DETERMINATION OF PERSISTENT ORGANIC POLLUTANTS IN COW'S MILK FROM NATIONAL PRODUCTION BY MEANS OF GAS CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY (GC/MS)

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Abstract

Persistent organic pollutants (POPs) are organic chemicals capable of persisting in the environment, being transported over long distances, bioaccumulating and biomagnifying in ecosystems. The harmful effects of these compounds on the environment and the health of living beings are of concern. In particular, humans can come into contact with POPs when consuming contaminated food of animal origin with a high fat content. In the Dominican Republic these compounds have been widely used and generated. However, the levels of POP exposure to which the population is exposed are unknown. Therefore, the objective of this study was to determine the presence of 34 POPs in five main brands of nationally produced cow's milk. The samples were

prepared using the QuEChERS extraction method and the analytical technique used was GC/MS. The results obtained indicated that no POPs were present in the samples.

The results obtained indicated that there is no presence of the POPs evaluated in any of the cow's milk samples, which suggests that their consumption does not represent a threat to the health of consumers. In addition, this study contributes to the knowledge on the evaluation of POPs in the Dominican Republic.

Keywords: contaminant; milk; Dominican Republic; chromatographic analysis; agricultural chemistry.

Introduction

Chemicals known as persistent organic pollutants (pops) are the focus of international attention because of growing scientific evidence that they can cause conditions such as cancer, damage to the central and peripheral nervous systems, immune system diseases, reproductive disorders, metabolic disorders and alterations in the normal development of infants and children (Secretariat of the Stockholm Convention, 2019). In addition, these compounds are capable of persisting in the environment, long-range transport through the atmosphere, bioaccumulate in human and animal tissues and biomagnify their concentrations in food chains (World Health Organization (WHO) Persistent organic pollutants (pops), 2014). Pops have been widely used in various human activities; as pesticides, in different pharmaceutical processes, and in the generation of chemical products and by-products in several industries. In particular, pops include different types of chemical species such as, organochlorine pesticides, polychlorinated dibenzo-dioxins (pcdds), polychlorinated dibenzo-furans (pcdfs) and polychlorinated biphenyls (pcbs) (Secretariat of the Stockholm Convention, 2017).

As a result, sustained exposure of many species, including humans, has been generated over periods of time spanning generations. In order to mitigate the adverse effects produced by these substances, a global treaty known as the Stockholm Convention was created in which the participating countries, including the Dominican Republic, agreed to eliminate or reduce the production, use and release of pops. Different activities, such as the use of organochlorine pesticides, waste burning and the use of electrical transformers and capacitors have contributed to the dispersion of pops in the country (Sbriz, Aquino, Rodriguez, Fowler, & Sericano, 1998; Secretaría de Estado de Medio Ambiente y Recursos Naturales (SEMARENA), 2008).

Specifically, human exposure to pops occurs mainly through contaminated foods, especially those of animal origin with a high fat content (Lee et al., 2006). Consequently, international organizations such as the United States Environmental Protection Agency (USEPA), the European Union (EU) and the Codex Alimentarius of the Food and Agriculture Organization of the United Nations (FAO) have created standards establishing maximum residue limits (mrls) of pops allowed in food (Codex Alimentarius of Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO), 2020; European Commission, 2020; United States Environmental Protection Agency (US EPA), 2020). Due to the need to study the adverse effects in humans produced by the consumption of food contaminated with such substances, several analytical techniques have been used for their detection and identification (Fernández-Alba & García-Reyes, 2008; Hernández, Grimalt, Pozo, & Sancho,

2009; Lacina, Urbanova, Poustka, & Hajslova, 2010; Polgár et al., 2012). However, the fast, easy, inexpensive, efficient, effective, robust and safe extraction method (quechers) and the analytical technique of gas chromatography coupled to mass spectrometry (GC/MS) are the most widely employed tools at present due to their outstanding advantages of flexibility, speed, economy, ease, efficiency and robustness, high sensitivity, accuracy and versatility (Chen, Lin, Dang, & Puschner, 2017; Jawaid, Talpur, Nizamani, Khaskheli, & Afridi, 2016; Rahman et al., 2018; Selvi, Paramasivam, Rajathi, & Chandrasekaran, 2012). In particular, abundant scientific studies evaluate the pops content in dairy products such as milk and even concentrations above internationally established mrls have been detected (Battu, Singh, & Kang, 2004; Chen et al., 2017; Dagnac, Garcia-Chao, Pulleiro, Garcia-Jares, & Llompart, 2009; Fromberg, Granby, Højgård, Fagt, & Larsen, 2011; Jawaid et al., 2016; Martins, Amaya Chávez, Waliszewski, Colín Cruz, & García Fabila, 2013; Rahman et al., 2018; Rawash et al., 2018; Selvi et al., 2012).

In contrast, in the Dominican Republic, the number of investigations on the presence of pops and their effects on the population are very scarce and old (Rodríguez, De Pratt, Peña, & Beltré, 2006; Sbriz et al., 1998; Subsecretaría de Estado de Recursos Naturales de la Secretaría de Estado de Agricultura (SEA-SURENA), 1999). It is believed that the release and use of these toxic substances has decreased over the years due to the implementation of regulations governing the management of pops. However, the real situation of pops in the country is unknown. For this reason, the objective of this study was to determine the presence of 34 pops in five brands of cow's milk produced nationally in order to obtain accurate and reliable information through the application of sensitive and effective analytical techniques on the degree of exposure to pops in foodstuffs in Dominican society.

