Immunoanalytical approaches for the control of xenobiotics and biotoxins in foodstuffs

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Abstract: Effective control of food quality and safety requires analytical methods that guarantee the reliable determination of any substance potentially harmful to the consumer that may be present in the food prior to its distribution and marketing. One of the analytical approaches that contributes to guarantee this objective encompasses a series of techniques that have in common the use of antibodies as essential elements for the detection of the target analyte, and which together are called immunochemical methods. This article aims to provide a basic overview of the biochemical principles underlying these technologies and their advantages and limitations in the determination of chemical contaminants, residues and additives in food matrices. The last part discusses some of our initiatives in this field that have resulted in commercially available rapid kits after transferring the corresponding technology to the industrial sector.

Keywords: Immunoassay; Rapid methods; ELISA; Immunoreactive strip; Hapten; Antibody; Food quality and safety; Contaminant; Residue.

Immunochemical methods encompass a set of analytical techniques in which the substance to be

detected (analyte) is identified and quantified through its molecular recognition by an antibody capable of binding to it with high affinity and specificity. Due to a number of characteristics, including simplicity, speed, sensitivity and portability, methods based on the use of antibodies as biomolecular receptors constitute a well-established set of techniques with applications in various fields, including the detection and quantification of different types of contaminants in food. Antibodies can be used to detect proteins and pathogenic or altering microorganisms, as well as to analyze low molecular weight substances, such as antibiotics, hormones, mycotoxins and pesticides, analytes on which our research group has focused its scientific activity in the last fifteen years. The great versatility of immunoanalytical techniques for the detection of xenobiotics and biotoxins is evidenced by the variety of assay formats that can be adopted to cover different analytical needs, such as (i) immunoaffinity chromatography, for the purification and concentration of an analyte in the sample prior to its determination by instrumental methods, mainly chromatographic; (ii) lateral flow immunoassays or immunoreactive strips, when what is needed is a simple and semiquantitative analysis that can be performed in any environment; (iii) biosensors, when automation is a priority; (iv) microarrays and systems based on detection by flow cytometry, such as Luminex, in cases where there is a need to determine several analytes simultaneously; and (v) enzyme-linked immunoassays or ELISA (Enzyme-Linked ImmunoSorbent Assay), undoubtedly the most widespread system due to its capacity to analyze a large number of samples quantitatively and affordably in a short time.

Compared to chromatographic methods, which are rightly considered the reference methodology for the analysis of organic molecules in the field of food safety, immunoanalytical techniques have some advantages, but also some drawbacks (see Figure 1). While an in-depth discussion of the pros and cons of the two methodologies can be very challenging, there is no doubt that the most significant discrepancies center on multi-residue capability and portability. Separative instrumental techniques are capable of simultaneously determining the presence of several compounds in a sample, and this is one of their great virtues. In contrast, immunochemical methods present an important limitation in this aspect, given the extraordinary specificity inherent to the antigen-antibody interaction, so that they can commonly only determine one analyte, or a few if they are structurally related. It could be argued that, while chromatographic methods are particularly well suited for analyzing a large number of compounds in a limited number of samples, antibody-based techniques are particularly well suited for analyzing a limited number of substances in a large number of samples. In terms of portability, chromatographic methods employ sophisticated equipment that must be operated by highly qualified personnel and in technically well-equipped environments. In contrast, immunoanalytical techniques, especially ELISA assays and immunochromatographic strips, require minimal equipment for their performance, which makes them ideal procedures for carrying out on-site analyses and thus obtaining practically immediate results wherever they are required.

As a consequence of their characteristics and conditioning factors, immunochemical methods are particularly suitable for a number of applications, including:

1. Food crises and food scares. The presence in a food of a contaminant or residue that is not permitted, or above the levels established in the legislation, usually triggers a temporary strengthening of controls directed towards the food-residue combination responsible for the alarm. In situations of this nature, immunoanalytical techniques allow rapid screening of a large number of samples in a short time.

Figure 1. Qualitative comparison of the analytical performance of chromatographic methods and

immunoanalytical techniques.

Sensibilidad	✓	✓
Selectividad	✓	✓
Rapidez	≈	✓
Robustez	✓	✓
Sencillez	≈	✓
Coste	≈	≈
Cuantificación	✓	✓
Capacidad	≈	✓
Portabilidad	X	✓
Multirresiduo	✓	X

Sensitivity
Selectivity
Speed
Robustness
Simplicity
Cost
Quantification
Capacity
Portability
Multiresiduo

