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Analytical Methods for Determination of Aflatoxin B_1 in Animal Feeds and Feedstuffs*

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Summary: Aflatoxin B_1 (AFB_1) is an *Aspergillus spp.* produced mycotoxins which is identified as one of the key contaminant of feeds and toxic to some degree to all species tested to date. Because of its hepatocarcinogenic nature, AFB_1 has obtained significant attention in past few years. A number of analytical, diagnostic and immunological procedures are available for assessment and estimation of AFB_1 in different feeds such as enzyme linked immunosorbent assay (ELISA), thin layer chromatography (TLC), high performance liquid chromatography (HPLC), liquid chromatography tandem mass spectrometry (LC-MS/MS) and electrochemical immunosensors (ECI). All available analytical methods for determination of AFB_1 principally included the same steps like, sampling, sample preparation, detection, confirmation, and finally risk assessment. HPLC approaches are most commonly used nowadays because of their high accuracy, precision and sensitivity. Since late 20th century, many countries including Turkey has imposed strict rules and regulations regarding AFB_1 in food and animal feed related industry to protect the consumer from the detrimental effects of the toxin. In this article, a number of analytical techniques for determination of AFB_1 in feeds and feedstuffs starting from sampling to risk assessment and international regulations are reviewed.
Key words: Aflatoxin B_1, analytical methods, international regualtions, risk assessment, sampling

Hayvan Yem ve Yem Hammaddelerinde Aflatoksin B_1'in Belirlenmesi İçin Analiz Yöntemleri

Özet: Aflatoxin B_1 (AFB_1) yemlerde ana kirletici olan *Aspergillus* türleri tarafından üretilen ve bütün türlerde belirli derecelerde zehirli olan bir mikotoksindir. Karaciğer karsinojeni olduğundan, son yıllarda ilgi odağı haline gelmiştir. AFB_1'in çeşitli yemlerden analizinde enzim bağlı immunosorbent assay (ELİSA), ince tabaka kromatografisi (İTK), yüksek performanslı sıvı kromatografi (HPLC), sıvı kromatografi tandem kütle spektrometrisi (LC-MS/MS) ve elektrokimyasal immünosensör (EKİ) gibi yöntemler kullanılmaktadır. Belirtilen yöntemlerin hepsinde örnek alma ve hazırlama, tespit, doğrulama ve risk değerlendirme aşamaları bulunur. HPLC yüksek doğruluk, kesinlik ve hassasiyetinden dolayı en yaygın kullanılan analiz yöntemidir. Türkiye dahil birçok ülke, 20. yy. sonundan beri, tüketiciyi zararlı etkilerinden korumak için gıda ve yem ile ilgili sektörlerde AFB_1 için katı kural ve düzenlemeler yürürlüğe koymuştur. Bu makalede, yem ve yem hammadelerinde, örneklemeden risk değerlendirme aşamasına kadar, AFB_1 analizinde kullanılan çeşitli yöntemler ve uluslarası düzenlemeler derlenmiştir.
Anahtar kelimeler: Aflatoksin B_1, analiz yöntemleri, örnekleme, risk değerlendirmesi, uluslararası düzenlemeler

Introduction

Aflatoxins (AFs) are organic chemicals and derived metabolites of different strains of fungi, like *Aspergillus flavus* and *A. parasiticus*. These toxins are mutagenic, teratogenic, carcinogenic, and immunosuppressive in their nature and usually found as contaminants in a variety of crops like cereals, oilseeds, tree nuts and spices (Marchese et al., 2018).

Animals are exposed to AFB_1 toxicty after consuming the contaminated feeds and feedstuffs. Liver metabolised the AFB_1 and AFB_2 by cytochrome p450 enzyme and excrete it as AFM_1 and AFM_2, such metabolites are reported in various milk and milk products (Aksoy et al., 2009a; Aksoy et al., 2010). In order to prevent the toxicity of AFs some absorbents like hydrated sodium calcium and aluminosilicate are mixed in feed which prevent the absorption of AFs by binding with it in gastrointestinal tract (Essiz et al., 2006).

