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RESEARCH OF SOME MYCOTOXINS IN AGRICULTURAL PRODUCTS

5A320501-Biotechnology (Production of food, feed, chemical products and biological products for agriculture)

DISSERTATION

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CONTENTS

INTRODUCTION

Today's time is important to provide the population with healthy and safe food. This requires full compliance with safety regulations at all stages of food preparation. In doing so, one must pay great attention to the condition of growing raw materials for products, the condition of storage, the condition of transportation, because many pollution can get in precisely these stages.

In addition, when preparing feed products for livestock enterprises, many pollution can form. Among these contaminants may be some microorganisms that produce low-molecular toxins which are called mycotoxins. These mycotoxins are so small that they are very difficult to detect. If the product gets some types of mycotoxins, consumers can get poisoned, in some cases can lead to disastrous consequences.

Actuality of work. The quality of food now determines the standard of living of people. One of the main principles of forming the quality of food products is their safety. Another priority principle is to ensure the nutritional value of the product, according to its purpose in human nutrition. These aspects of the quality of food provide for the further development and strengthening of the control and supervision system. The first President of the Republic of Uzbekistan, IAKarimov, on October 20, 2004, in his speech in the Kashkadarya region on food issues, said that "it is necessary to take up resolutely food safety". Nutrition is the most important factor determining human health. The solution of problems of food quality and safety, its processing is one of the priority directions in the implementation of the concept of state policy in the field of healthy nutrition of the population of the Republic of Uzbekistan.

The problem of the quality of food products can not be solved without modern methods of control. The deterioration of the ecological situation, the receipt of a large number of foreign products on the food market of Uzbekistan, put forward new requirements for food control methods. In this regard, the current situation requires the introduction of new methods for express control of food

products, the creation of universal, highly sensitive methods of control in the field of quality and safety of food products and industries.

Solving many of the issues encountered in food safety requires a multidisciplinary method, and requests the expertise of researchers and practitioners in various elements of food safety and food quality assurance, as well as monitoring, surveillance and other aspects of risk analysis at each stage. This approach requires a thorough knowledge of all food safety factors that apply to the phase before harvesting, processing and after the food phase of the food chain, thus providing the basis for recognizing and implementing effective intervention and risk management practices that protect the consumer against inherent hazards in Light of today's challenges.

It's no secret that high-quality imported goods create competition for our products, pulling it up to its level. There are enterprises that develop and apply modern quality assurance systems with the focus on the requirements of international standards.

The rather bad ecological situation, as a consequence of the consumer attitude towards nature, indicates the strongest impact on the safety and quality of food. Extremely dangerous are mycotoxins that contaminate food products due to disruption of the process in production or during storage of food products.

All of the above mentioned penetration vectors explain why the problem of food safety is now presented to the society so sharply and topically, especially it is glamorous for our region.

In connection with this, this thesis is devoted to the development of modern methods of HPLC analysis of mycotoxins of agricultural products in Uzbekistan.

Purpose and objectives of the study.

The purpose of this dissertation is to develop a modern standard method of HPLC analysis for mycotoxins contaminating food products.

The objectives of the study were:

1. Studying the methods of chromatographic analysis.
2. Study of methods for the release of mycotoxins.

3. Development of a standard method of HPLC analysis of acute mycotoxins in food composition, using standard samples of mycotoxins.

Scientific novelty.

For the first time using a chromatographic column Zorbax Eclipce XDB C8, standard HPLC analysis methods were developed to determine the content of the main mycotoxins in food composition.

Subject of study.

Standard samples of mycotoxins - afloxin G1, G2, B1, B2, Ochratoxina (Sigma USA,)

Objects of research.

The subjects of the study were barley malt and corn.

Practical significance of the work.

The results of the research conducted and the methods developed can be applied to the analysis of contaminants in food products, to control and determine the quality of food products in enterprises where food is produced, as well as in the certification and monitoring of food products produced and imported.

CHAPTER I. REVIEW OF LITERATURE

1.1. Origin of mycotoxins

Mycotoxins - secondary metabolites of the life of microscopic molds - contaminate food products of plant and animal origin and can cause serious damage to the health of both animals and humans. Most often they are not in the products individually, while the simultaneous presence of several species of mycotoxins leads to an increase in their toxic effects as a result of the synergistic effect.[1] All mycotoxins are divided into several classes and differ in their properties and structure, which leads to difficulties in their simultaneous determination. Methodological instructions for the determination of mycotoxins in products of plant and animal origin are based on the use of chromatographic analysis methods: TLC, HPLC with fluorimetric or UV-detectors, GLC with an electron capture detector. To extract mycotoxins from the analyzed objects, liquid-liquid extraction is mainly used. For the extraction of extracts from coextractable substances (proteins, fats, lipids, sterols, polar organic acids, carotenoids, chlorophyll), solid-phase extraction and commercial immunoaffinity columns are used to selectively extract mycotoxins (single or specific class). Typically, these methods are long (sample preparation can take from 3 to 5 hours), laborious, require large amounts of toxic organic solvents and expensive disposable immunoaffinity columns that are useful in determining only single classes or single standardized mycotoxins (aflatoxins Bl, B2, G1, G2, Ml , Ochratoxin A, T-2, deoxynivalenol, zearalenone, patulin).[3]

Recently, for the simultaneous extraction of mycotoxins and their determination by GLC (HPLC) -MS / MS methods, QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) is used. However, with such sample preparation, the determination of mycotoxins by more accessible chromatographic methods with fluorimetric, UV and electron capture detectors is difficult due to insufficient purification of the extracts.[5]

Mycotoxins

Mycotoxins are toxins, low-molecular secondary metabolites produced by microscopic mold fungi.

Mycotoxins are natural contaminants of grain cereals, legumes, sunflower seeds, as well as vegetables and fruits. They can be formed when stored in many food products under the influence of microscopic fungi developing in them.[10]

Origin

Mycotoxins are most often synthesized by imperfect fungi (formal class Fungi imperfecti) of the genera Fusarium, Aspergillus, Myrothecium, Stachybotrys, Trichoderma, Trichothecium, Penicillium, etc.[9]

Conditions for the development of mold fungi, the main sources of mycotoxins

Most fungi are aerobic organisms (that is, using oxygen for breathing). They are found almost everywhere in extremely small amounts and, in most cases, are microorganisms. They consume organic matter, where only the humidity and temperature allow, inside and outside the premises.[11]

Where conditions permit, fungi, reproducing, form colonies, increasing the concentration of mycotoxins. Some fungi produce dangerous toxins only at certain levels of humidity, temperature and oxygen content in the air.

1.2. Characteristics and distribution in nature

The presence of mycotoxins in feeds leads to a deterioration in the productivity, reproduction and immune status of animals. Mycotoxins differ in chemical structure, toxicity and mechanism of action. A common sign of all mycotoxins is toxicity, mostly for animals. The most often used classification of mycotoxins according to the molecular structure, according to which are distinguished aflatoxins, trichothecene mycotoxins, ochratoxins, fumonisin, zearalenone and its derivatives, moniliiformin, fusarchromanone, ergot alkaloids, cyclopiazonic acid, patulin, citrinein, etc.[12]

The term "mycotoxicosis" is first encountered in A. Kh. Sarkisov's article published in 1948. In NA Grandilevsky's work of 1938 to describe the poisoning of horses with straw stained with Stachybotrys alternans, the term "stachybotriotoxicosis" was used, and in the writings of Muratov , Preobrazhensky NG and Salikov GI, published in 1944, the poisoning of farm animals with fodders with ergot admixtures (Claviceps purpurea) was defined as claviclesotoxicosis. The term "mycotoxinium" (from the Greek words mikos - mushroom and toxic - poison) was first used in the early 60s of the last century. But the nature and toxicity of many substances that were later attributed to mycotoxins, as well as diseases resulting from poisoning by them, which were subsequently combined under the name of mycotoxicoses, were discovered and described long before the introduction of these terms.[13] The first mention of the poisoning of people and animals with bread and grain, contaminated with toxic metabolites of fungi, namely ergot alkaloids (Claviceps purpurea), are found in medieval chronicles. The nature of the alkaloid horns was first established in 1864, but the alkaloids were assigned to the mycotoxins much later.

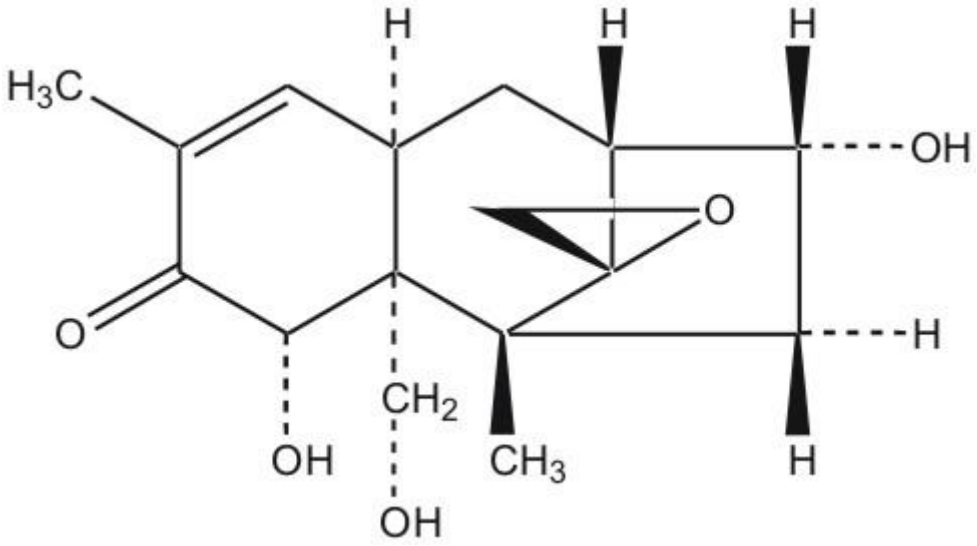
The attention of researchers to mycotoxins was attracted by aflatoxins discovered during the investigation of the cause of "X disease" - the case of 100 000 turkeys on England's farms in 1960. The disease was accompanied by apathy, loss of appetite, wings down, neck arching, head dropping and death within a week. During the autopsy, hemorrhages and necrosis in the liver were detected.

After careful and lengthy studies of peanut meal, which was fed to the turkeys, there was a colorless crystalline substance, the introduction of which to the ducklings allowed reproducing the signs of "X disease". It turned out that this substance is synthesized by fungi f the genus Aspergillus (A. flavus, A. parasiticus), which grow on peanuts, corn, soybeans and oilseeds in conditions of temperate climate. According to the name of one of the producers (A. flavus) the substance was called aflatoxin.[14]

The main representatives

Trichothecene mycotoxins are synthesized by the fungi of the genera Fusarium, Cephalosporium, Myrothecium, Stachybotrys, Trichoderma and Trichothecium; Contain a 12,13-epoxy-sesquiterpenoid residue (trichotecan); About 100 trichothecene mycotoxins are known.[18]

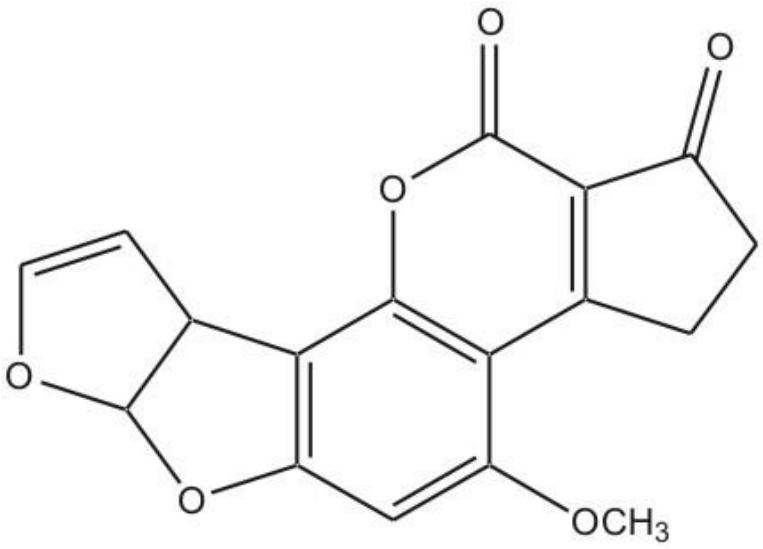
The mechanism of toxic action is based on the ability to inhibit protein synthesis.



Deoxynivalenol

Agaritin is a mycotoxin of some agaric fungi (Agaricales), including mushroom mushroom.

Aflatoxins are mycotoxins that produce fungi of Aspergillus flavus and Aspergillus parasiticus. They are pollutants of peanuts, corn and other grains and oilseeds; Characterized by a strong hepatocarcinogenic effect.[20]

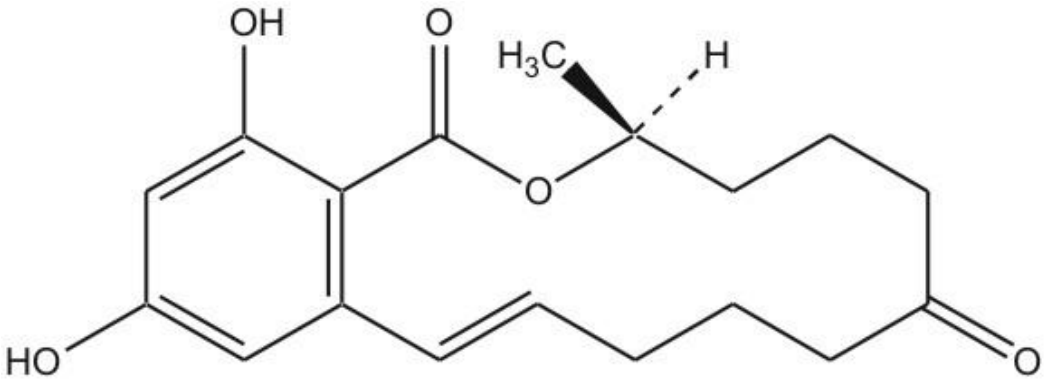


Aflatoxin B1

Ochratoxin is produced by the fungi of the genera Aspergillus and Penicillium. They contain an isocoumarin residue, linked by a peptide bond to L-alanine. They have a pronounced nephrotoxic and teratogenic effect.

