solution containing antigen; Enzyme-antibody conjugate specific for antigen.	Does not require purified antigen; a relatively long five-step test.
Direct cellular method	In the search for antigen-expressing cells; measurement of cellular antigen expression	Cells expressing the antigen of interest; Specific enzyme-antibody conjugate for cellular antigen.	It is a sensitive test in large amounts of scans; it is not sensitive in heterogeneous mixed cell groups.
Indirect cellular method	Searching for antibodies against cellular antigens	Cells used in immunization; Test solutions containing antibodies; Ig-binding enzyme conjugate in immunized samples.	May not detect specific antibodies for cellular antigens expressed in low amounts.

Generally, ELISA test approaches can be classified as competitive and non-competitive methods, focusing on either antigen or antibody detection.

4.5.1. Competitive ELISA Method

In the competitive ELISA method, either enzyme-bound antigens or enzyme-bound antibodies are utilized.

4.5.1.1. Competitive ELISA method using enzyme-antigen conjugate

In this method, antibody forms the solid phase. The sample antigen competes against the enzyme-antigen conjugate to bind to the antibody in the medium. Since the amount of enzyme-antigen conjugate added to the medium is known, the amount of free and bound labelled antigen in the medium can be easily determined. The amount of antigen in the sample is then calculated by using standard curves showing the concentration of the product (resulting from the enzyme-substrate reaction) against different concentrations of sample antigen (Turantaş and Ünlütürk 1999, Hosseini et al. 2018).

4.5.1.2. Competitive ELISA method using enzyme-antibody conjugate

In this method, the labelled antibody is reacted with the sample before the antigen is added to the medium. It is a method based on measuring the amount of antigen bound to

labelled (enzyme bound) antibodies and the solid phase is formed by antigen. After the antigen is bound to the solid surface, the sample antigen is inoculated into the wells with a certain concentration of enzyme-linked antibody. The binding rate of enzyme-linked antibodies to solid phase antigens depends on the amount of sample antigen in the medium. In the competitive ELISA method, as the concentration of sample antigen in the medium increases, the probability of the enzyme-linked antibody binding to solid phase antigens decreases. In essence, this method presents a competition between the sample antigen and the solid phase antigen for binding to the enzyme-bound antibodies (Fig. 22) (Turantaş and Ünlütürk 1999, Hosseini et al. 2018).

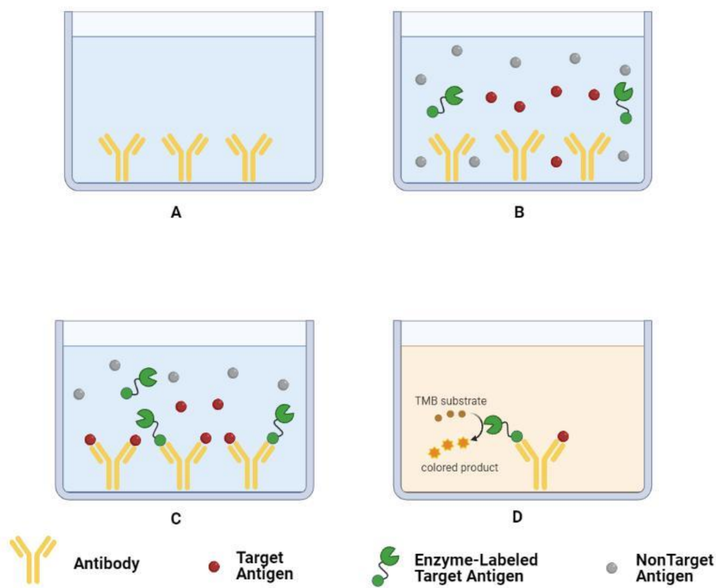

Figure 22. Competitive ELISA

4.5.2. Non-competitive ELISA Method

In non-competitive ELISA methods, the level of antigen-antibody binding is measured after the sample antigen is reacted with specific antibody. According to the valence of the antigen, it can be analyzed in two classes as single binding site non-competitive and two binding site non-competitive (sandwich or double antibody sandwich) ELISA methods. Sandwich method can only be applied to antigens with two or more binding sites (Turantaş and Ünlütürk 1999, Hosseini et al. 2018).

4.5.2.1. Single binding site non-competitive ELISA method

In this method, specific antibodies are first bound to the solid surface and the sample antigen is inoculated into the wells and left for incubation. It should be noted that the amount of solid phase antibodies should be more than the amount of test antigen. After incubation, unbound antigens are removed from the medium by washing. After washing, enzyme bound antigen is added to the medium to bind to the open areas of solid phase antibodies that are not bound to the sample antigen. In this method, the less antigen in the sample, the more enzyme-antigen conjugate will bind to solid phase antibodies. As a result, the concentration of the product formed as a result of the enzyme-substrate reaction will be inversely proportional to the antigen concentration in the sample (Turantaş and Ünlütürk 1999, Hosseini et al. 2018).

4.5.2.2. Non-competitive ELISA method with two binding sites

The antigen used in this method must have at least two binding sites for specific antibodies. The first binding site is occupied by solid phase antibodies, while the second binding site is occupied by enzyme bound antibody. Specific antibodies are bound to the solid phase and the microplate is prepared for testing. Sample antigen is then added to the medium. Unbound antigens are removed from the medium by washing. Then enzyme bound antibody is added to the medium and left for incubation. The

unbound enzyme bound antibodies are removed from the medium by washing. In this method, the concentration of the product formed as a result of enzyme-substrate reaction is directly proportional to the concentration of sample antigen in the medium (Hosseini et al. 2018).