Materials and methods

Reagents and samples

This research determined in the samples of Cow's milk the presence of the following pops: aldrin, chlordane, decabromobiphenyl, dichlorodiphenyldichloroethane (DDD), dichlorodiphenyldichloroethylene (DDE), dichlorodiphenyltrichloroethane (DDT), dieldrin, endosulfan, endrin, heptachlor, hexabromobiphenyl, hexabromocyclododecane (HBCD), hexachlorobenzene, hexachlorocyclohexane (HCH), lindane (gamma-HCH), mirex, toxaphene and the following PCB congeners: 2,3,3,3',4,5'-pentachlorobiphenyl (PCB 122), 2,2',3,3',4,4,4',5-heptachlorobiphenyl (PCB 170), 2,3',4,4',6-pentachlorobiphenyl (PCB 119), 3,3',4,4'-tetrachlorobiphenyl (PCB 77), 2,3,4,4',5-pentachlorobiphenol(PCB114),2,2',3, 4,4',5'

-hexachlorobiphenyl (PCB 138), 2,2',4,5,5'-pentachlorobiphenyl (PCB 101), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153), 2,2',5,5'-tetrachlorobiphenyl (PCB 52), 2,2',3,4,4,4'5,5'-heptachlorobiphenyl (PCB 180), 2,4,4'-trichlorobiphenyl (PCB 28), 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156), 2,3',4,4,4',5,5'-hexachlorobiphenyl(PCB167),2, 3,3',4,4'-pentachlorobiphenyl (PCB 105), 2,3,3',4,4,4'5,5'-heptachlorobiphenyl (PCB 189), 3,4,4',5-tetracyclobiphenyl (PCB 81) and 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169). The method created by the Association of Official Analytical Chemists (AOAC) called AOAC 2007.01 was used as the basis for the present experimentation (Association of Official Analytical Chemists, 2007). All

reagents and materials used were certified and of high purity, LC/MS grade, optimum and Fisher brand pesticide grade. In addition, five brands of cow's milk with a fat content of 3% (whole milk) produced in the Dominican Republic were selected as samples for the development of this study. The methodology was carried out through the following stages: sample collection, sample preparation and analytical technique.

During the months of May to July 2019, five different brands of nationally produced whole cow's milk marketed in the main supermarkets of the National District were selected. Three lots or productions of each whole milk brand were randomly selected. The analyses of each of the 15 lots were repeated three times, with a total of 45 samples (see Figure 1).

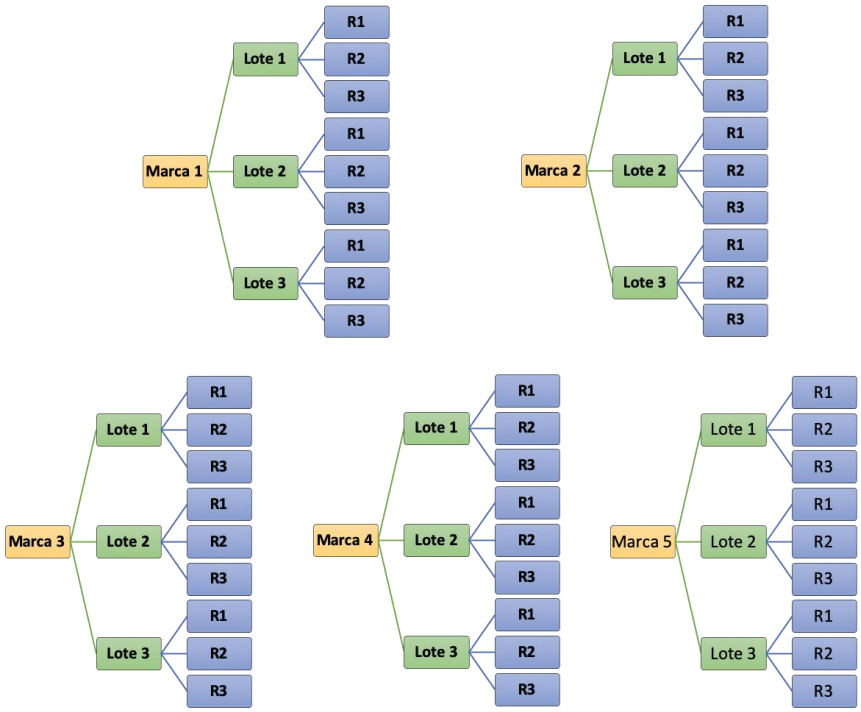


Figure 1. Schematic of the experimental design

Source: own elaboration.

