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Characteristics of Mycotoxin Analysis Tools for Tomorrow

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1. Introduction

Aflatoxins difuranocoumarin derivatives are produced by fungi *Aspergillus flavus*, *Aspergillus parasiticus and Aspergillus nomius* [1] and they are part of the group of mycotoxins. From the twenty metabolites that have been formed endogenously in animals, aflatoxins B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2) are the most common and the most toxic. The names of aflatoxins B_1, B_2, G_1, and G_2 are based on their florescence characteristics. Aflatoxin B_1 and B_2 show strong blue fluorescence under UV light, whereas aflatoxins G_1 and G_2 exhibit greenish yellow fluorescence [2]. All the aflatoxins have been classified as carcinogenic compounds for humans, but AFB_1 has been tagged as the most dangerous, highly toxic, immunosuppressive, mutagenic, and teratogenic compound and its effects have been identified as well. Also, malabsorption syndrome and reduction in bone strength may occur due to AFs consumption. Aflatoxins not only have adverse effects on human health but also cause serious economic losses when tons of foods have to be dropped or destroyed for being contaminated with AFs.

To ensure food's safety, the maximum level of aflatoxins in food has been set by international organizations. For each kind of aflatoxin a minimum quantity of concentration is allowed,

for instance, European Commission Regulation 2010/165/EC established limits of 8 and 15 µg/kg for AFB_1 and total AFs respectively. Several methods have been developed to determine AFs in foods, for instance: immunoassays techniques [3], Thin layer chromatography (TLC) [4]. High-Performance liquid chromatography (HPLC) with fluorescence detection [5]. Not long ago, analytical methods based on clean-up with immunoaffinity column and HPLC with postcolumn derivatization and fluorescence detection have gained much popularity. Even though, several works have been reported to determine AFs in foods by using these methods, only few validation studies are available which comply with certain regulations. There are immunochemical methods which are based principally on enzyme-linked immunosorbent assay (ELISA) that has a good sensitivity; speed and simplicity; however these kinds of instruments are expensive. An alternative of improving the disadvantages of the previous methods are trying to be solved by biosensors which are devices that enable identification and quantification of aflatoxins. Exists a variety of biosensors that base their performance in several principles, those are: optical, optoelectronic, electrochemical, piezoelectric, DNA and combined. In the same way, there are other methods not as common as the previous methods but they have a wide utility as well. The most important are those that base their principle on electrochemistry, spectroscopy and fluorescence.

The chapter has two main proposes. First, to give general description of the most common methods used for quantifying aflatoxin concentrations. And second, to give a perspective about the tendencies in the development of systems, based on the so far used methods, which could be employed in the near future to detect and quantify aflatoxins in food.

2. Chromatography methods

Chromatography is one of the most common methods for quantifying aflatoxins. This method started with Gas chromatography (GC). However, technology advancements allow the development of new chromatography-based techniques. Examples of these improvements are Liquid Chromatography (LC), Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC). The quantification of aflatoxins using chromatography relies principally on fluorescence detection depending of the compounds under analysis. So that, nowadays there are several works employing a variety of fluorescence detection in order to improve the sensibility of these techniques. In the same manner, UV visible (Vis) wavelength spectrum has been used to improve the detection and quantification of aflatoxins. Others methods employed to accomplish the chromatographic quantification of AFs are array of diodes and refraction index.

Before the chromatographic analysis, the toxic compounds must be extracted to remove the interfering particles; such extraction is commonly done by a solvent in a clean-up step that regularly uses an immunoaffinity column (IAC). This procedure increases the sensitivity and diminishes the necessary sample quantity in the analysis. Other system used to quantify single and multiple aflatoxins is the mass spectrometer which is coupled commonly with a HPLC system. This section explains the most common methods based on chromatographic principle and the steps before and after for accomplishing the analysis with better assessments.

2.1. Gas Chromatography (GC)

The instrumentation of gas chromatography comprises well defined components that accomplish specific functions of the overall process. GC almost reaches the complete development of technological level in 50 years. The Figure 1 shows the principal components that constitute a Gas Chromatography System.
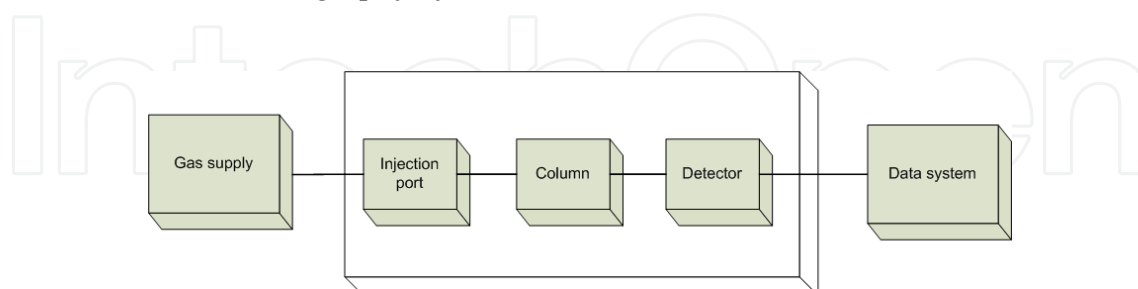

Figure 1. Block diagram of a gas chromatograph.

Gas supply means to move the sample through the column; the possible gases to choice are restricted and the most commonly used are nitrogen and helium. It is also necessary to control the gas flow because it can have impact on the separating performance. Tramps can be purchased to reduce or remove hydrocarbons and oxygen in the carrier gas. The chromatographic process starts when the sample is introduced into the column, ideally without disrupting flows in the column. Therefore, the deliberation of the sample into the column should be controlled, reproducible and rapid. The GC include an oven which is an important component in this process, because the vapor state must be maintained thought the GC separation, therefore, a good control of temperature must be kept. Another important component of the gas chromatography is the detector which has been evolved through the years. Nowadays, the mass spectrometer (MS) promises to be the most suitable method to be coupled with GC.

