Conducting tests on fish and seafood products by means of enzyme-immunoassay and molecular-biological methods

1. Introduction

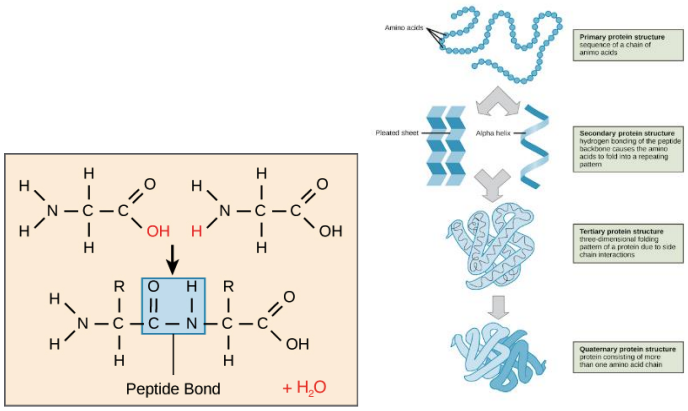
In addition to test methods based on analytical chemistry or traditional microbiological techniques, methods based on immunological reactions or on the properties of nucleic acids and their specific structures are increasingly used to identify either harmful substances or food pathogens and spoilage organisms. The advantage of both these approaches is their high specificity, rapidity and ease when compared with more conventional approaches. In the following chapter the theoretical background of these assays as well as their practical performance are summarized and outlined.

2. Important biomolecules

The complex biochemical phenomena characteristic to life are based on a huge number of different molecules that have specific functions in cells, tissues and organisms. These include carbohydrates, lipids, amino acids, and other small molecular weight compounds. However, the most important are the large macromolecules: proteins and nucleic acids.

2.1.The structure and functions of proteins

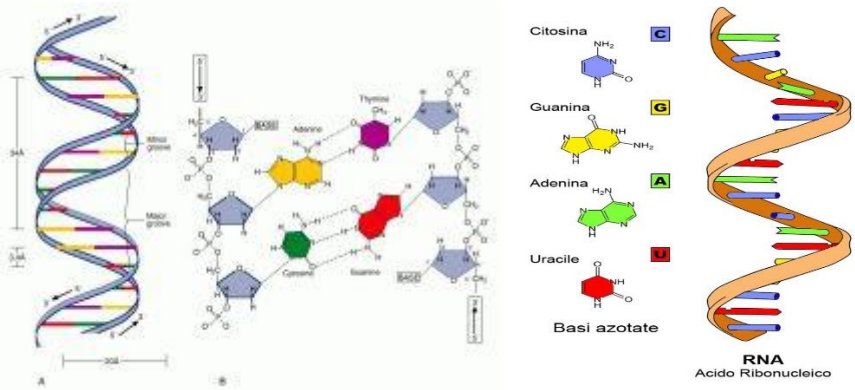
Proteins are polymers formed by amino acids. There are 20 different amino acids that occur in the living organisms. They all contain both a carboxylic group and an amino group, and the carboxylic group of one amino acid can react with the amino group of another forming the so called peptide bond. Proteins are polymers of hundreds of amino acids linked together by the peptide bonds and having a highly complicated, three dimensional structure (Figure 1) [Figure 1. Peptide bond and formation of proteins]. Proteins can serve as simple structural elements of the cell and tissue, but they generally have quite specific tasks such as acting as enzymes and hormones.



2.2. Nucleic acids

There are two types of nucleic acids: deoxyribonucleic acid or DNA and ribonucleic acid or RNA DNA is the carrier molecule for genetic information. It consists of two intertwined strands with phosphoric acid ester backbone, sugar moiety and the DNA bases (adenosine, guanine, cytosine and thymine). These four bases (abbreviated as A, G, C and T) keep the paired DNA strands together by the pairing of A with T and G with C in the opposite strands. DNA can exist as very large molecules containing millions of base pairs. Today it is possible both to elucidate the base sequences of large DNA molecules and to chemically synthetize small stretches of DNA with a defined sequence. Both the sequencing and synthesis of DNA are highly automated processes.