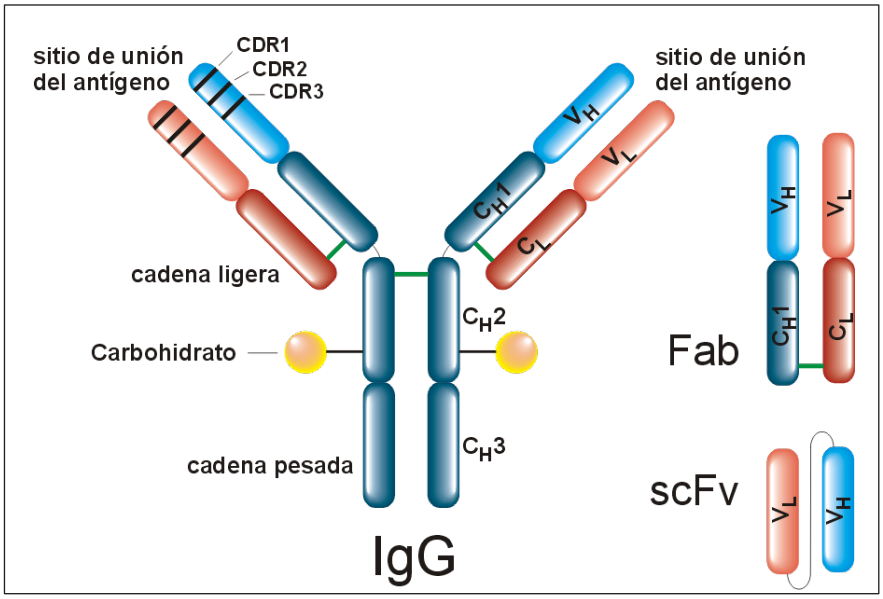
Source: own elaboration

2. Quality control departments. Agri-food companies, both fresh and processed products, often need to perform targeted analyses on certain substances to ensure that a certain process has been carried out correctly and to avoid the considerable economic and corporate image losses that the presence of a particular contaminant could entail, a situation in which rapid screening methods can be advantageous in economic and logistical terms.
3. Transfer studies during processing. While some chemical compounds, due to their structure and properties, are hardly transferred from the raw material to the final product (juices, wines, jams, etc.), others, on the contrary, do not suffer any reduction or even their concentration is higher in the processed product. Immunochemical methods can contribute to a better understanding of these processes and how they affect the sanitary quality of the food.

ANTIBODIES AS BIORECEPTORS IN IMMUNODETECTION

The fundamental component of any immunoanalytical method is the antibody, since its recognition properties (affinity and specificity) will largely condition the analytical performance of the method developed. Antibodies are glycoproteins produced by the immune system in response to exposure to a foreign element, such as bacteria or virus. The basic structure of antibodies, also called immunoglobulins, consists of two identical 25 kDa light polypeptide chains (L) and two identical 50 kDa heavy polypeptide chains (Figure 2). The heavy chains are linked to each other by disulfide bridges, and each is in turn linked to a light chain, also by disulfide bridges. Both the heavy and light chains are organized into variable and constant regions. Each antibody molecule has two antigen binding sites, and each binding site is formed by the association of the variable regions of a heavy chain and a light chain, located at the amino-terminal end. Depending on the type of heavy chain, immunoglobulins are classified into different classes and subclasses, with the IgG type being the most abundant.

Figure 2. Structure of an IgG immunoglobulin and two of the most common fragments: the Fab fragment, obtained by enzymatic digestion of the antibody, and the scFv fragment, obtained by genetic engineering.



antigen binding site
light chain
Carbohydrate
heavy chain

Source: own elaboration

In the context of antibody generation for analytical, biomedical or biotechnological purposes, antibodies can be classified into three types according to their origin: polyclonal, monoclonal or recombinant. Polyclonal antibodies are obtained from the blood serum of the immunized animal, and constitute a complex and heterogeneous mixture of all the types of antibodies generated against the same immunogen, each of them coming from a different B lymphocyte clone and therefore with a different affinity and specificity. Therefore, their characteristics represent an average of the animal's immune response, which can be useful to evaluate the suitability of the immunogen used for antibody generation. They are relatively simple, inexpensive and quick to obtain, but their main drawback is their limited nature and poor reproducibility due to the variability inherent in the immunization process.

Monoclonal antibodies are immunoglobulins obtained in vitro by the generation and culture of special cells called hybridomas, which are derived from the fusion of a B lymphocyte with a myeloma cell. Since every individual lymphocyte produces only one type of immunoglobulin, all antibody molecules obtained from a hybridoma line will be identical and have the same binding properties. Consequently, hybridoma technology guarantees an unlimited and reproducible source of antibodies with constant characteristics. They are more complex and costly to generate, but are more valuable from a biotechnological point of view.

Finally, recombinant antibodies are obtained by molecular biology techniques, either from synthetic or semi-synthetic gene libraries, or from antibody-coding genes from pre-established hybridoma lines. Subsequent cloning and expression of these genes in other organisms results in antibodies with well-defined properties whose binding site can be modified by mutagenesis. However, the use of recombinant antibodies for the immunodetection of substances of interest in food is still token from a commercial point of view.

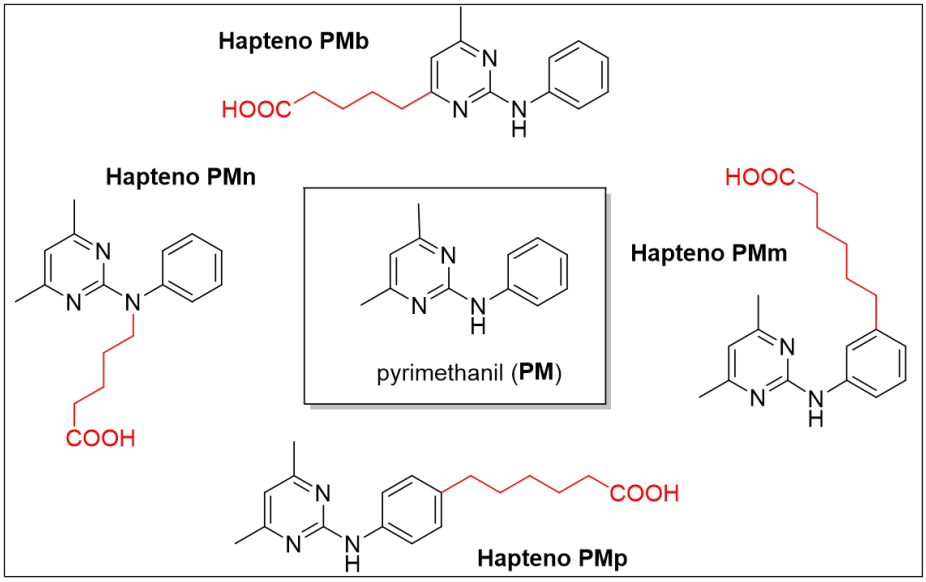
The immune system is specially adapted to generate antibodies against potentially pathogenic antigens of large size, such as viruses and bacteria, or exogenous proteins. However, low molecular weight organic compounds, such as drugs, pesticides or mycotoxins,

