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Common Methods to Detect Mycotoxins: A Review with Particular Emphasis on Electrochemical Detection

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Abstract: Mycotoxins are metabolites produced naturally, due to their presence in foodstuffs and their potential risk for human health, the food industries require analytical methods to secure the quality and stability of raw materials, processes and products. These methods must offer real time data that allow to control and to monitor each process to safeguard the innocuity of the nutritional products. The traditional analytical methods have to deal determinations with limited sensitivity levels and little specificity, they are expensive. Although the chromatographic methods show highly reproducible results and good limits of detection. Other alternative is the use of biosensors. In this review, some electrochemical methods about the detection of mycotoxins are presented with the objective to review and updated information of the methods employed to detect mycotoxins besides to know the regulation of the limits of mycotoxins in food.

Keywords: Mycotoxins; Electroanalytical methods.

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Introduction

Mycotoxins are produced by the secondary metabolism of different fungi, contaminated food, feed or raw materials used in their production, causing diseases and disorders in human beings and animals. Besides of its great variety of toxic effects, they also present extreme heat resistance. The majority of the agricultural products can be susceptible to contamination in almost any time so European legislation is becoming more restrictive in terms of the levels of mycotoxins in foods in order to ensure quality and health for consumers, hence maximum contents of these compounds have been established.

The most recent technical advances in terms of efficiency and miniaturization have been assessed with the first techniques such as capillary electrophoresis (CE), capillary liquid chromatography (capillary HPLC) and ultra-high performance liquid chromatography (UHPLC), coupled to very sensitive and selective detection techniques such as laser induced fluorescence (LIF). Nowadays, alternative sample treatments together with the use of biosensors [1,2] make an increment in efficiency and sample throughput as well as reducing contaminant waste possible.

For a long time, the chemistry has joined the advances in modern electronics (microcontrollers) due to the necessity of low cost analytical instrument that could provide qualitative and quantitative chemical information. Additionally, these technologies could provide great capabilities to acquire and process sensor signals. The development of semiconductor compounds manufacturing technology could also produce many light-emitting diodes (LED) with wavelengths from IR down to the 365 nm UV range. With its compact size, a wide range of selections of wavelengths and low power consumption are available and LEDs are used extensively in analytical instruments as light sources [3].

Mycotoxins

The mycotoxins produced by fungi are able to grow in great variety substrates and under the most diverse environmental conditions. These have been identified as dangerous agents for the human health. Humans are exposed to mycotoxins in consumption of polluted foods, a sharp intoxication manifests for vomit, abdominal pains, lung edema, fatty infiltration and necrosis of the liver. The excessive ingest is associated with hepatic cancer and renal damage.

The fungi kingdom comprises a group of organisms that can be classified in yeast and filamentous fungi or molds. The producers of mycotoxins, are multicellular eukaryotic organisms filamentous constituted by true mycelium. Besides lacking chlorophyll, they are formed by a series of aligned cells, called hyphae. The mycelium is the set of branching hyphae, and is visible on the food which is developed, on the surface or inside, by a characteristic color and appearance (Fig. 1). The molds are used for growing a series of chemicals called primary metabolites, such as nucleic acids, proteins, carbohydrates and lipids [4].

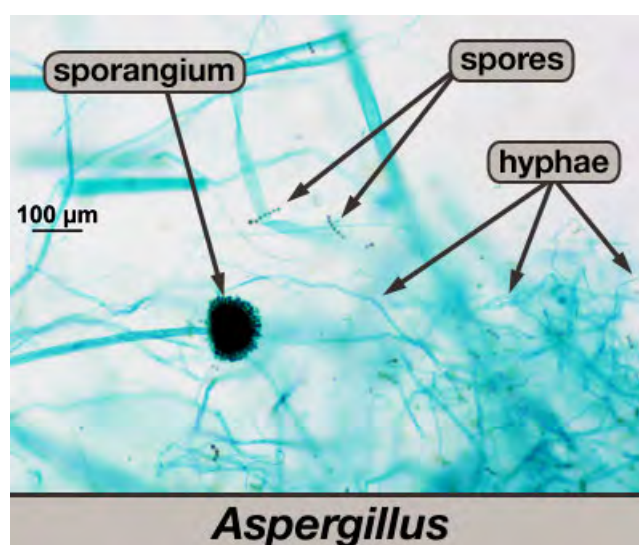


Fig. 1: *Schema of aspergillus mold.*

Mycotoxins, term derived from Greek *mikes* and *toxicon*, which mean fungi and poison respectively, are produced during the final stage of mold growth phase or the early stationary phase; moreover, these are associated with the differentiation and sporulation [5]. The chemical structure of the main mycotoxins found in foods is showed in Fig. 2. The general polyketonic chain for the formation of mycotoxins is $R-CO-CH_2-CO-CH_2-CO-CH_2-CO-CH_2-CO-ScoA$ [6]. The principal mycotoxins are showed in Table I with some important characteristics [7-9].

