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Techniques used for Detection of Aflatoxins in Milk: A Review

Rita Narayanan[1], M. Kirthy Reddy[2]

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ABSTRACT

Mycotoxins (aflatoxins) present in foods are serious health problems to consumers as they are highly toxic. The presence of high levels of AFM1 (aflatoxin) is an alarming issue in milk. So, the need of the hour is the development of a fast and reliable screening method for detecting trace aflatoxins in food. The analytical methods like HPLC and MS which have been used for aflatoxin detection are expensive, time-consuming and require many skills. Novel methods have been come up with high sensitivity, simplicity and capability of onsite monitoring which includes enzyme-linked immunosorbent assay (ELISA), immunosensors and lateral flow immunoassay (LFIA), *etc*. This paper reviews the techniques and methods used for the detection of aflatoxins in milk. Rapid technologies provide greater efficiency and sensitivity compared to conventional methods.

Key words: Aflatoxin, Dipsticks, ELISA, HPLC, Immunosensor.

Aflatoxins are secondary metabolite mycotoxins, naturally produced by molds such as *Aspergillus flavus* and *Aspergillus parasiticus*, and have adverse effects on humans, animals, and crops that result in illnesses and economic losses (Hussain and Anwar, 2008). These naturally occurring toxins can lead to multiple illnesses, from liver and kidney disease (including cancer) to immunosuppression disorders and birth defects (Subroto Mukherjee, 2016). As they pose a serious threat to humans and animals through consumption, most of the developed countries established maximum permissible levels for these compounds in food and feed (Hamid Mohammadi, 2011). Apart from raw milk aflatoxins can be seen in processed milk, yogurt, cheese, dry milk, infant formula, milk-based products ranging between 1-2%. As children consume larger amounts of milk and milk-based products, they face more risk of exposure to mycotoxins effects (Gratz *et al*. 2017).

Improperly stored feed sources such corn, soybeans, protein, minerals and vitamins (Dave Fischer and Mike Hutjens, 2007) are fed to animals to produce more milk, unfortunately, these may also contain mycotoxins, such as for fumonisin B1, a known carcinogen. If the cows or animals consume feed containing mycotoxins, they excrete these into their milk as aflatoxin M1 and lesser amounts of M2, both are known to be carcinogens. According to the study by Ardic *et al*. (2009) reported that the milk or milk products that are obtained from livestock that has ingested mycotoxin contaminated feed contain aflatoxin M1 (AFM1), which is a hydroxylated metabolite of aflatoxin B1 (AFB1).

An extensive and periodic analytical surveillance is clearly needed to find out the major sources of aflatoxin M1 contamination and to establish AFM1 regulations as well. HACCP (Hazard Analysis and Critical Control Point) system implementation and maintenance of records of all feeds, feeding practices, contamination levels, proper storage conditions are also highly recommended to achieve SDG-3 (Good health and well-being) by assuring the health safety issues (Tarannum *et al*. 2020).

[1]Department of Food Processing Technology, College of Food and Dairy Technology, Tamil Nadu Veterinary and Animal Sciences University, Koduvelli, Chennai-600 052, Tamil Nadu, India.
[2]Department of Food Processing Technology, Dr. NTR College of Food Science and Technology, Acharya N.G. Ranga Agricultural University, Bapatla-522 101, Andhra Pradesh, India.

Corresponding Author: M. Kirthy Reddy, Department of Food Processing Technology, College of Food and Dairy Technology, Tamil Nadu Veterinary and Animal Sciences University, Koduvelli, Chennai-600 052, Tamil Nadu, India. Email: kirthy88@gmail.com

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Studies by Lopez *et al*. (2001) showed that aflatoxin M1 in raw milk can be transferred to dairy products by adding 1.7 to 2 micrograms of aflatoxin to milk and production of cheese, and the amount of aflatoxin found in the final products (cheese and whey) are 40 and 60% respectively. It is very difficult to remove aflatoxin from milk and other dairy products, even after repeated processing, including sterilization and pasteurization (Alex *et al.* 2014). The main issue is that these aflatoxins cannot be detected if they are present in small amounts without proper instrumentation and scientific expertise. Another issue is nonuniformity in the permissible limits for mycotoxins in the foods as 0.05 parts per billion (ppb) was standard in the European nation for mycotoxin in milk whereas, the U.S. limit is 0.5 ppb (USFDA, 2015).