4.6. ELISA Test Methods for Antigen and Antibody Detection

As mentioned before, ELISA test methods can be classified as antigen and antibody seeking ELISA methods. This classification is more commonly used in practice.

4.6.1. ELISA Test Methods for Antigen Detection

In ELISA test methods aimed at detecting antigens, the fundamental principle involves initially binding a known antibody to the solid phase. Subsequently, this complex is exposed to the analyzed sample, and the presence of antigen-specific antibodies is examined.

4.6.1.1. Direct ELISA (sandwich) method

The surface of the wells in the microplate is coated by the binding of the antibody specific to the antigen to be investigated to the solid phase. Washing is performed to remove unbound antibodies from the medium. The material to be investigated for antigen is added to the medium and left for incubation. If there is antigen in the material being examined, it is bound to antibodies in

the solid phase. After incubation, unbound antigens are removed from the medium by washing. Next, enzyme bound specific antibody is added. If there is antigen in the sample being analyzed and this antigen is bound to the antibody coated on the microplate, the enzyme-linked antibody binds to the antigen bound to the antibody coated on the microplate (Fig. 23).

Unbound enzyme bound antibodies are removed from the medium by washing. Upon addition of the substrate, the enzyme linked to the antibody catalyzes a reaction, resulting in color development. The results of the reaction can be evaluated by reading the optical density values in the spectrophotometer as well as by visual evaluation of the color formed. A greater color intensity as a result of the reaction indicates a higher concentration of antigen in the sample (Coligan et al. 1994, Gülmezoğlu and Ergüven 1994, Bilgehan 1995, Hnasko 2015, Alhabbab 2018, Hosseini et al. 2018).

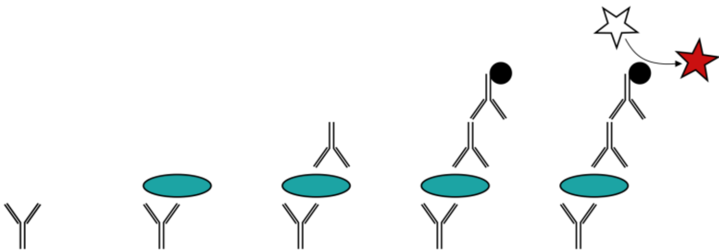

Figure 23. Binding reaction in microplate

4.6.1.2. Single-step sandwich method

Unlike the sandwich method, the direct method involves simultaneous addition of enzyme-linked antibodies with the antibody bound to the solid phase and the sample material for antigen detection. As in the sandwich method, it does not require an incubation period with the material to be tested for antigen. If antigens are present in the analyzed sample, they will bind to both the antibodies immobilized on the solid phase and the enzyme-linked antibodies.

With the washing process, enzyme-bound antibodies are removed from the medium together with antigens that cannot bind to the antibodies in the solid phase due to their non-specificity. The enzyme of the enzyme-bound antibody creates color by acting on its own substrate when substrate is added. The presence or absence of specific antigen in the examined sample can be interpreted visually as present / absent, or quantitatively by optical density evaluations in the spectrophotometer. The color intensity is expected to be higher in direct proportion to the amount of antigen in the sample (Bilgehan 1995, Coligan et al. 1994, Alhabbab 2018, Hosseini et al. 2018).

4.6.1.3. Indirect ELISA method

The material being tested for antigen and the enzyme-linked antibody are co-incubated for a while and then added to antibody-coated microplates. During this incubation, antigens and enzyme-linked antibodies form immune complexes. It's crucial to note that the enzyme-linked antibodies should originate from a different animal species than the antibodies bound to the solid phase in the microplates. If the antigen is present in the material analyzed, it will first bind to the enzyme-linked antibody during co-incubation and then to the antibody bound to the solid phase in the microplates in which they are inoculated. Once the enzyme-linked antibody is bound to the antigen, it cannot be removed by washing. The reaction caused by the addition of the appropriate substrate to the enzyme is evaluated by observing the color change. For a simple presence or absence determination, visual inspection is sufficient. However, when quantitative results are needed or the exact amount of antigen must be determined, spectrophotometric analysis is required (Bilgehan 1995, Coligan et al. 1994, Hnasko 2015, Alhabbab 2018, Hosseini et al. 2018).

4.6.1.4. Competitive ELISA method

The material to be searched for antigen is added to the wells in the antibody-coated microplates. After incubation, washing is performed. With the washing process, antigens that cannot bind

to the antibodies bound in the solid phase of the microplates are removed from the environment. The enzyme-bound specific antigen is inoculated into the micropillars. The main purpose of this ELISA method is to ensure that a competitive exclusion event is carried out in terms of binding points between the specific antigen known to be specific to the antibody bound to the solid phase and the antigen investigated to be present in the material to be examined. If specific antigen is present in the material, it will bind to solid phase antibodies. In this case, the solid-phase antibodies will bind to the sample antigens, preventing the enzyme-conjugated antigens from binding to the solid-phase antibodies. As a result, the enzyme-conjugated antigens will be washed away, leaving only the sample antigens bound to the solid-phase antibodies.