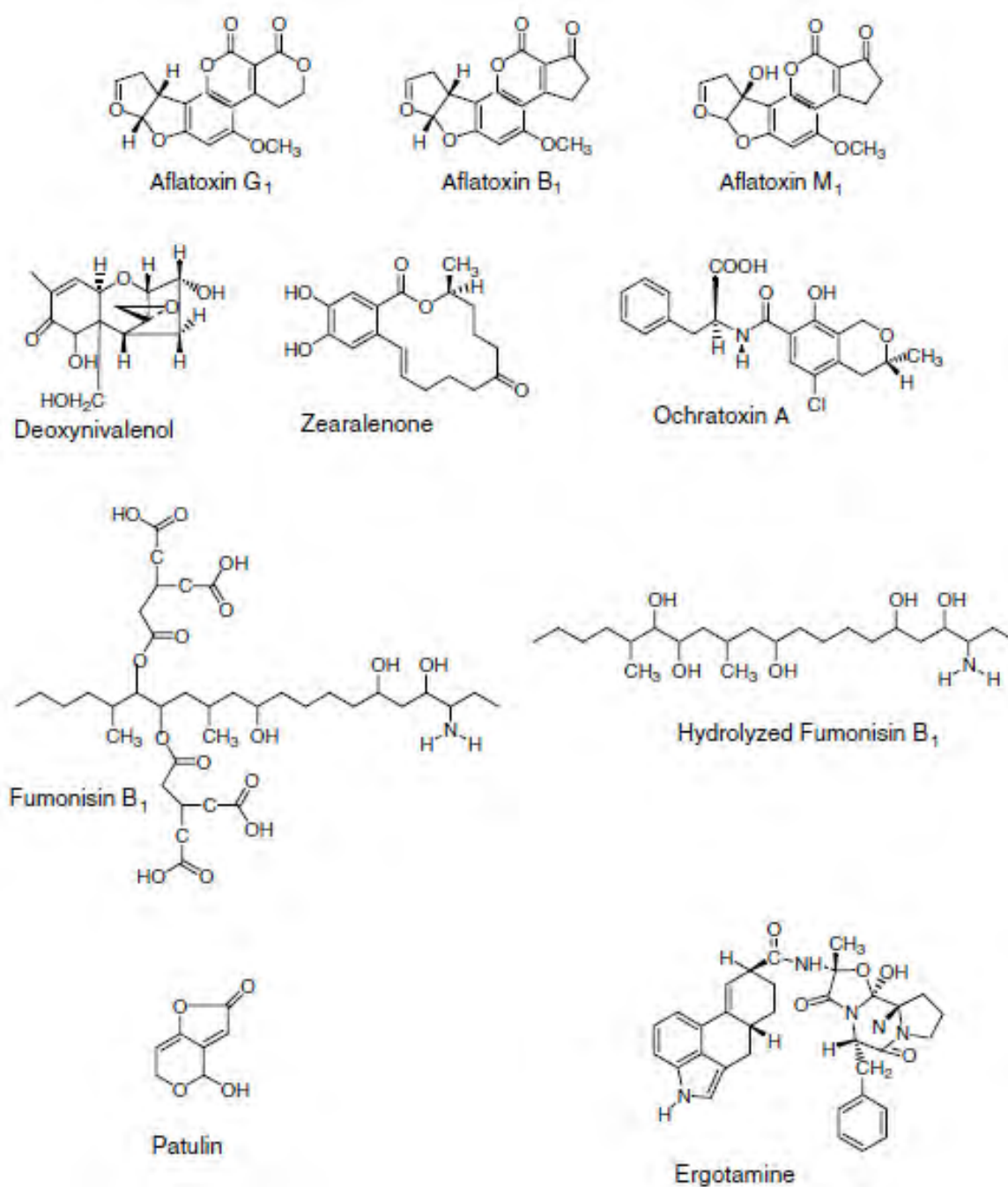


Fig. 2: Chemical structure of the main mycotoxins.

Classification of mycotoxins

The most important mycotoxins are toxins produced by molds of the family of *Aspergillus*, *Fusarium* and *Penicillium*, which can be broadly categorized into the following groups.

Table I: *Main mycotoxins or mycotoxin group and their features.*

Toxin	Source	Appearance	Molecular formula	Solubility	Symptoms	Affected species
Citrinin	<i>Penicillium citrinum</i>	Yellow crystalline solid	$C_{13}H_{14}O_5$	Clear yellow solution at 10 mg/mL DCM.	Isolated as a broad spectrum antibiotic.	Fruits, rice, cheese, wheat
Zearalenone	<i>Fusarium sp.</i>	White powder	$C_{18}H_{22}O_5$	DMSO, EtOH	Infertility	Corn, wheat, barley, oats, sorghum, hay and forage
Patulin	<i>Penicillium expansum</i>	White crystalline solid	$C_7H_6O_4$	DMSO		Fruits, rice, cheese, wheat
Ochratoxin A	<i>Aspergillus ochraceus</i>	Yellow crystals, blue fluorescence	$C_{20}H_{10}ClNO_6$	DMSO, MeOH, EtOH	Potent teratogen in mice, rats, hamsters and chickens, but no apparently in pigs when fed to sows during early pregnancy. Affects the immune system in mammalian species.	Corn, barley, hay
Fumonisin B1	<i>Fusarium moniliforme</i>	White to off-white powder	$C_{34}H_{59}NO_{15}$	Slightly soluble in water, soluble in acetonitrile. Insoluble in chloroform, petrol ether, hexane.	Lethargy, liver damage, pulmonary edema, respiratory problems.	Corn, wheat, barley, oats, sorghum, hay and forage
Moniliformin	<i>Fusarium moniliforme</i>	Yellow crystalline solid	C_4HO_3Na	Water, MeOH	Lethargy, liver damage, pulmonary edema, respiratory problems.	Corn, wheat, barley, oats, sorghum, hay and forage
Sterigmatocystin	<i>Aspergillus versicolor</i>	Pale yellow needles	$C_{18}H_{12}O_6$	DMSO, MeOH, EtOH, acetone		

Toxin	Source	Appearance	Molecular formula	Solubility	Symptoms	Affected species
T2 toxin	<i>Fusarium tricinctum</i>	White crystalline solid	$C_{24}H_{34}O_9$	DCM, DMSO, EtOH, ethyl acetate. Slightly soluble in petroleum ether; very slightly soluble in water.	Increases blood-brain barrier permeability in rats.	Corn, wheat, barley, oats, sorghum, hay and forage
Aflatoxin B1	<i>Aspergillus flavus</i>	Off white powder; blue fluorescence	$C_{17}H_{12}O_6$	DCM, DMSO, MeOH	Diarrhea, reduction of consumption, immunosuppression, liver damage, low growth and low efficiency.	Corn, peanuts and cottonseed
Aflatoxin B2	<i>Semisynthetic. Made from aflatoxin B1</i>	Off white powder; blue fluorescence	$C_{17}H_{14}O_6$	DMSO, MeOH	Diarrhea, reduction of consumption, immunosuppression, liver damage, low growth and low efficiency.	Corn, peanuts and cottonseed
Aflatoxin G1	<i>Aspergillus flavus</i>	Off white powder; blue-green fluorescence	$C_{17}H_{12}O_7$	DMSO, MeOH	Potent liver carcinogen and DNA damaging agent. Mutagenic, teratogenic and causes immunosuppression in animals.	Corn, peanuts and cottonseed
Aflatoxin G2	<i>Semisynthetic. Made from aflatoxin G1</i>	Off white powder; blue-green fluorescence	$C_{17}H_{14}O_7$	DMSO, MeOH	Potent liver carcinogen and DNA damaging agent.	Corn, peanuts and cottonseed
Aflatoxin M1	<i>Aspergillus flavus</i>	Off white powder; blue-violet fluorescence	$C_{17}H_{12}O_7$	DMSO, MeOH	Potent liver carcinogen and DNA damaging agent. It is also mutagenic, teratogenic and causes immunosuppression in animals.	Corn, peanuts and cottonseed

Toxin	Source	Appearance	Molecular formula	Solubility	Symptoms	Affected species
Aflatoxin M2	<i>Aspergillus flavus</i>	Off white powder; blue-violet fluorescence	$C_{17}H_{14}O_7$	MeOH, DMSO	Potent liver carcinogen and DNA damaging agent. It is also mutagenic, teratogenic and causes immunosuppression in animals.	Corn, peanuts and cottonseed

Abbreviations: DCM: Dichloromethane (methylene chloride); DMSO: Dimethyl sulfoxide; MeOH: Methanol; EtOH: Ethanol.