2.2. Liquid Chromatography (LC)

The principle of liquid chromatography is the separation process which is based on the distribution between two phases. The sample is propelled by a liquid which percolates a solid stationary phase. Thus a variety of stationary phases can be used in liquid chromatographic systems. The liquid chromatographic process and the separation of the sample may be achieved, both, in low and high pressure systems. And the correct selection of the separation mode stationary phase and mobile phase may be straight (normal) phase, reversed phase and size-exclusion (SEC) or ion-exchange (IEC) liquid chromatography respectively.

2.3. Thin-Layer Chromatography (TLC)

Thin-layer chromatography is a very commonly used technique in syntactic chemistry. This technique identifies compounds by determining the purity and progress of a reaction. Such reaction is fast and only requires a small quantity of the compounds. In TLC the mobile phase is liquid and the stationary process is a solid adsorbent. Several factors determine the

efficiency of a chromatographic separation. The adsorbent should show a maximum of selectivity toward the substances that are being separated so that the differences in rate of elution will be large. For the separation of any mixture, some adsorbents may be too strongly adsorbing or too weakly adsorbing.

2.4. High-Performance Liquid Chromatography (HPLC)

HPLC is now the most common used chromatographic technique for a detection of a wide diversity of mycotoxins, especially for aflatoxins [6]. The analysis sample cleanup can be performed by liquid-liquid partitioning, solid phase extraction (SPE), column chromatography, immunoaffinity clean-up (IAC) columns, and multifunctional clean columns [7]. Recently the utility of the IAC columns has become very popular because of its high selectivity. IAC columns can be used for sample preparation before HPLC analysis either in off-line or in-line mode [8]. While in the off-line immunoaffinity cleanup the purification step is done separately by an expert, the IAC column is directly coupled to the HPLC system in the in-line immnunoaffinity cleanup. A chromatographic process can be defined as separation technique which involves mass-transfer between stationary and mobile phase. HPLC utilizes a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid.

2.5. Combined methods

Nowadays there are combinations of the aforementioned methods with pre-process techniques. Such methods are able to detect in a better way, the concentration of aflatoxins in a solution. Immunoaffinity column sample clean-up followed by a normal or reverse phase of HPLC separation with fluorometric detection is mostly used for quantitative determination of AFM_1 because of the characteristics of specificity, high sensitivity and simplicity of operation [9].

There are several works that employ IAC combined with HPLC and fluorometric detection for detecting and quantifying precisely concentrations of AFM_1 [10]. In the reference [11] the authors employ IAC, HPLC and an optimized photo-derivatization to assess the concentration of mycotoxins of airborne from a house in Dalian, china. In [12] is employed, in the same manner, IAC and an HPLC equipped with fluorescence detector to determine the quantity of aflatoxins AFM_1 in milk used for preparing market milk, yogurt and infant formula products in South Korea. As can be seen, the HPLC process commonly needs a cleanup process with immunoaffinity columns before detection. Such sample preparations are multistage, expensive and time-consuming. The combination of GC with MS (GC-MS) for the analysis of aflatoxins can provide definitive, qualitative and quantitative results, but it requires a derivatization step, which lengthens the analysis time and may compromise analyte recoveries [13]. Post-column derivatization is a version of chromatography where the components that were separated eluting from the column are derivatized prior to entering the detector. The derivatization process is generally carried out during the process, during the transfer of the sample components from the column to the detector. The derivatization may also be accomplished before the sample enters into the column or the planar medium, thus it is called pre-column derivatization.

In the work presented by [14], aflatoxin B_1 was detected in animal liver (pig, chicken, turkey, beef, calf) and chicken eggs by a process consisting of sample immunoaffinity column clean-up and liquid chromatography with post column bromination in kobra cell and florescence detection, which was introduced and validated. The validation process was done based on the decision 2002/657/EC established by European commission. Figure 2 shows an exemplification of the common steps included in the detection and quantification of aflatoxins used nowadays. These steps include the processing of the sample before introduced it into the HPLC system. The most commonly pre-process technique is the IAC which permits to purify and decreases the quantity of sample needed in the experiment. The other problem that IAC tries to diminish is the non homogenization of the sample. HPLC is the most common based-chromatography technique used at this time; nevertheless, it needs of the steps shown in Figure 2 to work better. The principal advantages of IAC are the effectiveness and specificity in the purification of the extraction that provide the economic use of solvents and the improved chromatographic performance achieved with samples [15]. Also IAC can be used to analyze commodities that contain different aflatoxins. The fluorescence of aflatoxins make possible the use of a fluorometers to detect and quantify the concentration of aflatoxins in a sample, however, sometimes it is necessary to improve this property. Derivatization processes have been employed to improve the fluorescence of aflatoxins and by consequence the sensitivity of the system. Years ago, the quantification of the concentration of aflatoxins was accomplished by comparison of the sample and authentic standards using visual estimation of fluorescence of the separated spot long wavelength UV radiation [15]. A great advance in the detection and quantification of aflatoxins is the introduction of mass spectrometry as a viable detector system. The advantage of coupling mass spectrometer to LC is that, it allows improving the detection limits. For aflatoxins quantification, a number of instruments have been used including single quadrapole, triple quadrapole and lines ion single quadrapole and linear ion trap instrument.
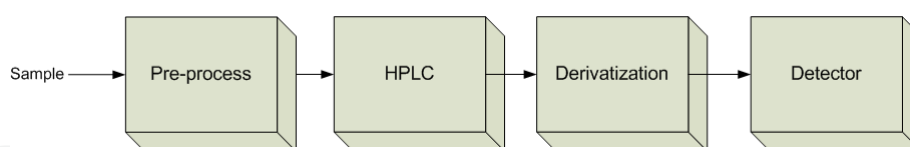
Sample → Pre-process → HPLC → Derivatization → Detector

Figure 2. Common steps followed actually to quantify aflatoxins using HPLC.