Citrinin is produced by the fungi of the genera Penicillium and Aspergillus; Characterized by nephrotoxic action, as well as antibiotic properties against gram-positive and gram-negative bacteria; Is involved in mycotoxicosis "yellow rice" in Japan.[21]

Zearalenone is synthesized by fungi of the genus Fusarium (F. graminearum, F. tricinctum); Refers to the resorcic acid lactones; Is characterized by anabolic and estrogenic action.

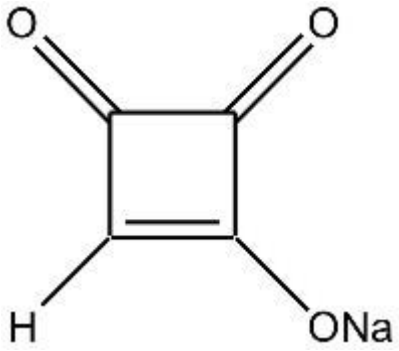


Zearalenon

Fumonisin is produced by fungi Fusarium moniliforme and F. proliferatum; The diester of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane; Corn and products of its processing; Cause a

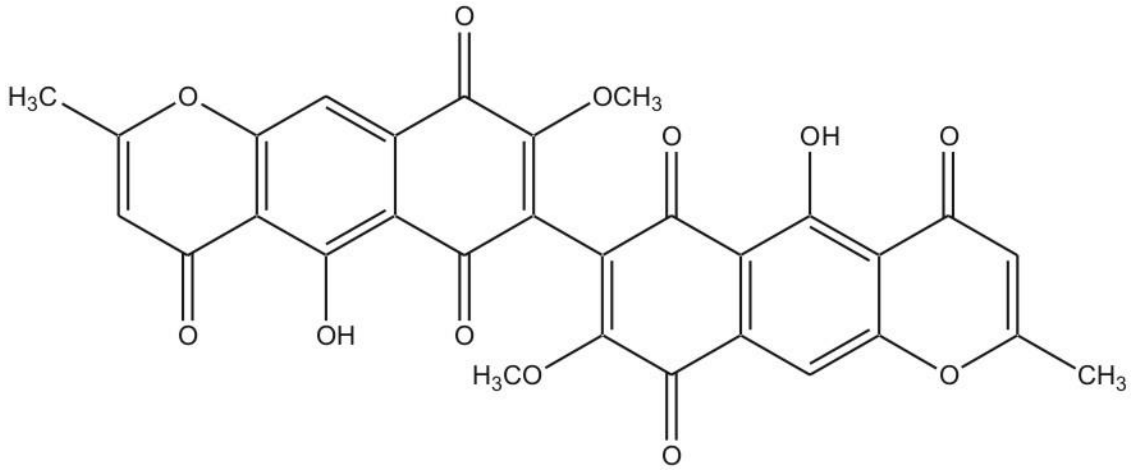
decrease in the serum of the sphingolipid complex with a simultaneous increase in sphingosine and sphinganine.[22]

Moniliformin is a mycotoxin produced by some species of the genus Fusarium (F. moniliforme, F. acuminatum, F. avenaceum, F. oxysporum, etc.); Is a mixture of K- and Na-salts of 3-hydroxy-3-cyclobutene-1,2-dione; Irreversibly inhibits pyruvate dehydrogenase complex.



Monoformin

Fusarhromanon - mycotoxin, which is found in fungi of the species Fusarium equiseti; Causes more tibial dyskondroplasia in chickens and turkeys and increases the mortality of chick embryos.



Aurofuzarin

Aurofuzarin refers to dimethyl naphthoquinones; Is produced by fungi of the genus Fusarium; Causes the chicken syndrome deterioration of the egg.

Patulin - mycotoxin, produced by various mold fungi from the genera Penicillium and Aspergillus and possessing pronounced toxic and mutagenic

properties. In high concentrations, patulin is found in fruit and vegetable processing products.[25]

Patulin acts as a broad-spectrum antibiotic and is tested for efficacy in common colds. However, efficacy has never been tested in practice and, due to its low toxicity, its use for medical purposes is not considered due to its irritating effect on the stomach and the ability to cause nausea and vomiting.

Symptoms of patulin-toxicosis include hemorrhages in the gastrointestinal tract of cattle (calves). In 1954, in Japan, Patulin killed 100 cows that consumed contaminated food.[26]

A deadly dose of patulin for rats is 15 mg / kg of body and 25 mg / kg after subcutaneous injection. In this case, death was associated with pulmonary edema. In chronic studies at low doses, no effect was observed. The immunotoxicity and neurotoxicity of patulin has been established. Some studies have established genotoxicity, for example, that it damages DNA or chromosomes in short-term experiments. However, these studies have been conducted on bacteria or on mammillary cell cultures with doses that are unimportant for humans.

Based on long-term studies in rats and mice on the study of reproduction and carcinogenicity, JECFA established a conditionally tolerated dose of weekly consumption of patulin at a level of 7 μg / kg body weight.

Prevalence

Mycotoxins are widely distributed in plant products stored under conditions favorable for the development of mold fungi.

Table 1.

Producer	**Mycotoxin**	**Amazes:**	**The main toxic effect**
Fusarium sporotrichioides F. poae	T-2 toxin	Corn, oats and products from it	Dermatoxin
	HT-2 toxin	Oats and products from it	
Fusarium graminearum	Deoxynivalenol (DON) vomitoxin	Wheat, corn	Neurotoxin

Fusarium tricinctum	Trichothecene	Corn, peanuts, rice	Neurotoxin
Fusarium moniliforme	Fumonisin B1 and other fumonisins	Sorghum, corn	Nephrotoxicity, respiratory disorders, neurotoxicity, probable carcinogen
Aspergillus flavus Aspergillus parasiticus	Aflatoxin B1 B2 G1 G2	Peanuts, corn	Carcinogen, hepatotoxin, mutagen and teratogen
Fusarium graminearum	Zearalenon	Corn, oats	Causes a genetic disorder, mutagen
Penicillium citrinum	Citrinine	Barley, corn, rice and walnut	Nephrotoxin, a mutagen, a probable carcinogen
	Aflatoxin M1	Milk and dairy products	Carcinogen, hepatotoxin, mutagen and teratogen
Claviceps purpurea (moederkoren)	Alkaloids of ergot	Rice, sorghum	Neurotoxin
Penicillium islandicum	Luteoskirin	Rice, sorghum	Hepatotoxin, a carcinogen and a mutagen
Aspergillus ochraceus Penicillium verrucosum	Ochratoxin A	Oats, coffee, meat, raisins	Nephrotoxin and teratogen
Penicillium aurantiogriseum Penicillium fennelliae	Penicillanic acid	Beans, corn	Neurotoxin
Aspergillus versicolor	Sterigmocysteine	Corn, wheat, coffee	Dermatotoxic, teratogen, a likely carcinogen
Penicillium expansumen andere Penicillium species	Patulin	Apples and other fruits, beans, wheat	Neurotoxin, causes disruption of the genetic apparatus, probable carcinogen, mutagen

Toxicity

Mycotoxins are poisonous mainly for eukaryotic organisms. In animals and humans, poisoning - mycotoxicosis - is caused by mycotoxins. The effect on plants has been little studied; Consider that mycotoxins reduce the resistance of the plant organism to fungal infection.

1.3. Mycotoxicoses. Methods of fighting with mycotoxicosis

Mycotoxicoses

Mycotoxicosis is called poisoning (usually in animals) due to eating fodders contaminated with mycotoxins.

Primary mycotoxicosis (acute or chronic)

Secondary mycotoxicosis - a consequence of the interaction of mycotoxins with other environmental factors

Diagnosis of mycotoxicosis

Diagnosis of the disease is based on etiological signs, combined with the identification and identification of mycotoxins in feed or tissues of a sick animal.[28]

Methods of fighting mycotoxicosis

In accordance with the Hazard Analysis and Critical Control Point (HACCP) system, seven critical control points were identified in the production and consumption of grain and mixed fodders by identifying and assessing the risk caused by the presence of mycotoxins, on which measures should be taken to prevent contamination: (1) The condition and quality of the seeds, (2) the quality of the soil cultivation, (3) the germination period, (4) harvesting, (5) the harvest period, (6) storage and (7) processing. In order to avoid contamination of grain and fodder with mycotoxins, it is necessary to adhere carefully to the technological norms in the first six critical control points. If the contamination does occur, measures should be taken to decontaminate (decontaminate) grain and feed substrates before use and to prevent poisoning (mycotoxicosis) of animals when using toxic fodder.[30]

Decontamination of grain and feed contaminated with mycotoxins

The process of grain decontamination is a directed action of physical, chemical or biological factors (agents), as well as their combinations, as a result of which the mycotoxins contained in the grain are degraded (destroyed). Grain is treated with decontaminating factors either in dry form or in an aqueous medium. In most cases, the second approach is more effective because, firstly, the prevailing

number of reactions leading to detoxification occurs in the aquatic environment, and secondly, in the dry substrate, mycotoxins are much less accessible to both physical and Chemical agents. The disadvantage of this approach is the need to remove the residues of chemical agents, whose presence in the food is undesirable, and the products of mycotoxin transformation in order to avoid the possibility of reverse reactions and activation reactions. In addition, after the completion of decontamination, the grain must be dried, which requires additional energy costs.[31]

Soaking

This is one of the earliest methods for disinfecting grain products. Two mechanisms are based on the method of detoxifying grain by soaking: (1) extraction of water-soluble mycotoxins and (2) transformation by enzymes contained in the grain. Many mycotoxins, whose molecules contain hydrophilic groups, are effectively extracted with water. Such mycotoxins include DON, nivalenol, NT-2 toxin, T-2 triol, T-2 tetraol. A method for neutralizing fodder grains is proposed, according to which the grain is poured with a four-fold volume of water and kept, stirring, for 6 hours, after which the water is changed. Thus, during the day the procedure is repeated four times. It was shown that the cultivated on the grain of the toxigenic strain Fusarium sporotrichiella 5750 thus lost the inherent ability to induce the formation of necrosis on the rabbit's skin [Kurmanov I.A., 1962].

Use of ammonia and carbon ammonium salts

The treatment with ammonia or monomethylamine is effective against aflatoxins, zearalenone and ochratoxins. The ether and lactone groups present in the zearalenone molecules and related compounds, as well as ochratoxins and aflatoxins, interact with primary and secondary amines, resulting in the formation of amides, which fundamentally changes the properties of mycotoxin molecules. However, breaking of the lactone ring under the influence of these substances occurs only when incubated from half an hour to several hours in a strongly alkaline medium, at a temperature of 100 ° C and a pressure of 3 to 10 bar. It has

been established that ammonium salts (UAS) are able to destroy aflatoxins B1 and G1, as well as T-2 toxin with the formation of T-2 triol and T-2 tetraol [Trufanova VA, Kotik AN, 2005]. At a concentration of 8% UAS in the grain and 4 weeks exposure, the concentration of aflatoxin B1 decreased by 75%, aflatoxin G1 by 94%, the initial concentrations of which were 40 and 12 mg / kg, respectively. UAS have a strong fungicidal, bactericidal and insecticidal action. Depending on the type and humidity of the grain, the concentration of VAS should be between 2.5 and 4.5%. In these concentrations, the UAS does not adversely affect the chickens.[32]

Oxidizing agents

With the contact of mycotoxins with oxidants, the functional groups that cause toxic properties are destroyed. The metabolites formed in this process are characterized by a high content of hydrophilic groups, as a result of which they are easily washed out of the processed substrate.[34]

Sodium hypochlorite

The active principle of sodium hypochlorite is active (reduced) chlorine and oxygen. Strongly expressed oxidative properties make HCN an effective disinfectant and detoxifying agent. A method has been developed to improve the quality of grain and seeds [Martinelli J.A. et al., 2005], which consists in processing grain with solutions of HCN. Depending on the intended purpose, the treatment is carried out by briefly immersing the grain in the working solution, spraying the solution over the surface of the grain or prolonged soaking. Sodium hypochlorite is used in the form of a pure solution or in a mixture with alkalis, hydrogen peroxide, organic solvents (ethanol, ethyl acetate) or organic acids (acetic, peracetic). When soaked for 7 hours in an alkaline 0.8% solution of hypochlorite, the concentration of DON, nivalenol, zearalenone, moniliformine, fumonisins, ochratoxin A, citrine and patulin decreases. As a result of the treatment, the intensity of the natural pigmentation of the grain (brightness) increases and the redness decreases, which is known to be due to the presence of pigments of mold fungi, many of which are highly toxic, for example, aurofusarin.

In addition, the percentage of seeds infected with phytopathogenic bacteria, including genera Xanthomonas and Pseudomonas, and fungi of the genera Fusarium (F. Graminearum, F. Solani), Alternaria, Helminthosporium, etc., is significantly reduced.[40]

Ozonation

An effective oxidant of mycotoxins is ozone. To decontaminate the grains use ozone-saturated water. Under the influence of ozone, the degradation of aflatoxins B1, B2, G1 and G2, cyclopiazonic acid, fumonisin B1, ochratoxin A, patulin, secal acid and zearalenone occurs [McKenzie K.S. et al., 1997]. Due to the modification of ozone, zearalenone loses its estrogenic activity [Lemke S. L. et al., 1999]. Treatment with ozone naturally contaminated with maize aflatoxin prevents the development of aflatoxicosis in turkeys [McKenzie K.S. et al., 1998]. It is shown that the trichothecene mycotoxins are also destroyed by the action of ozone. Most actively, the ozone molecule attacks the trichothecene molecule via a double bond between C9 and C10 atoms, resulting in unstable intermediates, lysonide and trichothecene ozonide, with concomitant hydrolysis of the C9-C10 bond [Young J. C. al., 2006].