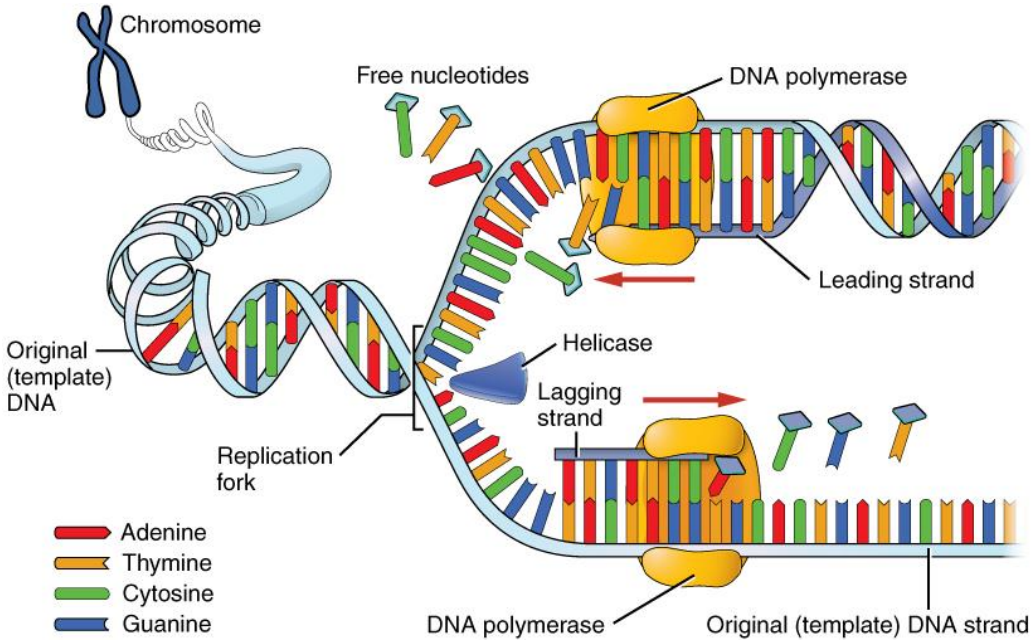
RNA is chemically very similar to DNA, differing in the sugar moiety (ribose instead of deoxyribose) and having uracil (U) instead of T as one of the bases. In addition, RNA molecules usually exist as single strands and are small in size compared to DNA (the size range from few hundreds to appr. 1500 bases) (Figure 2) [Figure 2. The structures of DNA and RNA] . There are several types of RNA with specific functions, such as messenger RNA, transfer RNA and ribosomal RNA.



The four bases of DNA form three letter “words” or triplets that contain the instructions for the synthesis of proteins. For example, the triplet TTT is “read” as the amino acid phenylalanine. In protein synthesis, the message of DNA is first copied into a messenger RNA (mRNA) molecule. This copy directs the actual synthesis of proteins in cell organs called ribosomes, where the amino acids are linked together by peptide bonds in the order specified by the sequence of triplets in the mRNA. In this process, the transfer RNAs guide the amino acids to the site of synthesis, while ribosomal RNAs (rRNA) are important structural components of the ribosomes. Thus, the information flow in the cell is from DNA to RNA to proteins, and the proteins direct the actual metabolism of the cell or organism. The section of the DNA containing information for a protein (or some other functional molecule) is called a gene.

An essential feature of DNA is that it can replicate or form copies of itself. The type of replication is called semiconservative, because for each new DNA molecule one of the old strands serves as a template for the new strand. This ensures that during the cell division, each daughter cell receives an exact copy of the parental DNA one strand of which is old and the other freshly synthetized..

The replication of DNA is mediated by specific enzymes called DNA polymerases, which direct the bases to correct positions specified by the pairing rules (Figure 3). The new bases are introduced in the form of nucleotides which consist of the actual base, the sugar moiety and three phosphoric acid moieties linked together. [Figure 3. Overview of the DNA -synthesis]



3. The immune system

Living organisms are constantly facing challenges from harmful bacteria, fungi, viruses and other environmental risk factors. Consequently, during the evolution a wide array of different defense mechanisms have been developed. In animals both the cell mediated and humoral immunity are the most important systems by which the animal is protected from infections or other harmful effects associated with pathogens or foreign proteins.