There are different methods available for different types and levels of mycotoxin detection and quantification, ranging from techniques used for regulatory purposes such as HPLC-MS (high-performance liquid chromatography-mass spectrometry) to rapid test methods such as ELISA (enzyme-linked immunosorbent assay). Novel technologies in aflatoxin-detection systems which are emerging include hyperspectral imaging, electronic noses, dip-stick kits,

molecularly imprinted polymers, aptamer-based biosensors, *etc*. (WHO, 2018).

Chromatography technique

Chromatographic techniques are separation techniques that rely on the interaction (physical) between a mobile phase and a stationary phase. The components which are to be separated are distributed between these two phases. The mobile phase is usually a fluid that penetrates through or along the stationary bed (liquid or solid component). Mobile phases can be liquid, gas, or supercritical fluid. Based on the mobile phase used, the advanced chromatographic techniques are categorized into liquid chromatography, gas chromatography, and supercritical fluid chromatography respectively. Generally used chromatography techniques for analysis of aflatoxins/mycotoxins are thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC).

Thin layer chromatography

Thin-layer chromatography was initially the most widely used separation technique in the analysis and detection of aflatoxins. It consists of two phases out of which the stationary phase is made up of alumina, silica, or cellulose immobilized on an inert material such as plastic or glass, which is called matrix. The mobile phase is comprised of an alcohol and water mixture, which acts as a carrier of the sample along with the solid stationary phase (Betina, 1985). TLC technique is highly sensitive and requires skilled technicians, pretreatment of samples and expensive equipment as explained by Stroka and Ankalm, (2002). Out of all techniques, TLC was the first one used to detect AFM1. Over time, it has been replaced by other chromatographic techniques, such as liquid chromatography (LC) and gas chromatography (GC) coupled with mass spectrometry (MS). Despite the fact, that TLC is still an accepted reference method for the detection of aflatoxins, the quantitative analysis of aflatoxins was replaced by HPLC and UPLC in most cases.

Gas chromatography (GC)

In gas chromatography, the carrier which is used as mobile phase is gas and the liquid coating onto inert solid particles acts as stationary phase. Gas chromatography is not so commonly applied in the commercial analysis of aflatoxins due to the existence of other cheaper chromatographic methods and being expensive (Liang *et al*. 2005). Besides, this technique also requires a preliminary cleanup step before analysis and it is therefore limited to analysis of a very few mycotoxins, such as trichothecenes (A and B). Even in this analysis, the GC has disadvantages of nonlinearity of calibration curves, high variation in reproducibility and repeatability, drifting responses and memory effects from previous samples (Petterson and Langseth, 2002).

Over-pressured layer chromatography (OPLC)

Over-pressured layer chromatography was developed by Hungarian scientists in the mid-70s. This is carried out on a TLC or HPTLC plate, applying forced flow in a pressurized ultramicro (UM) chamber, based on the principle of liquid chromatography. As a result, the following are aflatoxins were identified: 0.018, 0.100, 0.15 and 0.14 µg/kg for AFG2, AFG1, AFB2 and AFB1, respectively by Papp *et al*. (2000).

High-performance liquid chromatography

In the HPLC technique stationary phase is either a plastic tube or a glass and a mobile phase comprises an aqueous/ organic solvent, which flows through the solid adsorbent. At the top of the column, the sample which is to be analyzed is layered these flows through and distributes evenly between both the phases. Different components present in the sample are separated based on their affinities for the two phases and thus move through the column at different rates. HPLC mostly employs a stationary phase such as a C-18 chromatography column, a pump that moves the mobile phase(s) through the column, a detector that displays the retention times of each molecule and mobile phases.