Aflatoxins. Aflatoxins are a group of mycotoxins produced by strains of *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus parasiticus*, *P. Verrucosum* [10,11]. These molds can grow on peanuts, corn, cotton seeds, nuts, copra, cereals, oilseeds such as sunflower and soybeans, unrefined vegetable oils, spices (paprika and chili pepper), dried fruits (dried figs and raisins), coffee, cocoa, and feed [12]. Sixteen aflatoxins have been identified, but only AFB1, AFB2, AFG1, AFG2, and AFM1 are routinely analyzed [13]. The letter B indicates that these aflatoxins have blue fluorescence to ultraviolet light (365 nm), while the letter G indicates the yellow-green fluorescence. Aflatoxins occur at temperatures that are between 25 and 35 degrees Celsius and the maximum yield in relation to the Aflatoxin B is attained between 28 and 30 degrees Celsius [14, 15].

Ochratoxins. Ochratoxins are mycotoxins produced by certain fungi (*Aspergillus ochraceus* and *Penicillium verrucosum*). Other metabolites derived from ochratoxin A, ochratoxin α , ochratoxin β and the open form of the lactone were identified [16]. Structurally, they have a particularity of containing a chlorine atom. Naturally, they are found in many plant products, such as cereals, coffee, beans, cocoa, and nuts. They have been also detected in products made from cereals, wine, beer, and grape juice, as well as in animal products, such as pig kidneys [12]. Ochratoxin A (OTA) shows carcinogenic, nephrotoxic, teratogenic, immunotoxic, and neurotoxic properties. It has been also associated with nephropathy in humans. OTA is a small molecule soluble in water and it is chemically constituted by a combination of an amino acid (phenylalanine) and a polyketide to carbon 10, contains one chlorine atom necessary for its biological activity, and it is stable when exposed to heat [17].

Fusarium Toxins. These molds are the main contaminants in temperate regions; corn is the most contaminated product. It is also present in grains and malt brewing [18-20]. *Fusarium* toxins can be grouped as follows:

- *Estrogenic toxins* with lactone structure, such as zearalenone and zearalenol. The three dimensional structure of the lactone ring-OH group is formed at such a position that it can interact with the estrogen receptor.
- *Non-estrogenic toxins* known as trichothecenes are deoxynivalenol, nivalenol, T-2, and HT-2 and diacetoxyscirpenol. Among the trichothecenes, the most important and common toxin is deoxynivalenol (also known as “vomitoxin”), which is very stable in technological treatments. The T-2 toxin is produced in cold areas.

- *Fumonisin*s produced by *Fusarium* molds. At least 15 types of fumonisins are known, of which the most important is the fumonisin B1, produced by the fungus *Fusarium verticilloides* and *Fusarium proliferatum*. Such fungi are common contaminants of corn, and these toxins are related to the occurrence of esophageal and liver cancer, neural defects and poisoning.

Other Toxins

Patulin. Mycotoxin produced by several species of *Penicillium*, *Aspergillus* and *Byssoschlamys*. It appears in pear, grapes, cereal grains, and silage. Its highest incidence is in apple and apple products [21-24].

Citrinin. It is a mycotoxin produced by species of the genre *Aspergillus*, *Penicillium* and *Monascus*. Although characterized as antibiotic, antifungal and bacteriostatic demonstrated their capacity subsequently made responsible for swine neuropathy in several European countries. Their presence has been detected in various foods such as cereals, fruits, cheeses, acorns, nuts, carrots, tomatoes and meat. Citrinin has nephrotoxic and mutagenic effects [12,25].

Sterigmatocystin. It is a mycotoxin produced by *A. versicolor*, precursor of AFB1, AFG2, and AFG1. This related to gastric, liver and esophageal carcinomas. Their presence has been detected in cereals, coffee, ham, pepper and cheese [12,26].

Ergot Alkaloids. Commonly known as ergot alkaloids are produced in sclerotia of different fungi, which infect rye grains, wheat, barley, millet, and oats [27].

Toxicity of the mycotoxins

Mycotoxins are ubiquitous in a broad range of commodities and feed. They are toxic to mammals, poultry, and fish. They induce powerful and dissimilar biological effects. Some are carcinogenic (aflatoxins, ochratoxins, fumonisins), mutagenic (aflatoxins, sterigmatocystin), teratogenic (ochratoxin), estrogenic (zearalenone), hemorrhagic (trichothecenes), immunotoxic (aflatoxins and ochratoxins), nephrotoxic (ochratoxins), hepatotoxic (aflatoxins and phomopsins), dermatotoxic (trichothecenes) and neurotoxic (ergotoxins, penitrems, lolitrems, and paxilline). The ingestion of the mycotoxins is due to the consumption of plant-based foods

and their residues or metabolites in animal derived food. Diseases caused by the ingestion of mycotoxins are called mycotoxicosis [28].

Most cereal grains, oil seeds, tree nuts and dehydrated fruits are susceptible to fungus contamination and mycotoxin formation. Agricultural products are susceptible to contamination from production in the field, during harvest, transport or in storage as shown in Fig. 3 [29]. The impact of mycotoxins on health depends on the amount of the mycotoxin consumed, the toxicity of the compound, acute or chronic effects, the body weight of the individual, the presence of the other mycotoxins and other dietary effects [30]. Hsieh added other criteria as occurrence of the mycotoxins in food supplies, correlation between exposure and incidence, reproducibility of the characteristics symptoms in experimental animals, also the action in human [31].