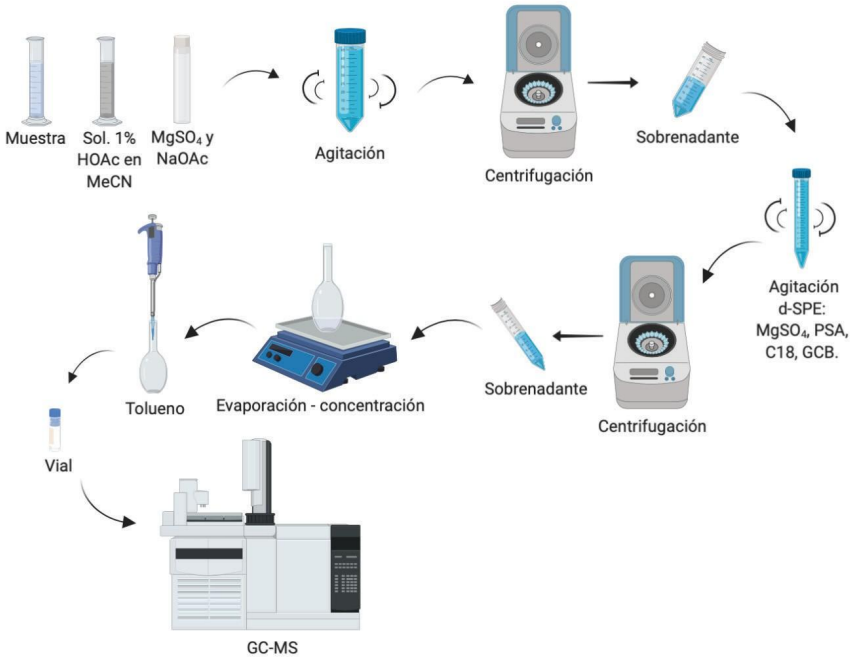
Sample preparation

Sample preparation was carried out in three parts: extraction, purification and concentration (see Figure 2). First, 15 ml of milk was poured into extraction tubes and 6 g of magnesium sulfate (mgso4) and 1.5 g of sodium acetate (naoac) were added, followed by the addition of 15 ml of a solution of acetonitrile (C2H3N) with 1 % acetic acid (CH3CO2H). It was shaken vigorously for one minute, and centrifuged at 3,900 rpm for 8 min.

Then, 8 ml of the supernatant was transferred to dispersive solid phase extraction (d-SPE) tubes containing 150 mg of magnesium sulfate (mgso4), 50 mg of ethylenediamine-N-propyl (primary/secondary amine, PSA), 50 mg of a C18 sorbent (octadecyl bonded silica) and 50 mg of graphitized activated carbon (GCB). It was then

shaken for one minute and centrifuged at 3,900 rpm for 8 min.

Subsequently, the separated upper phase was concentrated, and the solvent was exchanged for toluene (C6H5CH3). For this, the final extract was heated at a constant temperature of 60 °C until evaporation of the solvent and then 1 ml of toluene was added. Finally, that solution was transferred to 1.5 ml GC vials.



Agitation
Centrifugation
Toluene
Evaporation - concentration
Supernatant
Vial
Centrifugation
Sample

Figure 2. Workflow for sample preparation

Source: own elaboration.

Analytical technique

The determination of the presence of pops evaluated in the milk samples was carried out by the GC/MS technique which is the one recommended by the Codex Alimentarius international food standards, guidelines and codes of practice for the identification and confirmation of pops residues in food (Codex Committee on Pesticide Residues (CCPR), 2017). The equipment used was the Perkin Elmer brand Clarus SQ 8C GC/MS. 1 µL of the final extract was injected, in split mode, into the Perkin Elmer brand Elite 5MS column of film thickness 0.25 µm, inner diameter 0.25 mm, length 60 m and 1,4-bis(dimethylsiloxy)phenylene dimethylpolysiloxane phase. Hydrogen was used as a carrier gas and was maintained at a constant flow rate of 1.5 ml/min. The

temperature ramp of the column oven was started from 90 °C with an increase of 3 °C/min until 320 °C was reached. The injector was maintained at a constant temperature of 250 °C, for a total run time of 75 min.

The MS detector was used in full scan mode to evaluate the range of possible cops in GC/MS, selecting a mass range from 50 to 550 m/z, with an electron collision energy of 70 ev. The ionization source used was the electron impact source. The transfer line and ionization source were maintained at a temperature of 300 °C and 320 °C, respectively.

Reference standards

The compounds naphthalene-d8 and acenaphthene-d10 were used as internal standard with a concentration of 40 ppm.

For quality control purposes, a solution was prepared containing a mixture of external reference standards of the evaluated Sigma Aldrich pops with a purity between 95.8 % and 99.8 %, and these were studied under the same chromatographic conditions described above. In addition, they presented a limit of detection (LOD) of 0.010 ppm. Subsequently, the chromatograms and mass spectra obtained from these external standards were compared with those obtained from the samples.

Results and discussion

Sample selection

The selection of processed milk as a sample for the development of this study was satisfactory, because the usual treatments in milk for human consumption such as pasteurization, sterilization and UHT processing do not cause any appreciable effect on the content of POP residues in this matrix (Deiana & Fatichenti, 1992). In addition, evaporation and solvent exchange contributed to the reduction or elimination of interferences and concentration of target analytes.

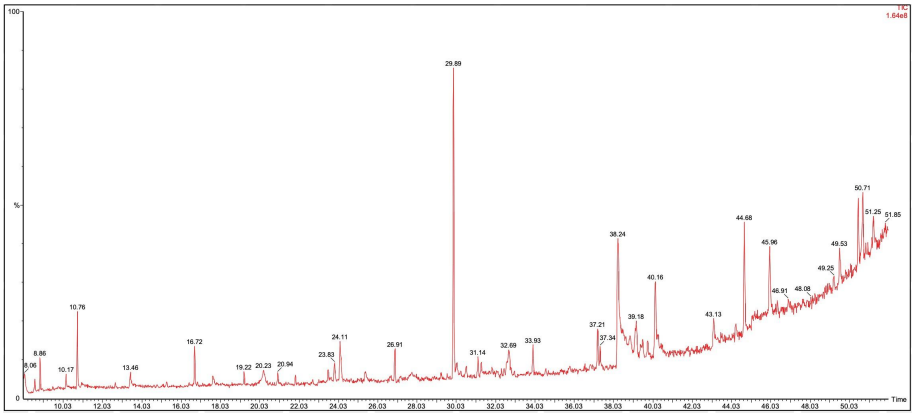


Figure 3. Chromatogram of a milk sample.