Until recently, thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) coupled with fluorescence detection (FL) or mass spectrometry (MS) were widely used for the analysis of aflatoxins (Campone *et al*. 2016). Liquid chromatography (LC) coupled with spectroscopy results in a high dynamic range, good sensitivity, and versatility. Detection by this method is made either by fluorescent detection (FLD), UV absorption or mass spectrometry. Both normal and reverse-phase chromatographic separations methods are used for aflatoxins determination. Out of which, the commonly used is reverse-phase HPLC with mixtures of methanol, water and acetonitrile as mobile phases. In reversed-phase eluents quench the fluorescence of mycotoxins like AFM1. Hence, pre or post-column derivations are used. The pre-column derivatization uses trifluoroacetic acid (TFA) to form the corresponding hemiacetals, while the post-column derivatization makes use of reactive halogens (iodine, bromine, *etc*) (Espinosa-Calderón *et al*. 2011). Furthermore, the limitations of the HPLC method are its complicated, time-consuming for sample preparation, needs a technician to operate, and is an expensive method.

HPLC analysis was also performed to validate. Interestingly, the chromatographic analysis of the effect of the plant extracts drastically diminished the fluorescence of AFB1 spot on the TLC plant under Ultra Violet (UV) radiation in the study by Mishra *et al*. (2019). The use of HPLC also enhanced the separation performance and identify the aflatoxins was based on the retention time (RT) in minutes (mins) of the peak areas (Kumar *et al*, 2019).

Spectroscopic methods

Frontier infrared spectroscopy

In an infrared spectrometer, IR radiations covering a range of different frequencies are passed through the sample and the radiant energy which is absorbed by each type of bond present in the molecules is measured. The spectrum which

is obtained normally consists of a plot of % transmittance against the wavenumber.

The models could successfully classify (>86%) and detect even 0.02 µg/l AFM1 in milk ($p \leq 0.05$) using SIMCA. AFM1 was best predicted in the wavenumber range of 1800-650 cm^{-1} with a coefficient of determination (R2) of 0.99 and 0.98, for calibration and validation, respectively, using partial least square (PLS) regression. The study by Jaiswal *et al*. (2018) indicated the feasibility of ATR-FTIR spectroscopy and chemometrics in the rapid detection and quantification of AFM1 in milk.

Fluorescence spectrophotometry

Molecules including aflatoxins emit energy at specific wavelengths and fluorescence is important in the characterization and analysis of the molecules in grains and raw peanuts (Babu, 2010). The aflatoxin amount which can be quantified in the sample ranged from 5 to 5000 ppb within less than 5 minutes in this method.

Mass spectroscopy

Mass spectrometry (MS) measures the mass-to-charge ratio of charged particles, allowing to quickly and accurately determine the molar mass, the sequencing of repeat units, and recognition of polymer additives and impurities. A spectrometer consists of three parts they are the ionizer, analyzer and detector. The ionizer produces ions in an unidentified sample, the analyzer differentiates into the characteristic mass components by the charge-to-mass ratio and then the detector identifies the ions in proportion to their relative abundance.

The introduction of mass spectrometry (MS) resulted in the development of many methods for AFM1 analysis in dairy products by subsequent coupling of LC have resulted in LC-MS or LC-MS/MS combinations (Skrbic *et al*. 2015). The MS technique has the advantage of producing spectra with characteristic fragmentation patterns which are used for confirmation purposes (Ketney, 2017). There are several types of instruments available for MS like single quadrupole MS, triple quadrupole (MS/MS) and linear ion trap (MSn). Among these Ion, trap instruments are better than triple quadrupole instruments in identification (higher MSn power), whereas triple quadrupole instruments are better for quantification which has faster scanning and higher sensitivity (Manetta *et al.* 2011).

Bioassays have become increasingly useful as a rapid screening procedure before chemical analysis for mycotoxin detection. For the detection of AFM1, immunochemical screening assays are mostly used, including enzyme-linked immunosorbent assays (ELISAs), immunochemical assays involving detection by electrochemiluminescence (ECL-IA), ELISA using fluorimetric detection and more recently, biosensor assays (Santos *et al.* 2019).