Chromatography has been one of the most common methods used for the detection and quantification of aflatoxins. This technique has been evolving through the years from GC to HPLC which nowadays is the most used chromatography-based technique employed for aflatoxin detection. TLC and LC are methods that have been going displaced by HPLC because of its sensitivity, specificity and facility of operation. At the present time, HPLC by itself is not enough and it is necessary to employ pre-process and derivatization techniques that, jointly with detectors, improve the purity of the sample and the fluorescence property of the aflatoxins. In consequence, the detectors, which generally take into account the fluorescence of the aflatoxins, accomplish better quantification and offer more sensitivity. The most common method for pre-processing the sample is IAC that allow having high specifici-

ty in the selection of aflatoxin in samples contaminated with more compounds. So that, it is clear that the tendency of the technology is principally to improve the stage of pre-processes and derivatizations in order to achieve a more precise quantification. The more sophisticated the technology of pre-process and derivatization the more high specificity and sensitivity the method gets.

3. Immunoassays for aflatoxins detection

Among aflatoxin detection methods, there are those that base their operation on antibody-antigen reactions (Ab-Ag), known as immunoassays. Different kind of Aflatoxin molecules (AF) can be considered as antigens from the immunological point of view, so that it is possible to develop antibodies against them.

Most of immunological the methods are based on enzyme-linked immunosorbent assay (ELISA), which require less expensive instruments, have good sensitivity, speed and simplicity. However, ELISA kits are expensive especially for third-world countries [16], so several studies have focused on developing less expensive methods, without losing the benefits they offer. Besides, other alternatives will have some advantages over ELISA, as the use of magnetic droplets together with RT-PCR (Reverse Transcription Polymerase Chain Reaction), which has sensitivity to 1000 times greater than ELISA [17].

3.1. Antibody-Antigen reactions

Immunoassays are based on antibody-antigen reactions, in which one of reactants is marked and the other is immobilized on a platform. There are several kinds of molecule markers, which may be radioisotope, enzyme, fluorescent compound and colloidal Au. Because of small size of AFs, they are bounded to proteins so they can be captured with antibodies (Abs).

There are three type of antibodies used in immunoassays: polyclonal, monoclonal and recombinant. Polyclonal Abs (pAbs) is produced by rabbit, horse or goat blood immunized with protein-AF conjugate. This type of Abs is low-cost for preparation and easily produced. Monoclonal Abs (mAbs) is produced from positive hybridomas, which are usually produced by fusing murine myeloma cells and spleen cells from immunized mice. In [16], the authors used an indirect-competitive ELISA to detect AFB_1 by using a platform coated with monoclonal antibody. Recombinant Abs (rAbs) is produced by cloning the functional gene of some Abs and transmitted it into a prokaryotic or eukaryotic genetically-modified organism to hybridoma or spleen cells with or without immunization.

3.2. Competitive and non-competitive assay

There are two types of immunoassays: competitive and non-competitive assays. Competitive assays in turn are divided into two types: indirect and direct assays. In an indirect competitive assay, aflatoxins are immobilized by a protein-aflatoxin conjugate (Ag). The set is

exposed to a buffer with the tested sample. Antibodies are released into the buffer; some of them will bind to the immobilized conjugate, while the remainder will join the analytes in the buffer. After a while, it is released a second group of enzyme-labeled antibodies, or other fluorescent-core kind of signal material; these are joined to the first antibodies, which in turn are attached to the protein-conjugate immobilized aflatoxins.

The process of a direct competitive assay does not require a second labeled antibody. For this type of analysis, either aflatoxins (aflatoxin protein conjugate) or specific antibodies can be immobilized. The complementary component to that immobilized is marked and added to the sample. A competitive reaction occurs between the Ab-Ag, so that some marked components remain adhered to the immobilized one, while others adhere to those present in the sample.

For non-competitive assays, Abs are immobilized. When immobilized Abs make contact with the sample molecules and AF bind to them because Abs are attached to a sensitive surface, the amount of analyte bounded by Abs will result in an electrical or optical variation. However, sandwich format is preferred for this kind of assay, in which the sample is mixed previously with Bovine Serum Albumine (BSA) because AF molecules are small. Previously it was carried out a treating of the sample with Bovine Serum Albumine (BSA) (carrier protein). BSA binds to the AFs and the conjugate is captured by immobilized Abs.

3.3. Enzyme-Linked Immunoabsorbent Assays (ELISAs)

Any type of assay involving Ab-Ag reaction, where one of the reactants is conjugated with an enzyme, is considered as an ELISA. Amplification and visualization of Ab-Ag interaction are achieved by this enzyme conjugation. ELISA is the most used immunoassays used in food-aflatoxin detection.

Antibodies or antigens are immobilized on a solid-phase matrix by linking them, either through adsorption or covalently. Reactants are usually adsorbed on to the wells of 96- or 384- microtitre plate of polystyrene, where adsorption is characterized by a strong hydrophobic binding and slow dissociation rate. After this coating process, the residual protein-binding capacity of solid matrix is blocked by exposing it to an excess of unrelated protein (e.g. gelatin or bovine serum albumin "BSA"). The next step is the addition of a test solution, which may be serum with an unknown concentration of antibodies against the immobilized antigen. After incubation and washing, binding of specific antibodies is visualized by the addition of antiimmunoglobulin-enzyme conjugate followed by a substrate, generating a colored product when hydrolysed. This change of color is proportional to the amount of antibodies bounded and may be recorded visually or spectrophotometrically. In case of an antigen measurement, the process is the same but may be done by using competitive- or sandwich-type assays. When using microarray format, ELISA may detect other toxins, such as AFs in a sample [18].

3.4. Recent advances

ELISA has been modified by using electrochemical techniques. Antibodies or antigens may be immobilized on an electrode with a free and enzyme-conjugate. So in a competitive assay, some enzyme-conjugate will bind to the electrode, and enzyme density can be shown by current produced from the catalytic oxidation reaction of the enzyme with the substrates. In a non-competitive assay, the formation of an Ab-Ag complex generates a barrier of direct electrical communication between the immobilized enzyme and the electrode surface.

