This part of the course will introduce underlying principles of ELISA, how it works and how it can be used to quantify or detect food contaminants and subsequently in Food regulation enforcement.

General advantages and limitations of ELISA methods will also be discussed in this section but they will be more developed in the lesson number 2.

ELISA

Principle

The enzyme-linked immunosorbent assay (ELISA) is a commonly used analytical biochemistry assay. It has been used in medicine, food sciences, agriculture, etc.

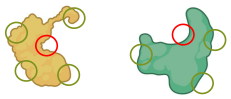
The assay uses the interaction between antibodies and an antigen (antibody-antigen binding) to detect the presence of the antigen in a liquid sample.

Enzyme-linked immunosorbent assays, or ELISAs, are commonly used analytical biochemical-based assay. ELISA is is a sensitive, accurate and rapid detection method when properly used. It is especially effective when large numbers of samples must be tested, and when results are needed rapidly. It is well positioned to serve as a screening method. It has been used in medicine, food sciences, agriculture, etc.

The basis of ELISA technique is the interaction between antibodies and an antigen to detect the presence of the **antigen** in a liquid sample. Due to this specificity, ELISA belongs to the broader group of immunoassays, of which we note a specific handheld or field devices that we name Lateral flow devices.

ELISA

Principle – Basic Terms



Antigen: Any molecule that elicits the production of antibodies when introduced into a body.

Antibodies: Proteins produced by the body in response to antigenic stimuli to neutralize the antigen. (Photo)

- Polyclonal antibodies: antibodies that are secreted by different B cell lineages within the body 
- Monoclonal antibodies: antibodies that are secreted by a single cell lineage 

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Extensive training and background in serology and immunology are not essential to use ELISA, but the understanding of some basic concepts is fundamental.
Like I said, ELISA test is based on the interaction between an antibody and an antigen.

The first question: what is an antigen? [CLICK FOR ANIMATION]
It is a molecule that elicits the production of antibodies when introduced into a body. It can be a protein, a chemical compound, a bacteria, viruses, etc.. Basically a lot of molecules can trigger this reaction with the exception of very small molecules. To give you an example, the minimal length of amino acids that triggers a reaction is about 5 or 6 amino acids. In ELISA assays, the antigen is the targeted compound that needs to be detected or quantify. In fact, ELISA is a serological technique based on the concept that many molecules are antigenic when injected into animals and that the immunized animal will develop antibodies against them.

When an antigen is big enough, several different parts can be recognized by different antibodies. These parts are called epitopes. Epitopes can be present several times on an antigen or can be unique. [CLICK FOR ANIMATION] This means that one antibody could recognize several locations on an antigen but that the parts that are recognized are the same or are very similar, as illustrated on the yellow molecule on the top of the screen.

The second question is: what is an antibody?
It is a molecule that is produced by the body of vertebrates to recognize an antigen and to trigger the immune reaction to neutralize this antigen. There is different kinds of antibodies but ELISA tests generally used Immunoglobulins G, which is the antibody that is presented on the slide.

In commercial ELISA kits, you may find two types of antibodies: polyclonal or monoclonal antibodies.
The difference is not fundamental for the application of ELISA but it will explain certain differences in the kit's response

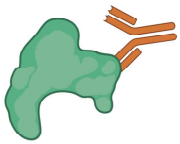
[CLICK FOR ANIMATION] Polyclonal antibodies are a pool of antibodies targeting the same antigen. In a body, there are several cells that can produce antibodies simultaneously. These antibodies are not exactly the same as they may target different epitopes even if they are produced against the same antigen. This pool of antibodies present an overall reaction against an antigen which is in fact the sum of the reaction produced by all the antibodies individually.

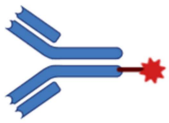
[CLICK FOR ANIMATION] The second option is monoclonal antibodies, which are antibodies targeting the same epitope. In fact, monoclonal antibodies are antibodies that are produced by a unique cell and they are all clones. A lot of commercial kits are based on monoclonal antibodies.

ELISA

Principle – Basic Terms

Primary antibodies: Recognition of the antigen (monoclonal or polyclonal)





Secondary antibodies: Recognition of the primary antibodies (polyclonal mostly)

Regarding antibodies, there is another point that needs to be explained.

Depending on the ELISA methodology that is used (which we will review later in the course), you will use primary antibodies and secondary antibodies. In commercial ELISA kits, both may be needed or present, depending on the format of the test.

What is a Primary Antibody? [CLICK FOR ANIMATION]
The primary antibody is the one that binds directly to the antigen. The primary antibody usually does not contain a marker to allow detection, meaning for example a fluorophore or an enzyme, so one cannot visualize the antigen without further reagents such as those brought by the use of a secondary antibody, as example or another reagent.

What is a Secondary Antibody? [CLICK FOR ANIMATION]
The secondary antibody binds to the primary antibody but not to any antigen that is present in the sample. Secondary antibodies bind to the heavy chains of primary antibodies, so they do not interfere with the primary antibody binding to the antigen. In most of commercial kits, the secondary antibody is in fact an anti-species antibody. For example, if your primary antibody is produced in mouse, the secondary antibody is an anti-mouse antibody. This minimizes non-specific binding that could lead to false positive results and high background noise. In most cases, this secondary antibody is the one that is linked

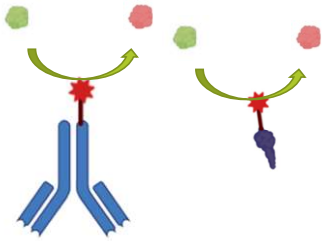
to an enzyme for further detection.

ELISA

Why this name?

Enzyme-linked immunosorbent assay

- All ELISA methods use either an antibody, or an antigen which has an enzyme attached (Enzyme-linked). A substrate will react with the enzyme to produce a colored product.



If the interaction between an antibody and an antigen is the root of ELISA test, why are they called Enzyme-linked immunosorbent assay ?

I will start to explain the Enzyme-linked part:
A fundamental characteristic of the ELISA test is the covalent link between an enzyme and an antibody or another conjugate that may be used. Various enzymes can be bound to an antibody to form a molecule that present enzymatic activity and is also serologically active. Since enzymes are highly active and are used to catalyze a reaction, meaning that the reaction will happen many times in a very short period of time. So if your reaction is producing something detectable like a colour, then this will represent an amplification of the signal. That is why enzymes linked to antibodies or antigens would be constitute an effective probe.

The complex where the enzyme is present can be detected when exposed to a substrate that enzymes can use. The reaction product of the enzymatic reaction like a colorimetric compound, or a product emitting a photon of light or a fluorescent product, will be used for the detection and quantification – with the signal made proportional to the presence of these enzyme complexes.

ELISA

Why this name?

Conjugate: Complex formed by an enzyme covalently linked to an antibody (Horseradish peroxidase (HRP) or Alkaline phosphatase (ALP))

Chromogen: Any substance that can become colored when in contact with the conjugate. In fact it is a substrate of the enzyme used in the conjugate.

- TMB (3,3',5,5'-tetramethylbenzidine) turns blue when detecting HRP
- ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) turns green when detecting HRP
- PNPP (p-Nitrophenyl Phosphate, Disodium Salt) turns yellow when detecting ALP

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At this point, I must introduce two terms that are commonly used in ELISA assays.

The first is “conjugate” which is the complex composed by the enzyme linked covalently to something (either an antibody), but we use this term more for an enzyme linked to another antigen. Different enzymes may be used in ELISAs : horseradish peroxidase and alkaline phosphatase are two of the most popular enzymes used for labeling antibodies or antigens. These enzymes are relatively inexpensive, and commercially available, while offering a high substrate turnover.

The second term I want to introduce is “Chromogen” which is any substance that reacts with the conjugate and more specifically the enzymatic part of the conjugate. The chromogen is another term to designate a substrate but it is the classical terminology used in ELISA assay. A chromogen that changes colour as a result of the enzyme action is used in commercial ELISA kits. For example TMB turns blue in presence of horseradish peroxidase. In the case presented on the slide, the green molecule becomes red after enzymatic interaction. The amount or rate of colour change is used to measure the amount of enzyme complex present.

ELISA

Principle – Basic Terms

Stopping: Addition of adding a solution to stop the action of an enzyme on a substrate. It is usually an acid or a base.

Reading: The spectrophotometric measurement of color developed during the assay. The wavelength depends on the substrate.

MANUAL READ PLATE

Left Plate	1	2	3	4	5
A	0.072	1.148	1.526	1.519	1.467
B	0.250	1.442	1.471	1.237	0.089
C	0.551	1.356	1.453	1.477	0.003
D	1.195	1.098	1.354	1.419	0.003
E	2.112	1.597	1.491	1.220	0.003
F	1.243	1.480	1.423	1.396	0.004
G	1.400	1.300	1.281	1.603	0.003
H	1.004	1.531	1.487	1.342	0.003

Obviously, the enzyme reaction needs to be properly timed and to be stopped to allow the comparison of different assays. The stopping process is performed by introducing compounds to the reaction that prevent any further change in the developed colors as a result of the enzymatic reaction. Application of stopping buffers should be done according to the recommended protocol for every kit. The temperature for the preservation of the stopping solution is of great importance. Stopping buffer has to meet the room temperature prior to the application in the assays. Therefore, it is important to observe that the solution is completely dissolved and has a clear appearance without signs of crystallinity before it is used in the assay. This is all the more important that this the last step before the reading.

