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# Aflatoxin Measurement and Analysis

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## 1. Introduction

Much research work has been devoted over the last 40 years for developing methods for detection and determination of aflatoxins in foods and agriculture commodities (Chu, 1991; Holcomb, et al., 1992). This effort is continuing and keeping pace with the progress in analytical chemistry. Methods for aflatoxins are required to meet the legislation, monitoring and survey work, and for research. Different highly efficient and sophisticated techniques have been developed in the recent years for the determination of aflatoxins in different commodities. Presently the most commonly used methods for detection of aflatoxins are: high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and enzyme-linked immunosorbent assay (ELISA) (Lee et al., 2009) and fluorometric method (Hansen, 1990). All analytical procedures include the steps: sampling, extraction, clean-up (purification) and determination (identification and quantification). The analytical detail in this chapter has been discussed in three sub-groups: sample preparation techniques, detection techniques and typical complete procedures.

## 2. Sample preparation techniques

Sampling and sample preparation is of utmost importance in the analytical identification of aflatoxins. It certainly affects the final conclusion. For the determination of aflatoxins at the parts-per-billion level, the systematic approaches to sampling, sample preparation and analysis are absolutely necessary. European Union has formed specific plans for certain commodities e.g. corn and peanuts. The performance of sampling plans for aflatoxin in granular feed products, such as shelled maize (Johansson, et al., 2000) and cotton seed (Whitaker et al., 1976) has been evaluated, while there has been little evaluation of sampling plans to detect aflatoxin in milk.

In case of sampling of solid commodities the entire primary sample must be ground and mixed so that the analytical test portion has the same concentration of toxin as the original sample. In case of sampling of liquid commodities like milk, due to homogeneous distribution of aflatoxins in liquid milk, there is less uncertainty in aflatoxin measurement in milk. After proper sampling, there are the steps of extraction and clean-up. Sometimes extraction and clean-up is the same step and sometimes extraction is different step and clean-up is a different step. Extraction of samples, together with effective clean-up step, is an essential step in the analysis of aflatoxins. The analyte migrates into the extraction solvent. The interfering compounds are removed by clean-up step. Common extraction solvents for aflatoxins are acetonitrile-water and methanol-water.

In addition to conventional technique of liquid-liquid extraction, there was need to develop new techniques due to its time consuming and tedious to apply nature. The new approaches have been developed to lessen the problems. A number of clean-up columns, using different principles such as solid phase extraction and immunoaffinity techniques, have been developed. The new techniques are easy to use and easily available. The immunoaffinity columns enhance selectivity, as only the analyte is retained in the column which can be eluted easily. On the other hand, in Mycosep columns the analyte is passed and all the other interfering contaminants are retained. A view of extraction techniques is forthcoming.

### 2.1 Liquid-liquid separation

The liquid-liquid separation is a conventional process and it is based on the partition of organic compounds between aqueous phase and immiscible organic solvent which may be non-polar or slightly polar. Extraction, in most cases, involves conventional procedures using acetone, chloroform and methanol *etc.* Small amounts of water give better extraction efficiencies. Hexane and cyclohexane are frequently used for compounds with aliphatic properties, whereas dichloromethane and chloroform are used for medium polar contaminants.

In a typical case of liquid-liquid separation, methanol and water were used as the extraction solvents in the first effective method for the determination of aflatoxin in fluid milk (Jacobson, et al., 1971). This method was modified by McKinney (1972) and others. Stubblefield and Shannon (1974) accomplished extraction with acetone and water, precipitation with lead acetate solution to de-proteinize the milk, and a de-fating step with hexane. TLC with fluorescence detection was applied for ultimate separation, detection, and quantification. The collaborative study proved the method to be successful and the method became an official AOAC method for aflatoxin M<sub>1</sub> (AOAC Official Method 974.17, 1990). AOAC is abbreviation for Association of Official Analytical Chemists.

In another case of liquid-liquid separation, extraction of aflatoxin from liquid milk was made with chloroform in a separating funnel and then extract was cleaned-up over a small silica gel column. Finally the separation was made by TLC and detection was made with fluorescence (Stubblefield, 1979). After modifications, this method was applied for determination of aflatoxin in cheese, in which two-dimensional TLC was applied to improve separation of the aflatoxin spots from the background. An AOAC/ IUPAC collaborative study evaluated the method (Stubblefield, et al., 1980) and it became an official AOAC method for aflatoxin M<sub>1</sub> in milk and cheese (AOAC Official Method 980.21, 2000).

The various mixtures of methanol - water (Masri, et al., 1969), acetone - water and acetone - chloroform - water (Purchase & Steyn, 1967) were used for the extraction of aflatoxins.

Liquid-liquid separation is a simple procedure and involves inexpensive equipment. Its disadvantages include contamination and loss of sample by adsorption to the glassware, as there are several steps. Large volumes of solvents are used and have to be disposed and these create pollution problems. In trace analysis, solvents with high purity have to be used which are highly costly.

### 2.2 Solid phase extraction (SPE)

The most significant recent improvement in the purification step, in aflatoxin analysis, is the use of solid-phase extraction (SPE). The use of solid-phase extraction with C-18 cartridges is now well established in aflatoxin determination. Solid phase extraction is suitable for the

analysis of aqueous samples. It can be performed on-line as well as off-line. Solid phase extraction process starts with conditioning of the column by activating it with the solvent. The sample is then applied and the analyte is trapped in the column. The interferences are removed by rinsing step. Finally, the analyte is eluted and then pre-concentration step is employed by evaporating excess solvent with nitrogen. A number of samples can be prepared simultaneously with the use of vacuum manifold. A vacuum manifold is shown in Photograph 1.