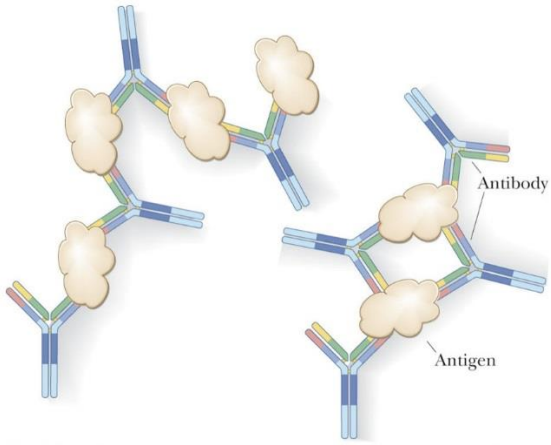
3.1. The immunological cells and their functions

In warm blooded animals, the immune system is based on different types of white blood cells or leucocytes. They are formed from the so called hematopoietic stem cells present in bone marrow and

further differentiated in a complicated process to different classes of immune cells (Chaplin, 2010). The most important of these are T- and B lymphocytes. T lymphocytes have the key role in the so called cell mediated immunity, meaning that they destroy cells infected by intracellular microbes. The B cells are responsible for the humoral immunity secreting different types of immunoglobulins or antibodies.

3.2. Antibodies

A typical B lymphocyte has on its surface receptors that recognize a protein, the so called antigen, that is foreign to the host animal, for example a protein derived from a bacterium or virus. This binding triggers the production of specific types of defense proteins called antibodies by the lymphocyte. An antibody specifically binds to the antigen that induced its synthesis marking it for further destruction by T-lymphocytes or other cells of the immune system. The ability of antibodies to quite specifically recognize and bind certain antigens makes them suitable tools for analytical purposes. [Figure 4, Reaction between the antibody and the antigen].



Antibodies to practically any protein of interest can be produced either in animals or cell cultures. In particular, the so called monoclonal antibodies have a particularly high specificity to a certain antigen, and thus are very often used in immunodiagnostics.

4. Immunodiagnostic tests

Diagnostic tests based on the antibody-antigen reaction are routinely used in medicine and in clinical settings. Pregnancy tests, which are based on specific antibodies, for example, against the human chorionic gonadotropin, a hormone produced during the pregnancy, are an everyday example

of the application of immunodiagnostics and also demonstrates the ease and speed of this methodology.

In food research immunological tests are used to detect antigens like microbial toxins and different allergens from complex matrices like blood, urine and different foods. They are all based on antibody-antigen reaction, but differ in the ways this reaction is demonstrated and quantitated.

4.1. Immunodiffusion tests

Immunodiffusion tests are perhaps the simplest types of immunological tests. Typically the antibody is mixed with a gel (such as agarose or agar). When the gel has solidified, a well is made in the gel, and the antigen sample is pipetted into the it. When the antigen diffuses into the gel, it reacts with the antibody and forms an insoluble complex, precipitin. At a certain precipitin concentration it forms a visible precipitate zone around the well, and the diameter of that zone is proportional to the antigen content of the sample (Staley, 2002). [Figure 5. Precipitates formed by the antigen-antibody complex (precipitin) around the wells containing variable amounts of the antigen]

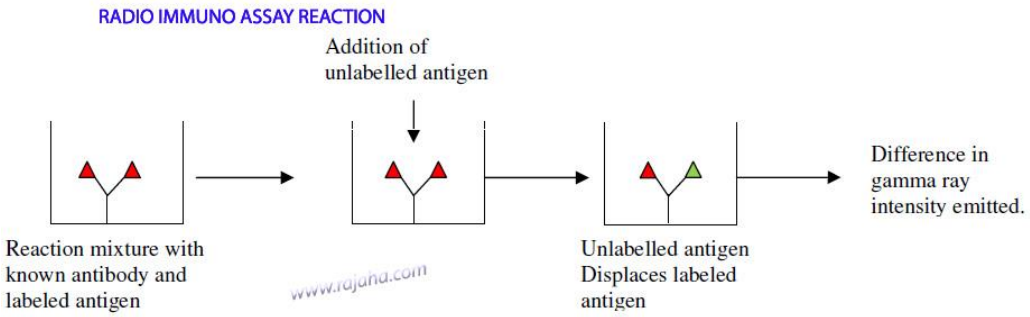
(EVDOTEC®, The **Biotechnology Education** Company)

Of this simple so called Mancini method there are also a bit more complicated variant, such as the Ouchterlony method, in which the sample can be tested simultaneously against several antibodies.

4.2. Radioimmunoassay

While the diffusion tests are still widely used, for example to demonstrate the presence of bacterial toxins in food samples, more advanced test methods have been developed. Typical modern tests include Radioimmunoassay (RIA) and Enzyme -Immuno-Assay (EIA).

For the RIA assay a known amount of radioactively labelled antigen is added into the sample, and the sample is allowed to react with antibodies immobilized in a small test tube or microtiter well. When the radioactive antigen reacts with antibodies, also the antigen-antibody complex becomes radioactive. If the sample contains natural, non-radioactive antigens, they compete with the radioactive antigen, and less radioactivity is bound (Berson, SA. and Yalov, RS: 2006). Thus, the amount of a certain antibody in the sample is inversely proportional to the bound, measurable radioactivity (Figure 6). [Figure 6. The principle of radioimmunoassay (RIA)].