are not capable of inducing the generation of antibodies by themselves, although they can be recognized by them; in other words, they are not immunogenic but antigenic substances. Karl Landsteiner, Nobel laureate in Physiology or Medicine in 1930 and considered one of the fathers of modern immunochemistry, baptized this type of substances with the name of haptens (Landsteiner and Simms, 1923). Thus, one of the difficulties in developing immunoanalytical methods for contaminants and chemical residues potentially present in food lies in the fact that their small size and low structural complexity make it very difficult to generate antibodies, an essential biomolecule for detection. The key to solving this apparent paradox lies in the fact that haptens can become immunogenic if they are covalently linked to a protein to give rise to what is called a protein-hapten conjugate. In this way, the hapten becomes part of the epitopes of that protein, which is capable of inducing the generation of antibodies after a vaccination or immunization process. However, it should be noted that in most cases in which it is desired to generate antibodies against a small molecule, direct covalent binding to a protein is not possible due to the lack of a reactive group that can be used for this purpose. It is therefore necessary to previously synthesize an analog of the target compound that mimics it as closely as possible and that incorporates a functional group that makes conjugation possible. From the immunological point of view, these synthetic derivatives are also haptens. Broadly speaking, a functionalized hapten consists of three basic elements: a main structure similar to the analyte of interest, a functional group for subsequent covalent binding to the protein, and a third element in between called the spacer arm. It should be noted that the synthetic derivative must have a structure as similar as possible to the analyte, preserving its main structural elements, conformation and electronic distribution (Mercader, Agulló, Abad-Somovilla and Abad-Fuentes, 2011). In this way, the antibodies generated towards the synthetic hapten will also recognize the analyte with high affinity and specificity. The purpose of the spacer arm is to improve the exposure of the molecule to the immune system by separating it from the carrier protein, facilitating its interaction with antibodies and other cellular receptors and avoiding partial or total masking of the hapten by excessive proximity to the protein. Typically, the spacer consists of a fully saturated linear hydrocarbon chain with the functional group at its end, such as a carboxyl group, which enables coupling to the protein through its reaction with the free amino groups of the basic amino acids, mainly lysines, by forming an amide bond. It is important that the spacer arm does not contain strongly immunogenic elements, such as aromatic rings, conjugated double bonds or heteroatoms, nor that it is excessively polar, which could change the electronic distribution of the molecule and divert the immune response towards undesired elements that are not present in the structure of the analyte (Vallejo, Bogus and Mumma, 1982). As for its length, it has been experimentally demonstrated that a chain of 4-6 carbon atoms is generally adequate to favor the exposure of the hapten to the immune system, resulting in the production of antibodies with the desired affinity and specificity towards the target compound.

Thus, although in any immunoanalytical method the antibody is a key reagent, in the case where the substance to be detected is a low molecular weight compound, the synthesis of haptens is considered a critical step because of its enormous implications on the affinity and specificity of the antibodies generated. Moreover, the introduction of the functional group at the desired position is often only possible by total synthesis strategies that require considerable experimental effort and a solid background in synthetic organic chemistry (Sanvicens, Pichon, Hennion and Marco, 2003). Even with experience in this area, and despite the advances that have been made in recent years in molecular modeling techniques that allow a more rational design of the most appropriate structures with a view to generating

antibodies with the desired characteristics, it is still difficult to predict which of all the viable alternatives will be the most suitable. Consequently, a common practice in our group and among some of the most active research groups in this area is to synthesize different derivatives of the analyte in which the functional group is introduced in alternative positions of the molecule's skeleton, thus maximizing the probability of success when presenting the molecule to the immune system through complementary approaches (see Figure 3) (López-Puertollano, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2018; Parra, Mercader, Agulló, Abad-Somovilla, & AbadFuentes, 2012; Suárez-Pantaleón, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011).

Figure 3. Structure of the pyrimethanil fungicide and different haptens synthesized for antibody generation. The spacer arm and functional group, located in alternative positions of the molecule, are shown in red.



pyrimethanil (PM)
Hapteno PMb

Source: own elaboration

The proteins most frequently used for covalent hapten conjugation are albumins and hemocyanins, with bovine serum albumin (BSA) being one of the most commonly used in the preparation of immunization conjugates due to its high tolerance to high hapten loads without loss of solubility, as well as its immunogenicity, physical and chemical stability, availability and low cost. In addition, this protein is particularly suitable for quantifying the number of hapten-coupled molecules (hapten density), and has a reasonable tolerance to organic solvents such as DMSO and DMF, in which haptens are normally soluble.

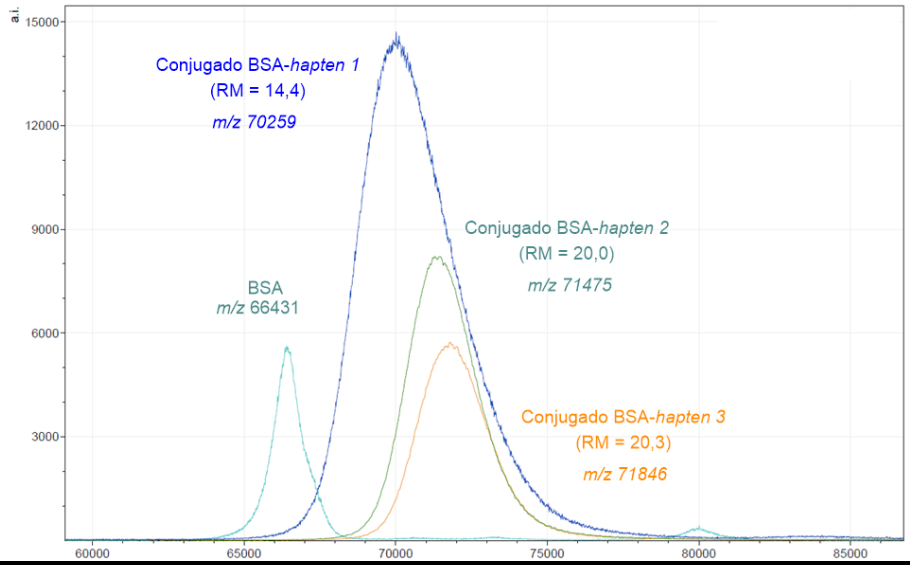
The nature of the functional group of the hapten determines the specific chemistry for conjugation. In the case of haptens with a carboxyl-terminal group, probably the most commonly used functional group, a prior activation step is required so that the reaction with the free amino groups of the protein can be carried out under mild conditions that do not affect its structural integrity. The most commonly used procedures for this purpose are the active ester method and the mixed anhydride method (Montalbetti and Falque, 2005). Once the carboxyl group has been activated, the hapten is reacted with the protein in a slightly basic medium, so that the amino groups of the protein are partially deprotonated and coupling with the carbonyl group of the activated chemical species is facilitated. A very remarkable feature of the active ester method is the