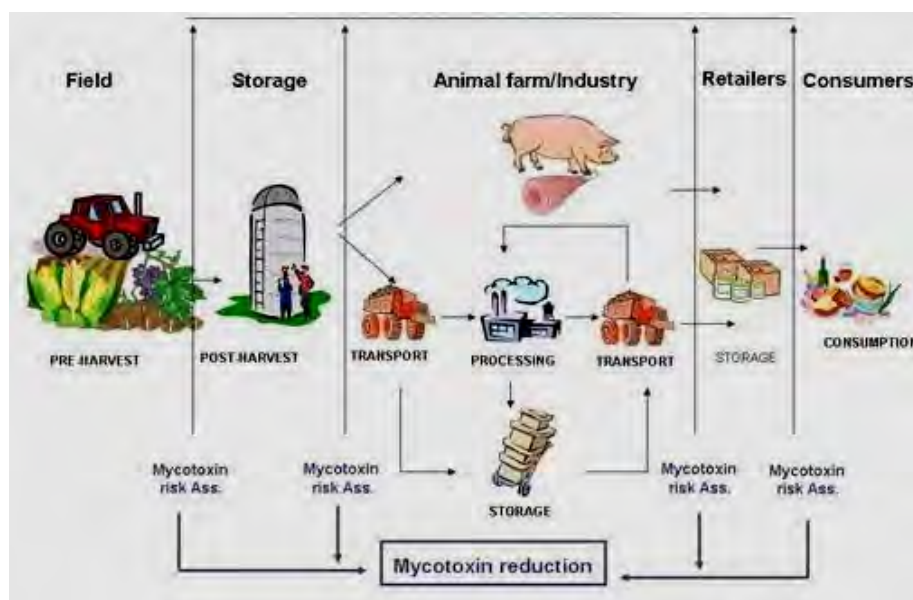


Fig. 3: *Spreading scheme for mycotoxins.*

Experience suggests that a definitive diagnosis cannot be made directly from symptoms, specific tissue damage or even feed analyses [31,32]. Mycotoxins can increase disease incidence and reduce production efficiency in cattle. They exert their effects through three primary mechanisms:

- 1) Alteration in nutrient content, absorption and metabolism.
- 2) Changes in the endocrine and neuroendocrine function.
- 3) Suppression of the immune system.

The International Agency for Research on Cancer (IARC) [33] classified several mycotoxins as carcinogenic or potentially carcinogenic to humans are showed in the Table II, according to the following groups:

- *Group 1*: The agent is carcinogenic to humans.
- *Group 2A*: Probably carcinogenic agent in humans, there is limited evidence in humans but sufficient animal.
- *Group 2B*: Possibly carcinogenic agent, the evidence in humans is limited and there is no sufficient evidence in experimental animals.
- *Group 3*: The agent is not classified as a human carcinogen, and cannot be included in another group.
- *Group 4*: The agent is probably not carcinogenic to humans; the available evidence from both human and animal studies suggests so.

Table II: *Classification of mycotoxins by IARC.*

Mycotoxins	Group
Aflatoxins, natural source	1
Aflatoxin M1	2B
Citrinin	3
Sterigmatocystin	2B
Fumonisin B1	2B
Ochratoxin A	2B
Patulin	3
Toxins of <i>Fusarium graminearum</i> , <i>F. culmorum</i> , <i>F. crookwellense</i>	3
Toxins of <i>Fusarium sporotrichioides</i> (T-2)	3

Yoshio Ueno [34] has ranked the most important toxins according to their affinity with cellular organelles, toxicological characteristics, and metabolic transformations. According to this criterion, mycotoxins are classified as:

- *Inhibitors of energy production*: inhibition of the activity of adenosine triphosphatase (ATPase), cell oxidative phosphorylation.
- *Inhibitors of protein synthesis*: inhibition of synthesis, elongation and termination of the protein, or competitive inhibition of the activity in the phenylalanyl-tRNA synthetase.

- *Cytoskeletal modifiers*: modification in the functions of cellular microtubules and microfilaments.
- *Estrogenic mycotoxin*: growth responses in the uterus, and abnormal hormone levels.
- *Generators of trembling*: induction generalized of tremors in animals.
- *Carcinogenic mycotoxins*: tumors in liver and renal cortex.

Current Legislation

In the late 90's, the regulations for mycotoxins were national laws. Nowadays, these regulations have been unifying and harmonizing for members of the European Union and other economic communities, increasing regulations in the countries. In 2003, several countries had regulations for mycotoxins in food products [35]

Currently, USA established the maximum levels permitted for certain contaminants in foods by the Regulation (EC) No. 1881/2006 [36]. In this regulation, the maximum levels for aflatoxins (AFB1, AFB2, AFG1, AFG2, and AFM1) in nuts, grains, milk, and baby food, OTA in cereals, raisins, coffee, wine, grape juice, food cereals, dietary foods for infants and young children, patulin in fruit juices, spirits made with apple, baby food and solid apple products made, peoxynivalenol in cereals, pasta, bread and baby food cereal, zearalenone in cereals, snacks and baby food cereal and fumonisins in grains and foods made from corn (levels are between 2-200 mg/kg) are included. This regulation has been amended in relation to the content of some mycotoxins due to increases the maximum allowable level in certain foodstuffs [37-41].

Moreover, the European recommendations for controlling corzuelo alkaloids (ergocristina, ergotamine, ergocryptine, ergometrine, ergosine, and ergocornine) in feed and food [42], together with T-2 and HT-2 toxins in cereals and cereal products [43] as to the requirements of methods of sampling and analysis for use in the control of mycotoxins in foods, these are set out in EC Regulation No. 401 /2006 [44,45].

If the Community does not require any specific method for the determination of mycotoxins in foodstuffs, laboratories may select any method of their choice provided that meets the criteria established by established regulation and to have a complete overview of the existing legislation for mycotoxins [46].

Legislation for Aflatoxins. The limits for aflatoxins may be controlled as the total aflatoxins referring to the sum of aflatoxin B₁, B₂, G₁, G₂, and/or aflatoxin B₁. In the European Union (EU), the limit for aflatoxin B₁ for products of infants is set as 2 µg/kg (groundnuts, peanuts, tree nuts, dried fruit and its processed products, cereals and products derived from cereals). The limit for aflatoxin B₁ is 12 µg/kg for foodstuffs (almonds, pistachios, and apricot kernels). To the total aflatoxins, the limit of food for infants is 4 µg/kg while the highest limit is set for groundnuts, almonds, pistachios, apricot kernel, hazelnuts, and Brazil nuts at 15 µg/kg. Other countries such as Turkey, Bosnia, Herzegovina and Switzerland seem to be influenced by the EU Regulation [47].