Photograph 1. A Vacuum manifold

Most frequently C-8 and C-18 bonded silica columns are used and these are very pressure resistant and give reproducible results. There is no significant drawback in case of SPE as compared to liquid-liquid separation. Its advantages include the consumption of less solvent, less time, and the possibility of automation. Photograph 2 shows some SPE C-18 columns.

A typical case example of C-18 cartridge use in aflatoxin analysis is that of the study of Bijl et al. (1987). They proposed a simple and sensitive method for the determination of aflatoxin  $M_1$  in cheese. The ground cheese sample is extracted with acetone-water mixture (3+1). Acetone is evaporated under vacuum, and the aqueous phase is passed through a C-18 disposable cartridge. After cartridge is washed with acetonitrile-water mixture (1+9), the toxin is eluted with acetonitrile. The extract is then cleaned up on a silica cartridge. Final analysis is performed by two dimensional thin layer chromatography combined with fluorodensitometry or by liquid chromatography on a reverse phase C-18 column with fluorescence detection. Recovery is greater than 90%, the coefficient of variation is 6% or less. The detection limit is in the range 10 ng/kg.

Application of C-8 (SPE) clean-up was shown by Manetta et al. (2005). They developed a new HPLC method with fluorescence detection using pyridinium hydrobromide perbromide as a post-column derivatizing agent to determine aflatoxin in milk and cheese. The detection limits for milk and cheese were 1 ng/ kg and 5ng/ kg respectively. The calibration curve was linear from 0.001 to 0.1 ng injected toxin. The method includes a preliminary C-8 (SPE) clean-up. The average recoveries of aflatoxin  $M_1$  from milk and cheese, spiked at levels of 25-75 ng/ kg and 100-300 ng/ kg, respectively, were 90 and 76%.

The precision ( $RSD_r$ ) ranged from 1.7 to 2.6% for milk and from 3.5 to 6.5% for cheese. The method is rapid and easily automatable and therefore is useful for accurate and precise screening of aflatoxin in milk and cheese.



Photograph 2. Solid phase extraction C-18 columns

### 2.3 Immunoaffinity columns (IACS)

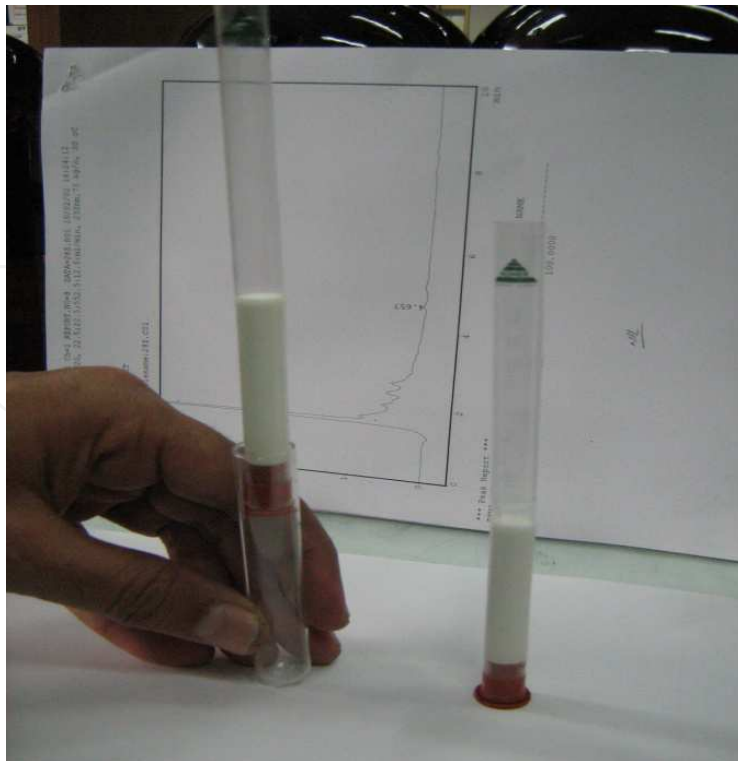
The immunoaffinity clean-up procedure was expanded in order to encompass successfully the determination of aflatoxins. Now, immunoaffinity columns have become increasingly popular in recent years for clean-up purposes, because these offer high selectivity and are easy to use. These can be applied for purification of samples that are contaminated with different aflatoxins. Aflatoxins are low weight molecules and they are only immunogenic if they are bound to a protein carrier. Antibodies are produced for aflatoxins. These antibodies are bound to an agarose, sepharose, or dextran carrier and packed in a column. The analyte molecules (aflatoxins) are bound selectively to the antibodies in the column. The matrix components do not interact with the antibodies and a rinsing (washing) step removes most of the possible interferences. The toxin can be eluted with a solvent causing antibody denaturation. Immunoaffinity columns have higher recovery than liquid-liquid partitioning. Single analyte columns are available and multifunctional columns for simultaneous determination of a number of mycotoxins are also available. Major disadvantages include the high costs and the fact that a column can be used once due to the denaturation of antibodies during elution step. Immunoaffinity columns are available commercially. Immunoaffinity column (AflaTest-Vicam, USA) is shown in Photograph 3.