Enzymes and microorganisms

Mycotoxins form a group of compounds heterogeneous in chemical structure. Therefore, enzymes that are able to transform mycotoxins are representatives of several classes and subclasses and are specific for various functional groups. Detoxification of mycotoxins occurs as a result of the action of enzymes that have oxidoreductase, hydrolytic (epoxide hydrolase, carboxyl esterase, lactone hydrolase) and transferase (UDP-glycosyltransferase) activity.[35]

Carboxylesterases

Catalyze the hydrolysis of ester bonds, and epoxyhydroglose-12,13-epoxy groups in the molecules of trichothecene mycotoxins. It is established that these two processes are carried out by bacteria inhabiting the intestines of chickens [Young J. C. At al., 2006]. Mixtures of microbes isolated from intestinal contents

are able to transform more than 12 different trichothecene mycotoxins. It was established earlier [Trufanova VA, 2004] that the inclusion of a culture of Escherichia coli isolated from the large intestine in feed for laying hens resulted in an increase in live weight and egg production against the background of T-2 toxicosis, compared with Group that received only T-2 toxin; The T-2 toxin concentration in the feed was 8 mg / kg.[36]

UDF glycosyltransferase

Conjugation of mycotoxins with activated forms of glucose. In the Arabidopsis thaliana genome, there are more than 100 genes encoding the various isoforms of this enzyme. Genes of the most specific DON-specific UDF glycosyltransferases are expressed in yeast cells [Poppenber-ger B. Et al., 2006]. The enzymes obtained from the yeast transformed in this way effectively destroy DON, 3-acetyl-DON and 15-acetyl-DON, but are less effective against the other trichothecene mycotoxins. Such yeast is recommended for use in the brewing industry. For decontamination of grain and mixed fodders, it is possible to use both immobilized UDF glycosyltransferase and yeast producing it.[38]

Lactone hydrolases

Convert zearalenone to 1- (3,5-dihydroxy-phenyl) -10'-hydroxy-1'-undecene-6'-one, which has no estrogenic activity. Clonostachys rosea isolated the gene zdh101, encoding lactone hydrolase. This gene was able to express in bacteria Escherichia coli, yeast Saccharomyces cerevisiae and rice cell culture [Takashi-Ando N. Et al., 2004]. Zearalenone effectively ("100%") was transformed in media with E. coli and rice cells, while transformed yeasts reduced the content of zearalenone in culture by 75%.[42]

Fumonisin hydrolase

From corn kernels isolated strains of microorganisms - the yeast Exophiala spinsfera and Rhinocladiella atrovirens, as well as the bacteria of the genera Xanthomonas and Sphingomonas, which could grow on media, the only source of carbon in which were fumonisins [Duvik J. Et al., 1998]. It was found that the first and key reaction of the biodegradation process of fumonisin B1 is the hydrolysis of

the ester bond to form a tricarballate and an amino alcohol derivative designated AP1. The enzyme that carried out this reaction was called fumonisin hydrolase. It is suggested that this enzyme refers to esterases specific for tricarbylate ester. None of the commercial esterases has this activity. In the same way, Ochrobactrum anthropi bacteria are isolated from corn grain, which can be utilized as a single carbon source by moniliiformin [Duvik J. Et al., 1999, 2000]. It is not yet known which enzymes are involved in the detoxification of moniliformin, but it is believed that hydrolysis of the double bond and rupture of the ring occurs first and then oxidation. A method for maize decontamination is proposed, according to which corn is ground, filled with an equal volume of a suspension of bacteria with a concentration of cells of 106 in 1 ml, and kept for two weeks at room temperature.[42]

Treatment with ultrasound

The method is based on the phenomenon of ultrasonic micro cavitation - a local wave-like formation of pores (bubbles) with a reduced pressure and a pressure increase of up to 100 kPa and a temperature of up to 1700 ° C. High-frequency oscillations, reported to the processed material by ultrasonic waves, contribute to the effective release of mycotoxins into the solution. In addition to physical impact, ultrasonic waves trigger so-called sono-chemical reactions, differing in thermodynamic and kinetic characteristics from similar reactions occurring under normal conditions, that is, in the absence of ultrasonic action. The grain is loaded into containers with ultrasound generators on their walls, poured into a reaction mixture and processed with ultrasound at a frequency of 35-100 kHz for 2-4 hours at a temperature of 12-50 ° C. This method allows to reduce the concentration of T-2 toxin, NT-2 toxin, DON, zearalenone, ochratoxins and aflatoxins in cereal grains by 70-80% [Lindner W., 1996]. The epoxy group of the trichothecene mycotoxins is effectively destroyed, which plays, as we know, a key role in the mechanism of toxic action. The hydrolysis of the epoxy group is facilitated by a shift in the acid-base equilibrium, both in the direction of decrease and in the direction of increasing the pH. To alkalize the medium, carbonates can

be used, as well as primary and secondary amines. The role of catalysts can be alcohols, for example, methanol, ethanol, propanol, glycerin or polyethylene glycol. In addition to participation in sleepy-chemical reactions of transformation of mycotoxins, the alcohols present in the reaction mixture improve the wetting of the grain and increase the solubility of mycotoxins and, consequently, their extraction from the grain. After completion of the ultrasonic treatment, the reaction mixture is drained and the treated grain is washed with water, if necessary re-subjected to ultrasound and dried.[44]

Treatment and prevention of mycotoxicosis in animals

Use of probiotic drugs

The prophylactic effect of probiotic drugs in mycotoxicosis is based on two main principles: (1) the synthesis of enzymes that transform mycotoxins to less dangerous products (2) the sorption of mycotoxins by the components of the cell wall. In addition, probiotic microorganisms have the ability to synthesize a number of substances that contribute to the improvement of the physiological state of the animal body and enhance productive qualities. Such substances include organic acids that normalize the pH of the gastrointestinal tract, antibiotics that inhibit the vital activity of pathogenic microorganisms, hydrolytic enzymes that increase the availability of feed nutrients, and vitamins.[46]

The use of sorbents

The action of sorbents is based on the ability to remove mycotoxins from the gastrointestinal tract. Sorbents must quickly bind and effectively retain mycotoxins at different acidity levels. Negative quality of sorbing materials is low specificity, due to which binding of nutrients (essential fatty acids, vitamins, amino acids) and medicinal preparations takes place. In advertising articles, which are replete with scientific and practical publications, you can find statements that a particular drug adsorbs exclusively mycotoxins and nothing else. However, one glance at the structural formulas of mycotoxins belonging to even one class, not to mention the representatives of different groups, will be enough to challenge such a claim. Mycotoxins are a group of heterogeneous compounds that have two common

attributes: first, toxicity to animals, and often to representatives of other kingdoms, and secondly, mold mycotoxins, with rare exceptions, mold fungi. It is unlikely that any adsorbent could selectively bind chemical compounds that are grouped into only these two common attributes that do not reflect their physico-chemical properties.[48] In addition, sorbents can cause mechanical damage to the intestinal epithelium, so an important criterion is their safety for animals. The process of developing preparations containing sorbent materials should include three steps: (1) a study of the adsorption activity of mycotoxins and nutrients in vitro; (2) animal experiments to study the prophylactic effect of the drug when a certain mycotoxin is introduced into the feed in various concentrations; (3) the study of prophylactic properties in feeding animals to feed naturally contaminated with mycotoxins. In the latter case, it is necessary to conduct the fullest analysis of the feed for the content of mycotoxins. When conducting animal experiments, attention should be paid not only to positive but also to negative effects of sorbent exposure. At present, it is known that for the optimal choice of a sorbent, its polarity must be taken into account. For example, aluminosilicates proved to be active only in relation to polar mycotoxins, in particular, to aflatoxins. Mycotoxins that do not contain polar groups, for example T-2 toxin, fumonisins and zearalenone, are adsorbed less efficiently by polar sorbents. The researchers failed to prevent the toxicosis of birds caused by trichothecenes of type A-T-2 toxin and diacetoxycirpenol - with the use of aluminosilicates [Kubena et al., 1990; 1993]. To bind hydrophobic mycotoxins, it is advisable to use nonpolar sorbents, such as activated carbon. The ability of activated carbon to adsorb ochratoxin A and T-2 toxin is quite effective when applied to the feed in a concentration of 5-10%, but it is found that some nutrients are also adsorbed.[50]

Soldering with sodium hypochlorite solutions

The presence of reduced chlorine in the molecule of sodium hypochlorite (HCN) gives it a number of unique properties that manifest themselves in a wide range of action on various body systems, including antimicrobial, antitoxic, immunomodulating, anti-inflammatory, antitumor and pro-apoptotic. One of the

key points in the expediency of using GCHN is that it is not a foreign compound for the animal organism. It is known that the hypochlorite ion is synthesized by the cells of the immune system of the animal organism during the development of the primary immune response, which is accompanied by inflammatory reactions. The role of hypochlorite in this case is to neutralize the toxins of pathogenic bacteria and oxidize toxic residues of bacterial cells and damaged cells of the body. In addition, hypochlorite enriches tissues with oxygen and specifically affects the activity of genes responsible for the development of the inflammatory process. An experiment was carried out on extracting chickens (140 days old) of sodium hypochlorite solution with feed feeding containing 40-70 μg / kg of T-2 toxin [Trufanova V.A., Kotik A.N. et al., 2005]. Number of groups: experience - 1 672; Control - 1 795. The experimental group was precipitated with a solution of HCN at a concentration of 30 mg / l for 25 days. By the results of the experiment, it was established that the solubilization of the HCN solution had a positive effect on the safety, the number of eggs on the first layer and the incubation characteristics of the eggs.[43]

Methods for combating mycotoxins in food and feed

- Farm management: quick drying, providing storage conditions to prevent the development of molds.
- Analysis at the exit of the louvre machine: rapid analysis is carried out using thin-layer chromatography, ELISA, HPLC.
- Sorting after peeling: discolored embryos are often infected with fungi, so automated sorting lines can prevent mycotoxins from entering animal feed and food for humans.

CHAPTER II. EXPERIMENTAL PART

2.1. Determination of mycotoxins in human foods

This work review deals with the analytical methods available for the determination of mycotoxins in food commodities. As the secondary metabolites of a range of fungal species, mycotoxins possess diverse chemical structures, presenting analytical chemists with a unique set of challenges in the μg kg^{-1} (ppb) range. A number of analytical methods have been applied to mycotoxin analysis. These include widely applicable HPLC methods with UV or fluorimetric detection, which are extensively used both in research and for legal enforcement of food safety legislation and for regulations in international agricultural trade. Other chromatographic methods, such as TLC and GC. are also employed for the determination of mycotoxins. whereas recent advances in analytical instrumentation have highlighted the potential of LC-MS methods, especially for multi-toxin determination and for confirmation purposes. Conventional chromatographic methods are generally time consuming and capital intensive, and hence a range of methods, mostly based on immunological principles, have been developed and commercialised for rapid analysis. These methods include, among others, enzyme-linked immunosorbent analysis (ELISA). direct fluorimetry, fluorescence polarization, and various biosensors and strip methods.

Mycotoxins occurring in food commodities are secondary metabolites of a range of filamentous fungi, which can contaminate food or food crops throughout the food chain. Although many hundreds of fungal toxins are known, a more limited number are generally considered to play an important part in food safety and for these a range of analytical methods have been developed. Fungal toxins of concern are generally produced by species within the genera Fusarium, Aspergillus and Penicillium, which frequently occur in crops in the field or during storage of major food agricultural crops, including cereals, groundnuts and various fruits. Besides the deleterious effect of the fungi themselves on agricultural productivity, the fungal toxins have a rangeof detrimental health effects in humans, including carcinogenesis, immune suppression, teratogenicity and growth retardation.

Similarly, mycotoxin-contaminated animal feeds can lead to animal toxicoses and the possible carry-over of mycotoxins or their metabolites into the human food chain.

Table 2.

Product group	Mycotoxins	Permissible levels, mg/kg, not more than
Food grain, seeds of leguminous plants, oilseeds, flour, cereals, pasta and bakery products, vegetable oils, nuts	Aflatoxin B	0,005
Food grain, cereals, oatmeal, flakes, wheat flour, rye, corn and cereals, pasta, bread	T-2 toxin	0,1
Barley grain, barley flour, cereals	Deoxynivalenol (vomitoxin)	
Wheat grain, wheat flour, cereals, pasta, wheat bread	Deoxynivalenol	0,7
Grains of wheat, barley, corn. Groats and flour wheaten, barley, corn, macaroni, bread	Zearalenon	1 0,2

Fungally-contaminated feed has long been associated with animal disease and cultured fungal species have been used since the I930's for the isolation of antibiotics. During World War II in the USSR, hundreds of thousands died of Alimentary Toxic Aleukia due to the consumption of over-wintered grain contaminated with *Fusarium sporotrichioides*. However, the defining moment for modern mycotoxicology is frequently given as the outbreak of Turkey-X disease in the United Kingdom in 1960. In this episode of mycotoxicosis, thousands of turkeys died after consumption of imported feed, which wassubsequently found to be contaminated with *Aspergillus flavus*, the producing fungus of a previously unknown carcinogenic mycotoxin, aflatoxin B_1. The realization that human food could be contaminated with mycotoxins led to a great expansion in research efforts into all areas of mycotoxicolog, especially into their biochemical modes of action,

their implications for human health and analytical methods for the chemical determination of these newly identified compounds at the levels found in human foods. Based ontheir production by fungal pathogens of major crops and their significant natural occurrence and implications for human health, most research has focused on the aflatoxins. fumonisins, trichothecenes, zearalenone, ochratoxin A and patulin. These mycotoxins represent a diverse range of chemical structures. For the aflatoxins, fumonisins and tricothecenes, each group contains a number of structurally-related analogues. Table 1 lists the important toxins in each group, their main producing fungi and some typical food commodities in which they can occur.

Introduction to analytical methods for mycotoxins

After the discovery of aflatoxin in 1960. the analytical methods for mycotoxins which typically occur in the μg kg^{-1} (ppb) range, have developed and expanded along with the general advances in analytical science. The earliest analytical methods were based on solvent extraction, crude clean-up on open-ended packed-silica columns and separation of the analytes of interest by thin layer chromatography (TLC). Such methods are still valid today and in the case of aflatoxins, TLC with either visual assessment or instrumental densitometry is routinely applied in many laboratories in the developing world.

The basic requirements of extraction, clean-up and separation for mycotoxin determination in food matrices remain the same in current methods. Advances have come in the areas of sample purification techniques and in separation science with the development of high-performance liquid chromatography (HPLC) and associated detectors. For mycotoxins with suitable chromophores (patulin) or fluorophores (aflatoxins), the increased sensitivity of modern detectors has allowed analysts to achieve lower limits of detection. Other mycotoxins. which lack suitable chemical groups (trichothecenes and fumonisins), can be initially derivatised and then separated either by gas chromatography (GC) or by HPLC.

Although investigated as a research topic, electrophoretic methods such as capillary electrophoresis have not found wide application in mycotoxin analysis.