Countries such as Austria, Germany, Denmark, Finland, Spain and Sweden their limits vary from 1 µg/kg in Austria, 5 µg/kg in Spain for aflatoxin B₁, 4 µg/kg in Denmark and Germany to 10 µg/kg in Spain for total aflatoxins. China, Brazil, and Mexico have the most comprehensive legislation on aflatoxins. Specific maximum limits are set for aflatoxin B₁ and/or total aflatoxin in several foodstuffs in these countries. Other countries such as the United States of America (USA), Canada, Australia and New Zealand, Gulf Cooperation Council (GCC), and Nigeria lay down specific limits for total aflatoxins in mainly nuts as indicated the Table III.

Table III: Total limits of aflatoxins in some countries.

Country	Foodstuffs	Total aflatoxins (µg/kg)
Australia,	Peanuts	15
New Zealand	Tree nuts	
Canada	Nut and nut products	15
Codex GCC ^{a)} ,	Peanuts, almonds, shelled Brazil nuts, hazelnuts pistachios	15
Nigeria	intended for further processing	
	Almonds, hazelnuts, pistachios, shelled Brazil nuts, “ready-to-eat”	10
India	Wheat, maize, jawar (sorghum) and bajra, rice, whole and split pulse (dal) masur (lentil), whole and split pulse urd (mung bean), whole and split pulse moong (green gram), whole and split pulse chana (gram), split pulse arhar (red gram), and other food grains	30
	Groundnut kernels (shelled) (peanuts)	30
USA	Brazil nuts, peanuts and peanut products, pistachio products	20
South Africa	Peanuts	15

^{a)} Members of GCC are Saudi Arabia, United Arab Emirates, Kuwait, Bahrain, Oman, Yemen, and Qatar.

One of the strictest controls is in Japan where the total aflatoxins level in all foodstuffs must be below 10 µg/kg. The limits for aflatoxin M1 are generally only laid down for milk and milk products and in some cases for infant and products for infants as illustrated in Table IV. All EU Member States other than Germany do not have any additional national limits laid down for aflatoxins M1. The German national legislation specifies an additional maximum limit for aflatoxin M1 for dietetic foodstuffs for infants and young children at 0.01 µg/kg.

Table IV: *Aflatoxin M1 limits.*

Country	Foodstuffs	Aflatoxin M1 (µg/kg)
EU,	Raw milk, heat-treated milk and milk for the manufacture of milk-	0.050
Bosnia and	based products	
Herzegovina,	Infant formulae and follow-on formulae, including infant milk and	0.025 ¹⁾
Turkey	follow-on milk	
	Dietary foods for special medical purposes intended specifically for infants	0.025 ¹⁾
China	Milk and milk products (for milk powder, calculated on a fresh milk basis)	0.5
	Formulated foods for infants (milk or milk protein based)	0.5 ²⁾
	Formulated foods for older infants and young children (milk or milk protein based)	0.5 ²⁾
	Formulated foods for special medical purposes intended for infants	0.5 ²⁾
Codex GCC, India, Kenya, USA	Milk	0.5
Argentina	Milk, liquid including milk used in the manufacture of milk and milk products and reconstituted milk	0.5 ³⁾
	Milk, powder	5.0
	Milk formulae	ND
Mexico	Pasteurized, ultrapasteurized, sterilized and dehydrated milk, milk formula, and combined milk products	0.5 ³⁾
South Africa	Milk	0.05

¹⁾ Products ready to use. ²⁾ Calculated on dry powder basis. ³⁾ Given in µg/l. ND: Not Detectable.

Legislation for Ochratoxin A. In the EU, the Commission Regulation lays down specific maximum limits for OTA to foodstuffs such as unprocessed cereals, dried vine fruit, coffee, beans, soluble coffee, wine, grape juice, spices, liquorice, and products for infants. The lowest maximum limit other than in products for infants is established for wine and grape juice at 2.0 µg/kg, while the highest maximum limits is set at 80 µg/kg for liquorice extract for use in foods. Additional national maximum limits for OTA are established in EU Member States such as Denmark, Hungary, Italy, and Germany in their national legislation. Table V present the maximum accepted levels for some food contaminated with ochratoxin A.

Table V: *Maximum accepted levels of Ochratoxin A in various foods in Europe.*

Products	Max. accepted level (µg/kg)
Crude cereals	5
Processed cereals	3
Dried raisin	10
Roasted coffee	5
Soluble instant coffee	10
Wine (red, white, rose) and raisin derived products	2
Raisin juice and derived products	2
Grape must	2
Baby foods and cereal based baby foods	0.5
Probiotics	0.5
Liquorice root	20
Liquorice extract	80
Spices	30

Egypt and Bosnia and Herzegovina refer to the Commission Regulation for setting up their national maximum limits for OTA. In Russia, the limit in wheat, barley, rye, oat and rice cereals and cereal products is set at 0.005 mg/kg while the limit for specific products for children is set at 0.5 µg/kg. In China a limit of 5.0 µg/kg for OTA is set for cereals, milled products from cereals, legumes and pulses. GCC, Nigeria and Kenya established OTA limit of 5 µg/kg is set for raw wheat, barley and rye. In India, the limit for OTA is established at 20 µg/kg. No specific limits for OTA in foodstuffs are set in USA, Canada, Australia and New Zealand, Japan, Mexico and South Africa [47].

Legislation for Deoxynivalenol. In the EU, the maximum limits are 200 µg/kg for processed cereal-based foods and baby foods for infants and young children and 1750 µg/kg for unprocessed durum wheat, oats, and unprocessed maize. For other international countries, maximum limits are mainly set for cereals, especially wheat. Table VI summarizes the maximum limits for deoxynivalenol in several countries [47].

Table VI: *Maximum limits for deoxynivalenol.*

Country	Foodstuffs	Deoxynivalenol (µg/kg)
Canada	Uncleaned soft wheat for use in non-staple foods	2000
	Uncleaned soft wheat for use in baby foods.	1000
China	Corn, cornmeal (coarse cornmeal, flakes)	1000
	Wheat, barley, breakfast cereals, wheat flour	1000
India	Wheat	1000
Japan	Wheat	1100 ¹⁾
Russia ²⁾	Wheat and wheat cereal, oatmeal and flakes; wheat flour; pasta products; bread; bakery products; wheat germ; food protein from the seeds of wheat	700
	Barley and barley cereal, oatmeal and flakes; barley flour; pasta products; bread; bakery products; barley germ; food protein from the seeds of barley	1000
	Flour confectionery	700
USA	Finished wheat products	1000 ³⁾

¹⁾ Provisional limit. ²⁾ Additional limits are specified for several types of products for children and products for pregnant and nursing women. ³⁾ Advisory limit.