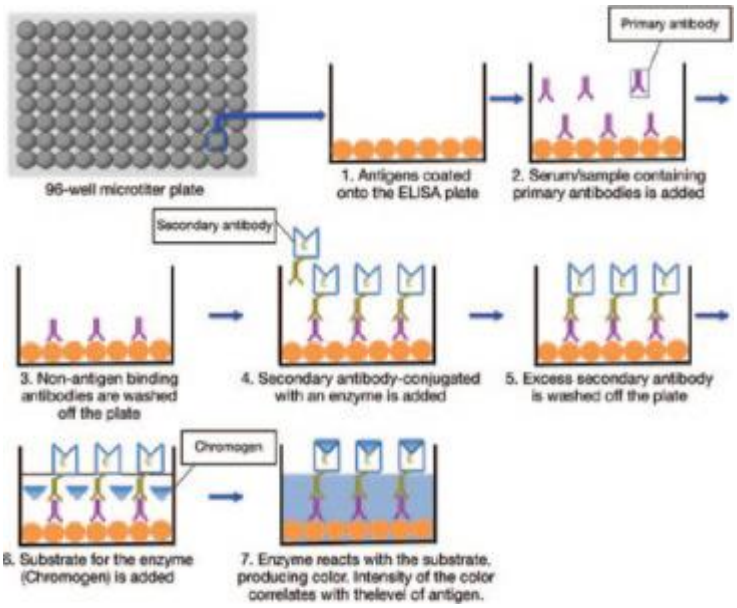


RIA is typically used, when the amount of antigen is supposed to be low. Despite of its sensitivity, the use of radioactive reagents and the need of special equipment limit is applicability. Therefore the basic the principles of RIA have been further developed to an assay method called Enzyme Immunoassay or EIA.

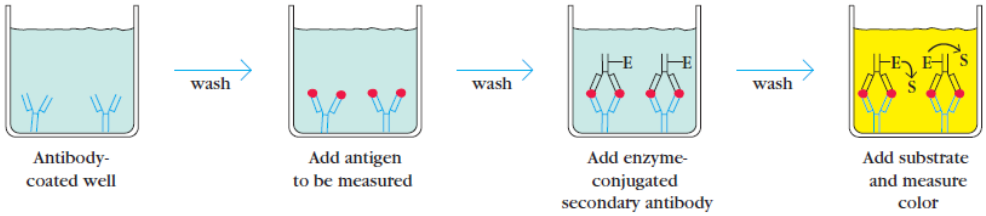
4.3. Enzyme Immunoassay (EIA)

EIA (also known as Enzyme-Linked-Immunosorbent Assay, ELISA) has rapidly become a routine immunodiagnostic method in food analysis and in clinical practice (Gahn and Patel 2013). In EIA the antigens in the sample are typically immobilized, and allowed to react with a specific antibody, which in this case is nor radiolabeled, but chemically linked to an enzyme molecule. Therefore, the reaction with the immobilized antigen results in a complex of three proteins, antigen, antibody and enzyme. Typically, enzymes like horse radish alkaline phosphatase or glucose oxidase are used, because for both of these enzymes so called chromogenic substrates are available. When a chromogenic substrate reacts with the enzyme, the reaction products have a colour the intensity of which is proportional to the amount of antigen-antibody-enzyme complex, and can be measured spectrophotometrically.

This basic EIA has been further elaborated to indirect EIA in order to increase the sensitivity. In this approach, the antigen is first allowed to react with unlabeled primary antibody. Subsequently, a secondary, enzyme-linked antibody is added. This antibody is not directed against the original antigen but against the primary antibody molecule. This results in a complex consisting of antigen -primary antibody - enzyme-linked secondary antibody. This complex can be visualized and quantified by the enzyme reaction with the chromogenic substrate (Figure 7). [Figure 7. The principle of indirect Enzyme-Linked Immunosorbent Assay].