As in many other branches of analytical chemistry, the introduction of bench top mass spectrometers as detectors for GC or HPLC instruments has impacted on mycotoxin analysis and has allowed detection at very low levels with simultaneous confirmation of the compounds of interest. Due to the range of chemical properties used for extract clean-up and chromatographic detection, analytical methods for mycotoxins are generally limited to a single toxin or a group of structurally-related toxins (fumonisins or aflatoxins) in a single analysis. The introduction of LC-MS instrumentation has made possible the development of multitoxin methods suitable for a range of structurally diverse toxins in a single chromatographic run. The need for such multitoxin techniques lies in the fact that a single fungal species can produce different toxins and that a single agricultural commodity can be contaminated with different fungal species resulting in the co-occurrence of a number of different toxins.

Another area of technological advance has been the introduction of solid phase extraction techniques using a range of chemistries (normal phase, reversed-phase. strong anion exchange). The development of immunoaffinity columns (IACs) containing antibodies specific to the analyte of interest has resulted in faster clean-ups and a greater degree of sample purification. This has been followed by the introduction of analytical methods which rely on direct fluorimetric measurement of the resulting purified extract (or a suitable derivatised product). When combined with HPLC separation, injected samples are cleaner and the resulting chromatograms are less complex, with attendant advantages to HPLC column life and analytical reliability.

Mycotoxin analytical methods need to have low limits of detection (generally in the $\mu g\ kg^{-1}$ range, but in the $ng\ kg^{-1}$range for aflatoxin M(in milk), be specific to avoid analytical interferences, be easily applied in routine laboratories, be economical for the laboratory involved and provide a confirmatory test for the analyte of interest. For official control and implemention of mycotoxin regulations,

a number of official methods, mostly based on HPLC. have been validated by inter laboratory collaborative studies conducted under the auspices of international bodies such as AOAC International. The validation process involves testing the within-laboratory repeatability, between-laboratory reproducibility, analytical recovery, and limits of detection and quantification. At the same time, the European Committee for Standardization (CEN) has published criteria for mycotoxin analytical method performance. However, aside from these official methods, a need exists for rapid screening methods which can be used for control purposes and in situations where rapid decisions are required, frequently in field situations at granaries, silos and factories. For this purpose, a number of screening methods and biosensors have been developed which are mostly based on immunological principles and use antibodies raised against specific mycotoxins. These methods range from quantitative ELISAs to qualitative tests based on obtaining a simple result of contamination above or below a set control level. Such tests, which are available in various formats such as lateral flow dipsticks, are evaluated by the level of false positive or false negative results. In terms of consumer protection, a level of false positives may be acceptable in that such batches will then be subject to more comprehensive testing. Thus in selecting a method for mycotoxin analysis, it is necessary to consider the purpose for which the results are needed, the matrix to be analysed, the detection limit required and the expertise and infrastructure available.

2.2. Methods for researching of mycotoxins

Sampling of commodities for mycotoxins

The mycotoxin contamination of agricultural products and food commodities, more than most other analytical tests, represents a substantial problem with respect to representative sampling. Mycotoxins are not produced homogeneously in the crop, but are the result of fungal growth in specific units, be they maize cobs, maize kernels, wheat kernels, groundnut pods. etc. For example, a large 360-kg crate of apples for juicing may contain only a limited number of apples that have been physically damaged and subject to growth of P. expansum and consequently contain high levels of patulin. The result is that there is a non-homogeneous distribution of mycotoxin and a single lot of the product will contain hot spots of contamination. This skewed distribution implies that most analytical samples drawn from the lot will contain lower mycotoxin levels than a mean formed by drawing many samples from the lot. An illustration of this is that analysis of 200 samples, each of 100 g, drawn from a batch of groundnuts found them to have a mean aflatoxin level of 1.0 μg kg^{-1}. whereas the range of analytical results for individual samples was 0 to 81 μg kg^{-1}. Processing of the agricultural commodity can improve the homogeneity of the material. Nevertheless, a study on aflatoxin contamination of peanut butter produced from a single batch of groundnuts illustrates the problem in that of the 377 jars produced. 300 had levels below 5 μg kg^{-1} but 7% of the jars were above 10 μg kg^{-1} and one jar was above 100 μg kg^{-1}.

The total error associated with mycotoxin testing can be split into three separate errors, namely the sampling error, sample preparation error and analytical error. The last term is the one most familiar to laboratory chemists and represents the error from the chemical analysis of the subsample extracted. The sampling error is the error introduced by withdrawing a random sample from the lot of material to be tested and the sample preparation error is the error introduced by milling or grinding the sample and removing the subsample for laboratory extraction. Each of these makes an independent contribution of variance to the total

testing variability. The worst case is the determination of aflatoxin in lots of groundnuts. In a trial in which 2.27 kg samples were withdrawn from a lot of nuts and a 100 g subsample of ground material was extracted and analysed, the sampling error contributed 92.7% to the total variability, sample preparation contributed 7.2% and the actual sample analysis only contributed 0.1%. The situation with respect to other mycotoxins in other agricultural commodities is not skewed to the same extent, although the problem remains. In the case of maize, 1.1 kg samples of kernels drawn from a single batch and 25 g subsamples analysed showed a sampling contribution to total variance of 61.0% and for the *Fusarium* toxin deoxynivalenol in wheat, 0.454 kg samples and 25 g subsamples gave a sampling contribution of 22.0%, the same as the contribution of the analysis. The reason for the difference between the mycotoxins and the commodities lies in a more general fungal contamination of the Fusarium species and in the fact that a unit mass wheat kernel sample represents a much greater number of kernels than the same unit mass of groundnuts.

The problem of sampling has been addressed by the development of sampling plans, which are based on statistical evaluations to balance consumer protection (by not accepting contaminated lots) and producer protection (by not rejecting clean lots). Such plans are a compromise between the statistical need for large samples and the practicalities and costs of such samples. For official food safety testing, sampling plans, which specify commodity type, number of increments sampled in a lot. size of the increments, place in the lot taken and total aggregate sample collected, are frequently specified by government regulation and can involve aggregate samples of up to 30 kg.

An important aspect of a sampling plan is the operating characteristics curve, which is generated from statistical evaluation of the mycotoxin distribution and the analytical variability. Such a curve plots the probability of a lot's acceptance against the mycotoxin level. It demonstrates that as the actual level of contamination increases and approaches the regulated level, so the probability of its rejection increases. This is known as the producer risk (of acceptable lots being

rejected). Similarly, lots contaminated at increasing levels above the regulated level have a diminishing probability of being accepted. This is known as the consumer risk (of contaminated lots being accepted). The ultimate aim of a good sampling plan is to reduce both risks, although they cannot ultimately be eliminated.

Extraction of food matrices

Parameters of importance in mycotoxin extraction are solvent type and composition of a mixture, solvent to sample ratio, type of matrix (processed or unprocessed), extraction method and physical aggregation of the sample. For optimum extraction efficiency, the analytical sample should be ground to a fine powder. Mycotoxins are polar compounds which occur naturally in the μg kg^{-1} up. to mg kg^{-1} range in diverse and complex food matrices, from which the analytes of interest must be extracted. Hence, mycotoxins are potentially extracted by a range of polar solvents or mixtures of solvents. For example, in the case of aflatoxins, a widely used early method relied on chloroform extraction of peanut products, which after shaking and filtration could be purified on packed silica columns. Similarly, current methods for patulin determination rely on the use of an ethyl acetate-n-hexane mixture to extract the mycotoxin from apple juice. Due to the cost and environmental implications of chlorinated solvents, the use of aqueous mixtures of methanol, acetonitrile or acetone have come to the fore. In the case of deoxynivalenol in cereals and cereal products, pure water has been used as an extraction solvent. For the other mycotoxins, the ratio of organic to aqueous solvent must be tailored to the toxin-matrix combination to achieve optimum efficiency. In the case of fumoni-sins, both pure methanol and pure water will extract fumonisins from maize, but the optimum is generally found at a methanol-water ratio of 3:1 (v/v). Alternatively, mixtures of water, methanol and acetonitrile have also been successfully employed.

Investigations of optimal extraction solvents for aflatoxins from a range of matrices highlighted a series of potential problems which need to be considered. The presence of salts or sugars in certain matrices has the potential to cause phase

separation of an extractant mixture with a non-uniform distribution of the toxin between the layers. Similarly, the extraction of very dry materials can lead to a variability associated with water uptake by the dry matrix, an effect that depends on factors such as the matrix, the organic solvent and its ratio in the aqueous extractant and the solvent-to-sample ratio used for the extraction experiment. A further consideration in selection of extractants is the extent to which the chosen mixture will also extract interfering matrix impurities. For this reason, acetone has been preferred over methanol for aflatoxin extraction from matrices containing citrus pulp. Further consideration must also be given to the next step of the analytical process, namely sample clean-up. It is desirable that the extractant mixture should be compatible with the extract purification process. Pure organic extractants are suitable for clean-up on silica columns (ethyl acetate-*n*-hexane for patulin from apple juice), whereas aqueous mixtures are suitable for reversed-phase or ion exchange clean-up. In the case of toxins for which immunoaffinity columns exist, aqueous methanol extractants allow, after suitable dilution, larger volumes to be used on the antibody column than would be advisable for acetonitrile or acetone.

The type of matrix that is analysed can strongly influence the analytical recoveries from the extraction process. This is particularly a problem with fumonisins. Extraction of fumo-nisins from maize is readily achieved as described above. However, once maize has been processed into a variety of commercial products such as breakfast cereal, muffins, extruded products and maize chips, analytical recovery can be a serious problem. Despite the application of a range of solvent mixtures and the use of acidic and alkaline extraction solvents, no single extraction solvent mixture appears to be of universal applicability to all these processed products. More recently, researchers have drawn attention to the fact that certain matrices contain both free mycotoxin and mycotoxin bound to protein or sugars such as glucose, which is not analysed or included in the conventional methods. Determination of such bound mycotoxins may require additional processing to release the toxin.

The physical process of extraction is generally achieved by-shaking of the matrix and extractant or by blending with a homogenizer for a shorter time period. The use of accelerated solvent extraction under increased pressure and supercritical fluid extraction (SFE) has been investigated, but the results have not justified the cost of adopting this instrumentation in place of simple shaking. In the case of SFE, the polar nature of mycotoxins and their poor solubility in carbon dioxide is a major problem which requires the addition of organic solvent modifiers, such as methanol or acetonitrile. Problems also arise with respect to extraction times, analytical recoveries and co-extracted impurities.

Extract clean-up

Original methods for mycotoxin analyses frequently relied on extract clean-up on open columns packed with materials such as silica or diatomaceous earth, which were eluted with various organic solvent mixtures. In some cases, liquid-liquid partition was used for sample clean-up and is still applied in a widely used method for the determination of patulin in apple juice, in which the original ethyl acetate extract is washed with a sodium carbonate solution to remove acidic impurities. These techniques are non-specific and require large volumes of organic solvents for column packing, clean-up and mycotoxin elution. In some instances, these methods can also involve multiple steps of defatting with hexane or petroleum ether and impurity precipitation with solutions of lead acetate. A number of alternative and simpler clean-up techniques have been developed and are available to the analyst. Solid phase extraction (SPE) using small prepacked cartridges containing up to 500 mg sorbent has been applied for a number of years to mycotoxin analysis. The general mechanism of this purification is the physical adsorption of the toxin on the packing surface, appropriate washing of the cartridge for removal of as many impurities as possible without loss of the analyte and then finally the complete elution of the analyte from the SPE cartridge. Packing materials are mostly based on silica particles, although a number of polymer resin packings have been developed commercially and applied to purification of mycotoxins. The SPE packings cover a range of separation chemistries, such as

normal phase (usually unmodified silica), reversed-phase (usually octadecylsilica. CI8) or ion exchange. This last category can comprise strong or weak anion or cation exchangers bound to a silica support material and can provide a more specific purification than silica or C18.

The sample extract solutions need to be compatible with the SPE chemistry to be used for clean-up. The example of patulin clean-up on silica has already been mentioned.[12] A further example is the conversion of an aflatoxin method from large open column to a SPE column containing 500 mg silica sorbent. Extracts from maize and peanut products were cleaned-up by application in dichloromethane solution onto the silica SPE columns, which resulted in a twenty-fold reduction in organic solvent use over the original open column method. The use of aqueous mixtures of methanol and acetonitrile, which have largely replaced polar organic solvents as extractants, results in extracts which are easily tailored for reversed-phase or ion exchange SPE clean-up. The fumonisin mycotoxins provide an example of this type of purification. These toxins are diesters of propane-1,2,3-tricarboxylic acid (tricarballylic acid) and various 2-amino-12,16-dimethylpoly-hydroxy-eicosanes in which the hydroxyl groups on C14 and CI5 are esterified with the terminal carboxyl group of the tricarballylic acid. They are extracted from maize with methanol-water (3:1, v/v) and can be retained on a strong anion exchange (SAX) SPE cartridge, provided the pH of the extract is such as to allow ionization of the carboxylic acids. This is generally achieved at around pH 6 and may require adjustment of the extract prior to application to the SPE cartridge. Such a system allows impurities to be washed off the cartridge with methanol and the fumonisins are only released on elution with dilute acetic acid, which suppresses the ionization of the carboxylic acid groups and allows elution of the mycotoxin. However, the fumonisins can also be purified on reversed-phase CI8 SPE cartridges. This clean-up is not as efficient as the SAX SPE method and requires the sample extract to be diluted with water so as to retain the fumonisin on the C18 cartridge. Elution of the mycotoxin occurs with methanol, so care must be exercised in the washing of impurites from the cartridge with aqueous methanol

mixtures. The toxin is a dlester. so under various conditions (nixtamalization with alkali in tortilla production), hydrolysis of one or both of the tricarballylic acid groups can occur. For samples of this nature. SAX clean-up alone is no longer possible and C18 cartridges are used to adsorb all the chemical forms. Alternatively, the two types of SPE cartridge have been consecutively employed to achieve a chemical separation, firstly the SAX for fumonisin itself and then the eluate is passed onto a C18 cartridge for isolation of the hydrolysed forms.

