Journal of Food Biosciences and Technology,
Islamic Azad University, Science and Research Branch, Vol. 14, No. 3, 31-42, 2024

A Review of Methods for Qualitative Detection of Aflatoxin

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Received: 12 June 2024 Accepted: 21 August 2024

ABSTRACT: One of the factors that is widely scattered in nature and causes contamination of food sources in humans and animals is aflatoxin-producing fungi, which can have dangerous effects on the consumer. It seems that aflatoxins produced by *Aspergillus* species are very dangerous and toxic and cause severe food contamination and high carcinogenic power. It seems that even a low concentration of aflatoxin has dangerous side effects, therefore the identification and quantification of this toxin in food and feed can have a high degree of sensitivity. In this field, there are strong methods for identification and quantification, which necessitates the development of aflatoxin research. The existence of appropriate methods for quantifying these poisons, accurate diagnosis and control can ensure the health of consumers and prevent the occurrence of dangers and side effects of poisons. There are various laboratory methods such as chromatography for the detection of aflatoxins. The conclusion of the study showed that fluorescence method with its high sensitivity and especially in combination with the HPLC technique is a good alternative for diagnostic laboratory uses. Usual FT-NIR and IMS techniques are also fast and inexpensive. Biosensors are among the common methods for detecting and quantifying aflatoxins, considering that they have fewer disadvantages than other methods.

Keywords: Aflatoxins, Method Validation, Quantification.

Introduction

One of the toxic metabolites of fungi that can contaminate food and feed are mycotoxins, and aflatoxins (AFs) are secondary and toxic metabolites of a type of mycotoxins such as *Aspergillus flavus, Aspergillus parasiticus* and the rare *Aspergillus nomius*. By contaminating food, mycotoxins can cause serious complications such as chronic toxicity, carcinogenic mutagenic toxicity and immune system disorders in humans (Akbar *et al.*, 2023). Aflatoxins can be divided into B1, B2, G1, B2a and G2a types with degree of toxicity B1 > G1 > B2. *A. flavus* only produces B aflatoxins, while *A. parasiticus* and *A. nomius* also produce G aflatoxins. Aflatoxins are found in various foods such as peanuts, pistachio nuts, copra, grains, fruits, oil seeds, dried fruits, cocoa, spices, grains, cottonseed corn, and beer, and are mainly produced in

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hot and humid areas. Because the temperature and humidity provide the necessary conditions for the growth of molds and toxins (Akhtar *et al.*, 2020). Although factors such as stress and drought, insect activity, poor soil and lack of storage conditions can be factors of damage to food products. Aflatoxins can become carcinogenic and cause hepatogenic, teratogenic and mutagenic effects in humans and animals when they are absorbed through the ingestion, inhalation or skin routes (Awasthi *et al.*, 2012). When aflatoxin B1 is ingested by cows, it is converted into hydroxylated products (AFs M1 and M2) that can be secreted in milk and stored stably during pasteurization of milk and dairy products. Among the 300 types of mycotoxins, aflatoxin seems to pose serious risks in the food sector all over the world. Many institutions in different countries are conducting research on the identification of aflatoxins and their food classification. Therefore, diagnostic and quantification methods have been developed to identify and classify aflatoxins (Azam *et al.*, 2020). Methods such as ELISA, chromatography, UV absorption, spectroscopy, fluorescence, and immunohistochemistry are among the methods that have good sensitivity and quantification, that has caused researchers to identify aflatoxins. The aim of this research is to introduce different methods for identification and quantification of aflatoxins in food.