After the enzyme antigens are added to the medium, the enzyme-specific substrate is added following the washing process after incubation. If the sample contains the target antigen, it will bind to the solid-phase antibodies, preventing the enzyme-labeled antigens from binding. Consequently, the enzyme-labeled antigens will be washed away, and no color will develop upon adding the substrate. In interpreting the results, wells in the microplate that show no color change will be considered positive for the presence of the target antigen (Hendry 1992, Coligan et al. 1994).

4.6.2. ELISA Test Methods for Antibody Detection

The basic principle of ELISA test methods that detect antibodies is to determine if there is an antigen-specific antibody in the analyzed sample. This is achieved by binding a known antigen to the solid phase and assessing whether the sample contains antibodies that specifically recognize and bind to this antigen. (except for the sandwich inhibition method).

4.6.2.1. Indirect ELISA (sandwich) method

After the binding of the known antigen to the solid phase, the unbound antigens are removed from the medium by washing and the microplate is made ready for the test. Sample serum, in which the presence of specific antibody is investigated, is added to the antigen-coated microplate wells and incubated. After incubation, the microplates are washed and antibodies that cannot bind to solid phase antigens are removed from the medium.

Enzyme-linked antibodies are added to show the complex formed by antibodies binding to solid phase antigens. Enzyme-linked antibodies must be specific antibodies capable of binding to the antibodies whose presence is being investigated. If the sample contains antigen-specific antibodies that bind to the solid-phase antigens, the enzyme-labeled secondary antibodies added to the medium will bind to these antigen-antibody complexes. The reaction caused by the addition of the

appropriate substrate to the enzyme is evaluated by reading the color change. The color change can be interpreted visually or quantitatively by determining the optical density values in a spectrophotometer (Bilgehan 1995, Coligan et al. 1994, Alhabbab 2018).

4.6.2.2. Sandwich inhibition method

In other tests that investigate the presence of antibodies, the antigen is bound to the solid phase. However, in the sandwich inhibition method, the antibody is coated on the solid phase. After the binding of the known antibody to the solid phase, the unbound antibodies are removed from the medium by washing and the microplate is made ready for the test. Antigens known to be specific for solid phase antibodies are mixed with sample serum and incubated for a while in a different medium than the microplate medium in which the test will be performed. The aim of this incubation process is to ensure that these antibodies block the antigens as a result of the presence of the investigated antibody in the sample serum. If the investigated antibodies are not present in the analyzed sample, the antigens will not be blocked. This incubated mixture is added to the antibody-coated wells in microplates.

If there are antibodies in the analyzed sample, antigens will not be able to bind to solid phase antibodies because they are blocked. After incubation, the complexes formed by the

blocked antigens are removed from the medium by washing. Then enzyme bound specific antibody and substrate are added to the medium respectively. Enzyme bound antibodies will bind to antigens in the presence of unblocked antigens that bind to solid phase antibodies. In this case, the presence of antibodies in the samples is interpreted in inverse proportion to the color intensity when the enzyme-specific substrate is added. If blocked antigens are present in large quantities, enzyme antibodies will not bind and no color will be produced after substrate addition (Bilgehan 1995, Coligan et al. 1994).

4.6.2.3. Competitive ELISA method

After the binding of the known antigen to the solid phase, the unbound antigens are removed from the medium by washing and the microplate is made ready for the test. Sample serum to which antibodies are searched is added to the antigen bound to the solid phase and left for incubation. After incubation, antibodies that cannot bind to solid phase antigens are removed from the medium by washing. Then, enzyme-labelled specific antibody known to be specific to solid phase antigens is added and left for incubation.

After incubation, unbound specific antibodies are removed from the test medium by washing. After washing, enzyme specific substrate is added. The main purpose of this ELISA method is to ensure that a competitive binding event between

the specific antibody known to bind to the solid-phase antigen and the antibody being investigated in the sample. This competition helps determine the presence of the specific antibody in the sample. If antibody is present in the sample serum, it will bind to the binding sites of the antigens before the enzyme-labeled antibody can bind. As a result, the enzyme-labelled antibody will not bind and will be washed away and no color will be formed when the substrate is added. In the interpretation of the reaction results; the samples in the microplate wells without color will be considered positive for the antibody sought (Baron et al. 1994, Coligan et al. 1994). Fig. 24 shows the basic reaction characteristics related to the principles of general ELISA methods that are routinely used in laboratory analyses.

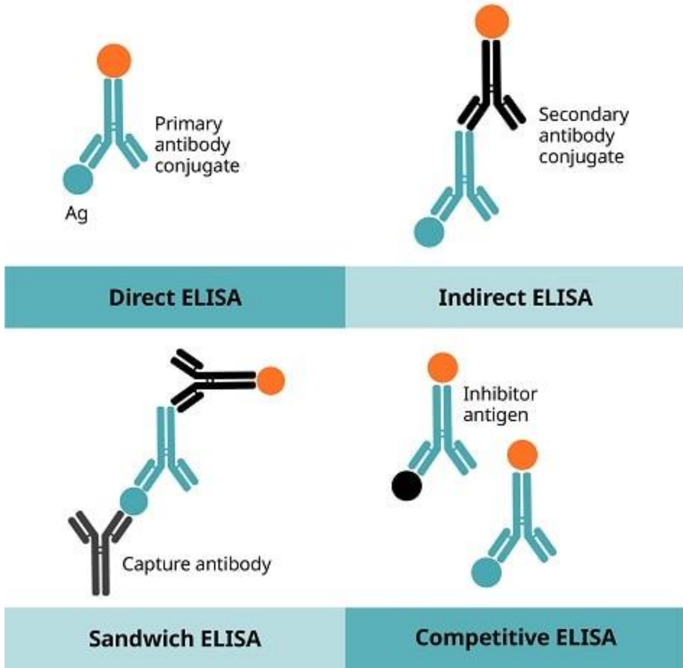

Figure 24. General ELISA methods

4.7. Evaluation of ELISA Test Results

If the desired response is yes or no at the end of the test steps, ELISA tests can be visually evaluated as shown below. Visual reading can be done by interpreting a series of dilutions of the test sample as shown in Fig. 25 (Roitt et al. 1998).