The signal measurement of the final product in ELISA can be done through several different strategies including colorimetric readings, fluorescent, or luminescent readings.
In colorimetric assays, the optical density value of the color produced in the enzymatic reaction is recorded and is typically proportional to the amount of enzyme complex. Depending on the format of the assay, we can then relate this to the concentration of the analyte of interest. The specific wavelength is generally provided by the manufacturers of the products. The signals are obtained in contrast to the recorded background signals (signal-to-noise).

In fluorescent assays, the enzyme conjugate converts the substrate to fluorophores that become excited with light of a specific wavelength and emits fluorescence when returning to the original energy state. Recorded fluorescence units are typically proportional to the concentration of the enzyme complex, which again can be correlated to the analyte of interest, depending on the format of the assay used.

In comparison to the colorimetric assay, a fluorescent assay is more sensitive. However, it typically produces a higher background signal due to the sensitive nature of the fluorescent assay.

In the case of luminescent immunoassays, the enzyme converts the substrate into a reaction product that emits light photons as it returns from an electronically excited state to the original state. Known as the most sensitive detection technique, luminescent detection is benefited from intense and prolonged light emission, low background signal, as well as signal multiplication and amplification. Luminescence is measured in relative light units, typically proportionate to the enzyme complex, which again can be related to the concentration of the analyte of interest.

ELISA

Why this name?

Enzyme-linked immunosorbent assay

- A sorbent is a material used to a**d**sorb something (liquid, gas or molecule). Immunosorbent means that this sorbent will be used in an immunoreaction. It will a**d**sorb antigens or antibodies. We are involving **a solid phase**

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After the Enzyme-linked part of the name, the immunosorbent part needs to be explained.

A sorbent is a material used to adsorb something (liquid, gas or molecule). Immunosorbent means that this sorbent will be used in an immunoreaction.
In ELISA assays, immunosorbent refers to antibodies (polyclonal or monoclonal) and/or antigens immobilized on a solid phase.

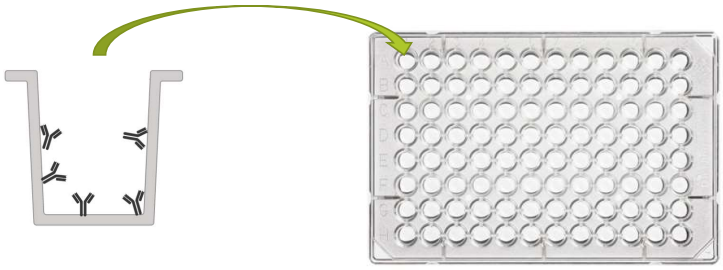
In most of commercial ELISA kits, antibodies are used as immunosorbents after a purification process through affinity chromatography techniques. By doing that, it will allow the kit manufacturer to prepare the plate in advance so food analysts can use them directly when they want to perform a test.

In some rare cases an anti-specie antibody has to be used instead of specific antibody. This will allow to immobilize the primary antibodies in a manner that is directed and more organized. It may also have the advantage to reduce the background noise.

ELISA

Principle – Basic Terms

Adsorption: Adhesion of antibodies (or an antigen) from a liquid to a microplate. It is already done in commercial kits.



The immobilization of an antigen, antibodies or any other compound on the well surface, for the purpose of a binding assay, is called "coating".
The bonding process can occur via different ways depending on the applied protocol, but generally through non-covalent binding – a process of adsorption

Immunosorbents are directly attached to the plate by passive adsorption, usually using a carbonate buffer at a pH higher than 9. Indeed, most of proteins tend to bind to the surface of microplates in alkaline conditions. As antibodies are proteins, they are no exception. After immobilization of antigens or antibodies, the surface is normally coated with a blocking agent to reduce the chance of non-specific binding in the next steps of the assay. The ideal blocking buffer will bind to all potential sites of nonspecific interactions, eliminating background altogether, without altering or obscuring the epitope for antibody binding. In principle, any protein that does not have binding affinity for the target or probe components in the assay can be used for blocking. In practice, however, certain proteins perform better than others because they bind to the plates surface more consistently. Some of the most commonly used protein blockers are: bovine serum albumin, non-fat dry milk or casein, whole normal serum, and fish gelatin.

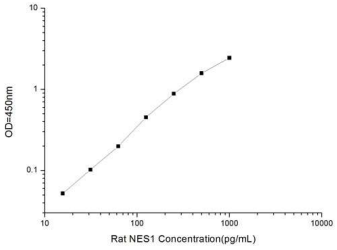
What about these solid phases ? The well-known supports used for immunosorbents, include microtiter well plates, which is used in most ELISA kits commercially available, but

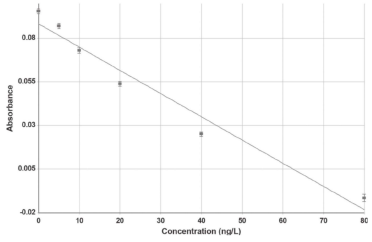
also small balls and small tubes. Usually, microplates are designed in the form of 96 well plates and, thanks to that, can be used for analysis of a large number of samples at the same time. The 96-well plates are typically made from polystyrene, which is a cost-effective, highly transparent, and relatively hydrophilic material suitable for protein adsorption. In commercial kits, solid supports are available in the pre-coated form containing specific types of antibodies or antigens present on the surface. Coated plates could be stored at low-temperature (2–8 °C) under dry condition up to six months.

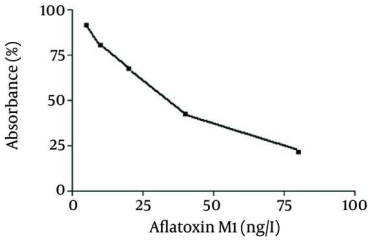
ELISA - QUANTIFICATION

Principle – Quantification

- The estimation of the analyte concentration depends upon the construction of a standard curve.
- The standard curve is prepared by making serial dilutions of one known concentration of the analyte across a range of concentrations near the expected unknown concentration.







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The protocol to perform an ELISA assay will be approached in another lesson, however, I would like to introduce now how ELISA is useful to quantify a targeted molecule.

When performing ELISAs, three different data outputs can be generated:
First, in a qualitative or semi-quantitative way. An ELISA can be used to determine whether the targeted compound is found within a particular sample by comparing the sample to a blank well or a sample that does not contain the target protein or targeted compound. Samples can also be compared to one another to determine the relative amount of targeted compound within each sample, as the intensity of signal can be correlated to the concentration of the targeted compound. By doing this, these tools may be less useful in food regulatory measures where the aim is to enforce regulations like for example a maximum level of a contaminant. This is because it is not possible to quantify the targeted food contaminant in this fashion. However, it would be possible to use this type of kits to enforce a yes / no response like a zero tolerance measure for example.

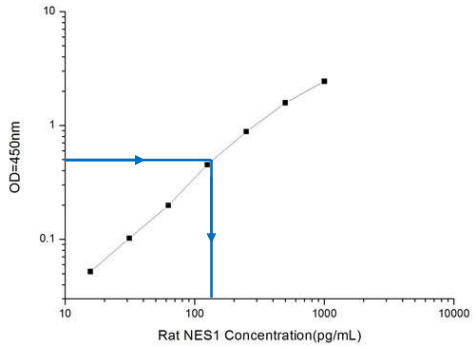
Finally, ELISA can be used for the quantification of analytes. The estimation of the analyte concentration depends upon the construction of a standard curve. The standard curve is prepared by making serial dilutions of one known concentration of the analyte across a range of concentrations near the expected unknown concentration. The shape of the standard curve is not always the same depending on the type of ELISA test and the targeted

molecules. It will be detailed in another lesson.

ELISA - QUANTIFICATION

Principle – Quantification

- The concentration of unknown samples is determined by interpolation which relies on a properly generated standard curve.



Standard curve run at different times will not yield the exact OD values for every run.

One test = one standard curve

One can ask how to use the Standard Curve to Quantify Samples. Once the intensity of each well has been measured on the plate reader, We generally calculate the average absorbance values for each duplicate/triplicate sample. Then generate a standard curve by graphing the mean absorbance for each sample (on the y-axis) vs. the standard concentration (on the x-axis).

In commercial kits, the proposed standard curve is always fit for purpose but there are several steps to follow:
First, you need to draw the best-fit trendline for the data.
Many ELISA plate readers have built-in programs for generating and analyzing standard curves or you can use Microsoft Excel, or similar graphing software.
Then, use the computer software to generate the equation of the line and the R2 value. The R2 value is an indication of how closely the data fit the trendline. An R2 value of 1 is perfection.
Finally, calculate the concentration of each sample by using the average of the duplicate/triplicate samples for x in the equation corresponding to your standard curve correlation

If the concentration of the sample exceeds the highest point of the curve, then dilute the

sample prior to measurement and do not try to extrapolate the results as it will with absolute certainty lead to inaccurate results. If a diluted sample is used, remember to multiply by the dilution factor to obtain the final value.