Source: own elaboration.

Quechers extraction method

The use of magnesium sulfate (mgso4) and sodium acetate (naoac) in the quechers extraction method were successfully used to remove the water present in the samples. Also, the use of ethylenediamine-N-propyl (primary/secondary amine, PSA) was successful in removing fatty acids, other organic acids and sugars, as well as the use of C18 sorbent (octadecyl bonded silica) in order to remove non-polar interferences contained in the samples.

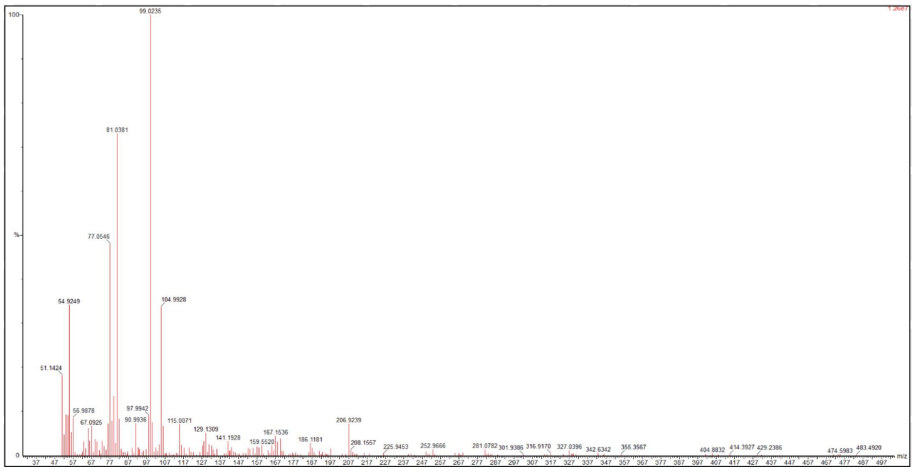
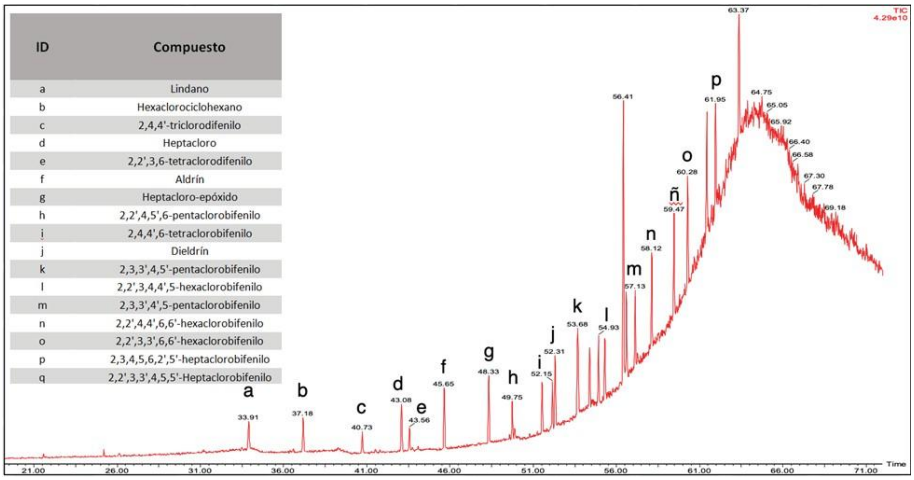


Figure 4. Mass spectrum of a component of a milk sample.

Source: own elaboration.

Determination of persistent organic pollutants in cow's milk of national production by means of gas chromatography coupled to mass spectrometry (GC/MS).



ID	Compuesto
a	Lindano
b	Hexaclorociclohexano
c	2,4,4'-triclorodifenilo
d	Heptacloro
e	2,2',3,6-tetraclorodifenilo
f	Aldrín
g	Heptacloro-epóxido
h	2,2',4,5',6-pentaclorobifenilo
i	2,4,4',6-tetraclorobifenilo
j	Dieldrín
k	2,3,3',4,5'-pentaclorobifenilo
l	2,2',3,4,4',5-hexaclorobifenilo
m	2,3,3',4',5-pentaclorobifenilo
n	2,2',4,4',6,6'-hexaclorobifenilo
o	2,2',3,3',6,6'-hexaclorobifenilo
p	2,3,4,5,6,2',5'-heptaclorobifenilo
q	2,2',3,3',4,5,5'-Heptaclorobifenilo

Figure 5. Chromatogram of a mixture of external reference standards of some of the pops evaluated.

Source: own elaboration.

GC/MS analytical technique

The use of the GC/MS analytical technique allowed the complete separation and identification of all the components present in the samples, yielding characteristic chromatograms and mass spectra (see Figures 3 and 4). After performing the analyses by means of this technique, none of the pops evaluated in any of the cow's milk samples were detected in this study.

Confirmation of results

The obtained mass spectra were corroborated with the NIST mass spectral library and the Wiley mass spectral data registry (National Institute of Standards and Technology, 2018; Wiley, 2016), confirming the absence of the evaluated pops in the samples.

In addition, the solution of the reference standard mixture of the pops evaluated was analyzed under the same conditions as the samples and showed retention times (see Figure 5), mass spectra (see Figure 6) and fragmentation ions (see Table 1) specific for

each compound. These data were compared with the respective chromatograms and mass spectra obtained from the samples and these did not coincide, confirming that none of the pops evaluated were present in the cow's milk samples.