Legislation for Patulin. China, GCC and USA have a maximum limit of patulin for apple products of 50 µg/kg. Other countries provided its limits according to the Table VII. In the EU, the universal limit 50 µg/kg also applies to products such as fruit juices, nectars, spirit drinks, cider, and other fermented drinks derived from apples or containing apple juice [47].

Legislation for Fumonisin and Zearalenone. In the EU, the limits for fumonisins and zearalenone are established for certain foodstuffs mainly in cereal and cereal-based foods products. Many countries take reference to the EU legislation, such as Turkey, Bosnia and Herzegovina, Norway, and Switzerland. In the USA, the Food and Drug Administration

(FDA) issued an advisory to industry on certain substances, including fumonisins. Specific maximum limits for zearalenone are laid down in the Brazilian, Chinese, and Russian national legislation. However, many other international countries including USA, Canada, Japan, Australian, New Zealand, GCC, and African countries do not establish specific maximum limits for zearalenone. Chile has a limit for zearalenone for all foodstuffs, which is set at 200 mg/kg [47].

Table VII: *Maximum limit for Patulin.*

Country	Foodstuffs	Patulin ($\mu\text{g/kg}$)
China	Fruit products containing apple or hawthorn (excluding Guo Dan Pi, a Chinese-style fruit snack)	50
	Fruit or vegetable juice containing apple or hawthorn juice	50
	Alcoholic beverages containing apple or hawthorn	50
Codex, GCC, Kenya, Nigeria,	Apple juice	50
India	Apple juice and apple juice ingredients in other beverages	50
Japan	Apple juice and food made of only apple juice as raw material	50
South Africa	Apple juice, apple juice ingredients in other juices	50
USA	Apple juice, apple juice concentrates and apple juice products	50

Methods to Detect Mycotoxins

Approximately a percentage of plant foods are contaminated with mycotoxins. Continuous analysis of contaminants in food requires methods able to detect the levels indicating the levels allowed to preserve the health of the population [48,49]. Therefore, the EU has regulated the minimum requirements to be met by an analytical method for use in the control of these contaminants in food [36-46].

Several recommended methods of analysis for the determination of mycotoxins in foods such as Official Methods of Analysis of the Association (AOAC) [50] which are validated for the determination of mycotoxins in various foods, or standard methods proposed by the International organization for Standardization (International Standard Organization, ISO) and the European Committee for Standardization (CEN).

There are different analytical methods for the determination of mycotoxins in foods and their applications [51-62]. The most numerous are the methods employing high-performance liquid chromatography (HPLC) with UV [63-66] or fluorescence (FL) [67-70] detection. There are numerous methods for OTA determination using HPLC-FL in different matrices such as wine, coffee or beer [52]. In the case of aflatoxins, their fluorescence is dimmed in the presence of certain solvents, therefore an affinity column or UV lamp and a reactor is required in some cases [71-76].

Other mycotoxins, which are not fluorescent, such as fumonisin [77,78] or T-2 and HT-2 [66], were determined using HPLC-FL after derivatization. The coupling of HPLC and mass spectrometry (MS) [53] using atmospheric pressure ionization (API), such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), has allowed the development of new methodologies for the determination of mycotoxins [79-81].

Using tandem MS (MS/MS) with detectors such as ion trap (IT) and triple quadrupole (QqQ), it is possible to identify and quantify the analytes in complex mixtures, *e.g.* methods for the determination of OTA in cheese [82], fumonisin in baby food [83], deoxynivalenol, zearalenone, and its metabolites in cereal products [84], Fusarium toxins in breakfast cereals and baby foods [85], wheat crackers [86], T-2 and HT-2 in cereals [87], zearalenone and its metabolites in traditional Chinese medicines [88], and enniatins producing mycotoxins (A, A1, B, and B1) and beauvericin bases in cereal products [89]. Recently, HPLC-MS/MS has been proposed for the simultaneous determination OTA, mycophenolic acid, and meat fumonisin B2 [90] mycotoxins and other secondary metabolites in corn, wheat, beer, and baby foods [91-95].

Screening Techniques

The purpose of these techniques is to do a fast analysis to discard the negative samples and reduce the number of analysis. These techniques are sensitive but not very selective. The common screening techniques employed for the analysis of mycotoxins are described in following paragraphs [12].

Immunoassays. Two techniques are used for the analysis of mycotoxins: radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA). The RIA adds to the reaction medium a specific antibody and a quantity of radiolabeled mycotoxin, which is incubated

with the test sample. The concentration of mycotoxins is determined by comparing the results of a straight pattern. The ELISA technique is based on the specific antigen-antibody reaction, it can be direct or indirect competitive. In direct technique, an extract of the sample is added to the solution and dissolution of the mycotoxin covalently linked to enzyme is observed. Indirect competitive uses a second antibody directed to the constant region of the first antibody. The binding of the first antibody depends on the amount of antigen in the sample [96].

Biosensors. The biosensor is a compact analysis device that incorporates a biological recognition element attached to a transducer system which can process the signal produced by the interaction between the recognition element and the mycotoxin.

When the mycotoxin reacts with the recognition element, it produces a variation in one or more physicochemical properties detected by the transducer. This system transforms the response of the recognition element to electrical signal that indicates the presence of the mycotoxin proportionally to its concentration in the sample. The most used transduction systems in the analysis of mycotoxins are optical and piezoelectric. The main characteristics are stated in Table VIII [12].

Table VIII: Applications, advantages and disadvantages of biosensors to the analysis of mycotoxins.

Transducer	Mycotoxin	Sample	Advantages	Disadvantages
<i>Optical</i>				
Surface plasmon resonance	Aflatoxins B1, Deoxinivalenol, Fumonisin B1, Zearalenone	Cereals	Easy to use. Direct detection in real time. High sensitivity. Unpurified samples.	Sensitive to temperature. High cost.
Evanescent wave	Aflatoxins, Fumonisin	Corn	Rapidity. Selectivity. Direct detection.	Labeling required.
<i>Piezoelectric</i>				
Surface acoustic wave	Ochratoxin A	Liquid samples	Direct detection Easy to use Low Cost Analysis on line	Long incubation. Calibration of each crystal. Interference.

Separation Techniques

The main techniques used to confirm the presence of mycotoxins in the selected samples are summarized below.

Thin Layer Chromatography. It is one of the most used for the investigation and determination of mycotoxins methods. It is a chromatography technique used to separate non-volatile mixtures. In thin layer chromatography (TLC), the stationary phase is polar and mobile phase is non-polar, allowing separation of the mycotoxin to be analyzed. Thin layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material (silica gel, aluminium, cellulose). The layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (mobile phase) is drawn up the due to capillarity. Because different analytes ascend the TLC plate at different rates, separation is achieved.