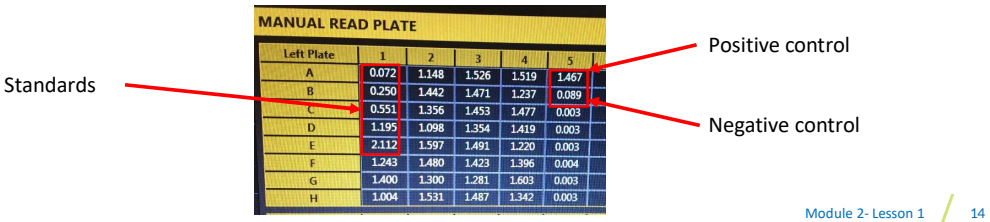
A very important point is that a standard curve run at different times will not have the same OD values for each dilution. This is due to operator differences and slight differences in pipetting, incubation times and temperature. Therefore, a new standard curve should be prepared for each experiment. Likewise, if analyzing more than one plate in an experiment, each plate should contain its own standard curve. We are talking about biological reactions and in order to correlate things accurately, they need to be performed under the same conditions.

ELISA - QUANTIFICATION

Quality assurance

Negative control: Sample without the targeted compound to assure that a positive results in a test sample is not an experimental artefact.

Positive control: Sample with the targeted compound to assure that the ELISA test presents good performance.



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A standard curve is not enough to use an ELISA test accordingly. Two kinds of controls must be used to confirm the validity of the results.

The first is a Negative control. It is a sample without the targeted compound to assure that a positive result is not an experimental artefact.

The second is a positive control, so a sample with the targeted compound to assure that the ELISA test presents good performance. Usually, positive control are samples that are similar to one of the tested food product.

ELISA - EXTRACTION

Importance of sample preparation

Extraction (depends on the sample)

- Sample homogenisation (grinding for solid samples, mixing for liquid samples)
- The targeted molecule has to be in a Liquid Sample.
  - Use of buffer (Type and Ratio)
  - Use of additives
  - Temperature
  - Mixing

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Food matrices contain a lot of compounds and interferents and because of that, food samples need to be prepared before analysis.

A published method like those provided by the AOAC International would offer details on the preparation of specific food samples for analysis, which depends on the nature of the food and the analysis to be performed. However, you can find sample preparation in others sources like scientific papers or national guidelines.

The selection of methods for extraction and cleanup of food samples is usually governed by three major factors: the chemical properties of the targeted molecules, the nature of the food matrix, and the detection method that will be used (ELISA, LFD, etc..).

Most liquid food samples such as milk, wine, and apple juice are subjected to liquid-liquid extraction to initially separate the wanted molecules. However, solid-liquid extraction may also be used, especially for extraction from grains, cereal foodstuffs, and other solid material. Numerous buffers (PBS, water, methanol, ethanol) and different additives have been used for extraction of food samples. Regarding ELISA assays, Phosphate buffered saline (PBS) in one of the most uses buffers in commercial kits. Sometimes, additives such as EDTA, D2-mercaptoethanol (2-ME) or dithiothreitol (DTT) may be useful depending on the targeted molecule.

The effect of additives must be tested before using them routinely as they can have potential impacts on the antibodies and consequently on the results of the assay. For example beta-mercaptoethanol that is used to unfold proteins can also unfold antibodies. When beta-mercaptoethanol is used during the sample preparation, one does need to be sure that the concentration of beta-mercaptoethanol will not have impact on the results on the assay. In the same way, the ratio of buffer to sample can also be important. Normally, the ratio of buffer to sample should be at least 10: 1. Higher concentrations may actually reduce reaction rates and make sample preparation more difficult.

As many samples are in solid state, grinding is most always necessary. There are many ways to grind samples. Pestle and mortar are fine for small numbers of tender samples but a grinder may be necessary for difficult samples and need to be cleaned between samples. Sometimes, samples need to be chilled prior to grinding to offset heating during the grinding process. It is normally not necessary to keep the sample on ice during grinding, unless unusually long grinding is required and the sample becomes warm to the touch.

In ELISA commercial kits, the sample preparation is always detailed but you have to keep in mind that kit manufacturers do not make tests workable on every foodstuff and that the sample preparation may need further optimization. For regulatory enforcement, the sample preparation is critical and need to be documented. This is especially the case when modifications are made compared to the official method of sample preparation.

ELISA - CLEANING

Importance of sample preparation

Cleaning

- Solid phase extraction, immune affinity columns, etc..
- Centrifugation
- Filter
- Dilution in adequate buffer (if necessary)

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Likewise, the cleanup of the extract is an important process to eliminate substances that may interfere with the subsequent detection of targeted analyte. By cleaning up the extract, the specificity and sensitivity is enhanced resulting in improved accuracy and precision.

A variety of cleanup methods have been implemented including solid phase extraction, immune-affinity columns, and many others but in most of commercial ELISA kits, the cleaning procedure consist of a simple centrifugation. Indeed, samples containing a lot of debris after extraction can be difficult to pipette. Sometimes, filtration can be used instead of centrifugation.

Finally, and depending on the additives that were used during the extraction, a dilution may be necessary before performing the test.

ELISA

Principle – Basic Terms

Washing: The simple flooding and emptying of wells with a buffered solution to separate bound from un-bound reagents in ELISA.

With a washing device:
Not mandatory but highly recommended for high throughput

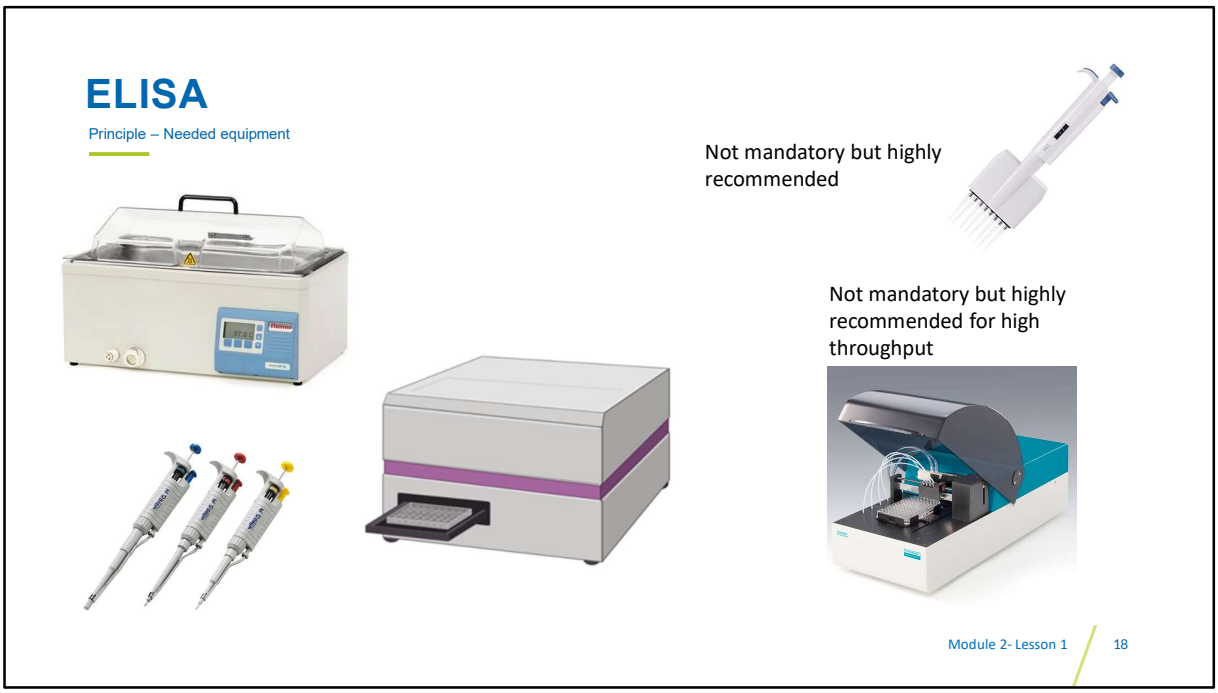
Another point that is often neglected in ELISA process is the washing steps.

Proper washing of the plates between steps is absolutely critical in every ELISA protocol. By charging and emptying the wells with specific amount of washing buffer, unreacted reagents can be removed from the wells hence the accuracy of the assay increases as only reacted analytes remain in the well plate. The wash buffer is always included in commercial ELISA kits.

The standard procedure is to wash the plate three times between each step with phosphate-buffered saline (PBS). Sometimes, detergents can be used in the washing buffer but it is important to note that only particular types of detergents with carefully controlled concentrations can be used. The excess of applied detergents dosage in preparation of the washing buffers can cause the denaturation of antibodies and interfere in the reaction between antibodies and antigens.

The two most critical wash steps are after sample incubation when cross-contamination must be avoided between wells, as they contain different samples, and after the conjugate incubation step. If even a minor residue of unattached conjugate remains, high background readings may occur.

Various plate washers are available which can promote consistent washing operations, but a plastic squeeze bottle will work well for small volumes of plates. Solutions in the plate wells can be removed by aspiration to avoid contamination, but usually the plate is inverted rapidly with a quick shake of the hand and tapped firmly on clean blotting paper or paper towels.



Even if ELISA is considered as a quick method of analysis, performing an ELISA test requires some small equipment.

To perform the test itself, pipettes and a plate reader are mandatory. The use of multichannel pipettes is recommended for the testing of 20 samples or more simultaneously. Do not forget that pipettes need to be calibrated and that they have a range of utilisation. Also, you have to change the tips between two samples.