Photograph 3. Immunoaffinity column (AflaTest-Vicam, USA)

#### 2.4 Mycosep™ columns

Mycosep™ columns, which remove matrix components with efficiency and can produce a purified extract within a short time, are also available. The Mycosep™ multifunctional clean-up columns (Romer Labs Inc., Union, MT, USA) consist of a number of adsorbents (charcoal, celite, ion exchange resins and others) which are packed in a plastic tube. On the



Photograph 4. Mycosep™ Columns of Romer Labs Inc., USA

lower end of the Mycosep™ column, there is a rubber flange, a porous frit and one-way valve which allow the extract to force through the packing material, when the column is inserted into the culture tube (glass tube). The purified extract appears on the top of the plastic tube within seconds. Almost all interfering substances are retained on the column, whereas the analyte does not show significant affinity to the packing material. No additional washing steps are required as in solid phase extraction. Columns are available for a range of mycotoxins and are usually suitable for one analyte. Photograph 4 shows Mycosep™ column of Romer Labs Inc., USA.

### 3. Detection techniques

After the extraction of the analyte (aflatoxin) from the sample and applying a clean-up procedure to remove interferences, then comes identification and quantification in the last in the analytical methodology. For the detection of aflatoxins, three main types of assays have been developed. These include biological, analytical and immunological methods. The biological methods were used when analytical and immunological methods were not available for routine analysis. Biological assays are non-specific and time consuming and are qualitative in nature.

#### 3.1 Analytical methods

Many analytical methods have been developed and are available for estimation of aflatoxins in agricultural commodities. These include: thin-layer chromatography, high performance thin-layer chromatography, and high-performance liquid chromatography.

##### 3.1.1 Thin-layer chromatography (TLC)

Thin layer chromatography is also known as flat-bed chromatography or planar chromatography and is one of the most widely used techniques in aflatoxin analysis. TLC is a chromatographic technique which is used for the separation, purity assessment and identification of aflatoxins. TLC can identify and quantify aflatoxins at levels as low as 1ng/g. Thin-layer chromatography consists of a stationary phase immobilized on a glass or plastic plate and a solvent acting as a mobile phase. The sample, either liquid or dissolved in a volatile solvent, is applied in the form of a spot on the stationary phase. Then the chromatographic plate is placed vertically in a solvent reservoir and the solvent moves up the plate by capillary action. When the solvent front reaches a certain limit of the stationary phase, the plate is removed from the solvent reservoir. The separated spots are then visualized with ultraviolet light or by spraying with a suitable reagent. The contents of a sample can be identified by running standards simultaneously with the unknown spots. The different components in a mixture move up the plate at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. The  $R_f$  value for each spot is calculated. It is the ratio of the distance (cm) from start to centre of sample spot and distance (cm) from start to solvent front.  $R_f$  stands for "ratio of fronts" or "retardation factor". It is characteristic for a given compound on the same stationary phase using the same mobile phase under same conditions of development of the plate. For identification purposes,  $R_f$  values of standards are compared to those of unknown samples. A number of methods have been developed for the determination of aflatoxins by TLC. Silica plates are mostly used with a number of solvent mixtures. Mostly the solvent systems are based on chloroform and small amounts of methanol or acetone. Now-a-days,

less toxic and environmental friendly solvent mixtures (e.g. toluene/ethyl-acetate or acetone/ iso-propanol) are also employed. Aflatoxins are strongly fluorescent (excitation  $\lambda = 365$  nm, detection or emission  $\lambda = 430$  nm) by themselves and can easily be detected by fluorodensitometry.

Thin layer chromatography is the standard AOAC method for aflatoxin analysis since 1971, AOAC Official Method 971.24, First Action 1971 and Final Action 1988 (AOAC Official Method 971.24, 2000). TLC separation of aflatoxins provided basis for sensitive analytical techniques. TLC quantification method gives a reasonable level of selectivity and sensitivity to separate aflatoxins from other interfering compounds. TLC is the method of choice for rapid screening of aflatoxins and for situations where advanced techniques equipments are not available.

A typical case application of TLC in aflatoxin analysis is that of Van Egmond et al. (1978). They confirmed the identification of aflatoxin  $M_1$  on thin layer plate by reacting aflatoxin  $M_1$  with trifluoroacetic acid (TFA). In the method the plate was developed with chloroform-methanol-acetic acid-water (92+8+2+0.8) mixture. The  $R_f$  value of the blue fluorescent derivative was compared with that of the aflatoxin  $M_1$  standard which was also spotted on the TLC plates.

### 3.1.2 High performance thin-layer chromatography (HP-TLC)

There is lack of precision associated with TLC procedures due to the introduction of possible errors during the sample application, plate development, and plate interpretation steps. High performance thin-layer chromatography methods improve the precision by automating the sample application and plate interpretation steps. This technique is less commonly used as compared to HPLC, which is more sophisticated as compared to this.

### 3.1.3 High performance liquid chromatography (HPLC)

Analytical laboratories moved away from TLC to HPLC determination with advances in HPLC methods in 1980s. High performance liquid chromatography is a very precise and highly automated quantification technique for aflatoxins analysis with high selectivity and sensitivity. Now-a-days, HPLC methods are widely used because of their superior performance and reliability as compared with TLC. HPLC methods have been developed for all major mycotoxins in cereals and other agricultural commodities. In the field of analysis of aflatoxins, HPLC is mainly used for final separation and detection of the analyte of the interest and extraction and clean-up techniques have to be applied prior to detection with HPLC.