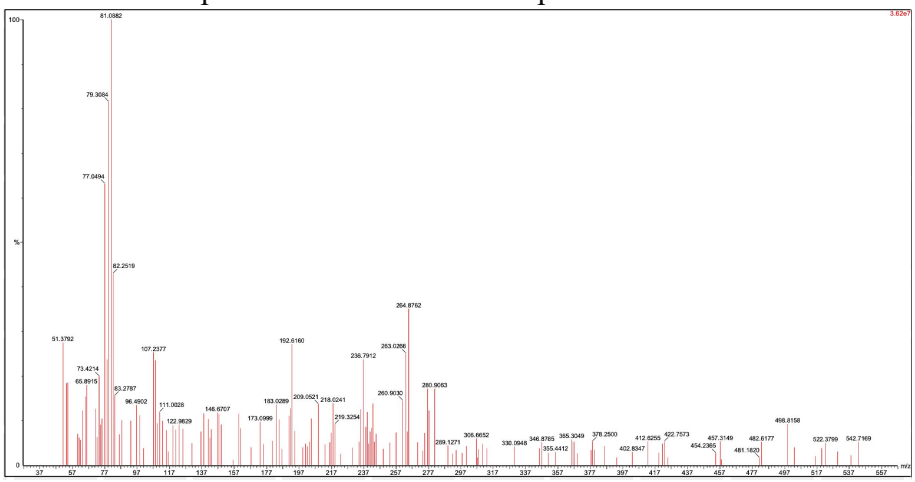


Figure 6. Mass spectra of the external reference standard dieldrin.

Source: own elaboration.

Table 1. Ion fragmentation mass/charge (m/z) values of external standards.

Compound	Molecular ion (m/z)	Primary fragmentation ion (m/z)	Secondary fragmentation ion (m/z)	Tertiary fragmentation ion (m/z)
Lindane	288	181	219	109
Hexachlorocyclohexane	288	183	181	219
2,4,4'-trichlorodiphenyl	256	256	258	186
Heptachlor	370	100	272	274
2,2',3,6-tetrachlorodiphenyl	290	292	220	290
Aldrin	362	66	263	265
Heptachlor-epoxide	386	81	353	355
2,2',4,5',6-pentachlorobiphenyl	324	326	324	328
2,4,4,4',6-tetrachlorobiphenyl	290	292	290	220
Dieldrin	378	79	81	82
2,3,3,3',4,5'-pentachlorobiphenyl	324	326	324	328
2,2',3,4,4,4',5-hexachlorobiphenyl	358	360	362	290
2,3,3,3',4',5-pentachlorobiphenyl	324	326	324	254
2,2',4,4,4',6,6'-hexachlorobiphenyl	360	360	362	145
2,2',3,3',6,6'-hexachlorobiphenyl	360	360	362	358
2,3,4,5,6,2',5'-heptachlorobiphenyl	392	394	396	324
2,2',3,3',4,5,5'-Heptachlorobiphenyl	392	396	394	324

Source: own elaboration.

Conclusions

In this work, it was determined that there is no presence of the pops evaluated in any of the cow's milk samples of the main brands of national production. These results may be due

to the reduction in the use and generation of pops in the country, as a result of the national and international legalizations that have been implemented in order to regulate the application of these compounds.

In addition, unlike other countries in the region such as the United States (Chen et al., 2017), Brazil (Ciscato et al., 2002), Argentina (Lorenzatti, Maitre, & Lenardon, 2003), Colombia(Lans-Ceballos, Lombana Gomez, & Pinedo-Hernández, 2018) and Chile (Muñoz, 2005) cow's milk samples produced in the Dominican Republic do not represent a source of exposure to the pops evaluated for people who consume this product. Similarly, this study plays a very important role in contributing to research on the current levels of pops in the Dominican Republic, as it is the first research study on the determination of pops in cow's milk produced and consumed in the Dominican Republic.

Recommendations

Based on the results obtained in this research, it is recommended that studies with similar objectives be carried out to evaluate other substances classified as pops, in order to have a better estimate of the POP content of cow's milk produced in the country.

It is also suggested that the number of samples be expanded to include other brands of milk produced nationally and with different fat content in order to obtain more complete and representative results.

Finally, it is recommended to carry out studies on other foods and to evaluate the presence of the different

Pops, in order to know the real exposure of consumers to pops-contaminated foods produced in the Dominican Republic.

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IMMUNOANALYTICAL APPROACHES FOR THE CONTROL OF XENOBIOTICS AND BIOTOXINS IN FOOD

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ABSTRACT: Analytical methods are required to effectively ensure food quality and safety. A reliable determination of any harmful substance potentially present in a food product is mandatory before it reaches the consumer. One of the analytical approaches that nowadays contributes to attaining this goal includes a series of techniques that use antibodies as essential elements for the detection of the target analyte, which collectively are known as immunochemical methods. This article aims to provide a basic overview of the biochemical principles underlying these technologies and their benefits and limitations for the determination of chemical contaminants, residues and additives in food matrices. In the last section, some of our initiatives in this field that resulted in commercially available rapid kits following technology transfer to the industrial sector are discussed.

KEYWORDS: immunoassay; rapid methods; ELISA; immunostrip; 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.

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

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

i) 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.

ii) 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.