Microplate readers are the equipment that provide with the final detection outcomes. Ideally, the plate reader should have high throughput performance, compatibility, cost-effectiveness as well as multi-mode operation. Some ELISA readers cover the entire ultraviolet-visible spectrum from 220 to 1000 nm. Usually, kits from the same company possess the same type of enzyme and chromogen so you may only use one specific wavelength. Incorporated software, varied types of plate formats, plate shakers etc. could be additional assets to the ELISA readout instruments.

For high throughput laboratory, an ELISA station could be useful as it is an automatic device that will perform the tests instead of the food analyst, reducing but not eliminating the risk of mistakes.

Also small equipment may be needed to perform the sample preparation. Water bath, grinder, centrifuge, vortex and scale are often necessary.
Finally, some equipment can be necessary for the treatment of the results. If the plate reader does not have any software to calculate sample concentrations, informatic equipment is mandatory.

In the end, the lack of adequate equipment may lead to inaccurate and not usable results and the availability of this equipment should be done before the sample preparation.

ADVANTAGES OF ELISA

- Reagents are relatively cheap and have a long shelf life.
- Equipment is widely available and cheap.

- Easy to perform and quick procedures.
- It is highly specific and sensitive (ppm or even ppb).

- Fit for a very large variety of molecules.
- Fit for **almost** all types of food sample.

ELISA techniques have several advantages.

There is no need for expensive equipment like for mass spectrometry methods. Components of ELISA kits are relatively cheap (enzyme, standards, antibodies, etc) and have a long shelf life.

The key advantage is that 96 determinations can be performed in a single run, providing results in usually less than 3 hours. This is both quick and cost effective. Compared to other immunoassay methods, like Lateral flow devices, ELISA tests are more accurate. They are considered highly sensitive, specific and compare favorably with other methods used to detect analytes in food matrices. ELISA tests typically offer a detection limit of a few ppm (mg/kg) but this limit of detection could be lower than that depending on the targeted molecules.

The Specificity of ELISA is owed to the selectivity of the antibody or antigen. Actually, the binding of antigen or antibody only occurs in the epitope of an antigen or antigen-binding site of an antibody. Since, there is a complementary relationship between epitope and antigen-binding site both in chemical structure and spatial configuration, the reaction between antigen and antibody shows a strong specificity.

These advantages of ELISA make it an useful biotechnical tool with many applications, either in scientific research or food monitoring.

DISADVANTAGES OF ELISA

- Kits are not cheap.
- Only one molecule at a time.
- Results may be affected by interferents.
- As specific as the antibody is. Possibility of cross reaction.

Unfortunately, ELISA also presents some disadvantages.

An obvious one is that the price of commercial ELISA kits could become a burden. Even if ELISA is considered as a cheap analytical method, the fact is that you can only target one molecule or a group of molecules per assay. For example, if you want to detect aflatoxins, ochratoxins, peanut and milk in a food sample, you will need 4 different kits. So far, multidetection with ELISA assay is still not available on the market. There is in fact, some multiplexing ELISA exists but they require most expensive equipment and highly trained personal.

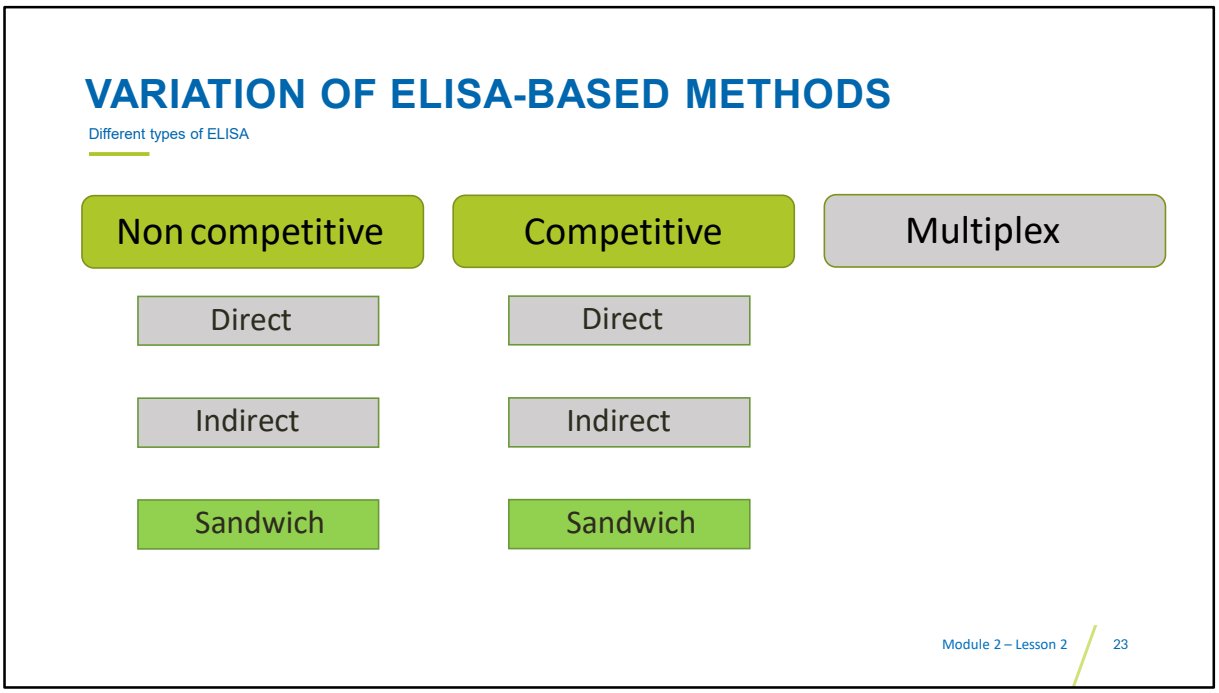
Another issue is the presence of interferents in food samples. This one is not particularly linked to ELISA assays as you can also find interferents in chromatographic methods. The presence of interferents may lead to an underestimation and sometimes an overestimation of the quantity of analytes in a sample. In the same way, even is antibodies are specific, there are possible cross reaction with similar proteins. When the similarity is known, these cross reaction could be predicted. However, sometimes, cross reaction occur with exotic or unusual compounds leading to false results.

Because of these two defaults, one can consider that quality control is very important in ELISA assays and that is why it will be more detailed in lesson 3.

You have reach the end of the This lesson. The basics of ELISA methods have been covered. In the next lesson, the different types of ELISA will be presented and discussed in relation with food regulatory enforcement and monitoring.

LESSON 2
INTRODUCTION OF THE VARIOUS FORMATS OF ELISA-BASED METHODS

Welcome to the second lesson of module 1. In this lesson, the different types of ELISA will be presented. Each type of ELISA offers some advantages and some limitations. Their use in food analyses will be discussed vis-à-vis food monitoring.



As presented in a previous lesson, there are in fact several kinds of ELISA.

[CLICK FOR ANIMATION] The first type of ELISA is non competitive ELISA which can be split in three different tests: direct, indirect and sandwich. They will be developed in the next slides.

[CLICK FOR ANIMATION] Then you have competitive ELISA, which can also be split in three different kinds of assays.

[CLICK FOR ANIMATION] Finally, there are multiplex assays which are not used currently for food regulatory purposes. As explained in a previous lesson, theses types of test are still in development and require a different type of equipment.

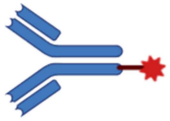
In a general way, it would very useful to visit a laboratory, where ELISA is practiced, in order to observe and practice the procedure under the guidance of an experienced user. Begin with a well-known system, and study the effects of adjusting reagent concentrations and test conditions.

NON COMPETITIVE TEST: DIRECT ELISA

Non Competitive Test

Direct ELISA:

- It uses a **primary labeled antibody** that react directly with the antigen.
- It is performed with the antigen that is directly immobilized on the microplate.



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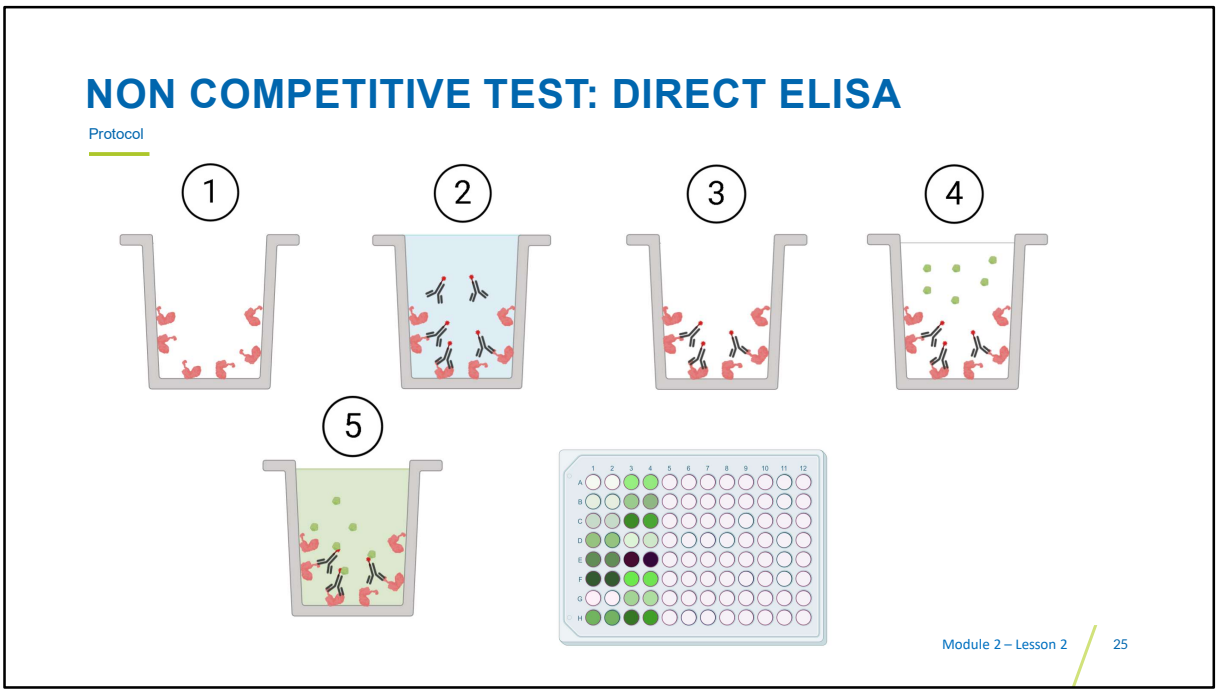
A direct ELISA is a plate-based immunosorbent assay intended for the detection and quantification of a specific analyte (e.g. antigens, antibodies, proteins, hormones, peptides, etc.) from within a complex biological sample. Of all the different ELISA formats, direct ELISA is the simplest and quickest to perform, but there are some disadvantages associated with this method.