In HPLC, a liquid mobile phase or solvent is used to move the sample through the column. An immobilized liquid stationary phase is packed in the column. The analyte is then partitioned between the two phases as it passes through the column and thus leading to the separation of compounds due to the difference in partitioning coefficients. Two types of HPLC methods are commonly used i.e., normal phase chromatography and reversed phase chromatography. In normal phase chromatography, a polar stationary phase e.g. silica gel and a non-polar solvent e.g. hexane are used. Whereas reversed-phase chromatography (RP-HPLC) employs non-polar stationary phase e.g., C-8 or C-18 hydrocarbons and polar mobile phase e.g. water, methanol or acetonitrile. In HPLC, detection is mainly accomplished by using ultra violet (UV) detector, diode array detector (DAD) or a fluorescence detector (FLD). Fluorescence detection utilizes the emission of light (435 nm) from molecules that have been excited to higher energy levels by absorption of electromagnetic radiation (365 nm) for aflatoxins. Fluorescence detection has superior



sensitivity than other detection systems and sometimes derivatization of the analyte has to be performed which enhances the sensitivity. Fluorescence detection is possible in the range of microgram/kg. Choice of detector usually depends on the nature of the sample.

RP-HPLC is commonly performed for determination of aflatoxins in foods. Stationary phase for aflatoxins include C-18 material. Pre- or post-column derivatization is necessary for low-level detection. For aflatoxins, derivatization is performed with strong acids or oxidants e.g., Br<sub>2</sub>, I<sub>2</sub> or trifluoro-acetic acid. This results in increase of fluorescence by a factor 20. Sometimes, a pre-column is employed to avoid heavy contamination or subsequent blocking of main separation column.

The HPLC systems of Shimadzu (Japan) and Agilent (USA) are very commonly used and these are highly sophisticated. All the HPLC systems are comprised of many components. The main components are: liquid pump, column oven, system controller, detectors (fluorescent detector, ultra violet (UV) detector, diode array detector i.e., DAD), communication bus module i.e., CBS and data acquisition software. Photograph 5 shows HPLC System.



Photograph 5. High-performance liquid chromatography system

HPLC system gives result in the form of chromatogram. A chromatogram gives two types of analytical informations: one is qualitative and the other one is quantitative. In the HPLC chromatogram, retention time is given on x-axis, while on y-axis height of the peak is given. Retention time is used for identification purposes and area of the peak is used for quantitative purposes. A sample HPLC chromatogram is shown in the Fig. 1. The Fig. 1 shows two graphs, i.e., "A" and "B". Graph "A" is for standard and graph "B" is for sample. By comparing the two graphs, identification of the unknown compound is made. After identification, quantitation is done from the area of the peaks.

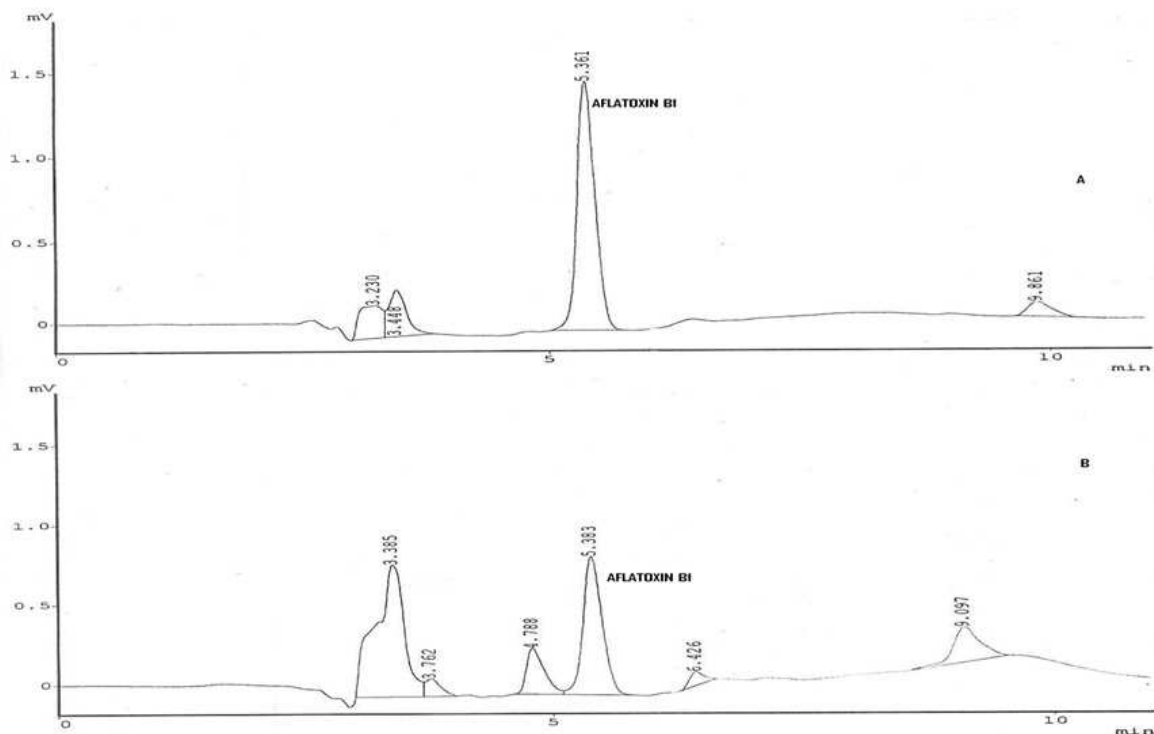


Fig. 1. A sample HPLC chromatogram, (A) for standard and (B) for sample

### 3.1.4 Liquid chromatography with mass spectrometric detection

Liquid chromatography with mass spectrometric detection (LC-MS) is fairly a recent development in aflatoxins detection and it is one of the most advanced techniques. It is time-consuming and requires expert knowledge. In mass spectrometric detection, extraction and clean-up techniques have to be applied before detection. In LC-MS, the HPLC effluent enters an ionization chamber via a nebulizer. There are several techniques for ionization, namely electrospray, thermo-spray, chemical and fast atom bombardment. Fragmentation takes place in a collision chamber. The fragments then enter the high vacuum region of the mass spectrometer, where detection takes place. Several set-ups are available for optimal identification and quantification. Ion trap instruments are more suitable for identification than triple quadrupole instruments (higher MS<sub>n</sub> power), whereas triple quadrupole instruments provide better information for quantification with faster scanning and higher sensitivity. There are also available hybrid instruments that provide a linear ion trap in a triple quadrupole instrument to get the best results out of both set-ups. LC-MS methods have their applications in determination of aflatoxins in corn, milk and samples of other commodities. In Selection-Ion-Monitoring (SIM) mode, detection can be made at pico-grams levels.