The development of antibodies raised against individual mycotoxins led to the introduction of immunoaffinity columns (IACs) in which a specific antibody is immobilized on a gel contained in a small column. The analyte is generally extracted with aqueous methanol and the resulting extract diluted with water or phosphate-buffered saline (PBS) prior to application to the IAC. The antibodies on the column will recognise and bind the specific mycotoxin and allow impurities to pass through the column, which is subsequently washed with PBS. The mycotoxin is eluted with a small amount (usually a few mL) of methanol, which denatures the antibody and releases the bound analyte. IACs have been commercially developed for most of the major mycotoxins of agricultural, trade and health interest. Recent developments have been the combination of different antibodies into one column which allows the determination of more than one mycotoxin per single sample extract and clean-up. The resulting solution can be analysed separately for each toxin or a suitable gradient HPLC separation can be developed to achieve a multitoxin determination in a single chromatographic analysis. IACs achieve a superior purification to that obtained with SPE cartridges, although it should be noted that impurities can still be retained on the column despite the specific nature of the antibody-toxin interaction.

In all the above examples of mycotoxin extract clean-up, the mechanism involved has been the linking of the toxin to the column or cartridge packing and the washing away of impurities prior to elution of the analyte with a stronger solvent. However, an alternative scenario is the passing of the extract through the cartridge without adsorption of toxin, but with adsorption of the interfering

impurities. This system has been the basis for a method developed for moniliformin, a highly polar acidic compound, which due to a low pK_a occurs naturally as an alkali salt rather than a free acid. In this method, the defatted sample extract in methanol is passed through a C18 reversed-phase SPE cartridge, immediately eluted with water, evaporated to dryness and the residue, after redissolving in HPLC mobile phase, is passed through a small alumina column. The multifunctional column is a similar concept, in which the extract is passed through a column containing a mixture of adsorbents such as charcoal, alumina, silica and Celite. This model has been commercialized into an easy to use test tube system for rapid purification. Specific commercial products are available for a range of mycotoxins in different matrices.

Molecularly imprinted polymers (MIPs) represent an area of research interest and have been investigated as a potential novel clean-up system for food analysis. The technique involves the creation from suitable monomers of a three-dimensional network (polymer) that retains a memory of the shape and functional groups of the template or analyte molecule around which the polymerization occurs. Once the template is removed, the resulting MIP is able to recognise the template (analyte) within a mixture, effectively functioning as an artificial antibody (biomimetic receptor). Although there has been some interest in developing MIPs for mycotoxin analysis, such methods are still confined to a limited number of research laboratories.

Thin layer chromatography (TLC)

Separation of the mycotoxin analyte from various impurities and interferences that may still be present after extract cleanup is mostly performed by chromatography. As TLC was a well known technique at the time of the first development of many analytical methods for mycotoxins. it was initially used for this purpose. In suitable cases, gas chromatography (GC) also found application in mycotoxin analysis, whereas HPLC methods were gradually developed as this separation technique itself matured. Despite the development of these instrumental techniques, TLC still has a place in some analytical laboratories, especially in

developing countries. TLC offers the advantage of testing a number of samples simultaneously and can also be used as a screening test prior to more sophisticated instrumental methods.

Mycotoxins are polar compounds and have mostly been separated on normal phase silica TLC plates using a range of organic solvent mixtures as mobile phase. The four major aflatoxin analogues (B_1, B_2, G_1 and G_2) are readily separated by TLC and are easily observed under long wavelength UV light at levels that are useful for quantification of naturally contaminated food samples. Quantification can be achieved by visual comparison of the intensities of sample spots with those of standards. A number of improvements over the conventional TLC analysis have been introduced and applied to aflatoxin analysis. These include the use of densitometry to improve quantification, the introduction of high-performance TLC plates (HPTLC) and the use of bi-directional TLC to improve the separation of the aflatoxin analogues from interfering impurities. In the case of mycotoxins that do not fluoresce, the plate must be sprayed after TLC separation to yield visible spots. In this manner a toxin such as deoxy-nivalenol is rendered visible after the drying of the developing solvent, spraying with $AICl_3$ and heating at 120^0C. Similarly, the fumonisins can be visualised on normal phase silica TLC plates by spraying with *p*-anisaldehyde solution. This type of reagent was suitable for the detection of fumonisins in fungal cultures, but did not allow the determination of fumonisins at levels suitable for investigations of natural contamination. To achieve this, reversed-phase (CI8) TLC plates were used and the mycotoxin was either separated and then derivatised to a fluorescent product by the spraying of borate buffer and fluorescamine onto the plate or a pre-derivatisation with fluorescamine was performed and then the fluorescent derivatised products were separated by reversed-phase TLC. This latter method achieved detection levels as low as 20 ng fumonisin B, spotted on the plate, allowed estimation of contamination levels down to 500 μg kg^{-1} and avoided the analytical interference from an unknown compound of similar R_f value to fumonisin B_1.

Given the low levels at which mycotoxins occur, confirmatory analyses are frequently conducted to provide confidence in the analytical result. In the case of TLC analyses, confirmatory methods have mostly involved alternative or additional spray reagents or the development of the TLC plate in an alternative solvent system. In the case of aflatoxins determined in cottonseed, blue fluorescent TLC spots can frequently be mistaken for aflatoxins G_1 and G_2. However, a sulfuric acid spray which turns the aflatoxins yellow or yellow-blue can be used to distinguish the aflatoxins from the interferences. Similarly, for the mycotoxin zearalenone, which shows greenish-blue fluorescence under shortwave UV light (256 nm), a confirmatory spray of A1CL can be used. After heating for 5 min at 130^0C, the putative spot for zearalenone should become visible under long wavelength UV light (365 nm) as blue fluorescence. For ochratoxin A. sodium bicarbonate. $AlCl_3$ and NH_3 vapour can all be used for this purpose. Ochratoxin A itself fluoresces greenish blue and under alkaline conditions of the confirmatory spray, changes to blue with an increase in intensity. Ochratoxin A has another confirmatory method in which the mycotoxin, which contains a carboxylic acid, can be derivatised by esterification with ethanol to the resultant ethyl ester. Confirmation is achieved by TLC separation of the derivatised solution and observing the disappearance of the OTA spot and the appearance of a spot corresponding to the ester.

Gas chromatography

GC has been applied to the analysis of a range of mycotoxins, although for many of these compounds, which possess strong fluorescence or UV properties. HPLC methods have been more successful. Nevertheless, for the trichothecenes, of which the B group possesses weak UV absorption and the A group does not have a suitable absorption band, capillary GC has been extensively used. The interplay between GC and HPLC methods is exemplified by the discovery of fumonisins and the need for a reliable and sensitive analytical method. The initial attempts to use GC analysis required the hydrolysis of the diester and the subsequent analysis,

after suitable sample preparation, of either the resultant tricarballylic acid or the aminopolyol backbone. The development of HPLC techniques with fluorescence detection for derivatised fumonisins caused the emphasis to shift away from GC and all natural occurrence data on fumonisins has been generated by HPLC methods.

The analysis of trichothecenes by GC has been extensively studied. These are oxygenated polar compounds which need to be derivatised prior to injection into a GC column. Most common GC detectors such as flame ionization (FID), electron capture (ECD) and mass spectrometry (MS and MS MS) have been used. The conjugated carbonyl group in group B trichothecenes and the use of fluorine-containing derivatising agents for group A compounds make them sensitive to ECD detection at low levels. Typically, the hydroxyl groups are converted to their corresponding trimethylsilyl (TMS) ethers or trifluoroacetyl (TFA), pentafluoropropionyl (PFP) or heptafluorobutyryl (HFB) esters. These derivatization reactions needed to be optimized so as to avoid multiple reaction products such as mono-, di- and tri-TMS derivatives of deoxynivalenol. Other aspects to consider include removal of excess reagents, removal of excess water and the stability of the derivative formed.

The fungal producers of trichothecenes frequently generate a number of these compounds simultaneously. Hence, a GC method capable of separation and analysis of a range of toxins in a single chromatographic run is desirable. Thus methods have been developed to simultaneously determine deoxynivalenol and nivalenol or members of the group A trichothecenes. T-2 toxin, HT-2 toxin and diaeetoxyscirpenol in cereals or in some cases members of both groups, as well as the 3- and 15-acetyldeoxynivalenol toxins. Mass spectrometry or tandem mass spectrometry provides the advantage of selective, multitoxin, quantitative data from a single analytical run. The ability of the MS detector not only to quantitate at low levels (down to approximately 5 μg kg^{-1} for deoxynivalenol). but to confirm the identity of the chromatographic peak by the production of characteristic fragment ions, has significant advantages for analytical chemists.

The analysis of trichothecenes by GC has produced a large number of publications in which researchers have varied the matrix (mostly cereals), the extraction solvents (mostly aqueous mixtures of methanol or acetonitrile). the clean-up methods (various adsorbents such as florisil, charcoal, alumina or commercial mixtures in multifunctional cartridges), deriva-tization methods, GC columns and detectors (mostly ECD or MS).

High-performance liquid chromatography

HPLC has found extensive application in the field of mycotoxin analysis. The polar nature of mycotoxins and their solubility in water and organic solvents such as methanol and acetonitrile implies that they are readily amenable to separation on reversed-phase HPLC columns and this has resulted in a diverse array of methods. The extent to which HPLC is suited to mycotoxin separation can be gauged from the compilation of a database of retention times, retention indices. UV absorption maxima and predominant mono-isotopic ions for 474 fungal metabolites. Chromatographic detection has mostly been achieved with UV and fluorescence detectors, although the relatively recent successful application of atmospheric pressure ionization techniques has resulted in the development of a range of LC-MS or LC-MS/MS methods capable of very low detection limits. In addition, the evaporative light scattering detector has been applied to solutions of relatively high concentration, for example in determining the purity of standards or the levels of mycotoxins in fungal cultures.

Although aflatoxin mixtures can be separated on normal phase silica columns using solvent mixtures consisting of chloroform, acetonitrile, cyclohexane and ethanol, reversed-phase columns have found a much wider application for these compounds. In an intercomparison study of methods conducted among European laboratories, only one out of the 24 laboratories involved reported a normal phase separation of the aflatoxins, whereas the majority were using columns with C18 packing material. In the case of the aflatoxins. which are most frequently detected by fluorescence, the quenching that can occur with chlorinated solvents is an additional factor in the selection of reversed-phase chromatography.

^ Other column chemistries, such as phenyl and C8 modifications of silica packing material, have found limited application. In the case of the mycotoxin moniliformin, which has a pK_a value of about 1.7 and is thus ionized at the pH levels normally used for HPLC. two other chromatographic approaches have been used. An older method employed a strong anion exchange HPLC packing with a sodium dihydrogen phosphate eluent at pH 5.0. whereas more recent methods have used ion pair chromatography on CI8 columns with aqueous methanol or acetonitrile mobile phases. In general, the mobile phase composition for mycotoxin determination by reversed-phase CI8 chromatography is chosen so as to match the column chemistry and the carbon loading of the column. In the case of the little studied mycotoxin tenuazonic acid, which is a metal chelating 8-diketone, a high carbon loaded CI8 packing material was required to achieve adequate peak shapes and prevent peak tailing. In the case of the separation of carboxylic acids such as the relatively nonpolar fumonisin analogues, which require an eluent of about 80% methanol and a buffer salt, the pH of the mobile phase is also adjusted so as to suppress ionization of the carboxylate moieties. For highly polar, uncharged mycotoxins such as patulin, eluents contain high percentages of water and typically have aqueous compositions with less than 10% acetonitrile in order to achieve adequate retention on reversed-phase packing materials.

For mycotoxins with useful UV absorption bands, UV detection has been universally applied. Thus mycotoxins such as patulin (wavelength maximum 276 nm), deoxynivalenol (wavelength maximum 219 nm) and moniliformin (wave-length maximum 229 nm) are routinely quantified by UV detection. However, a number of other mycotoxins, such as the aflatoxins, ochratoxin A and zearalenone, possess fluorescence bands. Fluorescence detection has a number of advantages. In measuring light emitted rather than absorbed, it can frequently achieve lower detection limits than UV detection and as analytical interferences may not absorb and fluoresce at the same wavelengths as the analyte of interest, the fluorescence chromatograms are frequently less prone to interference from co-eluting compounds.

A number of mycotoxins do not absorb in the UV range and for these, suitable derivatization methods have been developed to allow UV or fluorescence detection. Examples of these are T-2 toxin and the fumonisins. In the case of fumonisins, a number of fluorescent derivatives such as fluorescamine, *o*-phthaldialdehyde (OPA) and naphthalene-2,3-dicarboxaldehyde have been used. In the case of fluorescamine. two distinct chromatographically separable derivatives are formed. The majority of workers have settled on OPA as the most suitable derivatising agent, despite its inherent instability.39 The stability of the resulting compound can be improved by replacing the 2-mercapto-ethanol reagent with A'-acetyl-cystein.40 Although the aflatoxins are inherently fluorescent, quenching can occur in certain eluents. For optimum detection limits of the aflatoxin B_1 and G_1 analogues in eversed-phase chromatography, they are frequently deriva-tised either in a pre-column method with trifluoroacetic acid or post-column by a number of techniques. These include reaction with an elemental solution of iodine in a post-column reaction coil at 60^0C, reaction with bromine generated from potassium bromide in a post-column electrochemical cell (Kobra cell), reaction with pyridinium hydrobromide perbro-mide added post-column or hydrolysis using a post-column UV lamp and reactor coil.

As was mentioned in the section on TLC (see above), analytical confirmation is an important aspect of mycotoxin determination. The use of diode array UV detectors, which can collect spectral information of the chromatographic peak, enables a comparison to be made with the UV spectrum of the toxin standard for confidence in the analytical result. In the case of aflatoxins B_1 and G_1, if the method involves post-column derivatisation, then confirmation can be achieved by a switching off of the post-column pump, electrochemical cell or UV light. As was previously described for ochratoxin A by TLC, the derivatisation of ochratoxin A to its ethyl ester and re-injection allows confirmation by observation of the disappearance of the chromatographic peak due to the toxin and the appearance of one corresponding to the retention time of the authentic ethyl ester standard.