[CLICK FOR ANIMATION] It is performed with the antigen that is directly immobilized on the microplate and a **primary labeled antibody** reacts directly with the antigen.

This was the ELISA originally developed by Engvall & Perlmann in 1971. Direct ELISA was developed to avoid the use of radioactive elements like in radioimmunoassay. This ELISA format is simple and quick while minimizing potential user error compared to others ELISA methods as well as the Cross reactivity due to the secondary antibody being absent.

Direct ELISA is not widely used in food diagnostic or food monitoring, if not used at all for regulatory purpose. These kinds of method are suitable for qualitative and

quantitative antigen detection in samples of interest, antibody screening, and epitope mapping. Consequently, they are used for R&D purpose in most cases.



In a direct ELISA, the antigen is immobilized directly onto the surface of a multi-well microtiter plate and then complexed with an enzyme-labeled primary antibody specific for the antigen. Once the enzyme-labeled primary antibody binds to the antigen, the conjugated primary antibody catalyzes a reaction with its respective substrate resulting in a visible colorimetric output that is measured by a spectrophotometer or absorbance microplate reader.

1 Antigen is coated onto the wells by passive adsorption and incubation. The plates are washed to remove unbound antigens. Before using antibodies to detect antigens. The remaining binding surface must be blocked to prevent the nonspecific binding of antibodies. Otherwise, antibodies will bind to any remaining sites that initially served to immobilize the molecules of interest. The plates are washed to remove the unnecessary blocking buffer.

2 The conjugate (Complex Antibody-Enzyme) is added and incubated for a specific amount of time.

3 The wells are washed with an adequate washing solvent to remove unbound molecules.

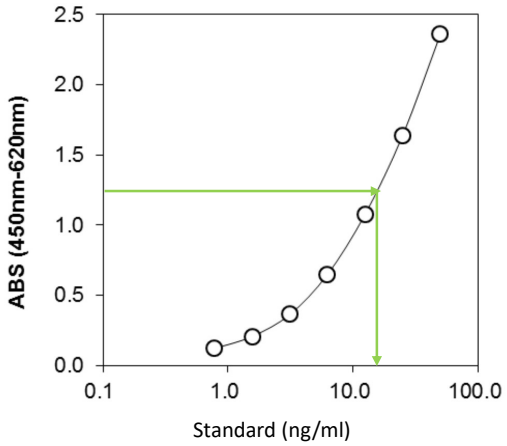
4 and 5 Chromophore (substrate) is added which allows the detection or estimation the

presence of the enzyme and thus the antigen. The enzyme modifies the chromogen to produce a colored compound (Green in the picture).

6 Plate reading and analyte quantification through informatic tool.

NON COMPETITIVE TEST: DIRECT ELISA

Standard Curve



- Quantification possible with standard curve
- Standards needed
- The sample as to be in the range of the curve

In order to quantify a targeted molecule, a standard curve has to be produced for each microplate. The standard curve is prepared by making serial dilutions of one known concentration of the analyte across a range of concentrations near the expected unknown concentration as discussed. In commercial kits, the range of the standard curve is already fixed. Consequently, the range of quantification of the kit must be verified before the start of the experiment.

In a direct ELISA, the more the concentration of the standard is, the more the OD is high. The shape of the standard curve could be slightly different depending on the kit.

You need to draw the best-fit trendline for the data and calculate the concentration of each sample by using the average of the duplicate/triplicate samples for x in the equation.

If the concentration of the sample exceeds the highest point of the curve, then dilute the sample and redo the ELISA test, with a new standard curve.

NON COMPETITIVE TEST: DIRECT ELISA

Disadvantages

- High background
- Potential reduction of the immunoreaction of the antibody du to the bond
- Labeling of the primary antibody is expensive
- Reduction of the flexibility (few antibodies available)
- Weak amplification signal

However, this type of technique presents various areas of improvement.

The first is high background due to the lack of specificity of antigen immobilization. The blockage step is really important to reduce this issue.

The second disadvantage is specific to this type of ELISA. Indeed, in this ELISA methodology, it is the primary antibody which is linked to the enzyme. In that case, immunoreactivity of the primary antibody may be affected by the labeling with enzymes, which would be different in other ELISA tests.
In the same way, from a commercial perspective, the production of direct ELISA tests is more complex from a develop stand point. Each type of primary antibody must be labeled according to a specific procedure, unlike the case where a secondary antibody is used which has been labelled and is applicable in several kits.

The two other disadvantages of Direct ELISA test are in fact limitations that were overcome with the creation of the other ELISA methodology, which will be explained subsequently.

NON COMPETITIVE TEST: INDIRECT ELISA

Indirect ELISA:

- It uses a primary unlabeled antibody in conjunction with a labeled secondary antibody.

In order to to overcome the impediments mentioned earlier, a second type of ELISA methods was developed. This one is called Indirect ELISA.

[CLICK FOR ANIMATION] This time a primary unlabeled antibody is used in conjunction with a labeled secondary antibody. [CLICK FOR ANIMATION] That is why this type of ELISA is called indirect.

The secondary antibody has specificity for the primary antibody. In most of commercial kits, the secondary antibody is an anti-species antibody. For example, if your primary antibody is produced in mouse, the secondary antibody is an anti-mouse antibody.

Indirect ELISA is widely used for the development of ELISA test. However, it is not widely used commercially as the coating of the plate needs to be done with antigens and cannot be prepared in advance.

NON COMPETITIVE TEST: INDIRECT ELISA

Advantages compared to direct ELISA

- Large variety of labeled secondary antibodies
- Fewer labeled antibodies are required (cost saving)
- The same secondary antibody can be used with several primary antibodies
- The immunoreactivity of the primary antibody is not affected by the labelling
- Better signal amplification

Compared to Direct ELISA, indirect ELISA offers better perspective for commercial outcome due to the introduction of the secondary antibody.

First, the production of secondary antibodies is really easy and allows a better flexibility for the development of ELISA tests. The fact that only one type of antibody is used in direct ELISA limits the number of possibilities of interaction with the antigen. The fact that there are two specific reactions, the non-specific binding leading to false positive results, is reduced as well as the background noise.

The same secondary antibody can be used with several primary antibodies, reducing cost of kits production. There is no need to prepare a dedicated conjugate for each ELISA tests. For example, if all your primary antibodies are produced from mouse, you can use an anti-mouse antibody as secondary antibody for all your ELISA tests or kits.

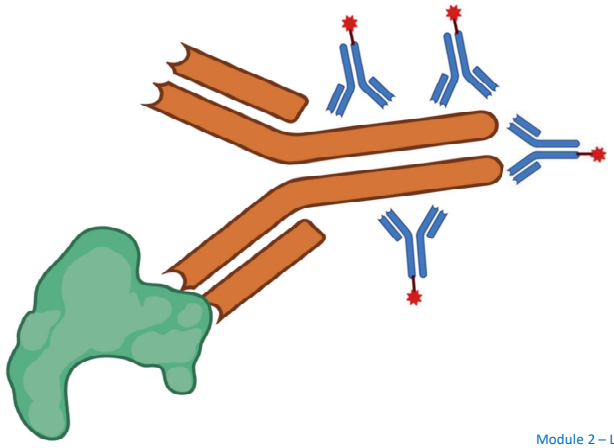
As the secondary antibody is the one that is labelled, the immunoreactivity of the primary antibody is not negatively affected which leads to more accurate results.

Finally, a huge advantage of indirect ELISA is the amplification of the signal compared to direct ELISA.

Better amplification because several epitopes may be targeted by the primary antibody.