Some authors have reported the use of electrochemical sensors. In [19] developed a sensor based on enzymatic silver deposition amplification to detect AFB_1 in rice. A linear sweep voltage was done in order to read the sensor response. In [20] the authors proposed the use of electrical impedance spectroscopy and free-labeled molecules.

Optical ELISAs often uses surface plasmon resonance (SPR). They are similar to electrochemical sensors, but in SPR, Ab or Ag is immobilized in an optical-sensitive surface. As AFB_1 changes, the angle of Spectral Power Distribution (SPD) varies. A combination of competitive-direct ELISA and an immunochromatographic assay was done by [21], in order to increase its sensitivity.

In recent years, some articles have developed modifications on ELISAs (e.g. with the using of nano particles). In [22] the authors refined the ELISA process for aflatoxin detection by using anti-AFB_1 single chain fragment variables, in order to detect only free AFB_1 instead of an AFB_1-protein conjugate. In the references [23] developed an ultra-sensitive ELISA by coupling a micro plate ELISA with sensitive magnetic particles. An important feature of this hybrid system is its small column size, high capture efficiency and lower cost over other reported materials.

A combination of a competitive direct ELISA and gold nanoparticle immunochromatographic strip was done by [21], with a detection limit of 1.0 ng/ml for AFM_1 in milk. Immunochromatographic assay (ICA) is rapid and simple, and can be carried out by untrained personnel without using electronic devices. However, this type of assay has low selectivity, so in [24], an improved ICA by using a new monoclonal antibody against AFB_1 was developed.

4. Biosensors for aflatoxins

Aflatoxins are harmful organisms. Their toxicity is due to their capacity to covalent binding DNA and proteins. The most acutely and chronically toxic aflatoxin is the B_1. The legal limits set for AFB_1 or for total aflatoxins vary from country to country [25]. The detection and quantification of aflatoxins are the first steps in the challenging task of controlling such organisms.

The rearing of livestock, the storage of grains, and the stock of their derivatives are daily life activities which are susceptible to be infected with pests and diseases. Such infections may cause human death and economical losses.

Every topic exposed in this chapter is so vast that could be by itself a single chapter. The aims of this chapter are to give a general overview of all the existing methods for the measurement and quantification of aflatoxins; to signalize their principles of operation; and to expose their tendencies.

Biosensors are multidisciplinary tools with an enormous potential in detection and quantification of aflatoxins. Thus, such devices have a huge impact in healthcare, food management, agronomical economy and bio-defense [26].

4.1. Biosensors

The International Union of Pure and Applied Chemistry (IUPAC) define biosensor as:

"A device that uses specific biochemical reactions mediated by isolated enzymes, immuno-systems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals".

4.2. Application of biosensors

Dr. Leland C. Clark established the concept of a biosensor as a biological sensing element whose change its properties when reacting biochemically with a specific compound or analyte [27]. Such reaction is converted into an electronic signal for its quantification. Dr. Clark developed a glucose oxidase enzyme electrode for detecting glucose.

There are two different approaches which can be carried out by biosensors.

a. The enzyme metabolizes the analyte, thus the analyte can be determined through the measuring of the enzymatic product.

b. The analyte inhibits the enzyme, thus the decrease of the enzymatic product formation is correlated with the analyte concentration. This case is called "biosensor based on enzyme inhibition".

Biosensors are tools basically conformed of a substrate (silicon, glass or polymers). Common polymers are: polymethyl methacrylate, polydimethyl siloxane, etc. The substrate is often coated with a conductive layer like: polysilicon, silicon dioxide, silicon nitrite, gold, and metal oxides. The specific recognition elements include: antigens, antibodies, nucleic acids, whole cells, proteins, enzymes, DNA/RNA probes, and phage-derived biomolecular recognition probes. The changes in these elements are detected via optical, electrochemical, calorimetric, acoustic, piezoelectric (quartz crystal, potassium sodium tartrate, lithium niobate), magnetic, and micromechanical transducers [28].

4.3. Biosensor based on optical techniques

Optical sensors are analytical tools that satisfy requirements as accuracy, precision and specificity in the selection of the analyte, allowing *in vivo* or *in vitro* investigations. Optical techniques provide a large realm of possibilities based on properties such as absorbance, reflectance and luminescence of single elements or groups of analytes [29].

Among the optical techniques used in biosensors it can be found: non linear optics (based on surface plasmon resonance) [30], resonant mirror, fiber-optics [31], complementary metal oxide semiconductors, fluorescence/phosphorescence [32], reflectance, light scattering, chemiluminescence, and refractive index [33].

Such advantages, plus their easy operation and wide detection capacity, have made of optical biosensors useful tools for the detection of dangerous organisms as aflatoxins.

4.4. Electrochemical biosensors

The first biosensor based on cholinesterase (ChE) inhibition for detection of nerve agents was developed by G.Guilbaut in 1962 [34]. Since then, many other enzymes have been used in biosensor for detecting and quantifying a huge realm of parameters.

Other important enzyme used in biosensors is the acetylcholinesterase (AchE). The principal biological role of AchE is the termination of the nervous impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine [35]. The AFB_1 inhibits AChE by binding at the peripheral site, located at the entrance of the active site (at the tryptophane residue) [36]. Even though, there other enzymes, as butyrylcholinesterase (BChE) that are also used for detection of AFB_1, AChE is preferred because it is more sensitive than BChE for this purpose [37].

Biosensors based on the amperometric method allow the detection of low aflatoxin concentration. For example, acetylcholinesterase (AchE) is measured using a choline oxidase amperometric biosensor [38]. In this example the decrease in the amperometric activity of AchE has a direct relationship with the quantity of aflatoxins. This method is commonly used when the aflatoxin concentration is too low, and it cannot be detected with the spectrophotometric method.