It should be noted that EIA can also be used by immobilizing the antibody (rather than the antigen) on solid support to detect antigens in the sample. In this case two antibodies are used, both developed against the antigen to be analyzed. The first is a capture antibody, which traps the antigen. Then a second, enzyme-linked antibody is added, which reacts with the capture antibody-antigen complex. The resulting "sandwich" structure is then quantitated by the reaction with the chromogenic substrate (Figure 8). [Figure 8. Sandwich ELISA]



Competetive EIA is another modification of the assay. The basic principle is the same as in RIA; the antigen present in the sample competes with the immobilized antigen in the antigen-antibody reaction. In competitive EIA the sample is first allowed to react with an excess of enzyme-labelled antibody, and this reaction mixture is then added to a tube or well containing immobilized antigen, which then reacts with the free antibodies in the sample. The more antibody-antigen complexes there are in the sample, the less antibodies are available for this reaction. After removing the sample, the bound antigen-antibody complexes are visualized and quantitated as in indirect ELISA.

4.3.1. The practical performance of EIA

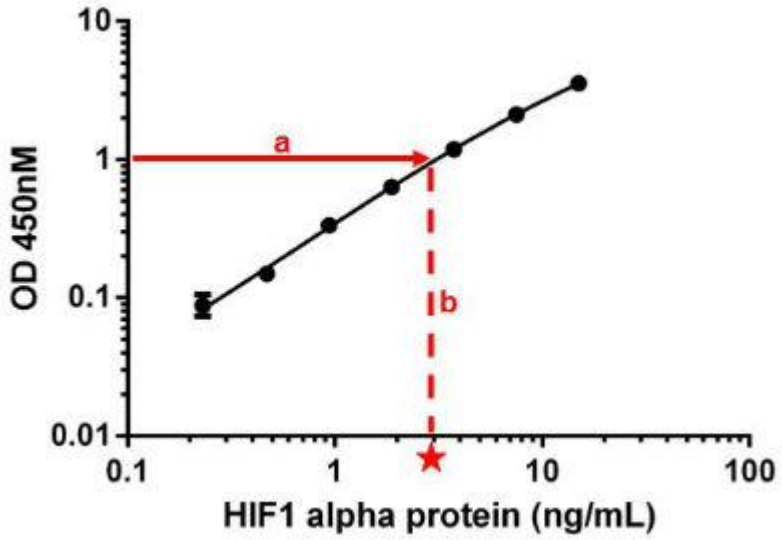
There are plenty of EIA/ELISA commercial kits designed for the detection of allergens, toxins and biogenic amines, antibiotics and other food contaminants. The assay can also be used for diagnostic purposes to identify food pathogens. The exact protocols are specific for each kit, but there are certain common steps that in any case should be taken into account.

The sample preparation is often critical for successful EIA. There are several publicly available internet protocols for sample preparation (for example http://www.abcam.com/protocols/elisa-sample-preparation-guide-1). When tissue or food samples are tested, the first step is to make a homogenate, usually in phosphate buffered saline (PBS) or Tris-buffer containing also detergents that dissolve and lyse cells and tissues. Alternatively, samples can be stored deep frozen for a later use. During the extraction the sample should be kept cold (for example in an ice bath) to prevent protein degradation. The recommended volumes of the extraction buffers may vary, but are generally in the order of 50 – 100 µl of lysis buffer per 10 mg of sample. Several methods can be used for homogenization, such as sonication, freezing and thawing and just mechanical ("pestle and mortar") disruption of the samples. A short centrifugation step to remove any insoluble debris should be performed. The expected protein contents of the extracts should be around 2000 µg/mL. If the sample is not tested immediately, it can be distributed to aliquots and stored deep frozen (at -20 or -80 °C).

The actual test protocols naturally differ, depending of the type of EIA to be applied (direct, indirect, sandwich, competitive). However, regardless of the assay type, the washings between the treatments are essential. Specific wash buffers are used to remove traces of the samples and/or antibodies between each of the steps from the antigen-antibody reaction to the final addition of the chromogenic substrates, and the washings are usually repeated several times.

The intensity of the color formed from the chromogenic substrate is measured spectrophotometrically. Because the assays typically are performed in microtiter plates, a specific microtiter plate spectrophotometer is routinely needed.

In order to quantitatively estimate the concentration of the analyzed antigen in the sample standards are needed. Typically the test kits provide standard samples with known antigen concentration, and on the basis of these a standard curve can be plotted showing the intensity of the colour formed as the function of the antigen concentration (Figure 9) [Figure 9. A typical standard curve for ELISA]



4.3.2 The applications of EIA for fish and seafood

EIA has been applied to detect fish protein in foodstuffs, in order to protect consumers with fish allergies (Chen and Chieh, 2014), and there are also commercial kits for the detection of crustacean-associated food allergens. Histamine is a strong physiologically active substance (decarboxylation product of amino acid histamine) formed as a result of microbial action in many protein-rich foods, fish included, and there are also several commercial systems for the analysis of this compound.