Commercially and for research purposes AFs are being detected by various techniques like enzyme linked immunosorbent assay (ELISA), thin layer chromatography (TLC). The more precise, sensitive and advance technique is high performance liquid choromatogarphy (HPLC) (Manetta, 2011). A number of analytical techniques to analyze AFB_1 in feed or feedstuffs are being reviewed in this study, starting from sampling, procedure/technique and relevant pros and cons coined with those analytical methods. Also the risk assessment of AFB_1 was summarized in this review.

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Sampling

Sampling is one of the most important steps in all analytical methods. Sample variation endured largest contributing factor for error in the analytical identification of AFB_1 in feeds and feedstuffs. Thus, systematic approaches have been designed and even adapted for sampling method, sample preparation, and analysis of AFB_1 at the parts-per-billion (ppb, ng g^{-1}) level (Saini and Kaur, 2012).

It is preferable to collect sample when lots are in movement as incidences like loading and unloading can be one of the predisposing factor of AFs content in a moving sample from trucks (FAO, 1993). Sample should be constituted by at least 100 incremental (small portions taken to compose an accumulatively bulk sample) grabbed in a systematic but random manner, manually or automatically from the maximum number of points from a lot. Then, It should be packed properly in an humidity free containers such as paper bags or cardboard boxes. Sample details, labelling and tagging should be done properly in next step according to the site of the lot from where it has been collected. Furthermore, sample must be protected against heat and moisture during transportation. It is important to keep it in its original form. Refrigeration can be a preferable practice until analysed. Proper protection from heat and humidity is needed in order to prevent from fungus development (Fonseca, 2002).

Accuracy of sampling can be improved by taking sample in large quantity and dividing them in to three parts. Weight difference have been also taken into consideration (Sinha, 1999). A study performed by Armorini et al. (2015) showed a high heterogeneity associated with the presence of AFB_1 in flour when sample was analyzed from a smaller weight as 5 g to 20 g. So it was concluded in their study that increasing the sample weight (start from 20 g) reduce the variability of AFB_1 level in multiple samples analyzed.

Fonseca (2002) also demostrated the impact of sample weight on the variation coefficient when sampled shelled peanuts. The bigger the sample, lesser variation noticed on graph. A lot of differences in sample size has been recommended worldwide. Different countries have different requirement in terms of sample weight. For example, The United Kingdom requires a weight of 10.5 kg per grain sample and The Department of Agriculture in United States consider 66 kg per grain sample for AF analysis. Generally, most of the countries accept 5 to 10 kg per grain sample weight. Sub-samples size varies from 20 - 100 g. Mostly methods need a 50 g sample for AF analysis, which seems to be economical in terms of solvents usage (Sinha, 1999).

Sample preparation

Reduction in particle size and homogenization are main aim of sample prepration to make subsamples which represent the parent sample for analysis. Reduction in particle size is carried out in mills/grinders that converts the sample to a paste while simultaneously homogenizing the sample (Davis et al., 2018). In case of non-granular product like milk there is no sample preparation, small portion of milk is taken for further processing. Blending or mixing is important in liquid samples before taking any portion for analysis. Sub-sample size also may vary, but usually it is in the order of 25 to 1000 g depending on particle size (Whitaker et al., 2005).

Extraction and clean-up procedures

Clean-up step is used to remove/reduce any disturbing substrate, that may result in any analytical error while extraction in quantitative step for the analysis. Different extraction methods have been used over the period of time by evolution of this subject (Sizoo et al., 2005). Currently, the extraction of the AFs is done by aqueous polar solvents, and most commonly used substances are methanol or acetonitrile. The solvent selection depends on the chemical composition and analytical of the mycotoxin (Pittet, 2005).