possibility of purifying the N-hydroxysuccinimidyl derivative resulting from the activation, an approach in which our group has pioneered (Esteve-Turrillas et al., 2010). Although these are rather unstable derivatives, their manipulation under anhydrous conditions is perfectly feasible, and undoubtedly the use of purified and perfectly characterized derivatives avoids possible undesired side reactions and facilitates a precise control of the ratio of hapten molecules conjugated per protein molecule, a parameter known as hapten/protein molar ratio (MR). Advances in mass spectrometry techniques, such as MALDI-TOF, make it possible to determine with excellent accuracy small mass differences in proteins, making it possible to calculate the hapten density per protein unit in conjugates (see Figure 4) (Esteve-Turrillas, Mercader et al., 2015; Ramón-Azcón, Sánchez-Baeza, Sanvicens, & Marco, 2009). An optimal immunizing conjugate should have a moderately high haptenic load. In the case of BSA, values between 10 and 20 hapten molecules per protein molecule are considered adequate for the generation of a good immune response and therefore for the production of antibodies.

COMMON IMMUNOCHEMICAL METHODS FOR FOOD CONTAMINANT ANALYSIS

The size of the haptens not only determines the procedure to be followed to obtain antibodies for this type of molecule, but also has a major influence on the configuration of the immunoassay. The immunoassay of proteins and microorganisms is usually performed by what is known as sandwich immunoassay, since due to their large size these antigens have several epitopes that allow them to bind to several antibody molecules simultaneously. However, low molecular weight compounds have only one epitope, so they can only interact with one antibody molecule. This circumstance determines that immunoassays for haptens are of the competitive type (González-Techera, Varell, Last, Hammock and González-Sapienza, 2007).

FIGURE 4. MALDI-TOF spectra of BSA and different conjugates with orthophenylphenol haptens.



BSA-hapten conjugate

Source: own elaboration

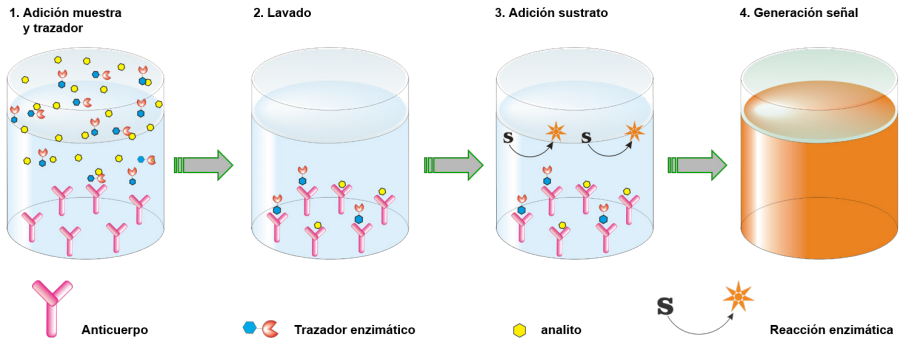
In this type of immunoassay, the analyte and a labeled form of the analyte compete for the binding sites of a limiting amount of antibody, so that the more free analyte in the sample, the more antibody will bind to it and less to the labeled derivative, thus generating less signal; conversely, in the absence of analyte, the maximum possible assay signal will be generated. Therefore, in a competitive immunoassay, the signal obtained will be inversely proportional to the concentration of analyte.

ELISA method

There are two basic competitive ELISA formats, the immobilized antibody format, also known as direct, and the immobilized conjugate format, known as indirect. In the direct format (Figure 5), the wells of a polystyrene microplate are coated with the specific antibody. Next, the sample containing the analyte and a predetermined concentration of the hapten covalently coupled to an enzyme, referred to as the enzyme tracer, are added. At this point a competition takes place, as the antibody can bind to the analyte or it can bind to the tracer. Depending on the concentration of analyte in the sample and the affinity of the antibody, the fraction of antibody bound to each of the two species will be different. After a washing step a substrate is added, which will be transformed by the enzyme into a product that will generate a signal.

As can be seen in Figure 6, when the signal is plotted against analyte concentration on a logarithmic scale, a decreasing sigmoid curve is obtained. The most important parameters of an inhibition curve are the maximum and minimum signal values, the slope, and especially the analyte concentration at the inflection point of the curve, called IC_{50}, which is a good estimate of the affinity constant of the antibody. Obviously, the lower the IC_{50} value, the more sensitive such an immunoassay will be. Other important parameters are the concentration of analyte that generates a signal equal to 90% of the maximum signal (IC_{10}), which is usually adopted as the limit of detection (LOD) of the assay, and the concentration that generates a signal equal to 80% of the maximum signal (IC_{20}), which is considered the limit of quantification (LOQ).