5. Molecular biological methods in food analysis

Molecular biological methods in food analysis are based on the reactions of nucleic acids, DNA and RNA. Because their structures are base compositions are highly characteristic to each organism, they are very useful in microbial diagnostics. In particular, because it is possible to produce large number

of copies of a certain nucleotide sequence by a technique called polymerase chain reaction (PCR), molecular biology forms an irreplaceable diagnostic tool in modern clinical and food microbiology (Law et al. 2015).

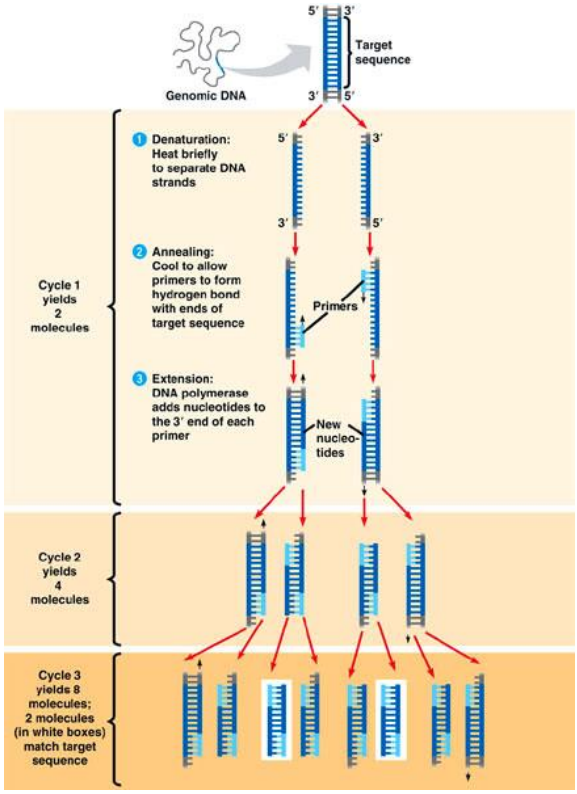
5.1.Polymerase Chain Reaction (PCR)

In PCR a certain sequence of DNA is multiplied to provide sufficient amounts of DNA for further research. There are many applications of PCR but one of the most important is to detect very small amounts of DNA in the sample (Garibyan and Avashia, 2013). In forensic studies DNA isolated from spots of, for example blood, saliva or from a single hair can be multiplied and compared with the DNA of either the victim or the suspects. In food microbiology the DNA isolated from a food sample may contain small amounts of the DNA of a pathogen, and this can be selectively detected and the presence of the pathogen thus confirmed. Alternatively, DNA isolated from an unknown bacterium or virus isolate can be compared to the known species-specific DNA or RNA sequences and the identity of the isolate can be confirmed. In order to perform PCR the nucleotide sequence of the targeted DNA should at least partially be known.

The basic PCR is based on the reversible thermal denaturation of DNA, the use of thermostable DNA polymerases and specific short DNA molecules called primers.

Thermal denaturation means the separation of the two strands of the DNA at temperatures of 97 – 99 °C, which results in two single strands. After lowering the temperature somewhat the primers can attach to the DNA-strands. Primers are short (up to 20 bases long) synthetic single stranded DNA molecules that are complementary to the target or template DNA. Primers can nowadays be synthetized by order, and the commercial PCR-kits specifically targeted against certain DNA contain the ready designed primers. Two different primers are used, one for each strand of the original DNA molecule, in such a fashion that the DNA to be multiplied is situated between them. The primers then act as a starting point for the DNA synthesis performed by the thermostable DNA polymerase. After each round of DNA synthesis, the thermal denaturation, primer attachment and synthesis is repeated, and the process is called as a cycle. At each cycle the amount of target DNA between the primers is multiplied by two. The outline of the PCR is shown in Figure 10. [Figure 10. The PCR process]

It can be seen, that the multiplication of the target DNA occurs exponentially. If in the samples there were originally N copies of the target DNA, the amount of copies after n cycles is $N \times 2^n$. For example, after 30 cycles the original amount has been multiplied by 2^{30} or 1,073,741,824 times! This demonstrates the power of the technique to detect even tiny amount of DNA in the sample.