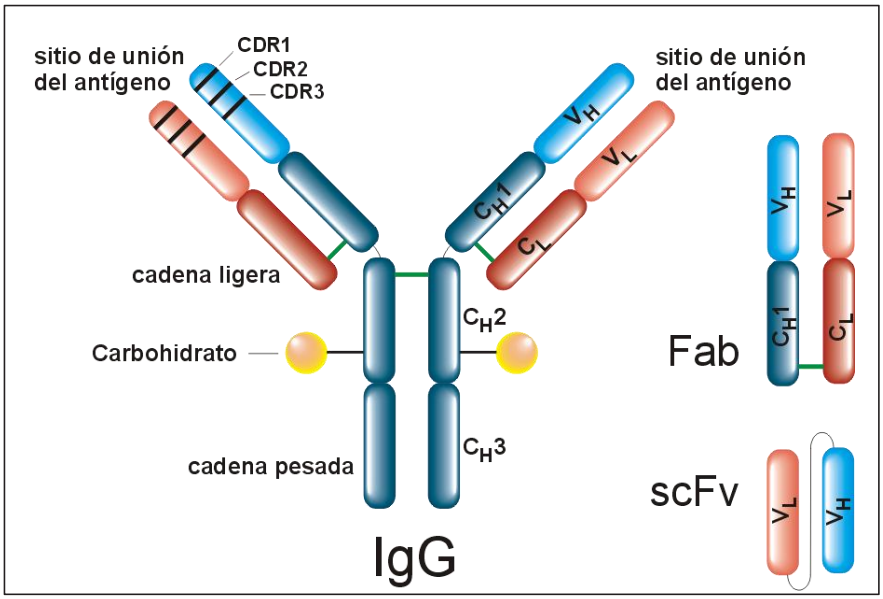
iii) Transfer studies during processing. While some chemical compounds, due to their structure and properties, are hardly transferred from the raw material to the final product (juices, wines, jams, etc.), others, on the contrary, do not suffer any reduction or even their concentration is higher in the 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.

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 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.

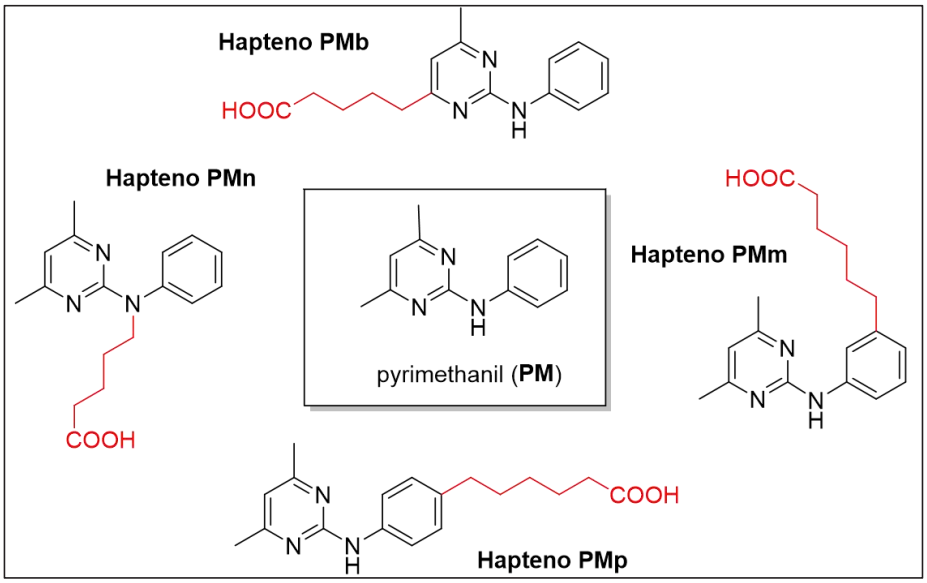
However, it should be noted that in most cases in which it is desired to generate antibodies against a small molecule, direct covalent binding to a protein is not possible due to the lack of a reactive group that can be used for this purpose. It is therefore necessary to previously synthesize an analog 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).

The proteins most frequently used for the covalent conjugation of haptens are albumins and hemocyanins, with bovine serum albumin (BSA) being one of the most widely 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.

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.



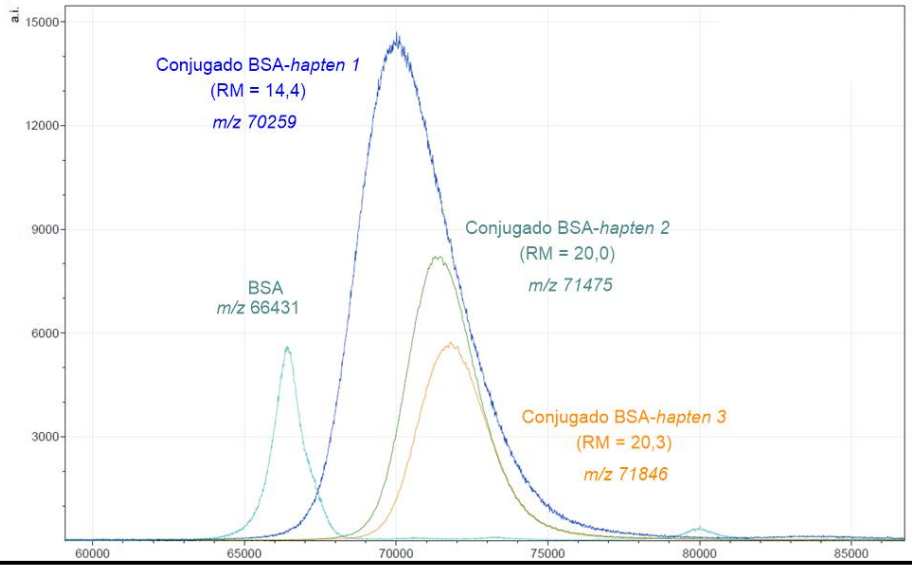
Pyrimethanil (PM)

Hapteno pmb

Source: own elaboration

The 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.