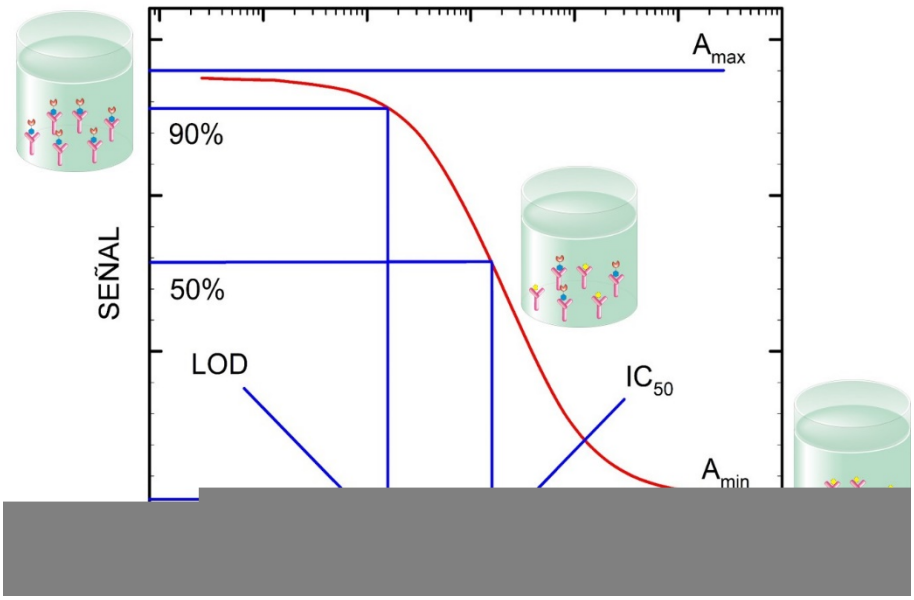
Figure 5. Schematic of a competitive ELISA in direct format.



1. Sample addition
2. Washing
3. Substrate addition
Signal and tracer generation
Antibody
Enzymatic tracer
analyte
Enzymatic reaction

Source: own elaboration

Figure 6. Typical inhibition curve of a competitive immunoassay.



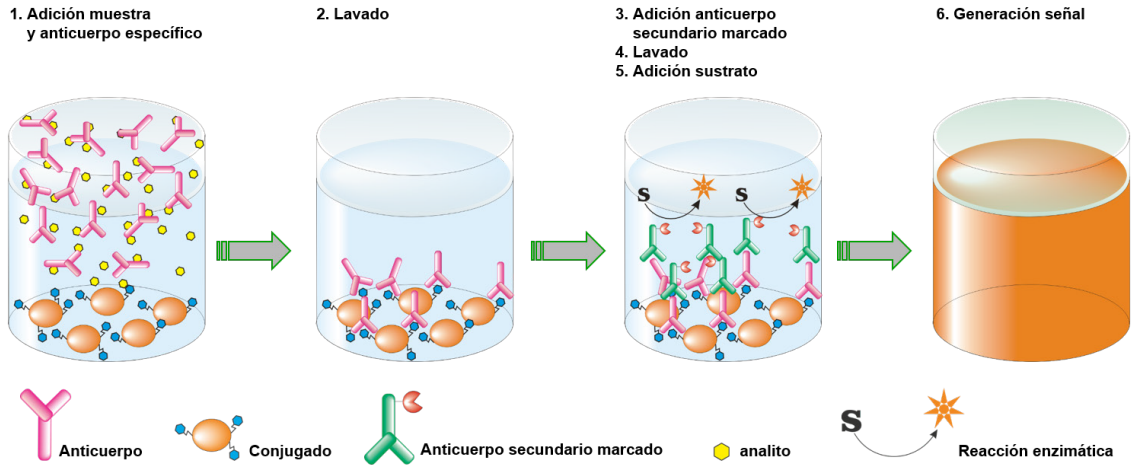
Source: own elaboration

Probably the most commonly used enzyme for tracer preparation is horseradish peroxidase (HRP). In the presence of H2O2, HRP promotes the oxidation of certain substrates to give rise to products with easily detectable optical properties, the most common being TMB (3,3',5,5'-tetramethylbenzidine) and OPD (o-phenylene diamine) among the chromogenic ones, and luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) among the chemiluminescent ones.

As for the indirect format (Figure 7), it is a non-enzymatic protein-haptene conjugate that is immobilized on the microplate. By adding the sample and a solution with a predetermined concentration of the specific antibody, a competition is also established,

since now the antibody in solution can bind to the immobilized conjugate or can bind to the analyte if present in the sample. After a washing step to remove excess reagents, an enzymatically labeled antibody that is able to recognize the primary antibody is added. The plate is washed again and the substrate is added, generating the signal, which as with any competitive ELISA will also be inversely proportional to the analyte concentration. The assay conjugate used in this format is essentially identical to the one used to generate the antibody, although the protein used is usually different (usually ovalbumin instead of BSA) and the degree of labeling is usually lower to enhance competition and sensitivity of the assay.

Figure 7. Schematic of a competitive ELISA in indirect format.



1. Addition of sample and specific antibody
2. Washing
3. Addition of labeled secondary antibody
4. Washing
5. Substrate addition
6. Signal generation
Antibody
Conjugate
Labeled secondary antibody
analyte
Enzymatic reaction