TLC can be used to monitor the progress of a reaction, to identify compounds present in a given mixture, and to determine the purity of a substance. Specific examples of these applications include: analyzing ceramides, fatty acids, detection of pesticides, assaying the radiochemical purity of radiopharmaceuticals, and identification of components in medical plants [97]. A number of enhancements can be made to the original method to automate the different steps, to increase the resolution achieved with TLC and to allow more accurate quantitative analysis. This method is referred to as high performance TLC (HPTLC).

Separation Process in Thin Layer Chromatography. Different compounds in the mixture travel at different rates due to their attraction to the stationary phase, and because of differences in solubility in the solvent. By changing the solvent, or using a mixture, the separation of components can be adjusted. Separation of compounds is based on the competition of the solute and the mobile phase for binding places on the stationary phase. If normal phase silica gel is used as the stationary phase it can be considered polar. Given two compounds which differ in polarity, the more polar compound has a stronger interaction with the silica and is therefore more capable to dispel the mobile phase from the binding places, so the less polar compound moves higher up the plate, if the mobile phase is changed to a more polar solvent or mixture of solvents, it is more capable of dispelling solutes from the silica binding places and all compounds on the TLC plate will move higher up the plate.

As the chemicals being separated may be colorless, several methods exist to visualize the spots. (i) Fluorescent analytes like quinine may be detected under black light (366 nm) or small amount of a fluorescent compound allows the visualization of spots under UV-C light (254 nm). (ii) Iodine vapors are a general unspecific coloring reagent. (iii) Oxidation using potassium permanganate.

Once visible, the retardation factor (R_f) value of each spot can be determined by dividing the distance the product traveled by the distance the solvent front traveled using the initial spotting site as reference. These values depend on the solvent used and the type of TLC plate and are not physical constants, and example of the TLC plate and how to measure the R_f is represented in Fig. 4 [98].

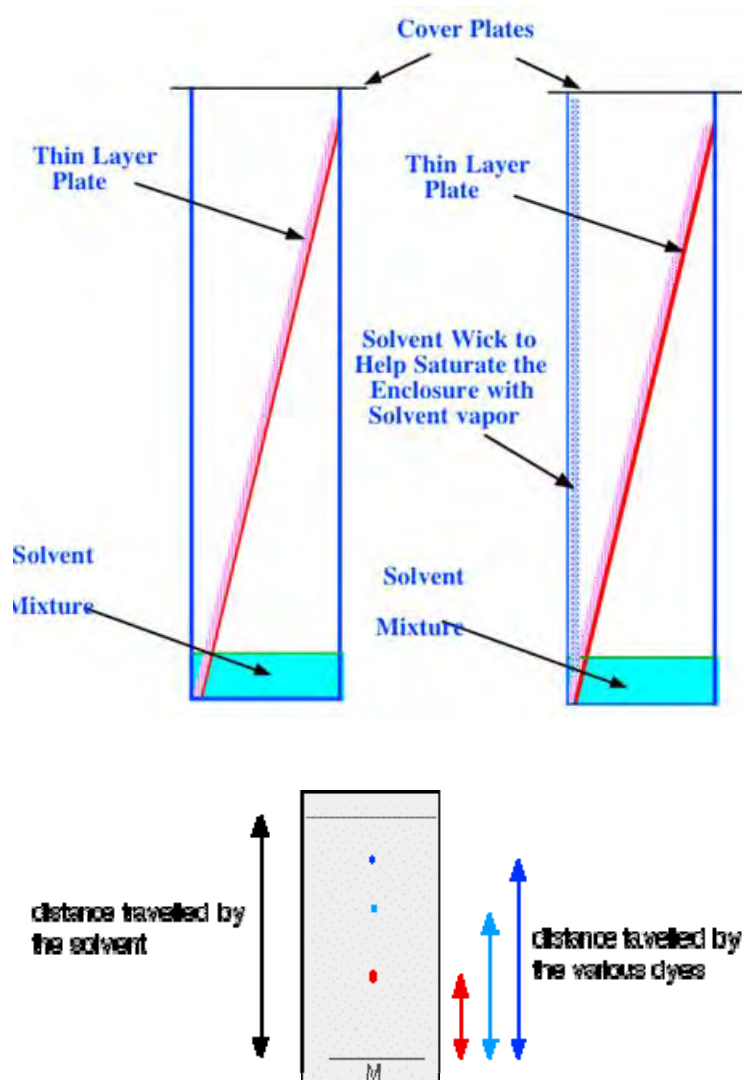


Fig. 4: Development of a TLC plate and its measure [99].

Capillary Electrophoresis. Capillary electrophoresis (CE) is a technique based on the migration of polar molecules in a capillary interior inside which flows buffer when an electric field is applied. The migration of a given ion depends upon the relationship load/size. The variants of electrophoresis include capillary zone electrophoresis (CZE), which uses a simple buffer, and electrokinetic micellar chromatography (MEKC) using a surfactant buffer to form micelles [12]. The electrokinetic micellar chromatography is the most widely used method for analyzing mycotoxins, mainly aflatoxin [100], fumonisin [101], zearalenone [102], and patulin [103]. Its main drawback is the difficulty in determining of low concentrations in real samples.

Instrumentation of Capillary Electrophoresis. The instrumentation needed to perform capillary electrophoresis includes sample vial, source and destination vials, a capillary, electrodes, a high-voltage power supply, a detector, and a data output and handling device (Fig. 5). The source vial, destination vial and capillary are filled with an electrolyte such as an aqueous buffer solution. To introduce the sample, the capillary inlet is placed into a vial containing the sample and then returned to the source vial (by the capillary via capillarity, pressure or siphoning).