ELISA

Among the rapid screening methods, ELISA has been widely used for detecting AFM1 due to its simplicity, sensitivity and adaptability in different food matrices, like in pasteurized and ultra-high temperature (UHT) milk, infant formula, powdered milk, yogurt, ice cream and cheese (Ketney *et al.* 2017). ELISA is a widely used biochemical technique for the detection of an antigen in a sample especially sandwich ELISA utilizes two antigen-specific antibodies, a capture antibody bound to a solid phase and an enzyme-linked detection antibody. Competitive ELISA is of direct competitive ELISA and indirect competitive ELISA. Despite its simplicity, ELISA shows some disadvantages, such as long incubation periods and several washing and mixing stages. Based on this, in recent years several modified ELISA methods have been developed for the improved detection of AFM1 in milk and dairy products (Matabaro *et al*. 2017).

Direct competitive ELISA

In this, the wells of the microtiter plate are coated with a specific antibody for the analyte to analyze. After the addition of the sample, the analyte starts competing with an enzyme-labeled analyte for binding as a restricted number of antibodies are present. After incubation, the unbound compounds are washed off and a chromogenic substrate is added for color development to discriminate. ELISA reader is used to determining the measurement photometrically. The enzymatic activity represented in each well is inversely proportional to the aflatoxin concentration or percentage in the sample, *i.e*., if the absorbance is lower, the aflatoxin concentration will be higher. This happens because a higher concentration of mycotoxin, conjugate (enzyme-labeled analyte) will react with the bound antibody will be less, leading to fainter color development in the sample.

The evaluation of both methods showed that this ELISA kit could be considered as a faster and equally reliable alternative method to HPLC in routine analysis for the determination of AFM1 in milk (Maggira *et al*. 2021).

Indirect competitive ELISA

The analyte or its analog, conjugated with a macromolecular carrier (*e.g*., BSA - Bovine Serum Albumin or OVA - Ovalbumin) is coated onto the well in the microtiter plate during incubation. When the specific antibody and the sample are added to each well, the immobilized analyte and the analyte present in the sample will compete for the antibody in solution. The washing step is followed to determine the amount of bound specific antibody is detected, through a secondary antibody, labeled with an enzyme. Due to the commercial availability of enzyme-labeled secondary antibodies [*e.g*., labeled with horseradish peroxidase (HRP) or alkaline phosphatase (AP)], The above-mentioned approach makes it possible to simplify immunoreagents preparation. However, it includes an additional step that can be eliminated by direct labeling of the specific antibody (De Saeger, 2011). Direct competitive ELISAs are usually used in aflatoxin analysis.

Radioimmunoassay (RIA)

This assay is the application of radioactively labeled molecules to form immunocomplexes. This assay is a very

sensitive method that is used to measure concentrations of substances, usually measuring antigen concentrations in the sample by the use of antibodies. Even though this technique is extremely sensitive and specific, requiring specialized equipment, it remains the least expensive method among all to perform such measurements. As radioactive substances are used this technique requires special precautions and licensing. To perform this radioimmunoassay, a known quantity of an antigen should be made radioactive, by gamma-radioactive isotopes of Iodine, such as 125-I, which is attached to tyrosine. Then this antigen is then mixed with a known amount of antibody for that antigen and as a result, these two specifically bind to one another. This method is mostly used in the case of agricultural samples (maize, soybean, wheat and rice), the LOD/LOQ of the method was 0.2/0.5 µg/kg for AFB1 (Korde *et al.* 2003).

Chemiluminescent assay

Chemiluminescent enzyme immunoassay (CLEIA) is a combination of chemiluminescence (CL) and enzyme immunoassay highly advantageous as it is highly sensitive and highly specific of all immunoassays with features of simplicity and rapidity, high throughput and low-cost equipment (Zhang, 2012). The substrates used for this assay are the luminol/ peroxide/enhancer system for horseradish peroxidase (HRP) or dioxetane-based substrates for alkaline phosphatase which detect labeled enzymes. Currently, these represent the most sensitive detection systems in immunoassay development (Magliulo *et al*. 2005).