The coupling of HPLC and MS via atmospheric pressure ionization (API) techniques, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), and the development of commercial bench-top instrumentation, has opened new methodologies for routine analysis of mycotoxins. Whereas TLC and HPLC techniques frequently require derivatization for sensitive detection, LC-MS provides a detection method independent of the formation of chemical derivatives or of the UV absorption or fluorescence properties of the molecule. The API ion sources generate quasi-molecular ions in positive or negative modes and can accommodate HPLC mobile phase flow rates. This method also combines sensitive quantification of mycotoxins with a confirmatory technique. The observation of specific fragment ions produced by collision induced dissociation (CID) of the molecular ion furnishes unequivocal confirmation. The selectivity and sensitivity of MS and MS/MS methods are enhanced by selected ion monitoring or selected reaction monitoring modes, which eliminate much of the chemical background interference found in other detectors. Methods have been developed for all the important mycotoxins using ion-trap and triple quadrupole instruments. As the detection method relies only on the ability to ionize the molecule in the API source, renewed interest has been shown in multitoxin methods. As a universal extract purification method is not available, use has been made of the sensitivity of the LC-MS instrument to avoid such clean-up and inject a diluted extract directly into the instrument. In avoiding the clean-up step, care must be exercised that impurities in the injected sample co-eluting with the analytes do not suppress the ionization of the compounds of interest. This technique has been applied to the determination of 39 mycotoxins in wheat and maize, with a single extraction step of acetonitrile-water-acetic acid (79+20+1) and subsequent analysis by LC-MS/MS. Because of the diversity of chemical structures, samples were run in both positive and negative ESI modes. Although ion suppression was negligible for wheat. 12 mycotoxins gave significant signal suppression in maize. This could be overcome by running matrix-matched standards. Further development of the method increased the number of metabolites

detected to 87, including most mycotoxins for which commercial standards are available and other metabolites produced by fungi involved in food storage.

Immunological methods

A range of analytical methods have been developed for mycotoxins that rely on immunological principles, i.e. the interaction between an antigen (the analyte of interest) and an isolated antibody raised against the antigen. Antibodies are IgG or IgY immunoglobulins with specific binding sites for the antigen. Recognition of the molecule is based on spatial complement of specific chemical groups (the epitope) on the antigen, not on the whole antigen. Cross-reactivity of antibodies results from different chemical compounds containing similar chemical groups interacting with the antibody to varying degrees. For the production of antibodies, mycotoxins are haptens (too small to elicit an immunological response), so they are conjugated to polypeptides or proteins to form an immunogen. Polyclonal antibodies are raised in selected animal species such as rodents and rabbits, whereas monoclonal antibodies are recovered from cloned cell lines. Because of these differences, polyclonal antibodies are easier to produce, but contain limited amounts in each batch and show significant batch to batch variations, whereas for monoclonal antibodies, it may be difficult to initially obtain the correct specificity, but they are preferred for commercial use as they have uniform affinity and specificity and can be produced repeatedly in sufficient quantity.

Enzyme-linked immunosorbent assays (ELISAs) have found a wide application for mycotoxin analysis and test kits have been commercially developed for the economically important mycotoxins. ELISAs can be developed in a number of formats, including direct assay, competitive direct assay and competitive indirect assay. In the competitive direct format, the antibodies are coated (immobilized) on the surface of wells in a microtitre plate or in strips. Crude extracts (unpurified, but usually diluted) or standards are then mixed with analyte that has been chemically conjugated with an enzyme and the mixture is allowed to interact with the bound antibody in the microtitre wells so that competition occurs for binding sites between the mycotoxin of unknown level in the extract and the

known (fixed) amount of conjugated mycotoxin standard. After reaction, the excess mixture is discarded, the wells are washed and substrate is added for reaction with the enzyme conjugate to produce a colour, whose intensity is dependent on the level of bound conjugate. In this format, the greater the sample toxin level, the less conjugate binds to available sites and the lower the colour intensity. In the competitive indirect format, the microtitre well contains bound toxin and a mixture of sample extract and specific antibody is incubated in the well. The analyte in the sample (or standard) reduces the amount of antibody available for binding to the immobilized toxin in the well. The bound antibody is detected by adding a second anti-antibody labelled with enzyme, together with substrate. The colour signal obtained is again inversely related to the mycotoxin level in the sample. ELISAs can provide rapid quantitative and semi-quantitative analytical results, although the presence of matrix effects can cause limitations. Matrix effects can arise from a number of factors including other co-extracted compounds (such as lipids, carbohydrates.tannins. polyphenols and pigments), extract pH. extraction solvent composition and sample processing (ELISAs for grains do not necessarily work for processed food commodities). These effects can elevate or decrease the analytical result and can be overcome by measures such as extract clean-up. extract dilution or addition of detergents. For this reason, detection limits of ELISA kits cannot be improved by concentration of sample extract.

As was described in previous sections, immunoaffinity columns have been developed in which specific antibodies are immobilized on a gel in a short column. Besides being used for purification of extracts prior to chromatographic separation, the eluate from the column may be used directly, or after suitable derivatization. for quantification in a fluorimeter. In such a system, mycotoxins which occur as a group of compounds (aflatoxins and fumonisins) are quantified as a total toxin level. This is the same situation as prevails for ELISA kits and the result depends on the cross-reactivity of the antiBody for the different toxin analogues, i.e. if the antibody is specific for the major afiatoxin or fumonisin analogue (afiatoxin B_1 or

fumonisin B_1). then the analytical result will not reflect the total but rather the level of that individual compound. For certain matrix-mycotoxin combinations, results compare well with HPLC determinations, although care needs to be exercised when investigating matrices for which the test has not been validated.

Mycotoxin antibodies have been used to develop methods for some mycotoxins based on fluorescence polarization. In this technique, the orientation of the fluorescence emission, which is related to the rate of molecular rotation, rather than total fluorescence is measured. The change in fluorescence polarization signal is obtained by competition for available binding sites on the antibody between the mycotoxin in the extract and an added fluorescently-labelled toxin standard.

Mycotoxin biosensors use antibodies attached to the surface of the sensor. These interact with the mycotoxin and produce a change in surface properties. This change can be monitored by evanescent wave techniques such as surface plasmon resonance or by fluorescence coupling with the evanescent wave using optical fibres. Both techniques have been used for the determination of fumonisins.

Immunoassay-based lateral flow devices or dipsticks allow rapid screening for detection of contamination levels above or below a pre-set limit. The mycotoxin sample extract is applied at the base of the dipstick to a conjugate pad containing colloidal gold conjugated anti-mycotoxin antibodies. Mycotoxin in the extract is bound by the antibodies and then both bound and unbound antibodies move up the dipstick membrane. On passing the test line of immobilized mycotoxin. any unbound colloidal gold conjugated antibody becomes immobilized and hence visible as a pink line, i.e. contamination levels below the set limit will be identified by the test line becoming visible. In order to check on dipstick performance, a control line, containing anti-antibodies, is included after the test line to ensure that the colloidal gold conjugated antibodies migrated along the strip.

Other analytical methods

Apart from the range of analytical methods that have been described above, a number of other techniques have been investigated as potential methods for

mycotoxin determination. However, these have found little application outside the research environment.

Capillary electrophoresis (CE) is an instrumental technique which achieves separation of components based on charge in solution rather than chromatographic interactions between solute and stationary phase. Separation of non-charged species can be achieved by the introduction of micelles in the technique known as micellar electrokinetic capillary chromatography (MECC). CE is performed in small volumes of aqueous buffers and thus avoids the large volumes of organic solvents frequently required for chromatographic separations. A wide range of mycotoxin standards, including aflatoxins, deoxynivalenol, fumonisins, moniliformin, ochratoxin A and zearalenone, have been separated by CE. As the separation mechanism differs from chromatography. CE separations can have their own problems with analytical impurities, as well as with achieving low limits of detection due to the limited sample amount that can be introduced into the CE capillary. The introduction of suitable lasers for detection, combined where necessary with appropriate derivatisation of the mycotoxin analyte. has lowered the detection limits to levels suitable for analysis of contaminated food samples. For example, a CE laser-induced fluorescence (CE-LIF) method has been developed for the determination of zearalenone in maize m which the addition of heptakis-(2,6-di-0-methyl)-P-cydodextrin enhanced the natural fluorescence of zearalenone and achieved a detection limit of 5 $\mu g\ kg^{-1}$.

All the methods discussed above have required the extraction of the mycotoxin from its matrix. This constitutes a time consuming and expensive exercise. In situations where rapid decisions are required, a method not requiring extraction but analysing the mycotoxin *in situ* would be advantageous. Because of this, researchers have investigated the possibility of using infrared (IR) analysers and principal component analysis for screening of mycotoxins directly from a grain sample. The advantages of these methods are the ease of operation, rapid result and nondestruction of the sample. Kernel rots and fumonisin contamination in maize were detected using near-IR reflectance spectroscopy which enabled a distinction

to be made between contaminated and clean lots. Deoxynivalenol contamination in wheat and maize has been investigated using IR spectroscopic techniques and the potential of the technique demonstrated. In the case of deoxynivalenol in maize, samples with levels as low as 310 μg kg^{-1} could be separated from uncontaminated samples. However, the application of near- or mid-IR presents a number of challenges, including the nonhomogeneous distribution of mycotoxin, the detection limits of the method, particle size distribution of the ground grain and the large calibration sets required. A further advance has been the use of mid-IR spectroscopy to sort dried vine fruit into different batches depending on the levels of ochratoxin A contamination. In this application, samples with high contamination (>20μg kg^{-1}) were separated from those with lower contamination (10 μg kg^{-1}) and from uncontaminated samples.

2.3. High-performance liquid chromatography

Fig.1. An HPLC. From left to right: A pumping device generating a gradient of two different solvents- a steel-enforced column and a detector for measuring the absorbance.

Fig.2. A modern self-contained HPLC.

High-performance liquid chromatography (HPLC; formerly referred to as high-pressure liquid chromatography), is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

HPLC has been used for manufacturing (e.g. during the production process of pharmaceutical and biological products), legal (e.g. detecting performance

enhancement drugs in urine), research (e.g. separating the components of a complex biological sample, or of similar synthetic chemicals from each other), and medical (e.g. detecting vitamin D levels in blood serum) purposes.

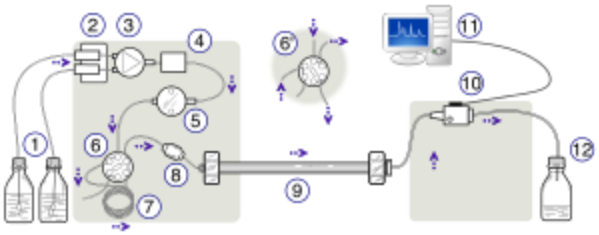


Fig.3. Schematic representation of an HPLC unit. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column (guard column), (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector.

Chromatography can be described as a mass transfer process involving adsorption. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with adsorbent, leading to the separation of the sample components. The active component of the column, the adsorbent, is typically a granular material made of solid particles (e.g. silica, polymers, etc.), 2–50 micrometers in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the adsorbent particles. The pressurized liquid is typically a mixture of solvents (e.g. water, acetonitrile and/or methanol) and is referred to as a "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination.

HPLC is distinguished from traditional ("low pressure") liquid chromatography because operational pressures are significantly higher (50–350 bar), while ordinary liquid chromatography typically relies on the force of gravity

to pass the mobile phase through the column. Due to the small sample amount separated in analytical HPLC, typical column dimensions are 2.1–4.6 mm diameter, and 30–250 mm length. Also HPLC columns are made with smaller sorbent particles (2–50 micrometer in average particle size). This gives HPLC superior resolving power (the ability to distinguish between compounds) when separating mixtures, which makes it a popular chromatographic technique.

The schematic of an HPLC instrument typically includes a sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components. A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or based on mass spectrometry. Most HPLC instruments also have a column oven that allows for adjusting the temperature at which the separation is performed.

Operation

The sample mixture to be separated and analyzed is introduced, in a discrete small volume (typically microliters), into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities, which are a function of specific physical interactions with the adsorbent (also called stationary phase). The velocity of each component depends on its chemical nature, on the nature of the stationary phase (column) and on the composition of the mobile phase. The time at which a specific analyte elutes (emerges from the column) is called its retention time. The retention time

measured under particular conditions is an identifying characteristic of a given analyte.

Many different types of columns are available, filled with adsorbents varying in particle size, and in the nature of their surface ("surface chemistry"). The use of smaller particle size packing materials requires the use of higher operational pressure ("backpressure") and typically improves chromatographic resolution (i.e. the degree of separation between consecutive analytes emerging from the column). Sorbent particles may be hydrophobic or polar in nature.

Common mobile phases used include any miscible combination of water with various organic solvents (the most common are acetonitrile and methanol). Some HPLC techniques use water-free mobile phases (see Normal-phase chromatography below). The aqueous component of the mobile phase may contain acids (such as formic, phosphoric or trifluoroacetic acid) or salts to assist in the separation of the sample components. The composition of the mobile phase may be kept constant ("isocratic elution mode") or varied ("gradient elution mode") during the chromatographic analysis. Isocratic elution is typically effective in the separation of sample components that are not very different in their affinity for the stationary phase. In gradient elution the composition of the mobile phase is varied typically from low to high eluting strength. The eluting strength of the mobile phase is reflected by analyte retention times with high eluting strength producing fast elution (short retention times). A typical gradient profile in reversed phase chromatography might start at 5% acetonitrile (in water or aqueous buffer) and progress linearly to 95% acetonitrile over 5–25 minutes. Periods of constant mobile phase composition may be part of any gradient profile. For example, the mobile phase composition may be kept constant at 5% acetonitrile for 1–3 min, followed by a linear change up to 95% acetonitrile.

Fig.4. A rotary fraction collector collecting HPLC output. The system is being used to isolate a fraction containing Complex I from E. coli plasma membranes. About 50 litres of bacteria were needed to isolate this amount.

The chosen composition of the mobile phase (also called eluent) depends on the intensity of interactions between various sample components ("analytes") and stationary phase (e.g. hydrophobic interactions in reversed-phase HPLC). Depending on their affinity for the stationary and mobile phases analytes partition between the two during the separation process taking place in the column. This partitioning process is similar to that which occurs during a liquid–liquid extraction but is continuous, not step-wise. In this example, using a water/acetonitrile gradient, more hydrophobic components will elute (come off the column) late, once the mobile phase gets more concentrated in acetonitrile (i.e. in a mobile phase of higher eluting strength).

The choice of mobile phase components, additives (such as salts or acids) and gradient conditions depends on the nature of the column and sample components. Often a series of trial runs is performed with the sample in order to find the HPLC method which gives adequate separation.