4.5. Biosensors that combine techniques

Commonly, polymerase chain reaction (PCR) has been used to accurately detect low numbers of different pathogens with multiple sets of primers. But, important disadvantages of PCR are: the inhibition of the polymerase enzyme by the contaminants from the sample; difficulties in quantification; false positives resulting from the detection of naked nucleic acids; and non-viable microorganisms or contamination of samples in the laboratory. Biosensors are useful tools that provide a rapidly detection of the presence and amount of microorganisms in any given environments [26]. Thus, the mixture of different techniques might overcome the exposed problem. For example, in [39] there was a decrease of contaminants by coupling PCR with a piezoelectric biosensor.

There are biosensors that combine biological and physical/physicochemical transducers (SPR, piezoelectric, acoustic, and amperometric biosensors). The related problems for these biosensors are: chemical/physical stability of the transducers in the biological samples, the difficulty in production of highly specific antibodies, poor signal, etc. Such problems are often overcome by: coating the surface to make the transducer compatible with the biological

samples; using of highly specific monoclonal antibodies; and incorporating amplification steps to generate stronger signals [26].

Latest researches on nanomaterials, such as carbon nanotubes, metal nanoparticles, nanowires, nanocomposite and nanostructurated materials reveal to be a key points in the design of the near future biosensing systems with applications in aflatoxin detection [40].

The aforementioned methods to quantify aflatoxins present several disadvantages, for instance those based on chromatography, however they have laborious and time-consuming process [41]. Therefore, a pathway to improve AFs detection is through biosensors. This term was first used by Cammaann in 1977 [42], who defined it as a device that enables the identification and quantification of the interest sample (e.g. water, air, food, solutions, among others). Nevertheless, the main characteristic in a biosensor is the biological recognition element that is capable to create a response of interest. Such element can be an antibody, an antigen or an enzyme [43].

There are many kinds of biosensors applied to detection aflatoxins, however they majorly work in conjunction with immunochemical methods. Such junctions are based on the high affinity of antigen-antibody interaction and have the aim of increasing the sensitivity and decreasing the detection time of the toxic element [41].

4.6. Immunochemical

These kinds of sensors use mainly immunological receptor units such as antibodies or antigens, and detection methods as optic effects (e.g. fluorescence and plasmon resonance), electrochemical, or acoustical readout [44]. The majorly of these techniques are comprised of three main steps: First, the extraction of the aflatoxin from de complex mixtures of materials in which it is found; then, the purification of the sample for removing pollutants; and finally, the detection and quantification of the toxins [45].

The main challenges of these types of biosensors are the design and construction of prototypes which minimize their handling. Besides, they must use the best immunochemical techniques, with the aim to generate automated sensors that replace the existing large, complex, cumbersome, and chemical laboratory analysis systems. Such immunochemical biosensors would offer the benefit of an increasingly developing of modular design that would permit the rapid substitution of other reagents to detect different toxics with the same platforms [45].

In [45] is reported a biosensor that it is based in the property of fluorescence. This fluorescence system consists on an arc lamp that generates a microsecond flash and a lens that focuses in the radiation into the sample. Such sample was previously treated, with process shown on the Figure 3 which in turn shows the three main steps before the antigen detection with the automated process placed in the arrows. Then the detection consists in using a filter which allows the passing of UV radiation, around 365 nm. This wavelength excites the fluorescence of aflatoxins.
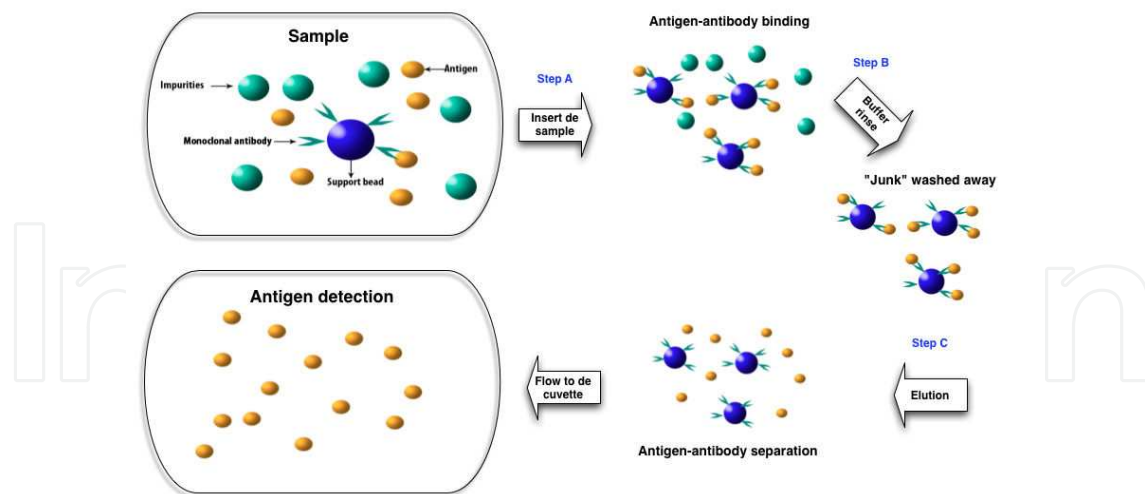
Figure 3. immunochemical-based capture, purification and detection process modify by [45].

After the excitation it is necessary the monitoring of the fluorescence response. This monitoring is carried out by a second lens that captures some of the light emitted by the sample through a photomultiplier tube and a filter centered around 455 nm. This is the wavelength where these AFs fluorescence. This device detects concentrations from 0.1 o 50 ppb in less than 2 minutes using a sample volume of 1 ml [45].

Another method used in conjunction with immunological techniques is named optical waveguide lightmode spectroscopy (OWLS). This technique is based in the precise measurement of the resonance angle of polarized laser light, diffracted by a grating in coupled into a thin waveguide. The incoupling resonance effect is very sensitive; such effect depends on the optical parameters of the sensor and the refractive index of the covering sample medium. The intensity effect response is carried out by a photodiode with the aim of determining the refractive index of the resonance incloupling angle detected with high accuracy [31].

There is another versatile technique named self-assambly structures that are considered as promising noble nanoscale systems with a several numbers of applications (solar cells, data storage, and biosensors). With this process it is possible to create biomarkers to exploit the absence of ligands on these nanoparticles surface that enhances the possibility of working better with molecules [46].