Fluro immunoassay

Fluorescent labels one is among the most widely used labels in immunoassays for AF detection. Good fluoro labels are water-soluble, chemically stable, less expensive, and highly fluorescent. The most used fluorescent markers include organic fluorescent dyes (for example, fluorescein and cyanine dyes), fluorescent marker proteins (for example, phycoerythrin), metal complex markers (for example, Eu3+, Tb3+), noble metal ions, and nanoparticles. Similarly, to increase its sensitivity, efficiency and easiness of manipulation, Kanungo and Bhand (2013) developed an ELISA using fluorimetric detection. This was performed in a 384-well microplate, in which there were AFM1-specific monoclonal antibodies and secondary conjugated antibodies. AFM1 was detected at a level of 0.001 µg/L in a testing volume of 40 µL.

Seyedjafarri, (2021) analyzed the milk and yoghurt samples by Aflascan (Immunoaffinity columns) method. The detection of AFM1 is semi-quantitative in this method. The aflascan system provides a rapid, economical semi-quantitative screening test for the detection of aflatoxins. This procedure is based on monoclonal antibodies bound in an affinity columns format. This method is very specific and sensitive. This system has the capability of detecting total aflatoxin levels to the fluorescence and intensity of captured AFM1 from the milk or yoghurt samples showed the level of AFM1 in samples.

Dip-stick assay/ immunotype dip stick/ lateral flow assay

This is a rapid test used for detection of Aflatoxin M1 molecules presence in raw milk samples on-site. This dipstick assay is a time-saving assay as it does not require any type of sample processing, cleaning or extraction. The results obtained can be directly interpreted using an instrument like a reading sensor or through visual observation of the test line. The sensitivity of Aflatoxin M1 detected in this assay is 0.1 ppb through a visual reading and 0.050 ppb with the read sensor instrument. Specific antibodies which have a high affinity for Aflatoxin M1 molecules are used for this competitive test. The test has two components: The first component is a predetermined amount of antibody in microwell linked to gold particles and the second is a dipstick two capture lines. The “control” line and the “Test” line.

When the reagent from the microwell is re-suspended with a milk sample, specific antibodies if any present during the first incubation will bind the analytes. When the dipstick is dipped into the sample, the liquid starts running vertically over the dipstick and passes through capture zones. A color development occurs at the “test” line, indicating the absence of Aflatoxin M1 in the milk sample and on the other hand, the absence of a colored signal at the “test” capture line indicates the presence of Aflatoxin M1 in the sample.

High-quality antibodies and antigens are primary guarantees for the high sensitivity and specificity of LFIA. However, the recognition of limited immunogenic targets by antibodies has hindered the development of LFIA in several fields of study. Secondly, there is a contradiction in the competitive detection mode itself. That is, within a certain range, the less quantity of antibodies makes the competition between free target analytes and the immobilized antigens more effective on the test zone. So, the lower the specific antibody concentration, the lower the detection limit. However, the amount of the label conjugated with antibody is reduced simultaneously with the low concentration of antibody, which decreases the signal intensity of the test line and hinders successful detection of the targets. To overcome these shortcomings above, researchers developed better-performed antibodies or signal labels with high luminescent intensity and stability (Foubert *et al.* 2017).

Immunosensors

A device named biosensor is used to determine the analyte based on the incorporation of bioactive materials with the physiochemical transducing element (Mosiello and Lamberti, 2011). Bioactive material can be classified into two types affinity or biocatalytic such as antibodies, DNA, receptor protein, enzymes, tissues, whole cells, or organs interrelating with a specific analyte. The interaction can then be converted with the help of a transducer into a quantifiable electrical signal. An antibody-based biosensor is also known as an

immunosensor. The basic parts of the immunosensor comprise a bioreceptor, a transducer and an electronic system that enables signal amplification, processing and display.