NON COMPETITIVE TEST: INDIRECT ELISA

The size of the primary antibody is exaggerated



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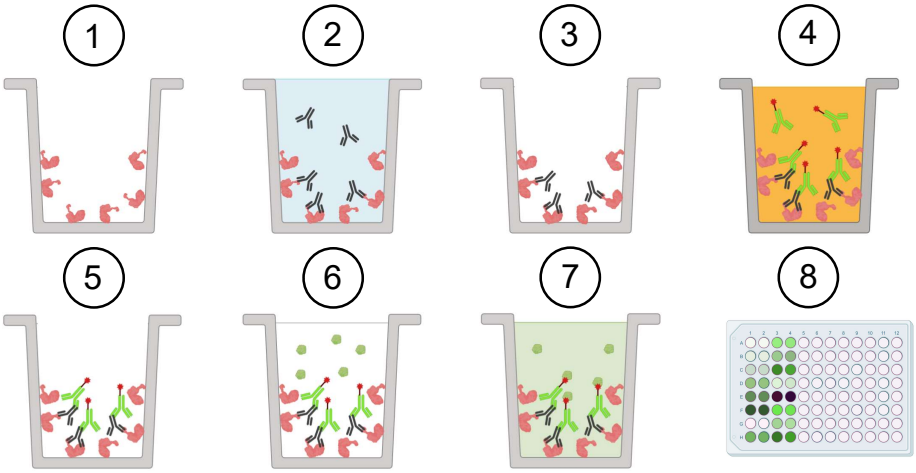
One may ask why there is more amplification is better thanks to the introduction of a secondary antibody.

In direct ELISA, you obtain a signal when the antibody reacts with an antigen. There is a direct correlation between the number of antibodies that are bound to the analyte depending on the number of epitopes that the antigen possess.
In fact, the number of epitopes on an antigen that an antibody can reach is limited, leading to a relative weak number of labeled antibody available for the reaction with the chromogen. In the end, the amplification is limited.

In indirect ELISA, secondary antibodies has several binding sites on the primary antibody, increasing the number of labelled antibodies for the enzymatic reaction. In the end, the signal will be greatly amplified compared to direct ELISA

NON COMPETITIVE TEST: INDIRECT ELISA

Protocol



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Like for direct ELISA, the antigen is coated onto the wells by passive adsorption and incubation. The blocking step is also needed. The plate is washed with the recommend buffer to remove unbound antigens first, and blocking agent next.

Then, the primary antibody is added to the plate and incubated with the immobilized antigen (step 2)

The plates are washed to remove unbound antibodies (step 3).

The secondary enzyme conjugated antibody is added and incubated with the antigen, as showed in the step 4

Wells are then washed to remove unbound secondary antibodies (step 5).

Chromogen is added detecting the presence of the enzyme and thus the antigen (step 6 and 7), and finally the plate is read (step 8).

NON COMPETITIVE TEST: INDIRECT ELISA

Disadvantages compared to direct ELISA

- Potential cross reactivity of the secondary antibody
- Longer than direct ELISA

Limitations

- Coating of microplates is necessary for each test
- Not suitable for complex samples

Compared to direct ELISA, indirect ELISA present two impediments which are the potential cross reactivity of the secondary antibody and that it is longer than direct ELISA. However, these limitations may very well be offset by the advantages discussed earlier.

However, both direct and indirect ELISA tests present a major flaw which is that coating has to be performed for each test because it is the a representation of the analyte or the analyte itself that has to be coated.
Each well has to be coated individually, increasing the risk of human error.

Consequently, these direct and indirect ELISA formats, discussed earlier are not suited for routine analysis and cannot allow a very high throughput of analysis.

The second limitation appears when complex samples are tested. The coating of the plate with the targeted antigen is influenced by the complexity of the sample. And do I really need to remind you that food samples are often complex matrices? In the end, this type of methodology requires a thorough sample preparation to purify the targeted molecule.

NON COMPETITIVE TEST: ELISA SANDWICH

- Plate is coated with a capture antibody.

- Antigens in the sample bind with the capture antibody.
- The detection antibody (primary antibody) binds with the immobilized antigen to form a sandwich.

Once again, another ELISA methodology, called Sandwich ELISA, was developed to overcome some of these limitations.

Contrary to direct or indirect ELISA, the plate is coated with a capture antibody. In this case, this antibody is not called primary antibody.
As a result the plate coating can be performed independently to testing and plates can be produced in anticipation of future analysis. The production of standardized commercial kits becomes more possible.

So obviously, this kind of kit is more widely used for food analysis.

[CLICK FOR ANIMATION] Once the coating is done, antigens or the solutions where the analyte is present are then added into the wells, and and the analyte would bind with the capture antibody.

[CLICK FOR ANIMATION] Another antibody is then added and bind with the antigen to form a sandwich. Each antibody is specific for a different and non-overlapping region or epitope of the antigen. It is important that matched antibody pairs are tested specifically in

sandwich ELISA to ensure that they detect different epitopes, to achieve accurate results.

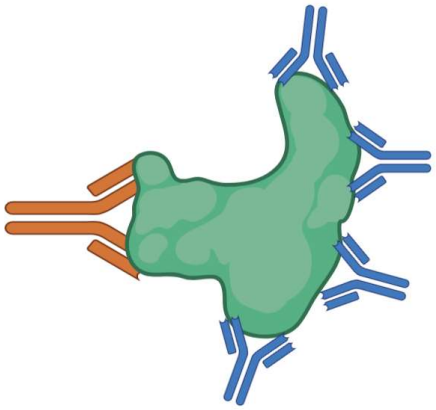
It is possible to use the same antibody for capture and detection but further developments may be necessary to obtain accurate results. Indirect detection is also possible. In that case, the detection antibody is not linked to an enzyme and another labelled antibody must be added to detect the sandwich. In that case, the antibody used for detection purposes is called secondary antibody.

Now you would note that there is a major condition for these assays to work. The analyte has to be big enough to allow the possibility of having enough epitopes to be covered by both antibodies : the capture one and the detection one – meaning to allow a sandwich to be formed.

NON COMPETITIVE TEST: ELISA SANDWICH

Advantages compared to direct or indirect ELISA

- High specificity: two antibodies instead of one, both against the targeted analyte.
- High sensitivity (up to 2 to 5 times more sensitive than direct ELISA)
- Suitable for complex samples

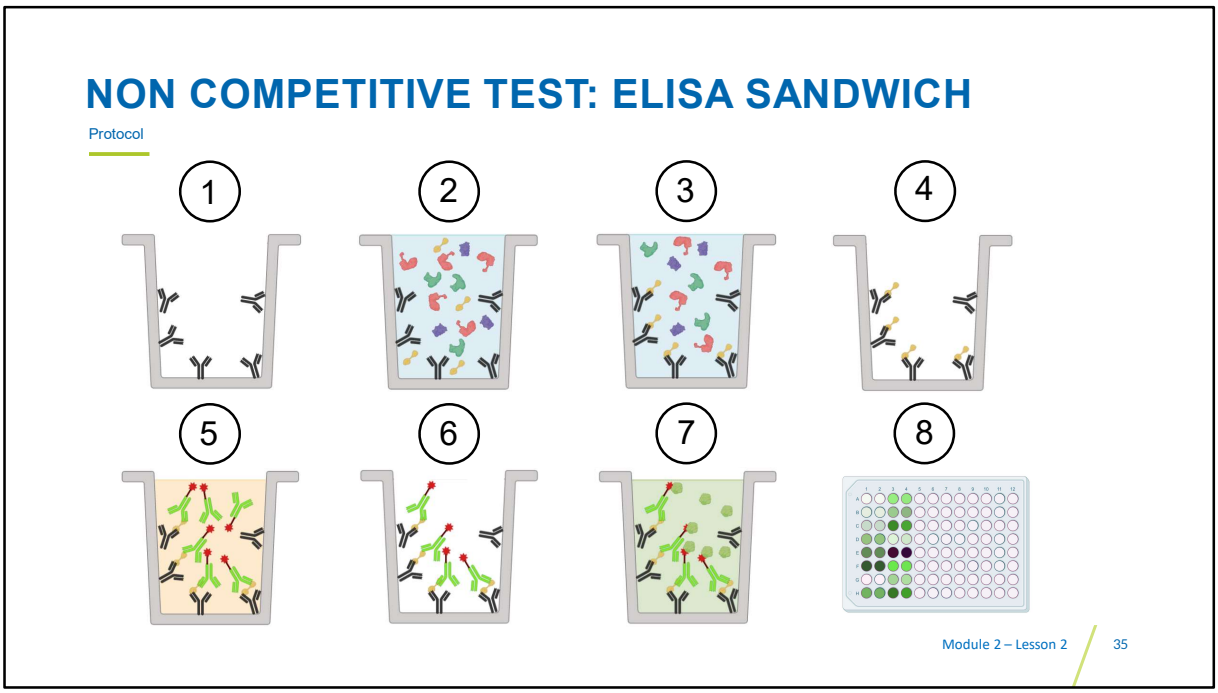


By applying this methodology several advantages are to be mentioned

The first is high specificity: two antibodies are used instead of one compared to direct ELISA. Compared to indirect ELISA which also used two antibodies, in this case, both antibodies (primary and secondary antibodies) target the analyte instead of one in the indirect ELISA format. It also offers flexibility since both direct and indirect methods can be used.

The second advantage is high sensitivity. Like for indirect ELISAs, the signal can be enhanced, as the same epitope can be present in the antigen and several detection antibodies can bind to the same analyte. Sandwich ELISAs are 2-5 times more sensitive than direct or indirect ELISAs.

And of course, Sandwich ELISAs are particularly suited to the analysis of complex samples, since the antigen does not need to be purified prior to the assay yet still delivers high sensitivity and specificity.
One again the condition is that the analyte has to be big, therefore this is not suited for the analysis of small molecules like pesticides.