The liquid-liquid extraction is based on the priciple of distribution of analytes in two non-miscible phases. The major downside of this process is, it demands organic and chlorinated solvents in significant amount. Consequently, this method has been replaced by solid phase exctraction (SPE) method (Sizoo et al., 2005). The SPE column includes a bonding phase which permits selective absorption of contaminants or the substances of interest such as an analyte. Silica traps the ingredient of interest which is analyte and the unwanted substances are washed off. A specific elution solution releases the analyte from the column. The analyte is eluted with specific solutions to it from the column (Yao et al., 2015).

The immunoaffinity column (IAC) are used to reduce the matrix effect which relly on princple that antibodies are raised against the AFB_1 is immobilised on a gel, packed in colmun. First conditioning of column is done with phospahte buffer solution (PBS) than sample extract is applied. During sample application AFB_1 gets bound to anitibody and than to IAC gel. After loading of extarct to IAC, gel is washed with PBS to detach any co-extractives and finally AFB_1 is removed from IAC by breaking antigen-antibody bond (Şenyuva and Gilbert, 2010).

Analytical techniques for determination of aflatoxin B_1

Enzyme-linked immune-sorbent assay

Enzyme-linked immune-sorbent assay is a simple, rapid and specific method used for detection and quantification of AFB_1 (as antigen) in a sample using an enzyme-labelled toxin and antibodies specific to AFB_1 test kit (Adi and Matcha, 2018). Along with rapid screening of toxins, these kits coined with some drawback also like cross reacitivity and matrix dependence which leads to extreme variation in results (Aksoy et al., 2016).

For the determination of AFB_1 in feeds and feedstuffs direct/indirect competitive ELISA has been used extensively. Low limit of detection (LOD) is one of main advantage of ELISA method based test. A study performed by Aksoy et al. (2009b) for the deterimantion of AFB_1 in compound animal feed in 40 samples by using ELISA. Reported percentage of AFB_1 in compund feed was 95%. In another study conducted by Aycicek et al. (2005) among 40 cacao hazelnut cream and 51 dehulled hazelnut samples AFB_1 existence was reported in 39 and 43 samples of cacao hazelnut cream and dehulled hazelnut with level of <1 to 13 ng $g^{-1}$and <1 to 10 ng g^{-1}, respectively.

Chun et al. (2007) validated 85 samples positive for AFs in nuts and nut products, where ELISA used for rapid screening and HPLC for further quantification. For AFB_1 the LOD was 0.08-1.25 ng g^{-1} and the limit of quantification (LOQ) was 0.15-2.50 ng g^{-1} respectively and recovery ranged from 83.4% to 102%. In this study, ELISA screening detected 31 positive samples, resulted above 0.06 ng g^{-1}. These samples were further quantified with HPLC, results shows contamination level up to 28 ng g^{-1} in nine samples. HPLC results demostrated that about 2/3 of the results were false positive in ELISA. As concerned with the advantage ELISA technique has high output and rapidity in screening.

Thin layer chromatography

Thin-layer chromatography, a technique used for purity assessment, separation, and identification of organic compounds, used quite extensively in the analysis of AFs. This method is quite sensitive and can quantify AFs as low as 1 ng g^{-1} (Rahmani et al., 2009). Ultraviolet light (UV) is used to examine the developed TLC plates. Visual comparison of the fluorescent intensity of the spots in the sample extracts gives estimation of concentration of AFs in sample being analyzed. These estimated spots are compared with AF standards chromatographed on the same plate (Sinha, 1999).

Stroka et al. (2000) developed a method which is able to detect maximum level of toxin set by European Union (EU). They use TLC for determination of AFs in various feed samples. IAC was used prior to TLC. Aflatoxins were analysed by comparing with them AF standards using densitometry. Result shows LOD for AFB_1 in peanut, paprika and pistachios were 0.6, 0.4, and 0.2 ng g^{-1} whereas LOQ were 1.5, 1.2 and 0.5 ng g^{-1} while recoveries ranged from 85% ,85% and 82% respectively.

High performance liquid chromatography

High performance liquid chromatography is the most widely and frequently used method with high precision, sensitivity and automation for mycotoxin analysis. Relative affinity is the key principle in this technique where a mixture of compounds present in an extract separates by relative affinity for a stationary column and a mobile solvent (Pittet, 2005).