The self-assembly nanoparticles of nickel and gold are widely used for biosensing applications due to their biocompability, high surface to volume ratio, strong adsorption, fast electron transfer, enhanced sensitivity, high selectivity, and large detection range [47].

4.7. Centralized testing of DNA

Due to the necessity of creating simpler and more user-friendly methods for aflatoxins detection, it has been developed centralized testing of DNA. This method allows the early detection of genes associated with human diseases [48]. In this case, it is interesting to denote

that the biosynthesis of aflatoxins has been extensively studied, and more than 25 genes arranged in a 70-kbp gene cluster were identified [49].

DNA biosensor has given out rapid and accurate measurements of aflatoxins in milk or dairy products [41]. The novel contribution of such system is its measurement technique based on electrochemical impedance spectroscopy (EIS) to analyze compounds that have restricted catalytic interaction activity such as aflatoxins.

The EIS method in recent years has become a powerful tool for evaluating many biochemical and biophysical processes. The biosensor's characterization and fabrication can be generated through EIS. Moreover, with the interaction between enzyme–substrate, biomolecule which have no reaction sequence after binding (such as antigen–antibodies), and DNA, among others, charge transfer changes occurred after affinity interactions can also be monitored with EIS.

To identify the aflatoxin M_{1A}, it is necessary the ss-HSDNA, which was specifically bounded to this aflatoxin. It is necessary to immobilize the ss-HSDNA on gold electrodes with the help of cysteamine and gold nanoparticles. The differences between before and after binding of aflatoxin M_1 to the HSDNA probe can be analyzed with a cyclic voltammetry and IES. An aflatoxin M_1 calibration curve was prepared by considering the differences in electron transfer resistances before and after aflatoxin M_1 binding [41].

However, in the most of the cases, the sample needs a pretreatment. For milk case, in order to remove the milk fat, the sample was centrifuged. Then three completely separated phases were obtained. The layer at the top was the fat; the cream was at the center; and the fat free milk was at the bottom. This last phase was used for the experiment as a sample, in order to avoid any possibly negative effect of fat on the EIS.

4.8. Piezoelectric biosensors

Biosensors based on piezoelectric effect are commonly used for aflatoxins detection because they have the property of providing sensitive measurements in air as well as in liquids. This kind of biosensors, based on piezoelectric quartz crystal (PQC), is usually combined with most of the above mentioned methods, like immunosensors with cells, bacteria, proteins (including antibody or antigen), DNA and so on [50].

Between the different existing kinds of immunosensors, the PQC has been extensively applied to biorecognition sensing due to its advantages of cost-effectiveness, direct detection, experimental simplicity, and real-time output. The principle of these sensors is based in the fact that the quartz is used as transducer, its resonance frequency changes with the change in the mass, according to the Sauerbrey equation.

In [49] the author reports a DNA-based piezoelectric biosensor with the aim of detecting a PCR-amplified 248-bp fragment of the aflD gene of *A. flavus and A. parasiticus* involved in the conversion from norsolorinic acid to averantin. Such biosensor was used for the analysis of DNA fragments coming from the amplification of DNA extracted from reference strains of *A. parasiticus.* Originally it was designed with the objective of researching about the influ-

ence of different parameters, such as amplicon concentration, dilution, and PCR specificity on the biosensor's response.

An important point of these kinds of devices is that the crystal only can be used for 25 measurements without losing sensitivity; this is because the devices that work with mechanical effects are majorly affected with the use. This is the reason for coupling the PCR protocol and the DNA piezoelectric biosensor. After its characterization with synthetic oligonucleotides, the piezoelectric-DNA biosensor led to the clear identification and quantification of contaminated feed samples with aflatoxins [49].

4.9. Optoelectronic

The principle of the optical waveguide light-mode spectroscopy (OWLS) technique is the measurement of the resonance angle of polarized laser light, diffracted by a grating and incoupled into a thin waveguide. Incoupling resonance occurs at very precise angles depending on the optical parameters of the sensor chips and the complex refractive index of the covering sample medium. The intensity of the incoupled light guided within the waveguide layer by multiple internal reflections is measured with a photodiode [31].The refractive index is determined from the resonance incoupling angle detected at high precision. Such index allows the determination of layer thickness and coverage of the adsorbed or bound material with high sensitivity. This method allows the construction of both chemical and biosensors. Therefore, it can be applied for direct sensing of various types of biomolecules.

Other optical based biosensor uses a high-tech semiconductor material–silicon for the efficient accuracy registration of narrow spectral bands or specific wavelengths. This biosensor is used for detecting and quantifying aflatoxins that are commonly found in a variety of agricultural products.

Based on the above mentioned techniques it was developed a structure with two oppositely directed potential barriers, the total current conditioned by these barriers depended on both, the external voltage and the wavelength of the absorbed radiation. A modification in these parameters resulted in the obtaining of high-accuracy data of aflatoxins contaminants in food and provender in natural conditions [51].

Detection and identification of harmful organisms, such as aflatoxins, in a cost and time effective way is a challenge for the researchers. Biosensors have proved to be useful tools for detection and quantification of such organisms. These sensors have advantages such as: fast response, relative easiness of use, a huge realm of applications, and flexibility for combination of techniques. Such advantages are derived from the involvement of multidisciplinary research activities. But, even though the vast research on biosensors, it is still needed the injection of economical funds to locate them in the commercial market, and impulse their use in real applications.

The research on biosensor has the aim to develop, at low cost an analytical approach simpler and faster. Being an alternative improving the classical techniques. It is necessary that the improvement of the processes is focused in autonomous measurement, in order to avoid, as much as possible, the human error. The mostly classical measurements are linked to the lab-

oratory; the biosensors must be the way to create embedded systems with the aim to detect the aflatoxins *in vivo*. Because of this, it is necessary to automate the whole process including the pretreatment of the sample to generate more efficient systems and manageable and better aflatoxin detection biosensors.