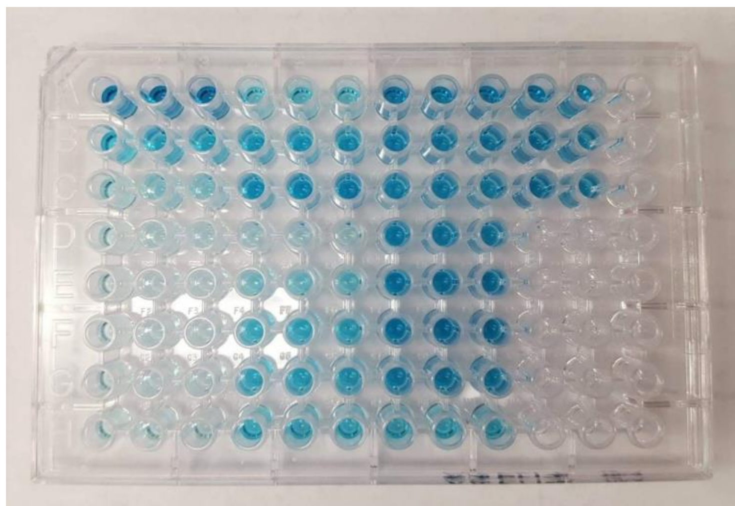

Figure 25. ELISA test visual reading results

For quantitative assessments, it's essential to measure the intensity after adding substrate/chromogen using a spectrophotometer. To date, there is no common measurement evaluation system used to quantitatively express the results, since quite different ELISA test procedures have been applied. However, in most applied methods, quantitative evaluation is based on optical density (OD) readings with a spectrophotometer (Voller et al. 1978).

Fig. 26 shows the general steps of an ELISA test procedure with a sample obtained from a patient specimen.

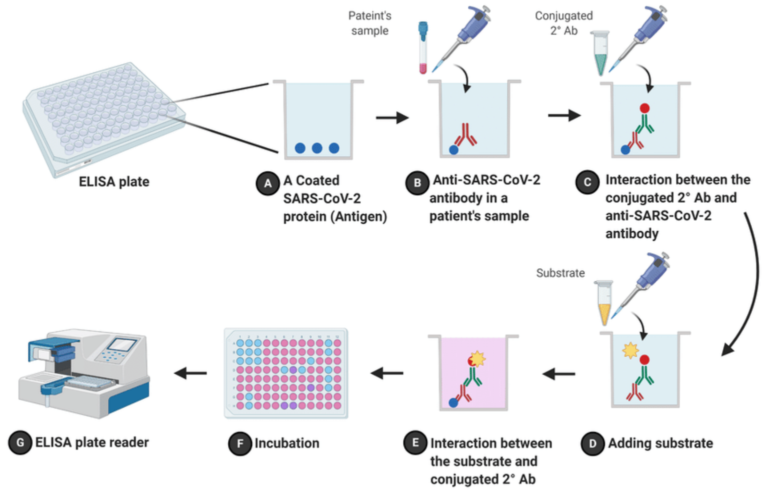

Figure 26. ELISA test reactions

5. ELISA APPLICATIONS

The ELISA technique finds widespread application across various fields including the food industry, vaccine development, immunology, diagnostics, toxicology, drug monitoring, pharmaceuticals, and transplantation.

5.1. Food Industry

The application areas of the ELISA technique in the food industry are quite diverse. It is applied as the basic standard for these analyses in food samples that are frequently analyzed for allergens such as milk, peanuts, walnuts, almonds and eggs. Monoclonal antibody-based sandwich ELISA method is applied for the detection of ovalbumin in foods, which is one of the most common causes of food allergy cases observed in predisposed individuals such as children. However, it can also be used to determine the authenticity of food products (Peng et al. 2014). In this way, it helps to protect consumer and public health as well as to prevent possible economic losses caused by fraudulent substitution (Asensio et al. 2008, Peng et al. 2014).

In meat and meat product analyses, the ELISA technique facilitates the identification of the sources of raw materials used in production by determining meat species. It has proven to be a reliable technique for the detection of adulteration in products (e.g. the use of soya and its products in meat products) and for the careful monitoring of the product when religious

considerations are involved in food selection. Additionally, it serves as a critical technique for quality control, particularly in monitoring harmful components that may be transmitted to humans, such as bovine spongiform encephalopathy (BSE). This includes various analyses of fish and fish products, milk and dairy products, as well as genetically modified and irradiated foods (Asensio et al. 2008, Peng et al. 2014).