Types

Partition chromatography

Partition chromatography was one of the first kinds of chromatography that chemists developed. The partition coefficient principle has been applied in paper chromatography, thin layer chromatography, gas phase and liquid–liquid separation applications. The 1952 Nobel Prize in chemistry was earned by Archer John Porter Martin and Richard Laurence Millington Synge for their development

of the technique, which was used for their separation of amino acids. Partition chromatography uses a retained solvent, on the surface or within the grains or fibers of an "inert" solid supporting matrix as with paper chromatography; or takes advantage of some coulombic and/or hydrogen donor interaction with the stationary phase. Analyte molecules partition between a liquid stationary phase and the eluent. Just as in Hydrophilic Interaction Chromatography (HILIC; a sub-technique within HPLC), this method separates analytes based on differences in their polarity. HILIC most often uses a bonded polar stationary phase and a mobile phase made primarily of acetonitrile with water as the strong component. Partition HPLC has been used historically on unbonded silica or alumina supports. Each works effectively for separating analytes by relative polar differences. HILIC bonded phases have the advantage of separating acidic, basic and neutral solutes in a single chromatographic run.

The polar analytes diffuse into a stationary water layer associated with the polar stationary phase and are thus retained. The stronger the interactions between the polar analyte and the polar stationary phase (relative to the mobile phase) the longer the elution time. The interaction strength depends on the functional groups part of the analyte molecular structure, with more polarized groups (e.g. hydroxyl-) and groups capable of hydrogen bonding inducing more retention. Coulombic (electrostatic) interactions can also increase retention. Use of more polar solvents in the mobile phase will decrease the retention time of the analytes, whereas more hydrophobic solvents tend to increase retention times.

Normal–phase chromatography

Normal–phase chromatography was one of the first kinds of HPLC that chemists developed. Also known as normal-phase HPLC (NP-HPLC) this method separates analytes based on their affinity for a polar stationary surface such as silica, hence it is based on analyte ability to engage in polar interactions (such as hydrogen-bonding or dipole-dipole type of interactions) with the sorbent surface. NP-HPLC uses a non-polar, non-aqueous mobile phase (e.g. Chloroform), and works effectively for separating analytes readily soluble in non-polar solvents.

The analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity. The interaction strength depends not only on the functional groups present in the structure of the analyte molecule, but also on steric factors. The effect of steric hindrance on interaction strength allows this method to resolve (separate) structural isomers.

The use of more polar solvents in the mobile phase will decrease the retention time of analytes, whereas more hydrophobic solvents tend to induce slower elution (increased retention times). Very polar solvents such as traces of water in the mobile phase tend to adsorb to the solid surface of the stationary phase forming a stationary bound (water) layer which is considered to play an active role in retention. This behavior is somewhat peculiar to normal phase chromatography because it is governed almost exclusively by an adsorptive mechanism (i.e. analytes interact with a solid surface rather than with the solvated layer of a ligand attached to the sorbent surface; see also reversed-phase HPLC below). Adsorption chromatography is still widely used for structural isomer separations in both column and thin-layer chromatography formats on activated (dried) silica or alumina supports.

Partition- and NP-HPLC fell out of favor in the 1970s with the development of reversed-phase HPLC because of poor reproducibility of retention times due to the presence of a water or protic organic solvent layer on the surface of the silica or alumina chromatographic media. This layer changes with any changes in the composition of the mobile phase (e.g. moisture level) causing drifting retention times.

Recently, partition chromatography has become popular again with the development of Hilic bonded phases which demonstrate improved reproducibility, and due to a better understanding of the range of usefulness of the technique.

Displacement chromatography

The basic principle of displacement chromatography is: A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities.

There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in some interaction between the biomolecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at the preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings. Displacement chromatography has advantages over elution chromatography in that components are resolved into consecutive zones of pure substances rather than “peaks”. Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentration.

Reversed-phase chromatography (RPC)

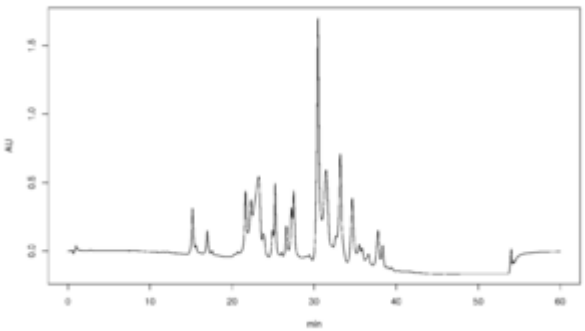


Fig.5. A chromatogram of complex mixture (perfume water) obtained by reversed phase HPLC

Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is a silica which has been surface-modified with RMe2SiCl, where R is a straight chain alkyl

group such as C18H37 or C8H17. With such stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily (early in the analysis). An investigator can increase retention times by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding more organic solvent to the eluent. RP-HPLC is so commonly used that it is often incorrectly referred to as "HPLC" without further specification. The pharmaceutical industry regularly employs RP-HPLC to qualify drugs before their release.

RP-HPLC operates on the principle of hydrophobic interactions, which originates from the high symmetry in the dipolar water structure and plays the most important role in all processes in life science. RP-HPLC allows the measurement of these interactive forces. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand on the stationary phase. This solvophobic effect is dominated by the force of water for "cavity-reduction" around the analyte and the C18-chain versus the complex of both. The energy released in this process is proportional to the surface tension of the eluent (water: $7.3\times10{-}6$ J/cm², methanol: $2.2\times10{-}6$ J/cm²) and to the hydrophobic surface of the analyte and the ligand respectively. The retention can be decreased by adding a less polar solvent (methanol, acetonitrile) into the mobile phase to reduce the surface tension of water. Gradient elution uses this effect by automatically reducing the polarity and the surface tension of the aqueous mobile phase during the course of the analysis.

Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C–H, C–C, and generally non-polar atomic bonds, such as S-S and others) is retained longer because it is non-interacting with the water structure. On the other hand, analytes with higher polar surface area (conferred by the presence of polar

groups, such as -OH, -NH2, COO− or -NH3+ in their structure) are less retained as they are better integrated into water. Such interactions are subject to steric effects in that very large molecules may have only restricted access to the pores of the stationary phase, where the interactions with surface ligands (alkyl chains) take place. Such surface hindrance typically results in less retention.

Retention time increases with hydrophobic (non-polar) surface area. Branched chain compounds elute more rapidly than their corresponding linear isomers because the overall surface area is decreased. Similarly organic compounds with single C–C bonds elute later than those with a C=C or C–C triple bond, as the double or triple bond is shorter than a single C–C bond.

Aside from mobile phase surface tension (organizational strength in eluent structure), other mobile phase modifiers can affect analyte retention. For example, the addition of inorganic salts causes a moderate linear increase in the surface tension of aqueous solutions (ca. $1.5\times10{-}7$ J/cm² per Mol for NaCl, $2.5\times10{-}7$ J/cm² per Mol for (NH4)2SO4), and because the entropy of the analyte-solvent interface is controlled by surface tension, the addition of salts tend to increase the retention time. This technique is used for mild separation and recovery of proteins and protection of their biological activity in protein analysis (hydrophobic interaction chromatography, HIC).

Another important factor is the mobile phase pH since it can change the hydrophobic character of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate, to control the pH. Buffers serve multiple purposes: control of pH, neutralize the charge on the silica surface of the stationary phase and act as ion pairing agents to neutralize analyte charge. Ammonium formate is commonly added in mass spectrometry to improve detection of certain analytes by the formation of analyte-ammonium adducts. A volatile organic acid such as acetic acid, or most commonly formic acid, is often added to the mobile phase if mass spectrometry is used to analyze the column effluent. Trifluoroacetic acid is used infrequently in mass spectrometry applications due to its persistence in the detector and solvent delivery system, but can be effective in improving

retention of analytes such as carboxylic acids in applications utilizing other detectors, as it is a fairly strong organic acid. The effects of acids and buffers vary by application but generally improve chromatographic resolution.

Reversed phase columns are quite difficult to damage compared with normal silica columns; however, many reversed phase columns consist of alkyl derivatized silica particles and should never be used with aqueous bases as these will destroy the underlying silica particle. They can be used with aqueous acid, but the column should not be exposed to the acid for too long, as it can corrode the metal parts of the HPLC equipment. RP-HPLC columns should be flushed with clean solvent after use to remove residual acids or buffers, and stored in an appropriate composition of solvent. The metal content of HPLC columns must be kept low if the best possible ability to separate substances is to be retained. A good test for the metal content of a column is to inject a sample which is a mixture of 2,2'- and 4,4'-bipyridine. Because the 2,2'-bipy can chelate the metal, the shape of the peak for the 2,2'-bipy will be distorted (tailed) when metal ions are present on the surface of the silica.

Size-exclusion chromatography

Size-exclusion chromatography (SEC), also known as gel permeation chromatography or gel filtration chromatography, separates particles on the basis of molecular size (actually by a particle's Stokes radius). It is generally a low resolution chromatography and thus it is often reserved for the final, "polishing" step of the purification. It is also useful for determining the tertiary structure and quaternary structure of purified proteins. SEC is used primarily for the analysis of large molecules such as proteins or polymers. SEC works by trapping these smaller molecules in the pores of a particle. The larger molecules simply pass by the pores as they are too large to enter the pores. Larger molecules therefore flow through the column quicker than smaller molecules, that is, the smaller the molecule, the longer the retention time.

This technique is widely used for the molecular weight determination of polysaccharides. SEC is the official technique (suggested by European

pharmacopeia) for the molecular weight comparison of different commercially available low-molecular weight heparins.

Ion-exchange chromatography

In ion-exchange chromatography (IC), retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Solute ions of the same charge as the charged sites on the column are excluded from binding, while solute ions of the opposite charge of the charged sites of the column are retained on the column. Solute ions that are retained on the column can be eluted from the column by changing the solvent conditions (e.g. increasing the ion effect of the solvent system by increasing the salt concentration of the solution, increasing the column temperature, changing the pH of the solvent, etc...).

Types of ion exchangers include:

Polystyrene resins – These allow cross linkage which increases the stability of the chain. Higher cross linkage reduces swerving, which increases the equilibration time and ultimately improves selectivity.

Cellulose and dextran ion exchangers (gels) – These possess larger pore sizes and low charge densities making them suitable for protein separation.

Controlled-pore glass or porous silica

In general, ion exchangers favor the binding of ions of higher charge and smaller radius.

An increase in counter ion (with respect to the functional groups in resins) concentration reduces the retention time. A decrease in pH reduces the retention time in cation exchange while an increase in pH reduces the retention time in anion exchange. By lowering the pH of the solvent in a cation exchange column, for instance, more hydrogen ions are available to compete for positions on the anionic stationary phase, thereby eluting weakly bound cations.

This form of chromatography is widely used in the following applications: water purification, preconcentration of trace components, ligand-exchange chromatography, ion-exchange chromatography of proteins, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, and others.

Bioaffinity chromatography

This chromatographic process relies on the property of biologically active substances to form stable, specific, and reversible complexes. The formation of these complexes involves the participation of common molecular forces such as the Van der Waals interaction, electrostatic interaction, dipole-dipole interaction, hydrophobic interaction, and the hydrogen bond. An efficient, biospecific bond is formed by a simultaneous and concerted action of several of these forces in the complementary binding sites.

Aqueous normal-phase chromatography

Aqueous normal-phase chromatography (ANP) is a chromatographic technique which encompasses the mobile phase region between reversed-phase chromatography (RP) and organic normal phase chromatography (ONP). This technique is used to achieve unique selectivity for hydrophilic compounds, showing normal phase elution using reversed-phase solvents.

Isocratic and gradient elution

A separation in which the mobile phase composition remains constant throughout the procedure is termed isocratic (meaning constant composition). The word was coined by Csaba Horvath who was one of the pioneers of HPLC.

The mobile phase composition does not have to remain constant. A separation in which the mobile phase composition is changed during the separation process is described as a gradient elution. One example is a gradient starting at 10% methanol and ending at 90% methanol after 20 minutes. The two components of the mobile phase are typically termed "A" and "B"; A is the "weak" solvent which allows the solute to elute only slowly, while B is the "strong" solvent which rapidly elutes the solutes from the column. In reversed-phase chromatography, solvent A is often water or an aqueous buffer, while B is an organic solvent miscible with water, such as acetonitrile, methanol, THF, or isopropanol.

In isocratic elution, peak width increases with retention time linearly according to the equation for N, the number of theoretical plates. This leads to the

disadvantage that late-eluting peaks get very flat and broad. Their shape and width may keep them from being recognized as peaks.

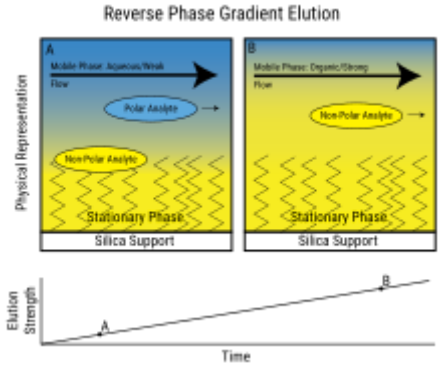


Fig.6. A schematic of gradient elution. Increasing mobile phase strength sequentially elutes analytes having varying interaction strength with the stationary phase.

Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower (and taller) peaks for most components. This also improves the peak shape for tailed peaks, as the increasing concentration of the organic eluent pushes the tailing part of a peak forward. This also increases the peak height (the peak looks "sharper"), which is important in trace analysis. The gradient program may include sudden "step" increases in the percentage of the organic component, or different slopes at different times – all according to the desire for optimum separation in minimum time.

In isocratic elution, the selectivity does not change if the column dimensions (length and inner diameter) change – that is, the peaks elute in the same order. In gradient elution, the elution order may change as the dimensions or flow rate change.

CHAPTER III. RESULTS AND DISCUSSION

3.1. HPLC analysis of aflotoxins in barley malt and maize.

Conducting analyzes using a new developed method for high-performance liquid chromatography (HPLC). The following experiment analyzed objects were the previous barley malt and corn.

Apparatus, materials and reagents:

Barley malt; Maize

HPLC devices Agilent 1200 Series HPLC

Laboratory mill

Scales technical

Shaker shaker

Rotary evaporator with trap

Water Pump

Agilent microsyringe # 5182-9619 at 10 μl

Cylinders measuring 100, 250 and 500 cm^3

Flat-bottom conical flasks of 500 cm^3 and round-bottomed to 250 cm^3 of NSH 11-29 with a ground stopper

Filter paper

Flasks measuring 100 cm3

Chemical funnels with a diameter of 150 mm

Dividing funnels for VD2-250

Flat-bottomed flasks for 100,250 and 500 m^3

Acetone "p.s."