Type of aflatoxins

Aflatoxin B1 has a molecular weight of 312 $g \cdot mol^{-1}$ and a formula of C17H12O2. When exposed to ultraviolet light, it exhibits a relatively strong blue fluorescence (Benkerroum, 2020). It is a colorless crystalline aflatoxin that forms crystals at temperatures between 269-268 °C, which is its melting point. It should be noted that aflatoxin B1 can also be synthesized in racemic form. Aflatoxin G1 has a molecular weight of 328 $g \cdot mol^{-1}$ and a formula of C17H12O7. When exposed to ultraviolet light, it emits green fluorescence (Chen *et al.*, 2015). Recent evidence suggests that the green fluorescence of aflatoxin G1 is likely due to impurities that can be removed. Aflatoxin G1 actually shows pure blue fluorescence. Its melting point is 246-244 °C. Aflatoxin P1 is a metabolite of aflatoxin B1 resulting from demethylation. It can be traced in the urine of animals such as monkeys. Aflatoxin P1 is derived from Aspergillus in vitro culture. Aflatoxin B2 has a molecular weight of 314 $g \cdot mol^{-1}$ and a formula of C17H14O6, while aflatoxin G2 has a molecular weight of 330 and a formula of C17H14O7 (García *et al.*, 2014). Both aflatoxins exhibit their own blue and green fluorescence when exposed to ultraviolet light. Their respective melting points are 240-247 °C and 289-286 °C. Aflatoxins B1, B2, G1, and G2 can be obtained through careful hydrogenation. When animals consume aflatoxin B1 alone or in combination with other aflatoxins, these toxins are transformed into the blood or other tissues. Two of these toxins, known as aflatoxins M1 and M2 (M stands for milk, as they are found in milk), have been identified in animal milk. Aflatoxins M1 and M2 are derivatives of 4-hydroxy-aflatoxin B1 and are three times more fluorescent than aflatoxin B1 (Chen *et al.*, 2021). They possess similar carcinogenic, mutagenic, and toxic properties as aflatoxin B1. The presence of aflatoxins can be detected and identified in urine, stool, muscle, liver, and kidneys of animals that have consumed or been injected with aflatoxin B1. Aflatoxin M1 is an oxygen molecular formula of aflatoxin B1 (in its hydroxy form).

Aflatoxin M1 has many structural similarities with aflatoxin G2 and is often referred to as aflatoxin GM1 (Hassan and Kassaify, 2014). The composition of aflatoxin M1 is very similar to that of aflatoxin M2. The optimal pH for the conversion of aflatoxin B1 to M1 in the liver of various animal species (such as mice, squirrels, monkeys, cows, chickens, and humans) is around 9.8, facilitated by the enzyme NADPH. The conversion of aflatoxin B1 to M1 may vary depending on factors such as pH and concentration (Anjum *et al.*, 2015). Aflatoxin M1 exhibits acute toxicity and inhibits RNA and protein synthesis, similar to aflatoxin B1. However, its effect on DNA is less pronounced. Aflatoxin M1 has carcinogenic properties, similar to aflatoxin B1, and is mutagenic (Akhtar *et al.*, 2017). Studies on natural and artificial milk contaminated with aflatoxin M1 have shown that it is resistant to pasteurization at a temperature of 64 °C for 2 hours. However, its stability decreases with higher temperatures (Chung *et al.*, 2018). The thermal processes used in the production of dairy products can also reduce the stability of aflatoxin M1. It is worth noting that the thermal stability of aflatoxin M1 during the process is not dependent on the type of product, and both natural and artificial milk show the same heat resistance. The hydroxy compounds of aflatoxins, aflatoxins B2, and G2 are derived (Fasoyin *et al.*, 2019). Aflatoxin M2 is an isomer with the hydroxyl group in position 2, while 2-hydroxy-aflatoxin G2 is actually Aflatoxin G2a. In 1966, these two derivatives were isolated from cultures of *A. flavus* in vitro. Additionally, the addition of an acid catalyst in suspension can also achieve aflatoxin B1(El Hadj-Khelil and Gacem, 2016). Aflatoxin B3 is a replacement of the cyclopentane ring in aflatoxin B1 with ethanol, resulting in 6-methoxy-coumarin on the 7th carbon. The chemical structure of Aflatoxin B3 has been identified. Aflatoxin B3, also known as Parazytykvl, is highly toxic to ducklings but less toxic than aflatoxin B3 for chicken embryos. Aflatoxin Ro or L, also known as Flatvksykvl, is the result of replacing the ketone cyclopentane of aflatoxin B1 with a hydroxyl group (Epifani *et al.*, 2016). This toxin can cause major changes in rat plasma and has carcinogenic properties. The reaction of converting Aflatoxin B1 to Aflatoxin Ro (hydrogenation of Aflatoxin Ro) takes place. Aflatoxin LH1 is a derivative of aflatoxin B1 through dehydroxylation. Aflatoxin Q1 is a mono-hydroxyl derivative of aflatoxin B1 with the hydroxyl group located on the carbon atom of the Carbonyl Cyclopentane Ring (Gong *et al.*, 2020). Its laboratory toxicity has been proven in rats, bovines, and mice.Aflatoxins B1 and B2 have been restored and are referred to as AFRB1 and AFRB2.Aflatoxin B1-2, 3-oxide or aflatoxin B1-8, 9-oxide is an intermediate composition and metabolism of aflatoxin B1. It is believed to be the ultimate outcome of aflatoxin B1 and is considered the active form or substance that is carcinogenic (Al Jabir *et al.*, 2019). This, combined with the fast and reliable covalent bond of epoxide molecules such as RNA, DNA, and proteins, is the main cause of toxicity and carcinogenicity of aflatoxin B1.Aflatoxin o-alky1 is the result of methoxylation of aflatoxins. (Alegbe *et al.*, 2017).