The procedure for a sandwich ELISA firstly requires the coating of the well with a capture antibody. The good news is that this step is already done and standardized in commercial kits (step 1).

The analyte or sample is then added (step 2) for a standardized amount of time to allow the interaction between the capture antibody and the analyte that we want to detect (step 3).

The plate is then washed to remove unbound molecules (step 4).

After the washing, the detection antibody is added (step 5) and incubated for a standardized amount of time.
The detection antibody can be enzyme conjugated, in which case this is referred to as a direct sandwich ELISA. If the detection antibody used is unlabeled, another enzyme-conjugated detection antibody is required. This is known as an indirect sandwich ELISA.

The plate is washed a second time to remove unbound antibodies (step 6).

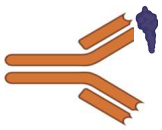
Finally, the chromogen is added and incubated for a standardized amount of time (step 7) and the plate is read once the reaction is stopped (step 8).

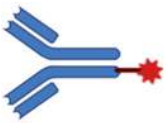
NON COMPETITIVE TEST: ELISA SANDWICH

ELISA sandwich

Disadvantages and limitations

- Potential cross reactivity of the secondary antibody.
- Easier to do with monoclonal antibodies (more expensive)
- Not suitable for small analyte, when several epitopes are not available.





As discussed before, Sandwich ELISAs offer a great number of advantages compared to previous ELISA methodologies discussed. Let us discuss their limitations.

If a standardized ELISA kit or tested antibody pair is not available, antibody optimization has to be worked out since it is important to reduce cross-reactivity between the capture and detection antibodies. The development of kits is more complex than for direct or indirect ELISA. As kits development is not into the prerogative of food analysts, this defect is often masked for most stakeholders – it is the responsibility of the kit manufacturer.

[CLICK FOR ANIMATION] However, an important limitation appears for small molecules. In sandwich ELISA, two antibodies interact with the antigen. This is a force as specificity is enhanced but this is also a defect for small molecules as several epitopes are not always available. Consequently, and as discussed earlier, this type of kit is not suitable for small molecules

Now where do we see this format used the most: it is for Allergens ! Where the analytes are large proteins presenting several potential epitopes on their surface.

COMPETITIVE ELISA

- Plate is coated with a capture antibody.
- Competition between an antigen and an labelled-antigen like for the site of the Antibody

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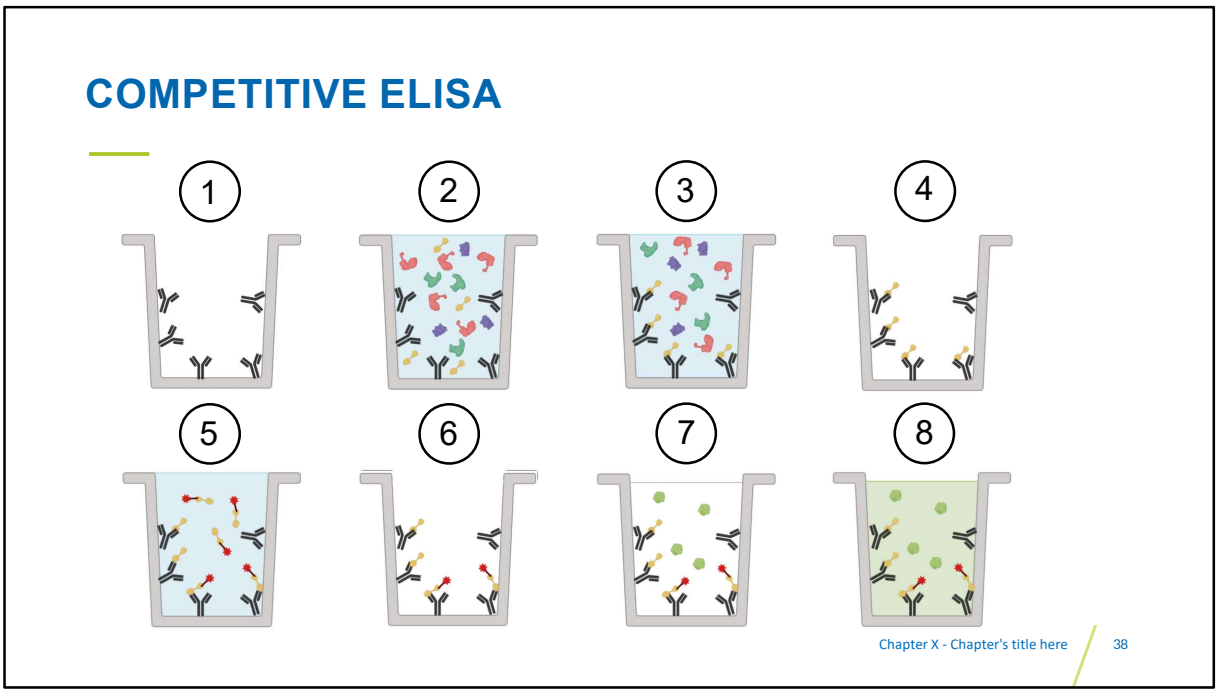
Another strategy called competitive ELISA was developed to allow the detection of small molecules.

In competitive enzyme immunoassays, the targeted analyte competes for limited antibody binding sites with an analogue of this analyte that is labelled with an enzyme for example. This type of reaction is one of the few methods possible for low molecular weight analytes acting as antigens with a limited number of epitopes or antibody-binding sites.

In this methodology, the plates are coated with a standardized amount of capture antibody. Then the sample and the antigen-like molecule (or the conjugate of the analyte) are added and compete for the capture antibody. Finally, the detection is performed by using the enzyme that is present or carried by the conjugate meaning the labelled analogue of the analyte.

You are able to note right away that the response or the signal to be measured in this case will inversely proportional to the amount of analyte in the solution.



The procedure for a competitive ELISA firstly requires the wells of an ELISA plate to be coated with a capture antibody (step 1). In commercial kits, this step is already done and the plate are ready to use.

The sample is then added (step 2). Targeted analyte binds with coated antibody (step 3).

After incubation, the plate is washed to remove unbound compounds (step 4).

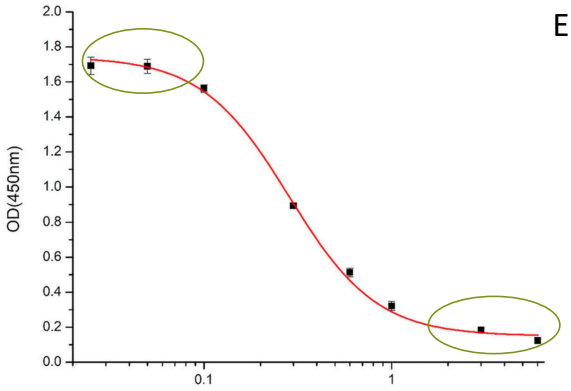
Then conjugate (antigen – enzyme) is added to bind with available antibodies which are antibodies that did not react with the sample because the quantity of the targeted analyte was not enough (step 5).
Sometimes steps 3 and 5 are combined, meaning that we add the analyte solution and the competitor at the same time.

The plate is washed to eliminate the excess of labelled antigen (step 6).

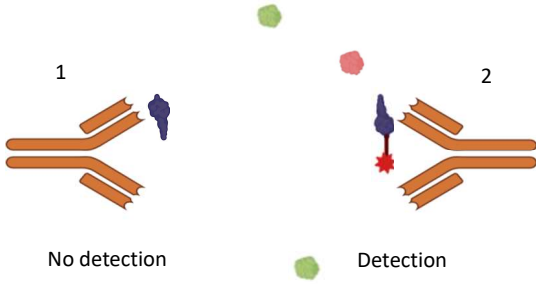
The substrate is added and react with the conjugate only, producing a colored compound (step 7 and 8).

The plate is then read and the quantification of analyte is done (not shown on the slide).

COMPETITIVE ELISA



Quantification curve for competitive ELISA test



Compared to other ELISA tests, the standard curve is different. In competitive ELISA, an inverse relationship between antigen concentration and substrate turnover is produced.

[CLICK FOR ANIMATION] During incubation, samples with high analyte content result in unlabeled antigen being bound in greater amounts than conjugated antigen. When chromogenic substrate is added to the assay to develop color, samples with a high analyte concentration generate a lower signal (number 1) [CLICK FOR ANIMATION] than those containing low antigen concentration (number 2), yielding the inverse correlation between analyte concentration in the sample and the color measured in the assay. This relationship can then be used to extrapolate analyte concentration in an unknown sample from a standard curve.

This standard curve can be treated mathematically to lead to an equation from which we extract the results of unknown samples

VARIATION OF ELISA-BASED METHODS

Why different types of ELISA

Direct and indirect ELISA

- Cheap and quick
- Fit for test development like discrimination of antibodies
- Mostly used for research

ELISA sandwich and competitive ELISA

- Need more optimization
- Fit for standardized production (kit distribution)
- Mostly used for regulatory testing

The description of the different ELISA kits allows making the difference between direct and indirect ELISA kits that are mostly used for R&D purposes, and Sandwich and competitive tests that are used widely for food analysis.

In fact, sandwich and competitive ELISA allow a standardized production, which is mandatory for analytical processes used in regulatory settings.