Source: own elaboration

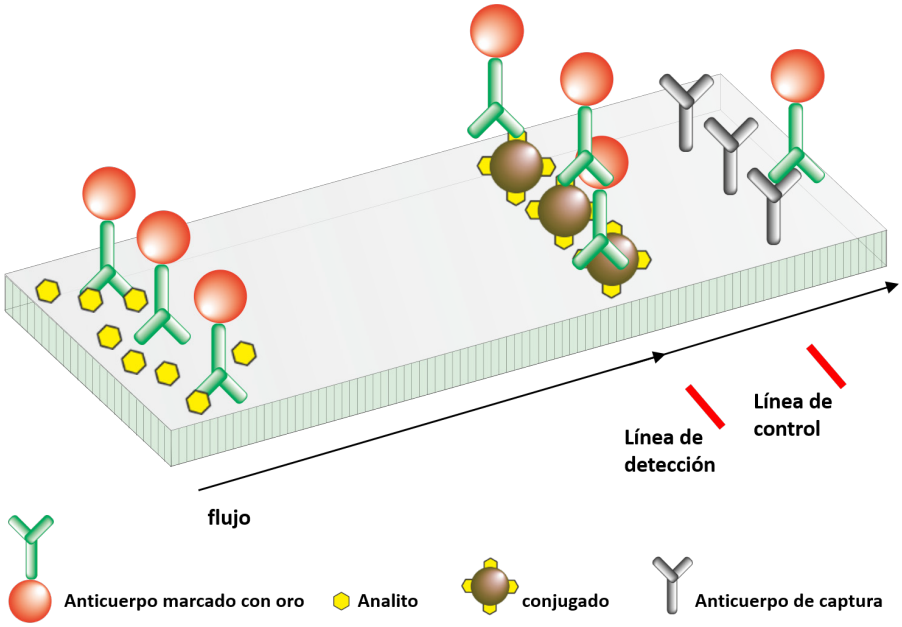
Lateral flow immunochromatography

Another widely used immunoanalytical method for the detection of xenobiotics and biotoxins in food is the lateral flow immunoassay or immunoreactive strips, the best known example of which comes from the field of clinical chemistry, the pregnancy test. In this system (see Figure 8) the polystyrene microplate typical of an ELISA is replaced by a nitrocellulose membrane on which a thin line of protein-haptene conjugate (test line) has been deposited. When the strip is placed in contact with the sample and with a previously established amount of specific labeled antibody, the solution begins to migrate by capillary action towards the area where the conjugate is located. There are many alternative ways of labeling the antibody, but colloidal gold nanoparticles are undoubtedly the most commonly used solution. Upon reaching the test line, antibody molecules that have not interacted with the analyte will bind to the conjugate immobilized on the membrane, generating a clearly visible signal in the form of a red band. In contrast, antibody molecules that have bound to the analyte will pass by.

A few millimeters above the test line is another line formed by immunoglobulins capable of recognizing the labeled antibody regardless of whether it is free or bound to the analyte (control line), so that the specific antibody that has not been retained in the test line will be retained in this zone. Logically, the amount of labeled antibody that remains bound to each line, and therefore the signal intensity of each of these two bands, will depend on the concentration of analyte in the sample being analyzed, which can be estimated from the ratio of signals in the test and control lines.

The great advantage of this immunoanalytical system is that it does not require any special equipment for sample analysis, so the assay can be performed in any environment, with the analytical advantage that this entails. If quantitative measurements are required, there are small, truly portable devices for reading the strips, or even cell phone applications based on the processing of the captured image. In addition, the strips can be stored after the assay, ensuring the traceability of the results.

Figure 8. Simplified scheme of a competitive immunochromatographic strip.



Gold-labeled antibody
Analyte
conjugated
Capture antibody
flow
Detection line
Control line

Source: own elaboration

DEVELOPMENT OF IMMUNOASSAYS AND THEIR VALIDATION IN FOOD SAMPLES

The stage prior to the development of immunoassays consists of the characterization of the antibodies against the homologous conjugate (that which contains the same hapten as that of the immunogen with which the antibody was obtained) and possible heterologues. In this way, the affinity of the antibody is studied, as well as its specificity using molecules

analogous to the analyte -commercial or synthesized in the laboratory- that allow studying the interaction between the antibody and the ligand (López-Moreno, Mercader, Agulló, Abad-Somovilla and Abad-Fuentes, 2014; Suárez-Pantaleón, Mercader, Agulló, Abad-Somovilla and Abad-Fuentes, 2008).

From the characterization data, antibody-conjugate pairs that provide adequate signal in the absence of analyte and higher sensitivity are selected. The development of immunoassays begins with the optimization of the concentration of each immunoreagent and the assay conditions (time, temperature, volume, etc.), and continues with the determination of the analytical parameters (A_{max}, IC_{50}, LOD, precision, accuracy, etc.). Next, it is necessary to study the selectivity of the assay against molecules that may potentially be present in the sample and that can be recognized by the antibody or interfere with the recognition. In addition, the influence of the pH and ionic strength of the assay buffer on the analytical parameters must be determined. Finally, it is also convenient to evaluate the tolerance to different organic solvents, mainly acetonitrile, methanol and ethanol, since they are the most frequently found in food samples, either because they are used for the extraction of the analyte or because they are part of them (Abad-Fuentes, Esteve-Turrillas, Agulló, Abad-Somovilla and Mercader, 2012; Esteve-Turrillas, Mercader, Agulló, Abad-Somovilla and Abad-Fuentes, 2015).

The final stage in the development of any immunoanalytical method is the characterization of its performance by analyzing real samples. The procedure to be followed does not differ essentially from that used with any other analytical method, and basically consists of establishing the most appropriate protocol for sample extraction and clean-up (in the case of solid matrices); determining the interferences of the food matrix, if any; performing recovery studies with samples fortified at different levels; and finally applying the assay to the determination of naturally contaminated foods and validating the results obtained by comparison with another analytical method (Mercader, López-Moreno, Esteve-Turrillas, Abad-Somovilla and Abad-Fuentes, 2014).

Over the last years, our research group has produced antibodies, polyclonal and monoclonal, and developed immunoassays for a wide range of compounds relevant to food quality and safety, among which are included:

1. Five strobilurin family fungicides: azoxystrobin, pyraclostrobin, kresoxim-methyl, picoxystrobin and trifloxystrobin.
2. Three fungicides of the anilinopyrimidine family: pyrimethanil, cyprodinil and mepanipyrim.
3. Four fungal succinate dehydrogenase inhibitors: fluopyram, penthiopyrad, fluxapyroxad and boscalid.
4. Seven fungicides from different families: orthophenylphenol, imazalil, fenhexamid, proquinazid, fludioxonil, quinoxyfen and fluopicolide.
5. Two insecticides: spirotetramat and imidacloprid.
6. A herbicide: dicamba.
7. An antibiotic: chloramphenicol
8. Two hormones: forchlorfenuron and melatonin.
9. Six mycotoxins: aflatoxin M1, aflatoxin B1, zearalenone, alternariol, ochratoxin A and patulin.
10. A cyanotoxin: anatoxin-a.

Tables 1, 2, 3 and 4 show the most relevant information about the immunoassays developed by our group that have been applied in different food matrices, and compare their LOQs with the maximum residue limits (MRLs) authorized in the European Union.