Sample preparation:

Because mycotoxins are toxic chemical compounds with low molecular weight and diverse chemical structures, therefore, there is no specific and specific method for their isolation and analysis (Ali *et al.*, 2016). One of the most important steps in

the analysis of mycotoxins is the preparation of the sample to be tested, the inaccuracy in this part has significant effects on the quantitative and qualitative diagnosis of mycotoxins isolation methods (Blaszkewicz *et al*., 2017). It should also be kept in mind that most of the foods that are contaminated with aflatoxin cannot be tested and analyzed without purification, and this is one of the reasons why researchers are looking for new ways of extraction and purification (Al-Jaal *et al*., 2019).

- ***Sample Homogenization***

There are different homogenization techniques, including dry, wet, and cryogenic grinding techniques. Since aflatoxins are found in various foods such as peanuts, oilseeds, cereals, dried figs, milk, cheese, and spices, they can be easily ground and mixed to obtain small particles and to minimize the effects of heterogeneous distribution of aflatoxins on quantification (Alvarez *et al*., 2020).

- ***Dry Grinding***

Dry grinding techniques are used for different food samples in order to reduce the sample volume and uniform distribution of the sample, which is mostly used for the detection and determination of aflatoxin B1 (Robertson, 2016).

- ***Wet Grinding***

One of the disadvantages of the dry grinding technique is that it causes heat during work, therefore it seems that the wet grinding method is more suitable. In this method, samples are mixed with water or other extraction solvents such as methanol. Homogeneity of the solvent/sample ratio is critical for extraction efficiency (Hameed *et al*., 2017).

- ***Cryogenic Grinding***

Cryogenic milling technique has effective applications in determining aflatoxins. This technique allows heat-sensitive samples to be ground into small sizes (Shrestha and Wang, 2019). This method is used in the determination of aflatoxin in fatty foods because the excessive heat produced in the dry milling method or the addition of water in the wet milling method changes the physical structure of the material (Asrani *et al*., 2019). It changes the primary and causes an error in the test. Today, this technique is widely used to ensure the homogenization of test samples for analytical analysis of mycotoxins in test samples. In this method, special grinding devices and dry ice or liquid nitrogen are used as cooling agents. In this method, it is recommended to keep the samples in the freezer overnight before grinding for better results. (Ayelign *et al*., 2018).

Extraction Methods:

- ***Pressurized Liquid Extraction (PLE)***

The PLE method, also known as accelerated solvent extraction (ASE), is performed under elevated pressure and temperature in a suitable pressure-resistant vessel. This method is used to check multi-gram test samples. Using this method reduces systematic errors caused by reducing the sample size (Bao *et al*., 2019). Because the extraction process in this method is automatic, one of the advantages of using this method is higher extraction efficiency in less time. This method is used to extract aflatoxin from (Benkerroum, 2020).

- ***Liquid–Solid Extraction (LSE)***

One of the simple methods of extracting aflatoxins is the liquid-solid extraction method (LSE) which is different for extracting aflatoxins from solid

matrices. In this extraction method, shaker, Ultra-Turrax and methods of extracting components of the desired sample extract are used. The most common extraction solvents are acetonitrile/water or methanol/water in different ratios. Among these solvents, methanol seems to be the best solution for separation using chromatography (Bischoff *et al.*, 2018). It should be kept in mind that the extraction efficiency is affected by the ratio of sample to solvent.

- ***Turbulent Flow Columns (TFC)***

One of the online and automatic sample analysis methods that also uses mass spectrometry is TurboFlowTM technology which extracts complex samples with considerable speed and efficiency and for separating AFB1 and AFM1 It is used in milk samples (Chen *et al.*, 2018).