GENERAL LIMITATIONS

- False positive/negative results, especially with mutated/altered antigen.
- Complexity of multiplex detection. One ELISA for one molecule.
- Antibody must be available (poor producer, interference).
- Not suitable for all food matrices

Now let us discuss the general limitations of ELISA techniques.

There are possibilities of false positive/negative results, especially with /altered antigens. As the interaction between the antigen and the antibody is the key of ELISA, a slight modification of the antigen can influence this interaction in positive or negative ways.

This can be illustrated with the case for antibodies detecting a group of molecules (for example aflatoxins). The interaction between an antibody and each aflatoxin can be different. The main obstacle is the development of an antibody able to binding many different congeners with an affinity that will yield sufficient assay sensitivity.

Another flaw is the availability of antibodies. For new molecules of interest, the production of antibodies can take several months of development. As the production of antibodies is a biological process, there are always uncertainties. Even if antibodies were produced against a specific molecule, they still could present cross reactivities or they could present weak interaction with the antigen which make them inefficiently equipped for food testing.

Finally, ELISA methods are not suitable for all matrices. For example, food matrices with extreme pH could modify the interaction between the antigen and the antibody, leading to inaccurate results. In this regard, some food matrices need specific sample preparation.

You have reach the end of the second lesson of module 2.

LESSON 3
APPLICATION IN THE DETERMINATION OF DIFFERENT FOOD CONTAMINANTS

Welcome to the third lesson of module 3.
In this lesson, the application of ELISA methods will be discussed from a regulatory perspective.

RELIABILITY OF TESTING

Fit for purpose approach

- Check if test is fit for purpose

- Fit for the food matrix?
- Potential cross reactivity with one of the ingredients?
- Potential interferents?

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Even before the use of commercial kits, several points have to be underlined.

First, the production of ELISA kits for food industry applications is challenging as a selection of adequate control and standard samples is necessary to carefully calibrate the assay. Secondly, ELISA tests are often validated by manufacturers for a limited number of food matrix, and further validation need to be done by the users, sometimes with the help of the manufacturer himself. Although firms manufacturing ELISA kits provide the assay validity parameters on their kit catalogs, it is sometimes difficult to obtain these data. Therefore, laboratories must test the assay validity parameters when they use a new ELISA kit.

In case of new food matrix, it is very important to test individually each ingredient individually to eventually report cross reaction of antibodies with those ingredients. In the pizza for example, each individual ingredient has to be tested. A database could be developed to avoid the testing of the same ingredients several times. Indeed, food ingredients could be found in several food matrices (salts, oils, etc..). Most of the time, manufacturers present the list of ingredients that can react and induce false positive results, but this list is always not exhaustive as new ingredients are develop on a regular basis. For example, mahaleb (a specie of prunes) induce false positive results with present in samples tested for almond detection (Walker et al., 2018).

Obviously, this validation is performed for each type of kits. If at some point, your laboratory switch from one kit manufacturer to another, this validation should be performed again. Use different kits from different companies without any validation is among the most common analytical errors. This is mostly du to the fact that different companies will use sometimes different antibodies, different buffer or protocol extractions and sometimes even different ELISA type, leading to slightly different results.

RELIABILITY OF TESTING

Fit for purpose approach

- Quality control
  - Reference material
  - Testing material
  - Negative and positive controls
- Analyst's experience
- Equipment (maintenance, calibration)

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Beyond the mandatory use of negative and positive controls, quality control is crucial to assure the good quality of results. So far, analytical methods have been calibrated against reference materials in which the origin and the properties of reference materials have significant effects on the resulting calibration curves and, subsequently, on the final results. However, these reference materials can be blank samples spiked with the targeted molecule, or incurred reference material, where the targeted analyte has been added to an ingredient mixture and processed as normal for that food matrix. These reference material are not always available or developed. For this reason, it is difficult to validate ELISA methods and evaluate their suitability for analyzing the presence of the targeted analyte in foodstuffs. At this regard, homemade testing material could be produce to validate the adequacy of the kit. It can also be used for the training of the personal as the experience of the analyst is also important as well as the equipment at disposal.

Nonetheless, errors are inevitable even in the well-established commercially available assays. There exist common errors in conducting ELISA assays that can be avoided by having a clear knowledge of the sources of such errors. The suppliers and distributors often provide the users with the necessary guidelines and troubleshooting strategies to overcome the errors and to carry out successful assays. This part will be developed in the last lesson of this module.

UTILISATION OF ELISA IN FOOD ANALYSIS

To assess the risk

- Screening method (cheap, quick and high throughput) for mycotoxins, food allergens, phycotoxins, pesticides, veterinary drugs, etc..

To check compliance with or efficiency of food regulations

- Screening method to detect non compliant samples. A confirmatory method will be needed.
- Utilisation as confirmatory method when it is the reference method

Regarding the use of ELISA assays for the detection or quantification of food contaminants, ELISA still plays a major role as analytical method.

As it is cheap, quick and allows the treatment of several samples simultaneously, ELISA assays are widely used as screening methods to assess the risk of a large number of food contaminants, but mostly mycotoxins, food allergens and phycotoxins. It is possible to use ELISA to screen the presence of pesticides or veterinary drugs even if chromatographic techniques are now widely used and allow the screening of several food contaminants at the same time. When the number of targeted analytes is high, like for dioxins and furans, operators have no choice but to use other techniques like UPLC or GC coupled with mass spectrometry.

ELISA is also widely used to verify compliance with food regulations. Food industries are majors users as ELISA is affordable compared to chromatographic technologies. On the other side, regulatory agencies have to used reference methods. The AOAC international provides a large number of official methods that can be used by every stakeholders and a few of them are ELISA methods. Currently, ELISA methods are endorsed for the detection of Domoic acid (a phycotoxins) in shellfish, and gluten in a large variety of foodstuffs. However, ELISA methods could be endorsed at a national level through national guidelines or compendium.

UTILISATION OF ELISA IN FOOD ANALYSIS

Food allergens and gluten

- Detection of allergen is mandatory in certains countries and the *Codex alimentarius* recommends it.
- Reference method
- No real alternatives so far (AOAC provides a HPLC method)
- One test for one source of allergen
- Sandwich and competitive ELISA

Currently, there is one area of food contaminants where ELISA is undisputed as reference method.

It is the main platform for identifying food allergens such as those present in milk, peanuts, walnuts, almonds, and eggs. Several other methods may be used but currently, the ELISA technique is the only one used in routine food analysis due to its high precision, simple handling and good potential for standardization.

So far, mass spectrometry methods are still inaccurate for the quantification of allergens in food samples and are not used on a regular basis by small or medium business. Food allergens are a particular type of food contaminants, as one terminology regroups in fact several molecules. For example, the term “peanut allergens” regroup at least 8 different proteins. As these proteins could present several levels of degradation (depending on food processes, presence of enzymes, etc..), ELISA assays present good results compared to other methods. Depending on the degree of hydrolysis of the food allergen, one may employ a competitive ELISA. This is the case at least for hydrolysed products like soy sauces or beers.

An alternative to the ELISA format has gained importance in recent years - lateral flow devices - as they are very inexpensive, rapid, and portable, do not require instrumentation

and are extremely simple to perform. However, the ELISA techniques are still the most commonly methods used in laboratories of the food industry and official food control agencies to detect and quantify hidden allergens in food.

UTILISATION OF ELISA IN FOOD DIAGNOSTIC

Mycotoxins and phycotoxins

- Detection of allergen is mandatory in certains countries and the *Codex alimentarius* recommends it.
- No the reference method – screening or preliminary tests
- One test for each toxins
- Sandwich and competitive ELISA

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Other areas of interest for the use of ELISA assays are mycotoxins and phycotoxins. Most of regulations propose MLs for a limited number of mycotoxins (6 different mycotoxins for the *Codex Alimentarius*) and phycotoxins (none from the *Codex Alimentarius*, but 5 from the European Union). In this context, ELISA methods are useful tools for early detection and screening.

Commercially available ELISA kits for the detection of mycotoxins and phycotoxins are normally based on a competitive assay format that uses either a primary antibody specific for the target molecule or a conjugate of an enzyme and the required target. These kits can be portable, rapid and are highly specific as well as simple to use. However, the disadvantage of these kits, compared to other potential methods like HPLC, lies in the fact that they are for single use, which can increase costs of bulk screening. Additionally, competitive ELISA suffers from having a limited detection range due to the narrow sensitivity of the antibodies.

When positive results are observed, a second analytical method (UPLC or GC-MS mostly) is used to confirm or invalid the results. However, it must be considered that in case of conflicting results, there is a need for a valid reference method.

UTILISATION OF ELISA IN FOOD DIAGNOSTIC

Pesticides and Veterinary drugs

- Developemnt of Maximal Residue Levels for a large number of molecules at the international level
- No the reference method – screening or preliminary tests

Finally, ELISA kits have been developed for the detection of pesticides and veterinary drugs. These kits have been wiped out by the development of chromatographic methods that allow the detection of several analytes at the same time on a routine basis. To the best of my knowledge, they are not widely used for regulatory purpose but they could be used on a regular basis by the food industry.

However, these kits could be useful in atypical situations, like for the risk assessment or the monitoring of a particular molecule, or for the follow up on a punctual incident involving pesticides or veterinary drug.

You have reach the end of the third lesson of module 2.
As explain, ELISA assays are widely used as screening and early detection methods which make them useful tools to support risk assessment.