5.2. Vaccine Development

ELISA technique is a frequently used method in vaccine development studies. It is especially used to determine the presence and quantity of antibodies against certain types of antigens transferred experimentally in serum samples taken from immunized animals and humans (Pizza et al. 2000). This technique is also utilized to determine a higher level of protective response with fewer side effects from different types of antigens selected to enhance immune reactions in hosts. The main difficulty in the application of ELISA technique in vaccine development research is the appropriate selection of positive and negative controls. It is particularly difficult to achieve high analytical precision in the experimental phase and when working with unknown samples (Miura et al. 2008). However, the unique qualities of the ELISA technique in the determination of immune response profiles have been reported (Smalley et al. 2016).

5.3. Immunology

Measuring and monitoring changes in the immune system, which protects the body through cellular or humoral responses, is the basis for understanding the pathogenesis of diseases. Various studies suggest that ELISA is a rapid and cost-effective gold standard method for such measurements and monitoring.

5.3.1. Autoimmunity

Multiple infections, environmental factors, immune system problems, uncontrolled immune system activation may be the causes of autoimmune response (Grammatikos and Tsokos 2012). The immune system produces antibodies in response to different types of pathogens. These external pathogens can be particles or epitopes that penetrate cells and then become part of the cell structure. In such cases, antibodies may react against the cells themselves, causing clinical symptoms resulting in immunodeficiency. Pulmonary alveolar proteinosis (PAP) is defined as an autoimmune disease characterized by the accumulation of surfactant in the alveolar system. Reactions related to auto-antibodies produced against granulocyte/macrophage colony-stimulating factor (GM-CSF) have been reported in the pathogenesis of PAP. GM-CSF is required under infectious conditions when the pathogen invades the respiratory system. Radiological and cytological analyses can be used to investigate and interpret cases of PAP disease. In

addition, ELISA method can be a technique used to determine the thresholds associated with PAP risk (Uchida et al. 2014).

Bullous pemphigoid, an acute or chronic skin disease, is another example of autoimmune diseases characterized by high mortality. The disease can be diagnosed with typical clinical features and histopathological examination. On the other hand, ELISA shows high sensitivity and specificity in the detection of circulating auto-antibodies against this disease-specific epitope. ELISA testing platforms are successful in providing a rapid, economical and specific diagnostic/monitoring method for bullous pemphigoid (Hsu et al. 2014).

The incidence of autoimmune diseases in individuals diagnosed with human immunodeficiency virus infection and acquired immunodeficiency syndrome (HIV/AIDS) has also been examined by various ELISA-based analytical platforms (Yen et al. 2017a). It has been reported that HIV-infected patients have a higher risk of developing Sjögren's syndrome, psoriasis, systemic lupus erythematosus, autoimmune hemolytic anemia and uveitis (Yen et al. 2017b). ELISA testing has revealed that low IgG antibody responses to pathogens may be the main disorder in such diseases. However, ELISA-based test platforms have also been produced for diagnostic purposes, especially for the detection of HIV (Terato et al. 2014).

5.3.2. Humoral Immunity

ELISA methods offer important clinical contributions in the study of the humoral response of the immune system to various pathogens with different characteristics. Humoral immune response is characterized by the presence of substances such as antibodies and other components in body fluids. Monitoring and measuring these components is of great importance for the interpretation and prognosis of diseases.

Although leprosy is a curable infection, it is still known to be observed especially in developing countries. ELISA technique is applied to interpret the course of the disease in infected individuals. In particular, paper-based ELISA platforms and lateral flow test strips have been very useful in clinical practice for the detection of this disease in endemic areas where access to centralized laboratories is limited (Bobosha et al. 2014).

ELISA has also been used to detect plasmatic antibodies against antigenic peptides of human endogenous retrovirus (HERV) in the etiology of multiple sclerosis. Monitoring HERV-related immune biomarkers is of great importance for analyzing disease progression, especially during interferon beta therapy (Mameli et al. 2015).

5.4. Diagnosis

In the field of diagnostics, ELISA has proven to be a useful platform applied worldwide to detect various types of diseases in humans and animals. It is known that various commercial ELISA kits are available for the detection of HIV, influenza, Dengue, Ebola, Chagas disease, Leishmaniasis, Lyme, West Nile virus and many other diseases. The ELISA technique is also widely utilized for diagnostic purposes in plant pathology, providing a reliable and sensitive method for detecting plant pathogens and diseases.

5.4.1. Pregnancy test

A number of different biomolecules are synthesized during pregnancy, including human chorionic gonadotropin (hCG), luteinizing hormone (LH), follicle stimulating hormone (FSH), estriol (E3) and thyrotropin stimulating hormone (TSH) (Wingeier et al. 2017). Using ELISA, some of these protein-based molecules can be detected from blood, saliva or urine sources in the early stages of pregnancy. Similarly, pregnancy tests are also used for animals (Karen et al. 2015).

In addition to the mentioned purposes, the ELISA technique can also be used as a reliable method for the diagnosis of congenital infections such as HIV or toxoplasmosis during pregnancy. In order to increase the sensitivity and accuracy of detection of complications in the first trimester of pregnancy,

marker panels capable of monitoring/measuring multiple markers in samples have been developed.

Examples of target biomolecules include; activin A, inhibin A, progesterone, A disintegrin and metalloprotease-12 (ADAM-12), pregnancy-associated plasma protein A (PAPP-A), pregnancy-specific B1-glycoprotein (SP1), placental-like growth factor (P-LGF), vascular endothelial growth factor (VEGF), glycodelin (Glyc) and human chorionic gonadotropin (hCG) (Makunyane et al. 2017).