Table 1. Examples of validated immunoassays for the analysis of strobilurinic fungicides in food.

Analyte	Antibody	Format	Sample	LOQ (µg/L)	MRL[a] (µg/L)
Azoxystrobin	mAb AZo#49	Indirect ELISA heterologous	Tomato juice	3	3000
			Peach juice	2	2000

			Red grape juice	2	2000
			Banana juice	5	2000
			Strawberries	4	50000
Pyraclostrobin	mAb PYs#11	Direct ELISA counterpart	Strawberry jam	50	100b
			Raspberry jam	50	3000
			Blueberry jam	50	4000
			Apricot jam	50	1000
			Peach jam	50	300
Picoxystrobin	mAb PCa#21	Indirect ELISA heterologous	Wheat flour	25	50
			Oat flour	50	300
			Rye flour	25	300
	mAb PCa#13	Indirect ELISA counterpart	Lager Beer	100	300
			Non-alcoholic lager	100	300
			Black Ale	100	300
			White ale beer	100	300
Kresoxim-methyl	mAb KMo#117	Indirect ELISA counterpart	Tomatoes	10	600
			Cucumbers	10	50[b]
			Strawberries	10	50[b]
Trifloxystrobin	mAb TF0#17	Indirect ELISA heterologous	Tomatoes	10	700
			Cucumbers	10	300
			Strawberries	10	50[b]

a The values are the latest values published by the EU for the corresponding original sample.

b Indicates the lower limit of the analytical method. Source: own elaboration

Table 2. Examples of validated immunoassays for the analysis of anilinopyrimidine fungicides in food.

Analyte	Antibody	Format	Sample	LOQ(µg/L)	MRL[a] (µg/L)
Pyrimethanil	pAb PMp#1	Direct ELISA counterpart	Carrot juice	40	1000
Cyprodinil	mAb PMm#31	Indirect ELISA heterologous	Strawberries	50	50[b]
			Tomatoes	5	1000
			Cucumbers	5	700
	Direct ELISA	Direct ELISA counterpart	Apple juice	20	1500
			Red grape juice	20	3000
			White wine	1	3000
			Red wine	5	3000
			Sparkling wine	1	3000
			Cider	1	1500
	mAb CDm#21	Heterologous direct ELISA	Strawberries	10	100[b]

a The values are the latest values published by the EU for the corresponding original sample.

b Indicates the lower limit of the analytical method. Source: own elaboration

Table 3. Examples of validated immunoassays for the analysis of fungicides from different families in food.

Analyte	Antibody	Format	Sample	LOQ(µg/L)	MRL[a] (µg/L)
Boscalid	pAb BLa#1	Direct ELISA counterpart	Red grape juice	10	5000
			Peach juice	50	3000
			Apple juice	10	2000
			Tomato juice	10	3000
Fludioxonil	pAb BLb#2	Indirect ELISA heterologous	Tomatoes	5	3000
			Cucumbers	5	3000
	mAb FDn#23	Homologous indirect ELISA	Apple juice	5	5000
			Red grape juice	10	4000
Fenhexamid	mAb FHo#27	ELISA direct homologous	Red grape juice	75	15000
			White grape juice	30	15000
			Red wine	75	15000
			White wine	75	15000
			Green kiwis	10	15000
Fluopyram	mAb FPb#12	ELISA direct homologous	Strawberries	25	50[b]
			Grapes	5	1500
			Wines	10	1500
			Plums	5	500

a The values are the latest values published by the EU for the corresponding original sample.

b Indicates the lower limit of the analytical method. Source: own elaboration

APPLICATIONS IN THE AGRI-FOOD INDUSTRY

A large number of companies worldwide focus their activity on the immunodiagnosis of substances of interest to the agri-food industry (Abraxis, Neogen, Envirologix, Zeulab, Tecna, Romer Labs, Unisensor, Prognosis Biotech, Randox, r-Biopharm, Vicam, Europroxima, Charm Sciences, Bioo Scientific, etc.), which gives an idea of the economic importance of this sector. In this sense, our group has participated in some initiatives that have finally led to immunoanalytical methods commercially available through different companies.

Determination of fungicides in fruit post-harvest

Because of their propensity to be infected by fungal pathogens, fruits are sent immediately after harvesting to fruit and vegetable processing plants, where they are treated with fungicides to prevent spoilage. These treatments must be carried out in accordance with current legislation so as not to exceed maximum residue limits, while at the same time being effective. Thus, if concentrations lower than those recommended are applied, there will be an increase in rotting at destination, which can lead to significant economic losses. If, on the other hand, the fruit is treated with an excess of fungicide, the residue in the fruit will increase and the legal limits may be exceeded.

Table 4. Examples of validated immunoassays for the analysis of various biotoxins in food.

Analyte	Antibody	Format	Sample	LOQ(µg/L)	MRLa(µg/L)
Ochratoxin A	mAb OTAf#223	Direct ELISA heterologous	White wine	0.5	2
			Red wine	0.5	2
Aflatoxin M1	Anatoxin-a	Heterologous indirect ELISA	Buffer	0.04[b]	0.05[c]
Anatoxin-a	mAb ANm#38	ELISA heterologous capture	Waters environmental	0.25	0.1-20[d]

a The values are the latest values published by the EU for the corresponding original sample.

b IC_{50} value in buffer.

c Maximum tolerable limit.

d The maximum regulated value depends on the country. Source: own elaboration

The usual way for a warehouse to check that the concentrations in the treatment broths and in the final fruit are adequate is to send samples to accredited laboratories. These analyses are carried out using chromatographic techniques, and can take days or even weeks in the case of countries with limited analytical infrastructures. In short, fruit and vegetable plants lack quick tools that allow them to effectively control a critical process, such as the treatments they carry out.