HPLC has several advantages in comparison to other analytical methods and most important is its potential for automation. Both normal and reversed-phase HPLC can be used in a sample processing. Normal-phase methods use ultraviolet (UV) monitoring at wavelengths of 254 and 365 nm for detection of AFs with native fluorescence and fluorescence with a silica packed cell while reversed-phase have been developed by using both UV and fluorescence detection (FLD). Recently, reversed-phase HPLC method for AFs detection have received significant attention because of fast and accurate detection and rapidity in final results (Wacoo et al., 2014). The only disadvantage with HPLC methods was the complexity in steps for extraction and clean-up, longer time and experience factor (Sinha, 1999).

A modified, reliable and rapid method has been designed based on the “Quick Easy Cheap Effective Rugged Safe” (QuEChERS) procedure by a single extraction step without exercising a clean-up step for AFB_1 analysis in four feedstuffs comprising fish meal, corn, broken rice and, peanut. Satisfactory results were achieved, including good linearity, specificity, accuracy, precision repeatability, reproducibility and analytical limits (Choochuay et al., 2018).

HPLC coupled with FLD is commonly used along with online post-column derivatization (PCD) for analysis of AFB_1. Kobra cell is typically used to electrochemically brominate the toxin and make it detectable even at low level. Atmaca et al. (2015) analysed the level of AFB_1 in 40 maize grain samples by HPLC using IAC for sample preparation. Samples were quantified with HPLC-FLD with Kobra cell. LOD and LOQ were 0.013 and 0.038 ng g^{-1}, respectively with recovery of 89%.

Liquid chromatography tandem mass spectrometry

The liquid chromatography tandem mass spectrome-

try (LC-MS/MS) is highly specific and widely applicable analytical method for both qualitative and quantitative analyses. The method have capacity of determining the toxins in a single run (Di Mavungu et al., 2009). Before MS, HPLC separates the sample into chemical compunds after that MS ionizes the molecules, afterward sort and identify the molecules according to their mass-to-change ratio (Mahfuz et al., 2018).

Njumbe et al. (2015) used LC-MS/MS method for the analysis of 23 mycotoxins including AFB_1 in different sorghum varieties. Result stated LOD for AFB_1 was 2.5 ng g^{-1} while LOQ was 5 ng g^{-1}. When this method was applied to small portion of samples (10 samples) obtained from different places 9 out of 10 samples (90%) samples were postive for AFB_1.

Souza et al. (2013) worked on 119 samples of poultry feed for the determination of different mycotoxins by using LC-MS/MS. Samples include 74 maize samples, 36 chicken feed and nine feed mill residues. AFB_1 was found in maize samples under value of 3.0 ng kg^{-1}.

Electrochemical immunosensors

In past, many electrochemical immunosensors (ECI) also known as antibody-based biosensor have been developed for the detection of AFB_1. Detection range reported to be as low as 0.03-0.15 ng mL^{-1} (Azri et al., 2018). For mycotoxin detection by ECIs rely on the use of specific antibodies and aptamers or artificial receptors as affinity ligands which permits binding of the analyte to the sensor for estimation with minimum interference from other components that can occur in a sample. Such affinity based sensors that consist of an electrode with the bioreceptors generate an electroactive signal which offer great selectivity and sensitivity for analyzed samples (Karczmarczyk, 2017).

Masoomi et al. (2013) designed a non-enzymatic sandwich form of an ECI that achieved a detection range of 0.6-110 ng mL^{-1} and LOD 0.2 ng mL^{-1} for AFB_1. Azri et al. (2018) worked on determination of AFB_1 in peanut sample. The developed ECI showed a linear range of 0.0001 to 10 ng mL^{-1} with LOD of 0.3 pg mL^{-1} and recovery of 80% and 127% in spiked peanut samples.

International legislations and regulations

Legally binding regulations have been issued for AFB_1 as this mycotoxin particularly has tendency to contaminate milk as AFM_1. The maximum level has been established by the European Commission to prevent the occurrence of AFM_1 and harmful effect to consumer health (Streit et al., 2012). According to performance criteria of EU, the recovery percentage should be between 50 to 120% in aflatoxin analysis at < 1 µg g^{-1} spiked level (EC, 2014).