5.4.2. Cancer Detection

Early diagnosis of cancer is of great importance for the patients. However, cancer biomarkers are among the most difficult biomolecules as target analyses. By applying gold nanoparticle layers in ELISA, the detection signal provided with a lower limit of detection (LOD) has been strengthened and a significant progress has been made in diagnosis. In this technique, sandwich ELISA based on gold nanoparticle layers, using plasma spiked with carcinoembryonic antigen as a biomarker, has proven to be suitable for obtaining clinically significant results. For a similar purpose, chemically engineered poly methacrylate microspheres have been integrated into routine ELISA to detect microRNA-21. Exogenous microRNAs in blood serum were found to be inversely correlated with breast cancer incidence in humans. It is reported that CA125

glycoprotein in serum is used as a biomarker for ovarian cancer detection in very low volume samples and thus early diagnosis is possible. As observed in the scientific research findings, advances in ELISA technique are promising for the use of cancer biomarkers (Scholler et al. 2006, Chin et al. 2016).

5.4.3. Detection of Infectious Diseases

A wide variety of ELISA-based techniques have been developed for the serological detection of infectious agents and diseases for accurate diagnosis and prognosis.

The ELISA technique offers a high throughput detection capability, especially in the following three infectious disease classes.

1. Sexually transmitted diseases : A class of infectious diseases targeting adults in developing countries. A number of different ELISA platforms have been designed and commercialized for the sensitive and selective detection of sexually transmitted diseases, including HIV, hepatitis, syphilis and chlamydia.
2. Regional or endemic diseases : These diseases, which have a high incidence in tropical and subtropical regions, can exhibit clinical pictures with serious and chronic consequences. Dengue fever, chagas, borreliosis and yellow fever are examples of these diseases. Although current techniques are insufficient for the early detection

of such diseases, advances in ELISA platforms have led to promising developments in the early and effective diagnosis of these conditions.

3. TORCH diseases : These diseases refer to a group of viral pathogens (Toxoplasma, Rubella-Rubella, Cytomegalovirus-Cytomegalovirus, Herpes simplex, other infections) that can cause prenatal infections. It is known that this class of infectious diseases can be a potential danger to fetuses. Other infections, such as syphilis, hepatitis B, Epstein-Barr virus, varicella-zoster virus and HIV, also have the potential to cause serious complications for the fetus. It is known that commercially developed and available ELISA platforms are used to detect these infectious agents with high sensitivity (Hosseini et al. 2018).

5.5. Toxicology

In the science of toxicology, where the undesirable effects of chemical compounds on organisms are investigated, the effective determination of these compounds is very important. Determination and improvement of the effects of toxins, which are antigenic agents of plant or animal origin, and toxic substances released into the environment, i.e. toxicants, constitute the basic scope of toxicology. The basic materials of toxicological studies are the correlations between the effects of

dosage and exposure rates of toxic substances on organisms, routes of exposure, the origin of toxic substances and the characteristics of the affected organs. ELISA is one of the main methods used in the conduct of these investigations. For example, it has long been known that ELISA has been used for the identification of aflatoxin B1 in rice. ELISA kits developed for aflatoxin testing are preferred because they are fast and simple with high specificity and sensitivity (Kolosova et al. 2006). On the other hand, Bio-Quant ELISA method has been used as a reliable method for the possible screening of amphetamine and methamphetamine in biological fluids, especially in forensic samples (Apollonio et al. 2007).

5.6. Pharmaceutical Industry

ELISA techniques also have various applications in the screening of specific classes of drugs in plasma. Traditional therapeutic drug monitoring (TDM) strategies are based on monitoring drug levels in plasma samples (Fraaij et al. 2004). However, it also provides data on the treatment procedure, allowing the examination of whether the drugs to be used for therapeutic purposes are present in the patient organism. However, the fact that the traditional TI technique is not economical and has technical difficulties causes the need for alternative strategies (Nso et al. 2010).

ELISA-based TIA is favored as a simple and economical method to measure drug concentrations in plasma samples. For this purpose, it has been used to assess the levels of plasma lopinavir (LPV) in young perinatal HIV-positive individuals and anti-drug antibodies in patients treated for rheumatoid arthritis and inflammatory bowel disease. Providing a high specificity in immunological assays for therapeutic proteins is of great importance in the evaluation stages of pharmacokinetic, bioequivalence and toxicokinetic studies (Prinapori et al. 2014, Hock et al. 2016).

5.7. Transplantation

Cross-compatibility testing is a critical step in organ transplantation, ensuring the compatibility of donor and recipient tissues when transplantation is medically necessary. The complement-dependent cytotoxicity cross-match (CDC-CM) assay has been used since its discovery to assess the compatibility of the organ with the recipient body. The selection of recipients without donor-specific antibodies is of great importance to increase the survival rate of transplant patients. CDC-CM plays a vital role especially for transplant recipients who are being treated with therapeutic antibodies or have autoimmune diseases. However, the CDC-CM test requires lymphocyte isolation from donors, which has some limitations. ELISA-based cross-match testing has proven to be an effective alternative procedure for transplantation analyses.