5. Miscellaneous methods

5.1. Electrochemical methods for aflatoxins determination

Aflatoxins measurement usually implies complex, expensive and slow methods. However, this determination can be carried out taking into account the response of aflatoxins to determinate electrical stimulus. These methods are called electrochemical, where immunosensors are applied to determine the presence of aflatoxins in a sample. Usually, these sensors are composed by two screen-printed-electrodes (SPE), the first one is made of graphite, platinum, or gold; and it is known as working, active, or measuring electrode. The second electrode is the reference and is commonly made of Ag/AgCl. In general, this technique involves two basic steps. In the first, the immunosensor working electrode is coated with an antibody; after an incubated time, the sample that contains the aflatoxins is added to this electrode, while the left one reacts for a determinate time; finally, a conjugated of aflatoxins and enzymes is added to the electrode, it is then when the competitive reaction begins. In this reaction, free aflatoxins compete to link to antibodies present in the working electrode. After a stabilization time, the measuring electrode is removed from the sample and rinsed with a buffer solution. The second step implies to apply an electrical potential (commonly 100 mV) to the electrode, which changes its electrical conductivity according with the aflatoxins concentration. After sampling the electrode; an increase or reduction in the electric current flow will appear according with the concentration of aflatoxins in the sample. This technique has received improvements; disposable immunosensors have been reached for measurement of aflatoxins M_1 (AFM$_1$) directly in milk following a simple centrifugation step but without dilution or other pretreatment steps. Exhibiting a good working range, comparable to the ones obtained in buffer; linearity between 30 and 240 ng/ml making it useful for AFM$_1$ monitoring in milk (maximum acceptable level of AFM$_1$ in milk is 50 ppt) [52]. It is easy to notice that electrochemical techniques offer some advantages over traditional methods for aflatoxins determination, among which it can be found: reliability, low cost, *in-situ* measurements, fast processes, and easier methodology than common chromatography techniques through a similar performance

Other improvements to this methodology involve the analysis of thermal stability given that the conductivity properties of materials also change with temperature variations and not only for the aflatoxins concentration in the electrode. SPEs with platinum as substrate for the working electrode have been used to achieve long-term stability. Probes have shown that this type of electrodes maintain a good biorecognition affinity for antibodies on its layer and a decrease in the detected signal of less than 10% after two weeks inside a refrigerator (5 °C) and less than 22% at laboratory temperature (25 °C), values that allow partial usability for

practical assaying [37]. Using this type of electrodes, a voltage of 50 mV, and a stabilization time of 1 minute are suggested to begin current measurements. Limits detection of 2.4 ppb has been reached in real capsicum spice samples, producing good correlations comparing with data from HPLC with fluorescence detector.

Working range for electrochemical immunnosensors from 0.1 to 10 ng/ml with a detection limit of 0.06 ng/ml has been achieved by using gold electrodes and enzymatic silver deposition amplification. In this procedure, an aflatoxin B_1-bovine serum albumin (AFB$_1$-SBA) conjugated is immobilized on the measuring electrode (gold electrode). An indirect competitive format between the selected analyte in solution and the AFB$_1$-BSA on the electrode is performed. After the competition step, monoclonal antibody against AFB$_1$ was bounded to the electrode and then conjugated to a secondary antibody-alkaline phosphatase (ALP) conjugated. The ALP could catalyze the substrate, ascorbic acid 2-phosphate, into ascorbic acid, and the latter could reduce silver ions in solution to metal silver deposited onto de electrode surface. Finally, the metallic silver deposited onto the electrode was determined by linear sweep voltametry (LSV). The peak current for this immunosensor exhibited a negative linear correlation to AFB$_1$ concentration [53].

As it can be noticed, electrochemical sensors and biosensors have, in some cases, the advantage of rapidity and sensitivity over the traditional techniques. Electrochemical sensors based on acetycholinesterase (AChE) inhibition by aflatoxins have been rapidly applied due to detection limits of 2 ppb. As reported by [53], the AFB$_1$ determination can be based on AChE inhibition, while the AChE residual activity is determined by using a choline oxidase amperometric biosensor coupled with AChE enzyme in solution. The amperometric detection of AChE activity is based on a second enzyme, cholesterol oxidease (ChOx), providing a consecutive conversion of the native substrate (acetylcholine) to an electrochemically active H_2O_2. Finally this component is measured at the screen-printed electrode previously modified with Prussian Blue (PB) at a potential of -0.05V versus screen-printed internal silver pseudo reference electrode. The linear working range was assessed to be 10-60 ppb.

Single electrode immunosensors have proved to be a reliable alternative to complex methods for aflatoxins determination. However, devices with multiple electrodes have been developed to offer the possibility to combine the high sensitivity of electrochemical SPE-based immunosensors with the favourable characteristics of high throughput ELISA procedures. An analytical immunosensor array, based on a microtiter plate coupled to a multichannel electrochemical detection system using the intermittent pulse amperometry technique is presented for detection of aflatoxins B$_1$ [54].

The device is composed by 96-well screen-printed microplated, their thick-film carbon sensors was modified according with a competitive indirect enzyme-linked immunoassay (ELISA) format for aflatoxins detection. Spectrophotometry and electrochemical procedures were both applied to determinate the reliability of the proposed system. The principal advantage of the aforementioned system is the possibility to separately apply the amperometric to each of the 96 sensing electrodes. The applied potential is +400 mV with a pulse of 1 ms and a selected frequency of 50 Hz. This immunoassay was applied for analysis of corn samples. AFB$_1$ could be measured at a level of 30 pg/ml and with a working range between

0.05 and 2 ng/mL. Aflatoxin AFM_1 was also quantified by this method. The suitability of the immunosensor for the direct analysis of the toxin in milk was assessed. AFM_1 was correctly measured with a working range of 5-250 pg/ml and a detection limit of 1 pg/ml was achieved. For this experiment, the intermittent pulse amperometry parameters were adjusted to -100mV with a pulse width of 10 ms and a 5 Hz frequency [55].