Depending on signal transduction, Biosensors can be divided into three groups *i.e.* electrochemical transducers, rely on an electrical signal measurement (amperometric, potentiometric, and conductometric) generated by a physicochemical change, Optical transducers, in which an optical signal (color or fluorescence) changes as a result of the formation of a complex and Piezoelectric transducers, which detect changes in mass. The most common transducers in AF detection are the electrochemical transducers and optical transducers. Immunosensors combine the most advanced technologies based on the binding properties of antibodies, which improve the sensitivity and the rapid detection during analysis (Rasooly and Herold, 2011).

Electrochemical immunosensors

This is a device that incorporates antibodies into a biorecognition layer to produce electroactive signals which can be detected by transducers (amplifiers), which generate measurable signals. As a result of ions binding to the sensing membrane, the signal is generated and measured in the form of membrane potential (Linting *et al.*, 2012). Masoomi *et al.* (2013) developed a nonenzymatic sandwich form of an electrochemical immunosensor and the sensor in the nonenzymatic sandwich-type was developed through modification of glassy carbon electrodes.

Abera *et al.* (2019) developed, a flexible, dispense-printed electrochemical immunosensor was developed to analyze AFM1 in both buffer and spiked milk samples, using MAb for molecular recognition. This system allowed the quantification of AFM1 in milk in an easy and cost-effective manner. The LOD of the sensor was 0.02 µg/L and 0.0259 µg/L, with a detection range of 0.01 to 1 µg/L for the buffer and milk samples, respectively. Both the sensitivity and detection range were in accordance with most of the existing limits imposed by law for milk and milk products.

Optical immunosensor

In optical immunosensor, surface plasmon resonance is used which relies on the measurement of changes in a refractive index produced by the binding of the analyte to its bio specific partner immobilized on the sensor surface. In this technique, an optical-sensitive surface such as a layer of gold or a glass surface is used to immobilize Ab or Ag. As the amount of AFM1 binding to the Au layer or glass surface increases, the angle of spectral power distribution (SPD) also increases (Li *et al*. 2012). When this analyte flows over the sensor surface, there will be a shift in resonant SPR wavelength, which is directly proportional to the refractive change at the sensor surface and can be calibrated to the bound analyte surface concentration (Vaisocherov *et al*. 2007). This sensor surface contains a layer that is biorecognition which selectively binds either an antigen or antibody, which, in turn, causes a parallel increase in the mass on the sensor surface that is directly proportional to an increase in refractive index. This increase in the refractive index will be observed as a shift in the resonance angle. Due to the dissociated binding of antibodies to antigen quantifiable changes are noted (Brusatori *et al.* 2003).

Photoelectrochemical sensors

Photoelectrochemical (PEC) sensors are emerging as an analytical technique that combines features of both optics and electrochemistry. In PEC sensors, light source, electrochemical workstation and signal acquisition system are the main components. Light is used as the excitation source and the generated photocurrent is used as the detection signal, which significantly reduces background interference (Zhou and Tang, 2020).

Piezoelectric quartz crystal microbalances (QCMs)

For detection of antigens this method uses label-free devices. It depends on changes in mass on the surface of the electrode when an antigen interacts with an immobilized cognate antibody on the quartz crystal surface. Since the change in mass is directly proportional to the concentration of the antigen-antibody complex, the reading is obtained. This method permits easy detection and quantification of the immune complex (Ab-Ag) (Zhou *et al.* 2011).

CONCLUSION

Compared to analytical methods, immunoassays are very simple, rapid, sensitive, portable and cost-effective. Most of them may even be operated at the field level. But still, these methods are having some limitations like low specificity of antibodies to antigens or matrix, and poor color change. Therefore, future research can be focused on automation. Thus, the use of immunological methods, such as the ELISA, could be used at a preliminary stage to select from a wide range of samples those that are contaminated with the toxin under study. Subsequently, other methods are used to confirm the results.

Conflict of interest: None.

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