For example, ELISA-based crossmatching has been reported to identify donor-specific anti-human leukocyte antibodies in frozen blood or spleen samples obtained from donors (Hosseini et al. 2018). ELISA-LATM application in kidney transplants is another area of use of ELISA-based technology before transplantation. The development of effective and rapid measurement of anti-A and anti-B antibodies in liver transplant patients is of great importance. It has been reported that the ELISA method is effective in measuring anti-A and anti-B antibodies at an early stage compared to the agglutination method and thus may contribute to the timely treatment of ABO-I-related humoral rejection (Satoh et al. 2007, Chacko et al. 2013).

6. ELISA APPLICATIONS IN FOOD ANALYSES

In the early 1970s, an important part of food analyses was the determination of food origin and drug residues. Analyses for this purpose constitute the beginning of the use of systems based on immunological methods in food analysis (Bonwick and Smith 2004). Most of the immunological method-based studies on the determination of the quality characteristics and reliability of foods are based on ELISA systems (Reid et al. 2006). At the same time, increasing consumer interest in the quality and safety of foods has led to the development of analyses applied in this field (Bonwick and Smith 2004). The routine ELISA methods are frequently used in various analyses of different foodstuffs.

In the food industry, ELISA methods are mainly used in the determination of the type of raw material used in food production, determination of the components that make up the nutritional elements, biochemical changes caused by applications such as fermentation and heat treatment in food, determination of antibiotic residue and pesticide contamination, as well as detection of contaminated pathogenic microorganisms.

6.1. ELISA and Food Analysis in the Literature

ELISA has been the most widely used and reliable method to detect the use of different types of raw materials in dairy and

meat products where counterfeiting and adulteration are intensively used. Moatsou and Anifantakis (2003) used indirect ELISA test for the determination of milk species and detected different types of milk added to the milk composition at 0.001% levels. Silva et al. (2000) analyzed hamburgers produced from different types of meat to determine the presence of low-value meat additions by dot-ELISA method. The researchers reported that different types of meat can be detected in amounts of 0.6%. Giovannacci et al. (2004) examined different meat products in terms of the detection of raw materials. The researchers reported that different types of meat present in amounts of 0.05% can be detected. Günşen et al. (2006) carried out similar studies to determine the type of raw material in meat products.

Ametani et al. (1987) and Mimmo and Pagani (1998) used the ELISA technique to determine the immunological properties of caseins in dairy products. Pizzano et al. (1997) and Senocq et al. (2002) similarly used ELISA systems in the detection of casein fragments in dairy products. Pizzano et al. (1999) used ELISA systems to determine the casein composition of heated or powdered milk. Boudjellab et al. (1994) for the determination of chymosin in cheese, Kaminogawa et al. (1989) and Jeanson et al. (1999) used ELISA to determine whey proteins. Pizzano et al. (2000) and Senocq et al. (2002) employed ELISA to assess proteolysis during the ripening of cheeses.

Instrumental methods for food analysis are gaining prominence today, with ELISA systems playing a pivotal role. These systems are highly regarded for their ease of application, cost-effectiveness, high sensitivity in terms of confidence intervals and detection limits, as well as their ability to provide rapid and efficient measurements. ELISA systems are widely used all over the world to detect antigens and antibodies and are adaptable for developing and modifying test procedures. As a matter of fact, many laboratories have continuously developed ELISA methods in the diagnosis of infectious diseases or in the field of endocrinology. However, the international standardization and validation of a large number of methods developed to meet the needs of these laboratories has not yet been fully realized. International standardization and validation of these methods is an important issue in ELISA applications today. ELISA applications, utilized for diverse purposes in food analysis since their discovery to the present day, serve as exemplary scientific studies of various qualities. They form the foundational method for research conducted over the past period and in the last decade, contributing significantly to the scientific literature.

Gilvanda et al. (1998), examined the presence of pesticide residues in foods; Kucharska and Leszczynska (2000) reported that the presence of antibiotics (e.g., chloramphenicol) in food products of animal origin; Rohner et al. (1985), penicillin and

its derivatives in milk; Sarımehmetoğlu et al. (2004) and Oruç (2003) reported that aflatoxin in various cheese and milk samples; Leduc et al. (1999), antigens and egg white antigens in meat products; Gutierrez et al. (1998), microorganisms that cause spoilage in meat products; Daly et al. (2002), *E. coli* in milk; Alexandre et al. (2001), enterohemorrhagic *E. coli* in meat products; Gümüş et al. (2003), The status of Ochratoxin A in beer fermentation and its effect on fermentation; Terzi (2005), protozoan contamination in water samples; Küplülü et al. (2002) the presence of staphylococcal enterotoxin in pasteurized milk; Aygün (2003), the presence of biogenic amines in milk and dairy products; Ekici et al. (2002), the presence of histamine in cheese; Günşen and Büyükyörük (2003), bacteriological quality of kashar cheeses; Luo et al (2006) used ELISA systems to detect the presence of staphylococcal enterotoxin C1 in water samples.