Variation of electrochemical immunosensors appeared recently to determinate aflatoxins through detection of a specific DNA [56]. The detection technique was optimized applying DNA sequences from *Aspergillus* gene *aflR* that codes a biochemical pathway of aflatoxins B_1 production. Then, voltametric detection of the specific *Aspergillus* DNA sequence is based on hybridization of adsorbed target DNA with a biotinylated probe and subsequent binding with streptavidin alkaline phosphatase conjugated. Then, the modified electrode surface of carbon paste electrode is incubated in a buffer solution with an electrochemically inactive substrate (1-naphthyl phosphate). Alkaline phosphatase converts 1-naphthyl phosphate into 1-naphthol, which is determinate by the selected voltammetric technique. The optimize procedure is capable to distinguish potentially aflatoxigenic fungi from other *Aspergillus* species.

5.2. Spectroscopy techniques

Spectroscopy techniques have been popularized because they present fast, low-cost and non-destructive analytical methods suitable to work with solid and liquid samples. This method involves the study of the interrelationships between the spectral characteristics of objects and their biophysical attributes, specifically, the interaction with radiated energy as a function of its wavelength or frequency [57].

In the particular case of aflatoxins, different studies have been carried out to determinate the wavelength in which these substances respond to radiant energy. The different spectroscopy systems available in the market have the facility to scan a sample over a determinate wavelength range and acquire the spectral data in different modes as reflectance, absorbance, or transmittance. The procedure to detect aflatoxins in a sample is quite similar to the aforementioned methods. The sample preparation implies extraction and clean up. However, some authors use the sample without any preparation. The samples are scanned with a spectrophotometer commonly over a wavelength range from 250 nm to 2500 nm at different steps (2 nm steps can be reached). Finally the results are shown in a graph of wavelength against reflectance or absorbance.

Near infrared spectroscopy (NIRS) is an excellent method for a rapid and low cost detection of aflatoxins in cereals [58]. Aflatoxin B_1 was successfully measured in maize and barley by applying grating and Fourier transform NIR spectroscopy instruments with multivariable statistical methods on intact, non-milled samples. This technique quantifies aflatoxins in order of 20 ppb. Variations to this method imply the use of horizontal attenuated total reflectance technique for determination of aflatoxin B_1, B_2, G_1 and G_2 in groundnut. The mid-band infrared attenuated total reflectance (ATR) spectra were obtained with a Fourier transform spectrometer equipped with a horizontal ATR accessory. This variant in the method generates rapid and substantial spectra of aflatoxins with a minimum sample size (>2 mL) and chemicals [59]. Other authors have incorporated a bundle reflectance fiber-optic probe to

NIRS system. Here, the fiber-optic probe is immersed in the sample without any previous treatment or manipulation of the samples. Then, NIR spectra are recorded direct from the fiber. This combination of technologies has proved to quantify aflatoxin B_1, ocharatoxin A and total aflatoxins in paprika successfully [60].

5.3. Fluorescence methods

Aflatoxins have a native luminescence due to their oxygenated pentaherocyclic structure. Thus, most analytical and microbiological methods for detection and quantification of aflatoxins are based on this feature. There are a number of microbiological methods that can be used for the direct visual detection of aflatoxin-producing *Aspergillus* strains. The aim of these procedures is to increase the production of aflatoxins and elicit at bright blue or blue-green fluorescent areas surrounding colonies under UV radiation. Complex agar media containing different additives to increase the production of aflatoxins have been implemented for this purpose. The addition of a methylated derivative of of β-CD plus sodium deoxycholate (NaDC) to yeast extract agar (YES) was found to be suitable for the identification of aflatoxigenic *Aspergillus* strains. This was achieved through the visualization of a beige ring surrounding the colonies. When this ring was examined under UV light, it exhibited blue fluorescence. Furthermore, it was observed that aflatoxigenic colonies grown in such environment also emitted room temperature phosphorescence (RTP), when examined in the dark, following excitation with a UV light lamp [61]. The main problem with this technique is related with the disturbance due to the background emission origination from matrix constituents, this because the emission maxima depends on the solvent and the pH. This problematic was addressed and solved by applying two-photon excitation conditions [62].

6. Conclusions

More than 300 micotoxins are discovered. They are toxic metabolites of a variety of fungi growing in a wide range of food and animal feedstuffs. Of all micotoxins, the aflatoxins are the major concerns as they are mutagenic, carcinogenic, teratogenic and immunosuppressive compounds. Consumption even at very low concentration may cause serious health problems. For the aforementioned reasons, it is important to develop new methodologies and systems able to quantify the aflatoxins concentrations that satisfy the restrictions proposed by the organizations in charge of control this compounds. To do this, several techniques have been employed such as: chromatography, immunological methods, biosensors and others methods. Through the paper can be noticed that almost all techniques need to combine efforts to accomplish precise quantifications. These combinations have depended greatly of technology development during the last years. In the case of chromatography, if the methods of pre-process, derivatization and detections improve their capabilities to achieve their functions, it can be developed new systems with higher sensitivity and portability than the so far developed systems.

In the case of immunological methods, there are several research papers reporting advances in the development and improvement of immunological techniques for detection of aflatoxins. Most of them are based on ELISA, although there are other techniques such as ICA and real-time PCR that have been used for this purpose; the objective of these studies is to achieve the development of rapid, simple, highly sensitive and low-cost techniques.

Future aflatoxins detection methods shall be guided by biosensors with mixed techniques, which have already proved their contribution, and utility in sensing and detection technology. Such sensors might be also used in biosecurity brigades along international borders. Biosensors may play a major role in this field as they provide rapid and specific detection compared to other techniques. A barrier that shall be overcome is the production of biosensors for harsh environments. Research on materials, techniques and working parameters need to be made to solve such problems. Portability is another obstacle to be defeated. The use of biosensors in small laboratories and the agricultural industry will increase as biosensors become more portable.

Tendencies in the development of new methods for quantifying the aflatoxins suggest a continuous combination among the different techniques. The combination of different techniques allows increasing the sensibility, portability and rapidness of analysis.

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