In Turkey and the EU, the maximum levels of AFB_1 for compound feeds for dairy cattle and calves, dairy sheep and lamb, dairy goat and kids, piglet and young poultry animals is 5 ng g^{-1}. The maximum level for AFB_1 in feed ingredients and compound feed of cattle, sheep, goat, pig, poultry (except above) is 20 ng g^{-1}. The maximum level of AFB_1 for complementary and complete feeds (except above) is 10 ng g^{-1}. The maximum levels of AFB_1 for all feeds above is for feedstuff with a moisture content of 12% (MAF, 2014; EC, 2015).

The international regulations for the maximum limit for AFM_1 in milk and dairy products range from 0 to 1 ng g^{-1}. The EU set the limit of AF levels to not more than 20 ng g^{-1} in lactating dairy feeds and 0.05 ng g^{-1} in milk (Iqbal et al., 2015).

The FDA action levels for total AFs in animal feed and feed ingredient were shown in Table 1.

Table 1. FDA (2019) action levels for total AFs in animal feed and feed ingredient

Feed or feedstuff	Concentration (ng g^{-1})
Corn and peanut products for beef cattle	300
Corn for immature animals and dairy cattle	20
Corn and peanut products for breeding beef cattle and mature poultry	100
Corn and peanut products for finishing swine (> 45.3 kg)	200
Corn and peanut products for finishing beef cattle	300
Cottonseed meal (as a feed ingredient)	300
Corn, peanut product, other feed ingredients and pet feed	20

Toxicological risk assessment

Risk assessment is bascially carried out on theoretical basis and technical support for quality and safety evaluation, standard establishment and risk management of agricultural and other consumable products (Ding et al., 2015). Toxicological risk is the toxic result of a particular activity/procedure in relation to the likelihood that it may occur which may characterised as a type of the hazard, magnitude of the hazard, and probability of the hazard. With respect to above mentioned definitions, toxicological risk assessment is basically a scientific activity using data from toxicological research. Finally, this leads to foundation of

risk management (Kleinjans, 2003).

Different animal species have different susceptibility for AFB_1 toxicity with oral lethal dose 50 (LD_{50}). The LD_{50} of AFB_1 differ with age, gender etc. For example LD_{50} for rabbit, cat, turkey (also puppies and young calves), horse (young foals, also sheep), chickens, mouse, female and male rats are 0.3-0.5, 0.55, 0.5-1, 2, 6.5-16.5, 9, 7.4 and 17.9 mg kg^{-1}, respectively (Dhanasekaran et al., 2011).

International Agency for Research of Cancer (IARC) classified AFB_1 as human carcinogen (group 1) and because of its structural similarity to AFM_1, is classified as carcinogenic (group 2B) (Atmaca and Aksoy, 2015). In this regard, In 1997 the Joint Food and Agricultural Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) concluded the dietary intake also a potential risk for AFs related carcinogenicity (Sugita-Konishi et al., 2010).

A recent study by Nugraha et al. (2018) on risk assessment for AFB_1 from contaminated maize and peanut was carried out by using margin of eposure (MOE) and estimation of liver cancer risk approaches. The MOE values of maize and wheat consumption were generally below 10.000 and for several occurrence data it was even below 1000. Consumption were above 0.1 cancer cases / 100.000 individuals / 75 years for liver cancer cases associated with AFB_1.

Conclusion

Mycotoxins are emerging issue worldwide, found in feeds and feedstuffs which is potential hazard for both human and animal health. AFB_1 received tremendous attention in several years due to its hepatotoxic and carcinogenic nature. Measures should be taken worldwide by implementing legislations and regulations to control toxic level of AFB_1 in consumable and agricultural products. Analytical methods are still considered a developing area where techniques are being designed to conduct analysis with time and cost efficiency and user friendly with highest possible accuracy.

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