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



BSA-hapten conjugate

Source: own elaboration

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).

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.

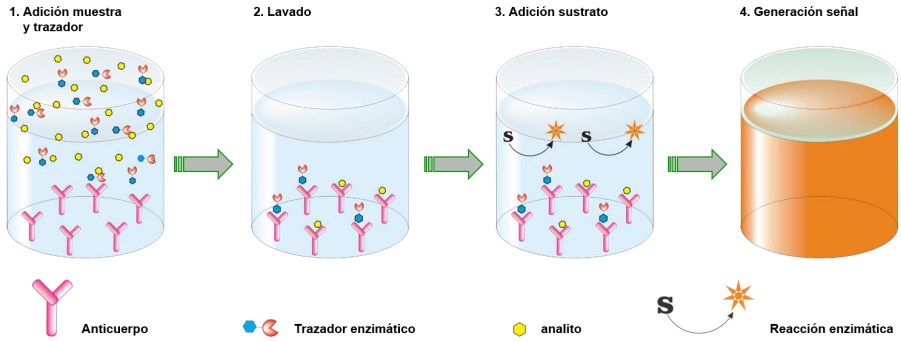
ELISA method

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 IC50, which is a good estimate of the affinity constant of the antibody. Obviously, the lower the IC50 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 (IC10), 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 (IC20), which is considered the limit of quantification (LOQ).

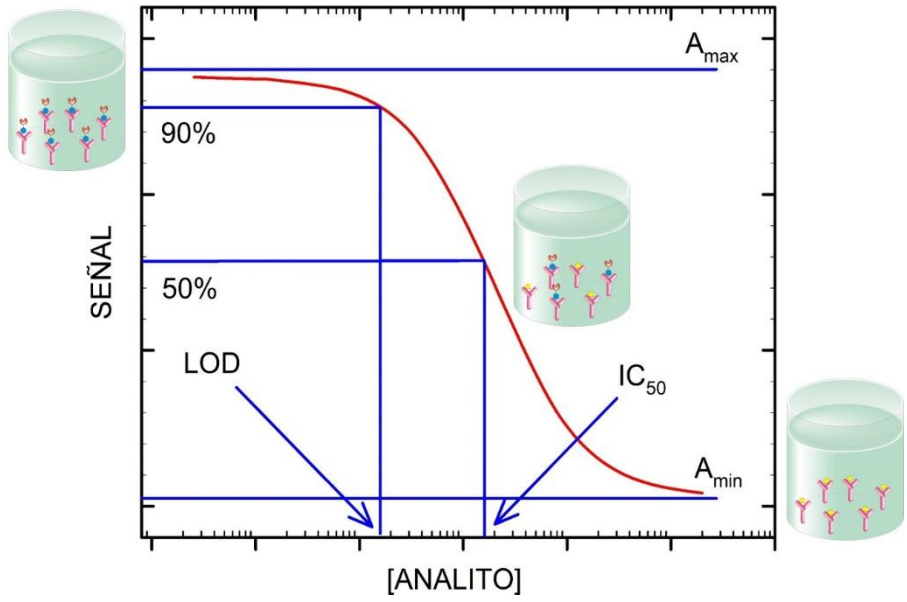
Figure 5. Schematic of a competitive ELISA in direct format.



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

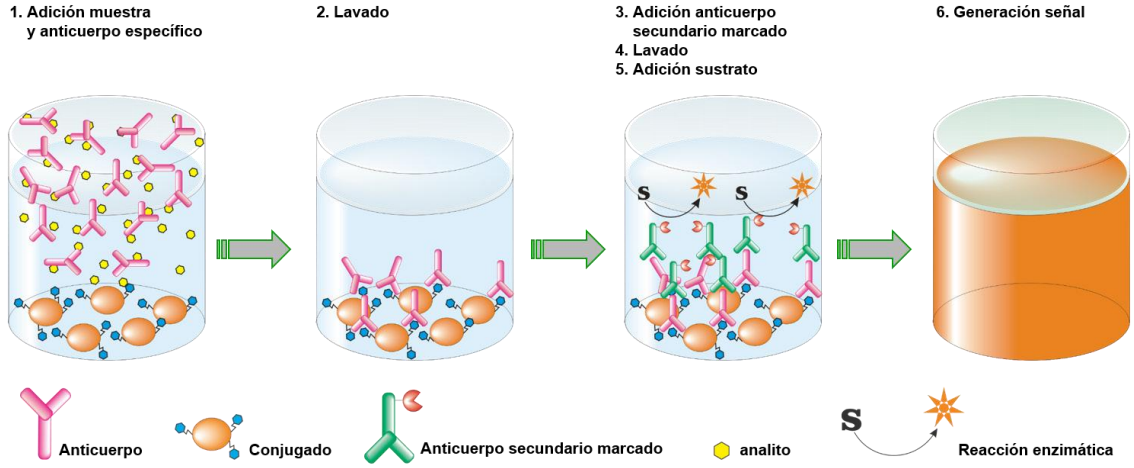
Source: own elaboration

Figure 6. Typical inhibition curve of a competitive immunoassay.



Source: own elaboration

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

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.