### 3.2 Immunological methods

Immunological methods are based on the affinities of the monoclonal or polyclonal antibodies for aflatoxins. Due to the advancement in biotechnology, highly specific antibody-based tests are now commercially available for measuring aflatoxins in foods in less than ten minutes. There are two major requirements for immunological methods. First requirement is high quality antibodies and second is methodology to use the antibodies for the estimation of aflatoxins. Being low molecular weight molecules, aflatoxins cannot

stimulate the immune system for the production of antibodies. Such molecules of low molecular weight, which cannot evoke the immune system, are called haptens. Therefore, before immunization, aflatoxins must be conjugated to a carrier molecule which is a larger molecule like proteins. Bovine serum albumin (BSA) is most commonly used as a carrier protein and hapten is conjugated with it. The three types of immunochemical methods are: immunoaffinity column assay (ICA), radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA). Immunoaffinity columns are mainly used for clean-up purposes and RIA has limited use in aflatoxins analysis. ELISA is most commonly used for the estimation of aflatoxins.

Many rapid tests, using specific antibodies for isolation and detection of mycotoxins in food have been discussed and applied by various workers (Newsome, 1987; Groopman & Donahue, 1988). Use of immunoaffinity cartridges is a more recent advance in quantitative extraction of aflatoxin. Monoclonal antibodies specific for aflatoxin are immobilized on Sepharose® and packed into small cartridges. The work of Mortimer et al. (1987) is very important as it is the first published method for aflatoxin M<sub>1</sub> with immunoaffinity columns. For the aflatoxin determination, a milk sample is loaded onto the affinity column. The antigen i.e., aflatoxin is selectively complexed by the specific antibodies on the solid support to form antigen-antibody complex. Then, the column is washed with water to remove all other matrix components of the sample. A small volume of pure acetonitrile is used to elute the aflatoxin and the eluate is concentrated and analyzed by HPLC coupled with fluorescence detection.

Many collaborative studies were done to develop the immunological methods; especially for aflatoxin M<sub>1</sub>. Immunoaffinity-based methods for aflatoxin M<sub>1</sub> were modified and subsequently published and studied collaboratively under the auspices of the International Dairy Federation and AOAC international by groups of mainly European laboratories that could determine aflatoxin M<sub>1</sub> in milk at concentrations equal to 0.05 µg/ L. The collaborative study of Tuinstra et al. (1993) led to International Dairy Federation Standard 171. Another collaborative study (Dragacci et al. 2001) was conducted to evaluate the effectiveness of an immunoaffinity column clean-up liquid chromatographic for determination of aflatoxin M<sub>1</sub> in milk at proposed European regulatory limits. The procedure included centrifugation, filtration, and application of the test portion to an immunoaffinity column. Then the column was washed with water and aflatoxin was eluted with pure acetonitrile. Aflatoxin was separated by reversed-phase liquid chromatography and detection was made with fluorescence detector. Liquid milk samples (frozen), both naturally contaminated with aflatoxin M<sub>1</sub> and blank samples for spiking, were sent to 12 collaborators in 12 different European countries. Test portions of milk samples were spiked at 0.05ng aflatoxin M<sub>1</sub> per mL. After the removal of two non-compliant sets of results, the mean recovery of aflatoxin M<sub>1</sub> was 74%. The relative standard deviation for repeatability (RSD<sub>r</sub>) ranged from 8 to 18%, based on results of spiked samples (blind pairs at 1 level) and naturally contaminated samples (blind pairs at 3 levels). The relative standard deviation for reproducibility (RSD<sub>R</sub>) ranged from 21 to 31%. As evidenced by HORRAT values at the low level of aflatoxin M<sub>1</sub> contamination, the method showed acceptable within and between laboratory precision data for liquid milk. The collaborative study resulted in approval of AOAC Official Method 2000.08 (AOAC Official Method 2000.08, 2005).

### 3.2.1 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay is most widely used test to detect aflatoxins, due to its simplicity, sensitivity and adaptability. There are two types of enzyme-linked immunosorbent assay, which are direct competitive enzyme-linked immunosorbent assay and