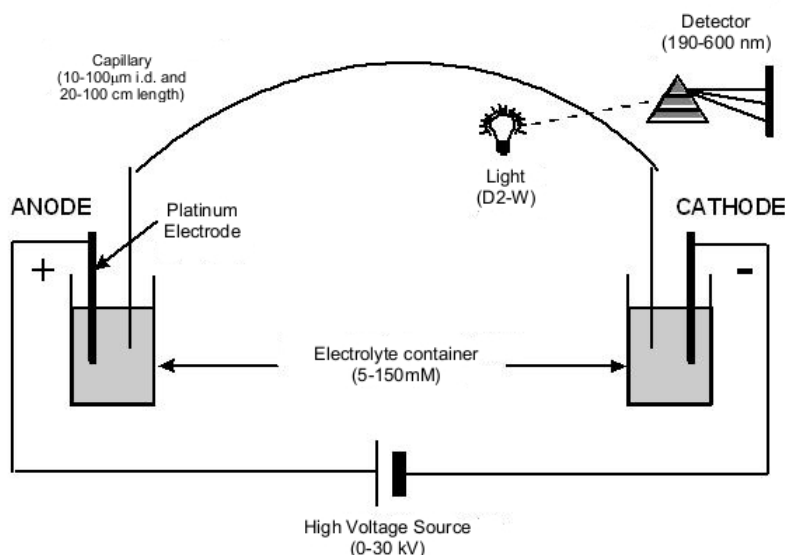


Fig. 5: Capillary electrophoresis instrument [104].

The migration of the analytes is initiated by an electric field that is applied between the source and destination vials and is supplied to the electrodes by the high-voltage power supply. Hence all ions (positive or negative) are pulled through the capillary in the same direction by electroosmotic flow. The separated analytes migrate due to their electrophoretic

mobility, the output of the detector is sent to a data output and handling device. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separated chemical compounds appear as peaks with different retention times in an electropherogram [105].

Detection in Electrophoresis. Separation by capillary electrophoresis can be detected by several detection devices; the majority of commercial systems use UV or UV-Vis absorbance. The portion of the capillary used for UV detection must be optically transparent. Bare capillaries can break relatively easily and capillaries with transparent coatings are available to increase the stability of the cell window. Fluorescence detection can also be used in capillary electrophoresis for samples with naturally fluoresce or that are chemically modified to contain fluorescent tags. This mode of detection offers high sensitivity and improved selectivity for these samples, but cannot be utilized for samples that do not be fluorescent.

Laser induced fluorescence has been used in CE systems with detection limits as low as 10^{-18} to 10^{-21} mol. The sensitivity of the technique is attributed to the high intensity of the incident light and the ability to accurately focus the light on the capillary. In order to obtain the identity of sample components, capillary electrophoresis can be directly coupled with mass spectrometers, or surface enhanced Raman spectroscopy (SERS). In most systems, the capillary outlet is introduced into an ion source that utilizes electrospray ionization (ESI) [105].

Gas Chromatography. Gas chromatography (GC) is a technique based on the separation of compounds as a function of its volatility and affinity for the stationary phase. Mycotoxins are volatile substances and require little preprocessing for analysis by GC. Some mycotoxins such as trichothecenes having reactive hydroxyl groups may be converted to trimethylsilyl, trifluoroacetyl and heptafluorobutryl derivatives that can be determined with a detector [99]. Typical uses of GC include testing the purity of a particular substance, or separating the different components of a mixture (the relative amounts of such components can be determined). In some situations, GC may help in identifying a compound [12,105].

In gas chromatography, the mobile phase is a carrier gas, usually an inert gas (helium or nitrogen). The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column. The gaseous compounds being analyzed interact with the walls of the column, which is coated with a stationary phase. This

causes each compound to elute at a different time; the comparison of retention times gives GC its analytical usefulness.

Firstly, the process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas mobile phase, whereas in column chromatography the stationary phase is a solid and the mobile phase is a liquid. After, the column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column has no such temperature control. Finally, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas.

The first application of gas chromatography with mass spectrometry (GC-MS) for the analysis of mycotoxins was performed in 1981 for deoxynivalenol [110]. Jiao et al performed the first analysis of ochratoxin A by GC-MS with the formation of the methyl ester O-metilocratoxin A. However, the most common use of GC is the analysis of trichothecenes for the group A (diacetoxyscirpenol, T-2 and HT-2) without fluorescence properties, which do not absorb light in the UV region of the spectrum [12].

Liquid Chromatography. Mycotoxins represent a range of compounds of different structure, polarity and acid-base properties. For thirty years, these are analyzed with liquid chromatography (LC). Liquid chromatography is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred to as high performance liquid chromatography (HPLC).

In HPLC, the sample is forced by a liquid at high pressure through a column that is packed with a stationary phase. HPLC is divided into two different sub-classes based on the polarity of the mobile and stationary phases. Its main advantage is that allows to separate non-volatile, polar, non-polar, and thermolabile substances. Several works on the analysis of mycotoxins using LC can be found in the literature [12,106].

Electroanalytical Methods

The electrochemical-based analytical methods are widely used in various disciplines, such as pH measurement methods influence in the detection of mycotoxins [107]. The electroanalytical methods are a class of techniques in analytical chemistry, which study an analyte by measuring the potential (volts) or current (amperes) in an electrochemical cell. The three main methods can be distinguished [105,108].

Potentiometry. The difference in electrode potentials is measured. It is the measure of the potential in solution between two electrodes; the potential is related to the concentration of one or more analytes. Potentiometry uses selective electrodes to the ion of interest. The most common potentiometric electrode is the glass-membrane electrode used in a pH-meter.

Coulometry. The electric charge needed to modify quantitatively the oxidation state of a sample is measured. It is a fast method, does not require calibration, and allows system automation. The total current passed is measured directly or indirectly to determine the number of electrons passed, other hand is to know the number of electrons passed can indicate the concentration of the analyte or the number of electrons transferred in the redox reaction when the concentration is known.

Voltammetry. The current in the cell is measured with respect to the variation of the potential in the cell. Constant or varied potential is applied at the electrode surface and the resulting current is measured with a three electrode system (work, auxiliary, and reference electrode). Chemically modified electrodes are employed for highly sensitive electrochemical determination of organic molecules.

These methods have been combined with other techniques to be used in the detection of mycotoxins; an example is the direct electrochemical detection ZEA due to its moderate electroactive character ZEA [109] by preconcentration on the electrode surface of vitreous carbon and subsequent detection by square wave voltammetry (SWV). Zougagh et al have developed a method for fast and simple screening to determine ZEA and derivatives in corn and flour samples [110], which consists of a supercritical fluid extraction followed by preconcentration and chromatography with electrochemical detection.

Nowadays, the researchers have developed various electrochemical immunosensors for the detection of mycotoxins in different types of samples. The main disadvantage of the conventional electrode-based immunosensor is the difficulty of regeneration of immunorecognition phase. To overcome this problem and get more sensitive disposables and devices, the described immunosensors are based on screen-printed electrodes. The advantages of electrochemical immunosensors are miniaturization, portability and low cost. Currently, immunosensors were developed for some of the major mycotoxins: aflatoxins [111-114], ochratoxins [115,116] and DON [117].

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