Kong et al. (2015) reported that different ELISA methods can be used to detect the presence of *Cronobacter sakazakii* in powdered baby foods. Sundararaj et al. (2019) used the ELISA method to detect the presence of S. aureus and staphylococcal enterotoxin B in milk, cake, cheese and chicken meat samples. Singh et al. (2018) used the indirect competitive ELISA technique to detect the presence of alternariol, one of the major mycotoxins produced by various *Alternaria* species, in bread and bran samples. Sajic et al. (2017) evaluated ELISA based

analysis methods for the detection of natural and processed gluten proteins in wheat, rye, barley, oats, other grains and grain products. Liu et al. (2017) used the ELISA method to detect *L. monocytogenes* in pork and pasteurized milk samples. Di Febo et al. (2019) used the ELISA technique to investigate the presence of *Salmonella enterica*, one of the important bacterial species responsible for foodborne epidemics, in dairy products, meat, pasta, flour, eggs and animal feed samples. Ma et al. (2018) used the microfluidic paper-based ELISA technique for the rapid detection of clenbuterol, a growth promoter prohibited in animals used for food production, in milk samples. Mandli et al. (2018) reported that an ELISA technique, which allows the detection of pork adulteration at the level of 0.1% in a very short time, can be used effectively for this purpose. Hendrickson et al. (2018) suggested that the ELISA method can be recommended as a universal analytical tool for the determination of zearalenone in wine samples. Du et al. (2019) stated that the ELISA technique is suitable for rapid screening of quinolones, tetracycline, lincomycin and streptomycin antibiotics in milk samples and for use in internal quality control programs in the dairy industry. Chen et al. (2021) reported that the sandwich ELISA method can be used successfully to detect egg white allergens in retail foods and will help reduce the incidence of egg allergy. Liu et al. (2022) used the monoclonal-antibody-based ELISA method to detect

forchlorfenuron, a widely used plant growth regulator, which is known to cause serious health problems in consumers with excessive exposure, in vegetables and fruits. Ren et al. (2023) suggested that the ELISA technique is a feasible and highly sensitive analytical method for the detection of ochratoxin A in corn, wheat and feed samples. Li et al. (2023) reported that the indirect competitive ELISA technique can be used practically for the analysis of the industrial colorant Red 2G, which is not allowed to be used in fruit drinks, red wine and yoghurts. Tsai et al. (2023) stated that the ELISA method is suitable for the detection of raw and heated edible mollusk species at 0.1 ppm. Buzas et al. (2023) used the ELISA technique to determine aflatoxin M1 contamination quantitatively in raw milk and drinking milk. Hu et al. (2024) used competitive ELISA to determine the IgE and cross-reactivity of allergens obtained from Sichuan peppers. Jaukovic et al. (2024) conducted an investigation into aflatoxin contamination in corn, feed mixtures, milk, and cheese samples obtained from producers, employing the ELISA technique.

7. SPECIES IDENTIFICATION IN MEAT AND MEAT PRODUCTS USING THE ELISA METHOD

Detection of different meat species adulterated in meat and meat products is important in terms of consumer protection, implementation of labelling information legislation and prevention of unfair competition. ELISA technique is one of the most widely used methods in the determination of animal species in meat and

meat products due to its high sensitivity, specificity and practicality of use alongside serological, histological, immunochemical and molecular biological techniques (Ayaz et al. 2006).

7.1. Preparation of Samples for Testing

25 g of small pieces of raw or heat-treated meat and meat product samples are transferred into sterile bags, followed by the addition of 225 ml of distilled water or physiological saline. The samples are homogenized in a blender or stomacher for 1 - 2 minutes. Then the samples are filtered through filter paper (Whatman No: 4) and subjected to heat treatment in a water bath (95 - 100 ˚C) for 15 ± 1 minutes. The filtrate is again filtered through filter paper (Whatman No: 4) and the samples are taken into tubes (Günşen et al. 2006).

7.2. Test Procedure

After the cold stored kit and reagents are brought to room temperature before use, 1 positive control and 2 negative control wells are separated from the wells on the strip for each animal species. Positive control (100 µl) is added to the positive well, while positive controls of other species (100 µl) are added to the negative wells. Sample filtrate (100 µl) is placed in the remaining wells, and the plates are kept at room temperature for 60 minutes. At the end of the time, the plates are poured and washed 3 times with diluted washing solution (Tris buffered saline). Then, 50 µl of species-specific antiserum (Anti-species Biotinylate) is pipetted

into the plate wells and the plate is kept at room temperature for 60 minutes. The plate is poured and washed 3 times again with diluted washing solution. Then, 50 µl of enzyme-labelled antibody (Avidin Peroxidase) is added to the wells, shaken gently and the plate is kept at room temperature for 30 minutes. At the end of the time, the plate is inverted and washed 5 times with washing water and dried. After the procedure, 100 µl of the Working ABTS solution obtained by dilution of the prepared substrate (ABTS Concentrate 2,2'azino-D-i-3-ethylbenzt azoline sulphonic acid) with peroxyde citrate buffer at a ratio of 1:24 is added to the wells. After 60 minutes on the shaker at room temperature, 50 µl of the stop solution (1.5% sodium fluoride) is added to all wells and the reaction is stopped. Absorbance values are measured at an average wavelength of 414 nm (405-420 nm) in an ELISA reader (ELX 800, Bio-tek Inst., USA) (Günşen et al. 2006).

7.3. Calculation and Evaluation of Results

The sum of the absorbance values of the negative controls applied in the test is divided by the number of negative controls and the result is multiplied by the F value (2.5) to obtain the cut-off value. The absorbance values of the samples are compared with the cut-off value. Samples with absorbance values equal to or higher than the cut-off value are determined as positive (Ayaz et al. 2006, Günşen et al. 2006).

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