Separation Techniques:

- ***Thin-Layer Chromatography (TLC) and High-Performance Thin-Layer Chromatography (HPTLC)***

One of the most common methods of aflatoxin isolation, that is considered in terms of simplicity and cost-effectiveness, is the TLC method, which is widely used in the laboratory to monitor the progress of the reaction and determine the purity of the tested sample and identify the compounds in the sample. In the flat chromatography technique, the stationary phase is an absorbent material with different thicknesses through which the liquid mobile phase passes. The most commonly used layers include silica gel, aluminum oxide (alumina) and cellulose (Chilaka and Mally, 2020). Another separation method is HPTLC, which allows more accurate measurement in the separation of aflatoxin. The difference between the TLC and HPTLC techniques is the difference in the size of the stationary phase particles and their sensitivity (Corassin and Sant'Ana, 2019). Despite these differences, TLC is still used as a common and reference method in the isolation of aflatoxins.

- ***High/Ultrahigh Performance Liquid Chromatography (HPLC/UHPLC)***

One of the more accurate methods for the separation of aflatoxins is the HPLC/UPLC method, which uses the principles of HPLC-fluorescent chromatography (FLD) and HPLC-MS/MS to separate AFB1 and AFG1 (Al-Zoreky and Saleh, 2019). In this method, the electrochemical reagents of trifluoroacetic acid (TFA), potassium bromide (KBr) or iodine are used, and it seems that electrochemical bromination is considered as a widely used method for the analysis of aflatoxins. This method is widely used in the separation of aflatoxin from children's food. Post-column derivatization (PCD) including electrochemical bromination can be considered an efficient method in the analysis of aflatoxins. Also, PCD together with pyridinyl hydrobromide perbromide is an effective derivatization method, especially for the analysis of baby food. In this regard, one of the more effective methods for detecting aflatoxins is PCD using azide with increased fluorescence properties. One of the disadvantages of using this method is the ability to derivatize iodine decreases over time, and as a result, the sensitivity of the technique also decreases (AlFaris *et al.*, 2020).

- ***Liquid Chromatography/Ultra-Performance Liquid Chromatography Mass Spectrometry (LC/UPLC-MS) and Tandem Mass Spectrometry (MS/MS)***

The Hyphenated technique is one of the separation methods based on mass

spectrometry, which includes the techniques of LC/UPLC-MS and MS/MS is used to determine aflatoxin. Two LC/UPLC-MS and MS/MS techniques are widely used in research laboratories for the analysis of mycotoxins because they are reliable techniques in quantitative and qualitative evaluation and determination of the exact content of mycotoxins, and in this field they can compete with the technique ELISA, but due to the expensive technique, they are not widely used in research or laboratory methods. In the LC-MS technique, all three atmospheric pressure ion sources are used to detect aflatoxins. Research has shown that atmospheric pressure electrospray source (ESI) is mainly used for LC-MS determination of aflatoxins because the ionization of aflatoxins is done by ESI and protonated molecules and ions created in the collision can be well measured (Al-Ghouti *et al*., 2020).

Rapid Test Methods

- ***Enzyme-linked immunosorbent assay (ELISA)***

In recent years, the use of rapid techniques for the identification of mycotoxins has been expanded, which are based on immunoassay and the reaction between antibody and antigen in an analyte (Amirkhizi *et al*., 2018). The basis of the ELISA method is based on the use of antibodies that are used in the analysis of compounds, and it is based on a color reaction related to an enzyme, the color intensity of which is inversely proportional to the concentration of the desired compound for measurement (Ayelign and De Saeger, 2020).

- ***Radioimmunoassay (RIA)***

The radioimmunoassay method uses radioactively labeled molecules in immunoassays and is a highly sensitive method that is used to detect aflatoxin in agricultural samples such as soybeans, wheat, and rice. Although this method is very expensive and requires expensive laboratory equipment, it is used more for aflatoxin analysis due to minimizing the side effects caused by gamma rays and preventing health risks (Bakirci, 2020).

- ***Flow cytometry based competitive fluorescent microsphere immunoassay (CFIA)***

The fluorescent microsphere immunoassay method is a competitive flow cytometry method that uses monoclonal antibodies with high affinity and can identify AFB1, FB1, DON, T2, ZEA mycotoxins with high sensitivity compared to the ELISA method(Choochuay S *et al*., 2018).