5.1.1. A typical PCR experiment

In order to perform the PCR one need some special instrumentation. A basic requirement is a programmable PCR machine, with which one can define the parameters of the PCR run (Figure 11). The machine typically consists of a lid-covered thermobloc, into which the reaction tubes can be placed [Figure 11. A typical PCR machine]

For the actual experiment the DNA has to be isolated from the sample. There are several commercial DNA isolation kits designed for different types of samples (food, tissues, soil, feces etc), and the kits usually contain all the buffers and reagents needed for the isolation. Regardless of the sample, the steps in the isolation typically include the total or partial lysis of the cells, separation of the proteins and other components except the DNA (often using chromatography minicolumns) and the final

elution and purification of the DNA. The quantification can be done spectrophotometrically at the wavelength of 260 nm. The 260/280 absorbance ratio is also an indicator of the purity of DNA, ideally it should be around 1.8. For the quantification of very small amount of DNA there are nowadays several instruments, which can measure the DNA content from a few µl sample.

For the set up of the PCR reaction one needs to mix the reaction components, which include the suitable buffer, the nucleotide triphosphate mixtures, required amount of Mg^{2+} ions (as $MgCl_2$), the primers, the template or target DNA, and the thermoresistant DNA polymerase. The total volume of the reaction is usually up to 50 µl. For the successful reaction sterile conditions and protection of the sample/reaction from any contamination are absolutely necessary. The work should be performed in a special space designed for PCR with automated pipettes and sterile tips, and wearing laboratory gloves during the operation (Figure 12) [Figure 12. Setting up the PCR reaction mixture].

After placing the reaction tubes in the PCR machine the actual PCR cycles can be started. A typical run could be as follows:

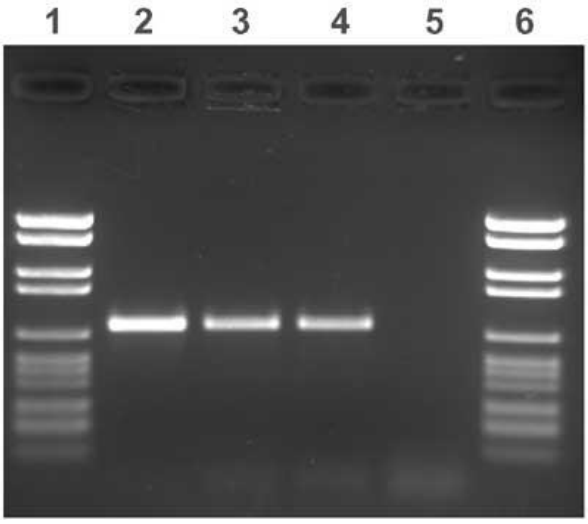
1. Initialization: A rapid heating up to 94 – 96 °C for a few minutes
2. Denaturation: Raise of the temperature to 94 – 98 °C for 15 – 30 seconds
3. Annealing of the primers: A rapid drop of temperature to 50 – 64 °C for 20 – 40 seconds to allow the primers to bind to the template single strands (at this temperature short primer sequences can pair with the DNA, but the actual reannealing of large DNA sequences is still inhibited)
4. Elongation: Temperature is raised to 72 – 80 °C for a couple of minutes to allow the actual DNA synthesis by the thermostable polymerase

5. Final elongation (optional): Several minutes at 70 – 74 °C

The steps 2 - 4 (the PCR cycle) are repeated 15 – 40 times, the whole process taking a few hours.

It should be noted that several primer pairs targeting different genes can be simultaneously applied to the sample. This approach is called multiplex PCR.

The PCR products generated can be analyzed by gel electrophoresis. The DNA molecules have a net negative electric charge. Therefore, if they are placed in electric field, they migrate against the positive electrode (katode). Electrophoresis is typically performed in solid gels (usually agarose gel) placed between the anode (negative electrode) and katode, and the DNA is applied in wells near the anode. In these conditions the speed of the DNA migration towards the katode is inversely correlated with the size of the molecule. Larger DNA molecules are slower than the small ones. By using DNA fragments with known sizes the size of the PCR product can be estimated. For the visualization of the DNA different DNA reeacting dyes that are fluorescent at UV illumination are typically used (Figure 13). [Figure 13. A typical DNA electrophoresis gel after a run, the molecular weight standards on lanes 1 and 6 and the samples on lanes 2, 3 and 4].



Because of the extreme sensitivity of PCR a series of negative controls are usually included. At least a reaction mixture without the template DNA is usually run along the samples to detect any contaminating DNA.