indirect competitive enzyme-linked immunosorbent assay. In direct competitive enzyme-linked immunosorbent assay method, specific antibody is coated to a solid phase such as a microtiter plate, whereas in indirect competitive enzyme-linked immunosorbent assay method, toxin-protein conjugate is coated onto the microtiter plate. In aflatoxin analysis, direct competitive enzyme-linked immunosorbent assay is used. The enzyme-linked immunosorbent assay is detection and quantification of an antigen (aflatoxin) in a sample by using an enzyme labeled toxin and antibodies specific to aflatoxin. The enzyme-linked immunosorbent assay is based on antigen-antibody reaction (Aycicek et al., 2005). Antigen is that substance which can elicit production of antibodies when introduced into warm blooded animals. Whereas antibodies are glycoproteins which are produced as a result of an immune response, after introduction of antigens, leading to the production of a specific antigen-antibody complex. In the direct competitive enzyme-linked immunosorbent assay, specific antibodies for aflatoxin are coated on to the wells in the microtiter strip. The test samples or aflatoxin standards are added to the wells. After incubation and washing, enzyme conjugate (a conjugate of aflatoxin and bovine serum albumin is attached with an enzyme molecule, such as, horseradish peroxidase or penicillinase or alkaline phosphatase) is added to the wells. Free aflatoxin and aflatoxin enzyme conjugate compete for the aflatoxin antibody sites in the wells. Washing step removes any unbound enzyme conjugate. Then substrate/chromogen is added to the wells and incubated. The bound enzyme conjugate converts the colorless chromogen into a blue product. The stop solution is added which leads to color change from blue to yellow. Then measurement is made photometrically at 450 nm in an ELISA reader. The absorbance is inversely proportional to the aflatoxin concentration in the sample i.e., the lower the absorbance, the higher the aflatoxin concentration.

The main instrument used in enzyme-linked immunosorbent assay is the ELISA reader. It is basically a photometric instrument which gives the absorbance of the solution at the end of the process. The whole process has been described with complete details in the past paragraph. An ELISA reader is shown in Photograph 6.



Photograph 6. An ELISA reader

A sample enzyme-linked immunosorbent assay calibration curve is shown in the Fig. 2. The ELISA reader gives absorbance readings from which % absorbance is calculated. For standard solutions, the % absorbance is plotted against aflatoxin concentration to get the calibration curve. The aflatoxin concentration is on x-axis and % absorbance is on y-axis. From the calibration curve, aflatoxin concentration is calculated for samples.

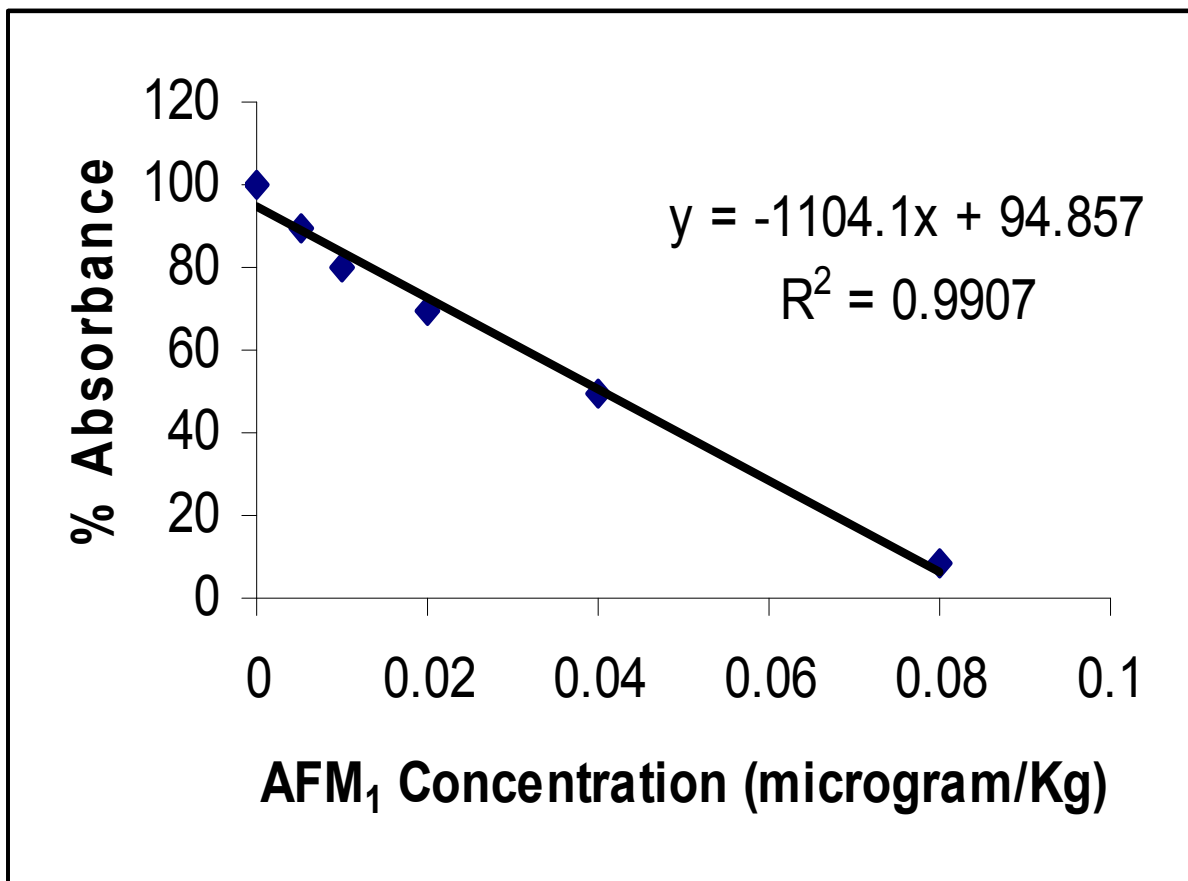


Fig. 2. A sample enzyme-linked immunosorbent assay calibration curve

#### 4. Typical complete methods

Some typical methods are given completely to make the understanding of the process of aflatoxin analysis.