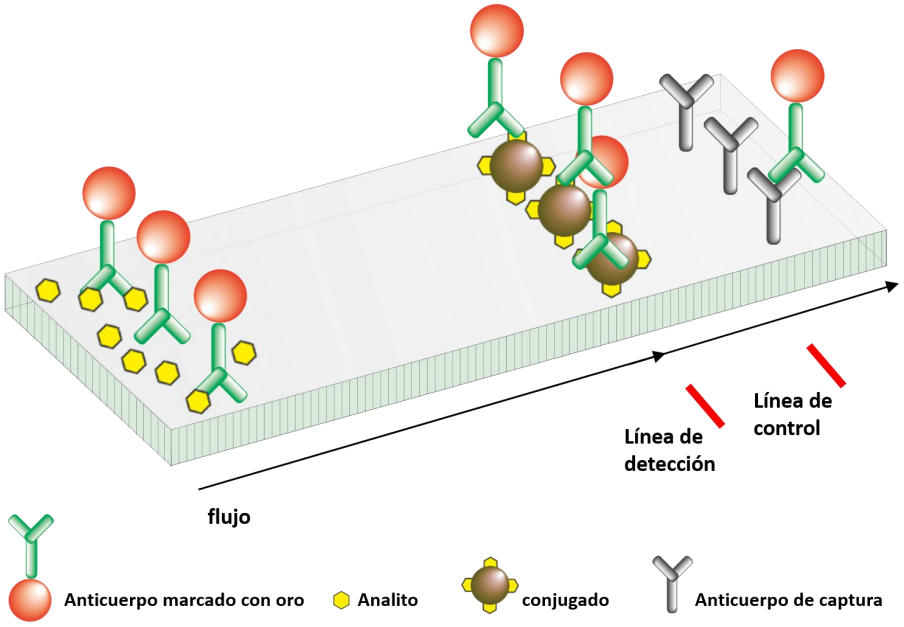
Lateral flow immunochromatography

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. In case 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 (Amax, IC50, 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 method of analysis, 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:

- Five strobilurin family fungicides: azoxystrobin, pyraclostrobin, kresoxim-methyl, picoxystrobin and trifloxystrobin.
- Three fungicides of the anilinopyrimidine family: pyrimethanil, cyprodinil and mepanipyrim.
- Four fungal succinate dehydrogenase inhibitors: fluopyram, penthiopyrad, fluxapyroxad and boscalid.
- Seven fungicides from different families: orthophenylphenol, imazalil, fenhexamid, proquinazid, fludioxonil, quinoxyfen and fluopicolide.
- Two insecticides: spirotetramat and imidacloprid.
- A herbicide: dicamba.
- An antibiotic: chloramphenicol
- Two hormones: forchlorfenuron and melatonin.
- Six mycotoxins: aflatoxin M1, aflatoxin B1, zearalenone, alternariol, ochratoxin A and patulin.
- 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 their loqs are compared with the maximum residue limits (mrls) authorized in the European Union.

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.

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

Analyte	Antibody	Format	Sample	LOQ	Mrl[a]

				(μg/L)	(μg/L)
Azoxystrobin	Mab azo#49	Indirect ELISA	Tomato juice		3000
		Heterologous	Peach juice		2000
			Red grape juice		2000
			Banana juice	5	2000
			Strawberries		50000
Pyraclostrobin	Mab pys#11	Direct ELISA	Strawberry jam		100b
		Counterpart	Raspberry jam		3000
			Blueberry jam		
			Apricot jam		1000
			Peach jam		
Picoxystrobin	Mab pca#21	Indirect ELISA	Wheat flour		
		Heterologous	Oat flour		
			Rye flour		
	Mab pca#13	Indirect ELISA	Lager Beer		
		Counterpart	Non-alcoholic lager		
			Black Ale		
			White ale beer		
Kresoxim-methyl	Mab kmo#117	Indirect ELISA	Tomatoes		
		Counterpart	Cucumbers		50b
			Strawberries		50b
Trifloxystrobin	Mab TF0#17	Indirect ELISA	Tomatoes		
		Heterologous	Cucumbers		
			Strawberries		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		1000
	Mab Pmm#31	Indirect ELISA Heterologous	Strawberries		B
			Tomatoes	5	1000
			Cucumbers	5	

Table 2 continued

Analyte	Antibody	Format	Sample	LOQ(μg/L)	Mrl[a] (μg/L)
Cyprodinil	Direct ELISA	Direct ELISA Counterpart	Apple juice		
			Red grape juice		3000
			White wine	1	3000
			Red wine	5	3000
			Sparkling wine	1	3000
			Cider	1	

	Mab cdm#21	Heterologous direct ELISA	Strawberries	[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			Red grape juice		5000
	Pab	Direct ELISA	Peach juice		3000
	Bla#1	Counterpart	Apple juice		2000
			Tomato juice		3000
	Pab	Indirect ELISA	Tomatoes	5	3000
	Blb#2	Heterologous	Cucumbers	5	3000
Fludioxonil	Mab fdn#23	Homologous indirect ELISA	Apple juice	5	5000
			Red grape juice		
Fenhexamid			Red grape juice		15000
			White grape juice		15000
	Mab fho#27	ELISA direct homologous	Red wine		15000
			White wine		15000
			Green kiwis		15000
			Strawberries		[B]
Fluopyram	Mab fpb#12	ELISA direct homologous	Grapes	5	
			Wines		
			Plums	5	

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

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	White wine	0.5	
		Heterologous	Red wine	0.5	
Aflatoxin M1	Anatoxin-a	Heterologous indirect ELISA	Buffer	0.04[b]	0.05[c]
Anatoxin-a	Mab anm#38	ELISA heterologous capture	Waters	0.25	0.1-20[d]

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

B IC50 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 carry out effective control over 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 for the dairy 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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