- ***MALDI-TOF-MS***

MALDI-TOF-MS is a method for detecting the amount of aflatoxin AFB1, AFB2, AFG1 and AFG2 in various agricultural products. a-Cyano-4-hydroxycinnamic acid (Et3N-a-CHCA) was used as MALDI matrix and NaCl was added to the matrix to increase the sensitivity (Chu *et al*.,2018). This method seems to be applicable for high-throughput screening of not only aflatoxins, but also other mycotoxins.

- ***Near-Infrared Spectroscopy (NIRS)***

One of the new methods that is widely used in the chemical, food and pharmaceutical industries is the near infrared spectroscopy technology, which is an effective analytical method for the qualitative and quantitative evaluation of organic substances (Akçael *et al*., 2019). Studies have shown that this method has been effective and successful in identifying mycotoxins, but the low sensitivity of NIR spectroscopy is not

suitable for the quantitative measurement of chemical residues in food, and for this reason, the further development of this method for the accurate measurement of chemical pollutants in food and feed needed. One of the benefits of this method is food analysis without any preparation, which makes this method a priority even for measuring aflatoxins (Fan *et al.*, 2020).

Results and Discussion

Mycotoxin contamination, especially aflatoxin, can cause serious health concerns due to global diversity, which factors such as climate change and health-regulatory policies make this issue more complicated (Rodríguez-Cañás *et al.*, 2019). The need for analysis methods and methods for the detection and determination of mycotocins, especially aflatoxins, causes the effective development and evolution of methods such as chromatography and mass spectrometry to determine aflatoxins. Since aflatoxins are heterogeneously distributed in foods and feeds, the stages of sample preparation, extraction, purification and determination of the extraction method and detection and determination play an important role in aflatoxin analysis (Alfonso *et al.*,2021). Methods such as TLC, LC-MS have been able to provide reliable improvements in the field of detection and extraction of aflatoxins from samples. The results of future research can be effective in the ability to replace and improve the current techniques in monitoring aflatoxins in foods. It is of particular importance and researchers are constantly searching for analytical methods therefore that today with the development of immunoanalytical methods, the identification of aflatoxin in food can be done easily and quickly. Although traditional methods such as purification and HPLC are routine methods of measuring aflatoxins, but strong methods such as ELISA or antibody-based methods are also strong and effective in addition to traditional methods (Garrido Frenich *et al.*, 2021). Today, one of the concerns of mycotoxin contamination is multi-mycotoxin contamination, which poses a serious risk to human and animal health. Research in recent years has shown that multiple mycotoxin infections require more advanced diagnostic methods. LC-MS/MS is an accurate and highly sensitive technique for the analysis of multimycotoxins in recent years, which can detect several mycotoxins simultaneously. Another point that is being considered today due to technological advances in laboratories is the use of biosensors and chips for safety-based diagnosis and analysis of multitoxins. Methods based on chip technology are simple, fast and cost-effective methods (Kimanya *et al.*, 2021). Today, the ELISA method is known as a fast, reliable and common method that is widely used in the identification of aflatoxins in food. One of the things that should be taken into account in the evaluation and quantitative measurement of food contaminated with aflatoxins is the amount of sample volume tested in order to correctly interpret the measured concentration and estimate the legal limits and determine the sources of error in order to expose future consumers (Bazalou *et al.*, 2017). This is the case that when laboratory methods are evaluated from a technical point of view, error sources and performance parameters must be identified (Ji *et al.*, 2018).

Conclusion

Investigating mycotoxin detection methods and techniques is effective in recognizing and diagnosing types of mycotoxin contamination of food and also

helps in more detailed analysis and recognition of the advantages and disadvantages of the techniques. The results of the study showed that the most common methods are ELISA, electrochemical safety sensors, chromatography and fluorescence, that can have advantages and disadvantages such as the need for an advanced laboratory with trained personnel and harmful chemicals and solvents. Detection and determination of aflatoxins using an electrochemical immunosensor is an efficient and easy method for detection at very low concentration.

Acknowledgments

The researchers hereby would like to thank the research deputy of medical university of Islamic Azad University of Zahedan for approval and financial support of this project.

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