##### 4.1 Determination of aflatoxin M<sub>1</sub> with fluorometer

This is very simple and efficient method. It is also a specific method. The analysis is carried out with Fluorometer along with the use of affinity chromatography columns for clean-up step according to the method described by Hansen (1990). Before analysis, sample is brought to room temperature. To remove cream from the milk sample, it is centrifuged at 2000×g for 10 minutes. The 10 mL sample of skim milk is passed through AflaTest affinity column of the Vicam, USA. These affinity columns contain antibodies to aflatoxin. The column is then washed twice with 10 mL portions of 10% methanol and the aflatoxin M<sub>1</sub> is eluted from the affinity column by passing 1.0 mL of 80% methanol. All the sample eluate (1.0 mL) is collected in a glass cuvette.

The concentration of aflatoxin M<sub>1</sub> is measured in a fluorometer (Vicom, USA) with the option of 360 nm excitation filter and 440 nm emission filter. The results are recorded using digital Fluorometer readout with automatic printing device.

#### 4.2 Determination of aflatoxin M<sub>1</sub> by HPLC

A very competent method used for determination of aflatoxin M<sub>1</sub> is that of the AOAC Official Method 2000.08 (AOAC Official Method 2000.08, 2005). Details of the method are given in coming lines.

##### 4.2.1 Extraction procedure

After warming at about 37°C in water bath, liquid milk is centrifuged at 2000×g to separate fat layer and then filtered. The prepared test portion of 50 mL is transferred into syringe barrel attached with immunoaffinity column (IAC) and passed at slow steady flow rate of 2-3 mL/ min. The washing of column is done with 20 mL water and then it is blown to dryness and afterwards aflatoxin M<sub>1</sub> is eluted with 4 mL pure acetonitrile by allowing it to be in contact with the column at least 60 seconds. The eluate is evaporated to dryness using gentle stream of nitrogen and at the time of LC (liquid chromatography) determination it is diluted with the mobile phase.

##### 4.2.2 LC Determination with fluorescence detection

The HPLC system of Agilent 1100 series (Agilent, USA), equipped with an auto sampler LAS G1313A and a fluorescence detector FLD G1321A with excitation and emission wavelength of 365nm and 435nm respectively, may be used for aflatoxin M<sub>1</sub> determination. Any other suitable system may be used instead of the above mentioned system. The ZORBAX Eclipse XDB-C18 (Octadecyl silane chemically bonded to porous silica) column (Agilent, USA), 4.6×150 mm with particle size 5 μm in diameter, may be used. Acetonitrile in ratio of 25% with 75% water is used as mobile phase. The flow rate is 0.8 mL/min. Calibration curve is determined using a series of calibration solutions of aflatoxin M<sub>1</sub> in acetonitrile. The concentrations of calibration curves may be in the range of 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 μg/ L. The retention time for aflatoxin M<sub>1</sub> may be in the range of 6.1-6.5 min.

##### 4.2.3 Calculations

Calculations are made according to the following equation:

$$W_m = W_a \times (V_f/V_i) \times (1/V_s)$$

Where  $W_m$  = amount of aflatoxin M<sub>1</sub> in the test sample in μg/L;  $W_a$  = amount of aflatoxin M<sub>1</sub> corresponding to area of aflatoxin M<sub>1</sub> peak of the test extract (ng);  $V_f$  = the final volume of re-dissolved eluate (μL);  $V_i$  = volume of injected eluate (μL);  $V_s$  = volume of test portion (milk) passing through the column (mL).

#### 4.3 Determination of aflatoxin B<sub>1</sub> by HPLC

An important method for the determination of aflatoxin B<sub>1</sub> is that of the AOAC Official Method 994.08 which has been described here with small modifications (AOAC Official Method 994.08, 2000).

#### 4.3.1 Extraction and clean-up procedure

A test portion of 50.0g and 100mL extraction solvent (850mL acetonitrile with 150mL deionized water) is taken in 250mL Erlenmeyer flask and placed in a shaker for 1 hour at high speed. After filtration, 8mL extract is taken with pipette in 10mL glass tube. MycoSep® column (rubber flange end) is pushed slowly into the tube. As column is pushed into the tube, extract is forced through frit, through 1-way valve, and through packing material and is collected in column reservoir. The purified extract (2mL) is transferred quantitatively from top of column to screw cap vial (derivatization vial) and is evaporated under nitrogen.

#### 4.3.2 Aflatoxin derivatization

After adding n-hexane (200 $\mu$ L) in the derivatization vial to re-dissolve aflatoxin, 50 $\mu$ L of trifluoroacetic acid is added and it is mixed on vortex mixer for 30 seconds. After five minutes, 1.95mL of deionized water: acetonitrile (9:1) mixture is added and again mixed on vortex mixer for 30 seconds. Layers are allowed to separate and aqueous layer (lower layer) containing aflatoxins is removed, filtered through 0.45 $\mu$ m syringe filter and then injected onto LC column.

#### 4.3.3 LC Determination with fluorescence detection

The high-performance liquid chromatography equipment (LC-10, Shimadzu, Japan), comprising liquid pump LC-10AS, column oven CTO-10A, system controller SCL-10A, fluorescence detector RF-530, communication bus module CBM-101, and data acquisition software class LC-10A may be used for aflatoxin B<sub>1</sub> determination. Any other suitable system may also be used instead of the above said one. The excitation wavelength of 365nm and emission wavelength of 435nm is set during analysis. The stainless steel column Discovery® C-18 of Supelco (Bellfonte, PA, USA) with dimensions of 25cm $\times$ 4.6mm (id) and with particle size of 5  $\mu$ m diameter may be used. The mobile phase (acetonitrile: methanol: deionized water in the ratio of 20:20:60) is degassed with sonicator before use. The flow rate is 1.0 mL/ min. Calibration curve is determined using a series of calibration solutions of aflatoxin B<sub>1</sub> in acetonitrile. The concentrations of calibration solutions may be 0.5, 1.5, 2.5, 5.0, 10.0, and 15.0  $\mu$ g/ L. The retention time for aflatoxin B<sub>1</sub> is near to 5.36 minutes or may be slightly different by changing conditions or instrument.