In view of this situation, and in collaboration with the company Productos Citrosol, we decided to develop an immunoanalytical system that would make it possible to determine in the fruit and vegetable plants themselves the three most commonly used fungicides in citrus post-harvest, i.e., imazalil, orthophenylphenol and pyrimethanil. The key features of the system are its speed (results are obtained in thirty minutes), its simplicity (no specialized training is required), its cost (less than €10 per sample) and above all its portability (analyses are performed in the warehouse itself, since no sophisticated equipment is required). The system, based on ELISA kits, provides results comparable to those obtained in accredited laboratories, enabling quality systems in processing warehouses to be considerably improved. The system represents an important innovation for the post-harvest sector, in that it allows corrective measures to be taken in real time, thus avoiding unexpected and undesirable situations. Since the beginning of 2018, Productos Citrosol has been marketing these tests exclusively worldwide under the name Easy Kit, with a level of acceptance by the sector that is exceeding the company's initial forecasts.

Chloramphenicol and aflatoxin M1 analysis in milk

One of the fields where immunoanalytical methods are most widely used is in the determination of antibiotics and mycotoxins in food. Antibiotics are substances widely used in veterinary practice for the treatment of diseases and microbial infections.

Excessive use of antibiotics can lead to the presence of their residues in milk, which is not only a public health problem due to the appearance of allergic reactions and resistant bacteria, but also an industrial problem due to the problems it generates for the dairy derivatives industry. Chloramphenicol is a broad-spectrum antibiotic banned in the European Union, the United States and other countries for veterinary use in animals intended for human consumption. However, due to its high efficacy and low cost, this drug continues to be used fraudulently, especially in countries with laxer legislation and less rigorous food safety systems, and many food products with chloramphenicol residues are often destined for Europe.

In collaboration with the immunodiagnostic company Zeulab, we have generated monoclonal antibodies with an extraordinary affinity for chloramphenicol. These antibodies, and the conjugates prepared to obtain them, have enabled the development of a rapid immunochromatographic test that makes it possible to detect the presence of chloramphenicol in milk and milk products in less than ten minutes and at levels below 0.3 µg/L, the most demanding permitted level in the legislation in force, and which corresponds to the European Union. This test is currently in the large-scale production phase with a view to its immediate commercialization. In this same line of collaboration, it should be noted that a system, also of the immunochromatographic type, is currently under development, which will allow the detection of aflatoxin M1 in milk at a level as analytically demanding as 0.05 µg/L, in accordance with the regulations promulgated by the European Union for this mycotoxin in dairy products.

Detection of anatoxin-a in waters

Cyanobacteria are photosynthetic prokaryotic organisms that under favorable conditions are capable of proliferating very rapidly. These massive proliferations are considered to be more frequent and intense than in the past due to human activity and global warming. Some of these cyanobacteria are capable of producing toxic metabolites known as cyanotoxins, which pose a serious threat to ecosystems and human health, as well as to the welfare of wildlife, livestock and domestic animals. The main routes of exposure to cyanotoxins are ingestion of contaminated water, consumption of fish and shellfish, and the presence of unwanted cyanobacterial species in dietary supplements.

Anatoxin-a is a cyanotoxin identified in the 1960s as the causative agent of cattle deaths in Canada. It is a very potent neurotoxin that binds irreversibly to the acetylcholine receptor, causing death by paralysis due to overexcitation of the nerve impulse. In some regions the problem is recurrent and incidents are recorded every year, such as the shutdown of drinking water supplies for human consumption, or the death of wildlife and domestic animals due to accidental consumption of contaminated water. Anatoxin-a is a very small molecule and structurally not very complex, so despite the efforts made during the last thirty years, it had not been possible to produce antibodies against it and consequently to develop immunoanalytical methods that would allow its detection quickly and easily. In 2015, our group was able to achieve the generation of monoclonal antibodies capable of recognizing anatoxin-a with high affinity and enantioselectivity. This development led to the filing of an international patent that was exclusively licensed to the US company Abraxis Inc (https://digital.csic.es/bitstream/10261/176373/1/ES2612751R1.pdf), who currently markets the only ELISA-type immunoassays and immunochromatographic strips available worldwide for the detection of this cyanotoxin. In addition, this company markets ELISA kits for the insecticide imidacloprid and for the fungicides azoxystrobin and pyraclostrobin, based on immunoreagents developed by our group.

Determination of ochratoxin A in wines

Ochratoxin A is a toxic secondary metabolite produced mainly by fungi of the genus Aspergillus that can contaminate agricultural products and processed foods throughout the food chain. The International Agency for Research on Cancer classifies ochratoxin A within group 2B of substances potentially carcinogenic to humans. This mycotoxin is found in various foods contaminated by toxigenic fungi, mainly cereals, but also in beverages such as coffee, beer and wine. In fact, ochratoxin A is the only mycotoxin for which the European Food Safety Authority (EFSA) has established maximum permitted levels in wines and musts, namely 2 μg/L. The available data suggest the need to monitor the presence of ochratoxin A in wine, not only for obvious food safety reasons, but also for quality reasons, since its presence in this matrix is an indicator of poor raw material selection. Recently, our group has generated what are probably the monoclonal antibodies with the highest affinity for this mycotoxin produced to date. This has been possible thanks to the use of innovative synthetic strategies not previously explored that have allowed the preparation of functionalized derivatives of the mycotoxin, leaving free, and therefore accessible, the carboxyl group that ochratoxin A possesses in its structure. With these immunoreagents we have developed an immunoassay capable of reliably determining the presence of ochratoxin A in wines at a concentration of 0.5 μg/L, a level four times lower than that established by European legislation. As an example, the analysis of a certified material (ERM-BD476) provided by the Federal Institute for Materials Research and Testing of Germany, consisting of a red wine naturally contaminated with 0.5 μg/L ochratoxin A, gave an ELISA value of 0.45 ± 0.07μg/L (n=7). These developments have been duly patented, and we are currently negotiating with food immunodiagnostic companies interested in their licensing and commercialization. We believe that, as has happened in the post-harvest sector mentioned above, access to an analytical tool of this type by wineries would allow them to control the quality of their wines and the raw materials, grapes and musts with which they are made, in situ, in a simple, fast and economical way.

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