5.1.1. Reverse PCR and real time PCR

There are several modifications of the basic PCR-procedure described above. When the intention is to use PCR not as a diagnostic tool but as a method to monitor the metabolic activity of an organism, cell or tissue, one can use reverse PCR. In this technique the target is not DNA but the mRNA present in the sample. As pointed out above (section 2.2.) the message of DNA is first translated in mRNA. Thus, the number of mRNA molecules containing a specific copy of a certain gene in the sample indicates the relative activity of this gene or its expression.

Reverse PCR utilizes a specific enzyme, reverse transcriptase, which uses RNA as a template in DNA synthesis. Thus, the usual information flow is reversed. By isolating mRNA from the sample and applying reverse transcriptase, one first creates the cDNA molecule, in which one strand is parental mRNA and the second complementary, newly synthetized DNA. This cDNA can be subjected to standard PCR, and the intensity of the resulting PCR product is the indicator of the gene activity. There are various methods to estimate this either in relative or quantitative terms.

Real time- or quantitative PCR (qPCR) is used, when one needs to know the exact number of the target DNA molecules. As the name indicates, in real-time PCR the multiplication of the target DNA is continuously monitored by using fluorescent signal molecules. The intensity of the fluorescence is proportional to the number of DNA copies generated (van Guilder et al. 2008) . One of the simplest systems is based on a fluorescent dye called SYBR green, which has a special affinity to double stranded DNA and thus to the PCR-products generated. The quantification is usually based on a standard curve obtained by plotting the number of cycles required to get a fluorescent signal against variable concentrations of the standard DNA (identical to the DNA to be screened).

Because of the need to use fluorescence technology and of the continuous monitoring of the signal real-time PCR, it requires quite special type of equipment and software thus being a much more sophisticated technique than ordinary PCR. Despite of this, it is increasingly used in routine food analysis.

5.1.2. Examples of the use of PCR in food analysis

PCR has found a widespread use in bacterial diagnostics. Ribosomal RNA, a building block of ribosomes, has variable sequences that are species specific. They can be easily multiplied by PCR using either general or species specific primers. These primers detect the genes for rRNA (in particular the so called 16 S RNA), and the PCR product obtained can further be sequenced and the sequence compared with databases containing thousands of 16 S rRNA sequences from known bacteria. Instead of the 16 S rRNA sequences, one can also target other genes that are known to be unique for a certain bacterium.

Commercial PCR kits for the detection of food pathogens like *Salmonella*, *Campylobacter*, *Staphylococcus* and *Listeria* are commonly available. Some are based on conventional PCR, but many on real-time PCR. As with EIA these kits usually contain all the buffers and reagents that are needed for the analysis as well as the necessary controls and standards.

PCR can also be applied to viral diagnostics, for example for the detection of hepatitis A virus in seafood. In this case, because the virus only contains RNA, reverse PCR should be used. Commercial kits exist also for this purpose.

6. Concluding remarks

Immunological and molecular biological methods are an essential part of modern food analysis. The rapid development of commercial kits and instrumentation have made once very sophisticated and demanding techniques like EIA and PCR routine procedures. No doubt the repertoire of commercially available specific test kits will further expand in the future increasing their areas of application. Although the test procedures may be complicated, the theoretical basis of both EIA and PCR is simple and should be a part of the training of food scientists and laboratory technicians

References

Berson, SA., Yalov, RS. (2006) General principles of radioimmunoassay. Clinca Chimica Acta 369: 125 – 143.

Chaplin DB. (2010) Overview of the immune response. Journal of Allergy and Clinical Immunology, 126:S3-S23

Chen, YT., Hsieh, Y-HP. 2014. A sandwich ELISA for the detection of fish and fish products. Food Control 40:265-273.

Gahn, SD, Patel, KR. 2013. Enzyme Immunoassay and Enzyme-Linked Immunosorbent Assay. Journal of Investigative Dermatology, 133: 1-3.

Gariabyan, L., Avashia, N. (2013) Research techniques made simple: Polymerase Chain Reaction (PCR). Journal of Investigative Dermatology 133 e6

Law, JW-F., AbMutalib, N-S., Chan, K-G., Lee, L-H. (2015) Rapid methods for the detection of foodborne bacterial pathogens:principles, applications, advantages and limitations. Frontiers in Microbiology, 15: Article 770, 19 p

Stanley, J. (2002). 12. Precipitation: Laboratory Technique 12-1: Radial Immunodiffusion Test. Essentials of Immunology & Serology. Albany, New York*:* Delmar Division of Thomson Learning. pp. 172–174.

van Guilder, HD., Vrana, KE:, Freeman, WM. (2008) Twenty-five years of quantitative PCR for gene expression analysis. BioTechniques 44: 619-626.