#### 4.3.4 Calculations

Aflatoxin B<sub>1</sub> peak is identified in derivatized extract chromatogram by comparing its retention time with corresponding peak in the standard chromatogram. The quantity of the aflatoxin B<sub>1</sub>, 'C' is determined in the derivatized extract (injected) from the respective standard curves. The concentration of aflatoxin B<sub>1</sub> is calculated in test sample as follows:

$$\text{Aflatoxins B}_1 \text{ ng/g} = C/W$$

$$W = 50\text{g} \times (2\text{mL}/ 200\text{mL}) \times (0.02\text{mL}/ 2\text{mL}) = 0.005\text{g}$$

Where  $W$  = equivalent weight of test portion (in 20 $\mu$ L) injected into LC;  $C$  = ng aflatoxin (in 20 $\mu$ L) injected into LC.

#### 4.4 Determination of aflatoxin M<sub>1</sub> in cheese by ELISA

An ELISA method for the determination of aflatoxin M<sub>1</sub> in cheese is given here as described by the protocol provided with RIDASCREEN® ELISA kit (RIDASCREEN® Aflatoxin M<sub>1</sub> 30/15, 2007).

##### 4.4.1 Sample preparation

Cheese (2.0g) samples are first of all triturated. Extraction is completed with 40 mL dichloromethane by shaking for 15 minutes. The suspension is filtered and 10 mL of the extract is evaporated at 60°C under weak N<sub>2</sub>-stream. The oily residue is re-dissolved in 0.5 mL methanol, 0.5 mL PBS buffer and 1 mL n-heptane. After mixing thoroughly, it is centrifuged for 15 minutes at 2700 × g. The upper heptane layer is removed completely. From the lower methanolic-aqueous phase, 100µL is taken and diluted with 400 mL buffer 1 and 100µL of it is used per well in the test.

##### 4.4.2 Test procedure

The standard solutions (100µL) and prepared samples (100µL) are added into the microtiter well placed in the microwell holder. Gentle mixing is accomplished by shaking the plate manually and incubated for 30 minutes at room temperature (20-25°C) in the dark. The liquid is poured out of the wells and microwell holder is tapped vigorously upside down against adsorbent paper to ensure complete removal of liquid from the wells. The wells are washed by adding 250 µL washing buffer in each well and poured out the liquid again. Washing step is repeated for two times. Then 100 µL of the diluted enzyme conjugate is added and mixed gently by shaking the plate manually and incubated for 15 minutes at room temperature in the dark. After incubation the wells are washed again. The 100 µL of substrate/chromogen is added and mixed gently by shaking the plate manually and incubated for 15 minutes at room temperature in the dark. Now stop solution (100µL) is added in each well. Mixing is done by shaking the plate manually. The absorbance is measured photometrically at 450 nm against an air blank with in 15 minutes after the addition of stop solution.

##### 4.4.3 Calculations

The following formula is used to measure the % absorbance.

$(\text{Absorbance of standard or sample} / \text{absorbance of zero standard}) \times 100 = \% \text{ absorbance}$

The zero standard is made equal to 100 % and absorbance values are taken in percentages. A calibration curve is obtained by plotting %absorbance values for the standards against the aflatoxin M<sub>1</sub> concentration (µg/L). The concentration of aflatoxin M<sub>1</sub> in samples is calculated from the calibration curve.

## 5. Conclusion

The methods of measurement and analysis of aflatoxins have been discussed in this chapter. Some photographs were taken by the author himself, while others were downloaded from internet. Some analytical studies in the aflatoxin analysis have been included to have the insight of methods' application and their development. Typical complete methods have been included as exemplary methods, so the understanding of the process of aflatoxin analysis may become clear.



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## 7. References

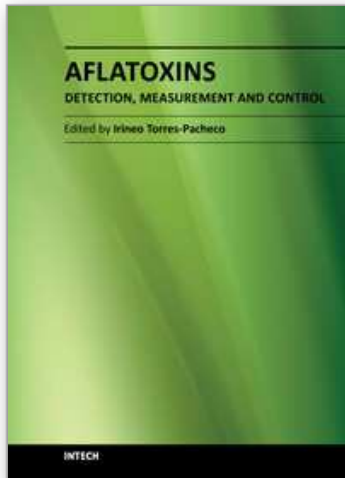
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## **Aflatoxins - Detection, Measurement and Control**

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This book is divided into three sections. The section called Aflatoxin Contamination discusses the importance that this subject has for a country like the case of China and mentions examples that illustrate the ubiquity of aflatoxins in various commodities. The section Measurement and Analysis, describes the concept of measurement and analysis of aflatoxins from a historical perspective, the legal, and the state of the art in methodologies and techniques. Finally the section entitled Approaches for Prevention and Control of Aflatoxins on Crops and on Different Foods, describes actions to prevent and mitigate the genotoxic effect of one of the most conspicuous aflatoxins, AFB1. In turn, it points out interventions to reduce identified aflatoxin-induced illness at agricultural, dietary and strategies that can control aflatoxin. Besides the preventive management, several approaches have been employed, including physical, chemical biological treatments and solvent extraction to detoxify AF in contaminated feeds and feedstuffs.

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