Hexane TU6-09-3375-73

Benzene according to GOST 5955-75

Acetonitrile (Sigma, USA)

Methanol (Sigma, USA)

Chloroform is a medical device.

Ether diethyl medical according to GOST 6265-52

Water is bidistilled.

Sulfuric acid according to GOST 4204-77

Sodium sulfate anhydrous according to GOST 4166-76, calcined.

Sodium chloride according to GOST 4233-66

Sodium acetate h.p.

Lead in acetic acid according to GOST 1027-67

Alumina for column chromatography (neutral)

Conducting the analysis.

The analysis of the procedure is divided into 3 main parts: 1) extraction, 2) purification of the extract 3) detection and quantitative determination of the Aflotoxins by HPLC. Extraction was also carried out according to paragraph 2.3, but the purification of the extract was carried out a little deep.

Cleaning the extract: To 50 cm^3 of the filtrate, 20 cm^3 of a 15% solution of lead acetic acid and 30 cm^3 of distilled water were added, mixed and allowed to stand for 10 minutes in the dark. The precipitate formed was filtered off through a paper fold filter, 80 cm3 of filtrate was collected. Transferred to a separatory funnel, 30 cm3 of hexane was added, shaken and after separation of the layers the lower full-acetone layer was separated. The upper hexane layer was discarded. 30 cm^3 of hexane was added to the water-acetone layer, shaken in a separatory funnel, the upper hexane layer was discarded. Then it was extracted again, 30 cm^3 of chloroform was added to the water-acetone solution, shaken in a separatory funnel. After separation of the layers, the lower chloroform layer was separated, and 30 cm3 of chloroform and 15 cm^3 of acetone were added to the upper water-acetone layer. After shaking and separation of the layers, the lower chloroform layer was separated. The combined chloroform extracts were placed in a 100 cm^3 flat bottom flask with ground stopper, 7 g of anhydrous sodium sulfate was added, shaken and left to Z0min in the dark. The solution was filtered through a piece of cotton wool, a kneaded and glass chemical funnel, into a pear-shaped flask 100 cm3 with a sprout.

The chloroform solution was evaporated on a rotary evaporator in a water jet pump vacuum at a bath temperature of no higher than 500 dry to a volume of 1 cm^3. Next, the extract was purified by column chromatography with silica gel:

A small lump of cotton wool was added to the bottom of the column, then anhydrous sodium sulfate (layer thickness 5 mm), a suspension of 2 g of silica gel in chloroform was poured, a layer of anhydrous sodium sulfate (20 mm) was poured on top. The chloroform was allowed to drain, then chloroform extract of the sample was applied onto the column, eluted with a mixture of chloroform -acetone (9: 1) -60 cm3. The eluate was evaporated to dryness on a rotary evaporator, the residue was dissolved in 2 cm3 of chloroform filtered through a paper filter into a 5 cm3 tube, the solvent was poured out in a stream of nitrogen, and the residue was dissolved in 400 μl of chloroform. And further, the resulting solution was analyzed by high performance liquid chromatography (HPLC).

Hyperspil ODS 100x2. 1-mm id,

Dispersion 3 mkm

HP 799160D-352

Water-methanol-acetonitrile (63:26:11) as an isocratic mixture.

Flow rate 0.3 ml / min at 25 ° C

Detectors: Fluorescent lex 365 nm,

lem 455 nm.

Thin layer chromatography can be replaced by reversed-phase HPLC, while increasing the accuracy of the analysis, and significantly reducing the time required for determinations. For example, using TS chromatography, the analysis of aflotoxin B1 is carried out for three, and M1-hours of hours. Due to the fact that in fluorescence analysis the sensitivity of the method for B2 and B1 differs significantly from G1 and G, respectively, the quantum yield of fluorescence for B2 is approximately 60 (times higher than for B1). If only a fluorescent detector is used, it is desirable to improve the quantum yield of fluorescence for aflotoxin.

Fig 7. Chromatogram in the determination of aflotoxin B1 in the product with the application of purification of the extract on the immune-affinity column. The contents of aflotoxin B1 - 0,1 mkg / kg

The system given is isocratic: during the chromatographic process the composition of the mobile phase does not change. If in the course of chromatographic analysis it is necessary to change the concentration of one or several components of the mobile phase, then so-called gradient systems are used, consisting usually of two or more pumps. In the case of gradient elution, each solvent is fed from a separate vessel to a special mixing chamber with a magnetic stirrer, where a certain program mixes them with a predetermined volume ratio.

For the analysis of mycotoxins gradient HPLC systems are often used, where solutions of acetonitrile in water with a linearly varying concentration of time are used as the mobile phase.

Chromatographic column is a metal tube length from 150 to 250 mm with an internal diameter of 4.6 mm, filled with a special sorbent on the basis of silica gel with grafted hydrocarbon radicals. The precolumn serves to protect the chromatographic column from contamination.

UV-photometric detector is the most common type of HPLC detectors. The principle of operation of the detector is similar to the principle of the action of a conventional spectrophotometer: it records the optical density of the solution. The difference is that the UV detector is a flow detector, instead of a cell with a solution, a photometric cell is used in it. The eluent stream flows through the working cell, and a pure mobile phase flows through the reference cell. The source of light is a mercury lamp, which gives intense UV radiation. Light with the desired wavelength is extracted with the help of suitable optical filters, passes through the cells, is partially absorbed by the molecules of the mobile phase and the components to be separated and captured by the photo receiver. Absorbance (optical density) of the eluate continuously registers the chart recorder or computer, recording a chromatogram. The components of the mixture to be separated (eg, mycotoxins) are shown in the chromatogram as peaks. The position of the peak on the chromatogram is used to identify the substance, and the peak area for quantification.

A more complex device is a fluorescent (fluorine-metric) detector. Such a detector uses the ability of organic compounds, in particular aflatoxins and zearalenone, to fluoresce under the influence of UV or visible radiation. The fluorescent detector has a proprietary cell with two mutually perpendicular optical channels. One of them serves for the supply of exciting radiation, the other allows one to measure the intensity of fluorescence. In the case of analysis of aflatoxins B and M, the wavelength of the exciting radiation is 360 nm, and the wavelength of the emitted radiation is 420 nm.

It should be noted that an UV detector can also be used to analyze aflatoxins, but its sensitivity is an order of magnitude lower than that of a fluorimetric detector, so fluorescent detection is preferred for the analysis of low concentrations of aflatoxins (at the MPC level and below).

3.2. Use of high-performance liquid chromatography to assess airborne mycotoxins. Aflatoxins and ochratoxin A

1. An HPLC analytical method combining methanol-deionised water (80:20, v/v) extraction, methanol-acetonitrile (50:50, v/v) extraction and fluorescence detection was implanted to analyse ochratoxin A and aflatoxins B1, B2. G1 and Ql of air samples collected during the usual production process in a number of workplaces of a coffee factory to assess the occupational exposure of the engaged workers. The average levels of airborne ochratoxin A and aflatoxins were less than 1.2 and 0.4 ng/m^3 respectively, using 50 L air samples. When 150 L air samples were used, levels lower than 0.04ng/m^3 ochratoxin A and 0.013 ng/m^3 for aflatoxins Bl, B2, Gl and G2. could be detected.

Aflatoxin Bl (AFB1). Afiatoxin B2 (AFB2), aflatoxin Gl (AFG1). Aflatoxin G2 (AFG2) and ochratoxin A (OA), are metabolites that may be produced by molds such as Aspergillus and Penicillum. Their biosynthesis depends on the temperature and humidity conditions.

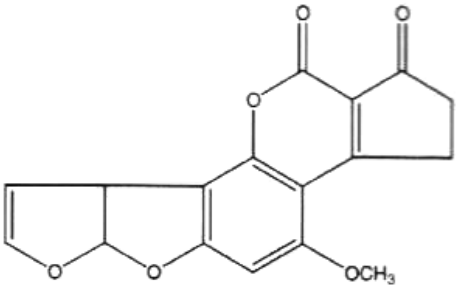
Aflatoxins and ochratoxin are found in agricultural products such as cereals, cacao, coffee, wine, fruits, peanuts, cotton seed, corn and rice as a consequence of unprosper-ous storage conditions (humidity of 70-90% and a minimum Temperature of about 10°C).

As a matter of fact, components of heat, ventilation and air conditioning (HVAC systems) may be reservoirs of fungi and bacteria. In this regard, the occurrence of aflatoxins in homes and buildings has been reported, owing to the growth of mycotoxins producing moulds in the air conditioning systems.

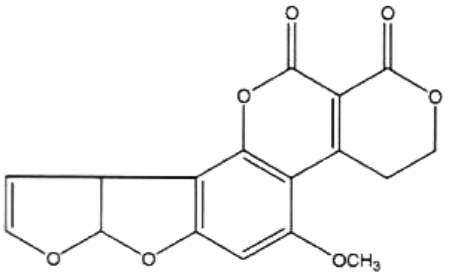
AFBl, AFB2, AFGl, AFG2 and OA(Fig. 1) are mycotoxins whose absorption represents an important health hazard. Epidemiological studies have shown a correlation between liver cancer and the prevalence of aflatoxins in the food supply. Aflatoxin B1 is a powerful hepatocarcinogen and ochratoxin A is a well-recognised nephrotoxic.

The available information on airborne particulate aflatoxins and ochratoxin A occurrence is scarce, and the majority of the airborne particulate matter studies have been focused on aflatoxins durine processing and handling of corn.

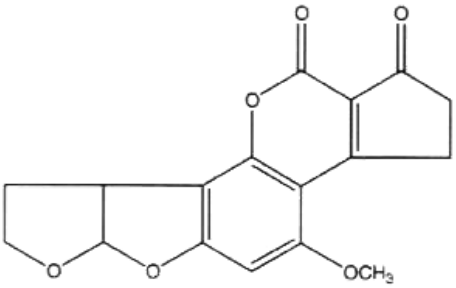
HPLC is a common method used for determination of aflatoxins in contaminated food. To carry out this study, the method by Brera et al. was modified to estimate the levels of aflatoxins and ochratoxin A in the air sampled in a coffee factory during the production process. The main modifications of Brera's method were as follows: inmunoaffinity columns were not used, and aflatoxins were extracted with 3 mL of methanol-acetonitile (50:50. v/v) rather than methanol-deionised water (80:20, v/v).



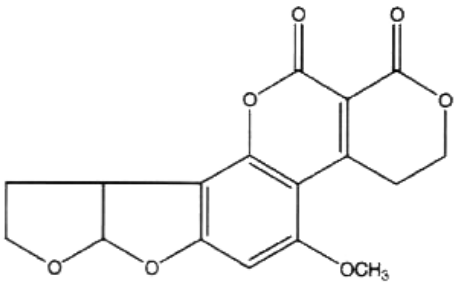
Aflatoxin Bl (AFB1)



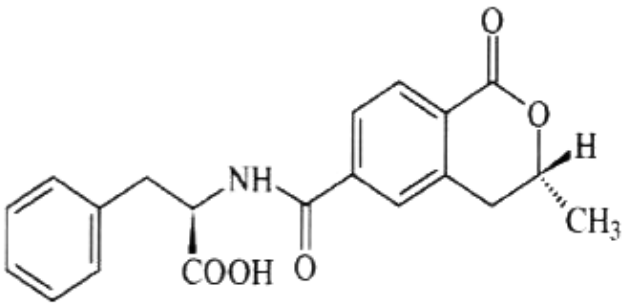
Aflatoxin Gl (AFG1)



Aflatoxin B2 (AFB2)



Aflatoxin G2 (AFG2)



Ochratoxin A (OA)

Fig 8. Chemical structures of aflatoxins and ochratoxin A

The aim of this paper is to describe an HPLC-fluorescence detection (FLD) method for the determination of particulate aflatoxins and ochratoxin A in air samples intended to assess the exposure of the workers to these substances by inhalation at their workplaces.

2. Experimental

2.1. Sampling of airborne particulates

Eleven air samples were collected using aircheck pumps at a flow rate of 1.0 L/min during the coffee production process while workers were handling raw materials, tools and machinery in the factory. The sampling device through which the airflow passed was provided with a microfibre filter (Whatman 3-7 cm GF/C), in order to retain the particulate matter. The sampling time at each of the eleven workplaces in the factory was set in accordance with the length of the specific handling activity, between 50 and 150 min, approximately.

2.2. Reagents

Acetonitrile, acid acetic (glacial), n-hexane, methanol and trifluoroacetic acid (HPLC grade) were supplied by J.T. Baker (Barcelona, Spain).

Deionised water was obtained using a Milli-Q purification system (Waters Millipore QTUM 000EX).

Aflatoxin primary standards to check the linearity were provided by Tecnova (Madrid, Spain). Six milliliters vial of a methanol solution with the following specifications: 1000 ng/mL total aflatoxin, 250 ng/mL of AFB1. AFB2, AFG1 and AFG2.

Ochratoxin A primary standard for linearity was provided by Tecnova (Madrid, Spain). Six milliliters vial of methanol solution with the following specifications: 1000 ng/mL.

Aflatoxins and ochratoxin A primary standards for a recovery appraisal were provided by Sigma (Spain). Solid primary standards with the following specifications: 5 mg of AFB1, 5 mg of AFB2 and 5 mg of OA.

2.3. Working standard solutions

Working standard solutions of 0.25, 0.5, 1.25 and 2.5 ng/mL off each aflatoxin (AFB1, AFB2, AFG1. AFG2) were prepared by diluting the primary standard solution with methanol-acetonitrile (50:50, v/v). Ochratoxin A working standard solutions of 0.04, 0.2 and 2 ng/mL were prepared by diluting the primary standard solution with methanol-deionised water (80:20, v/v).

2.4. Instrumental analysis

Experiments were performed on a Waters 717 plus autosampler liquid chromatograph coupled to a Waters 474 scanning fluorescence detector, equipped with isocratic pump (Waters 1525). The system was controlled by a Waters Millennium 32 chemstation. The analytical column was a 150 x 4.6 mm. i.d. Synergi Max-RP (Phenomenex, Spain), thermostatted at 27 °C.

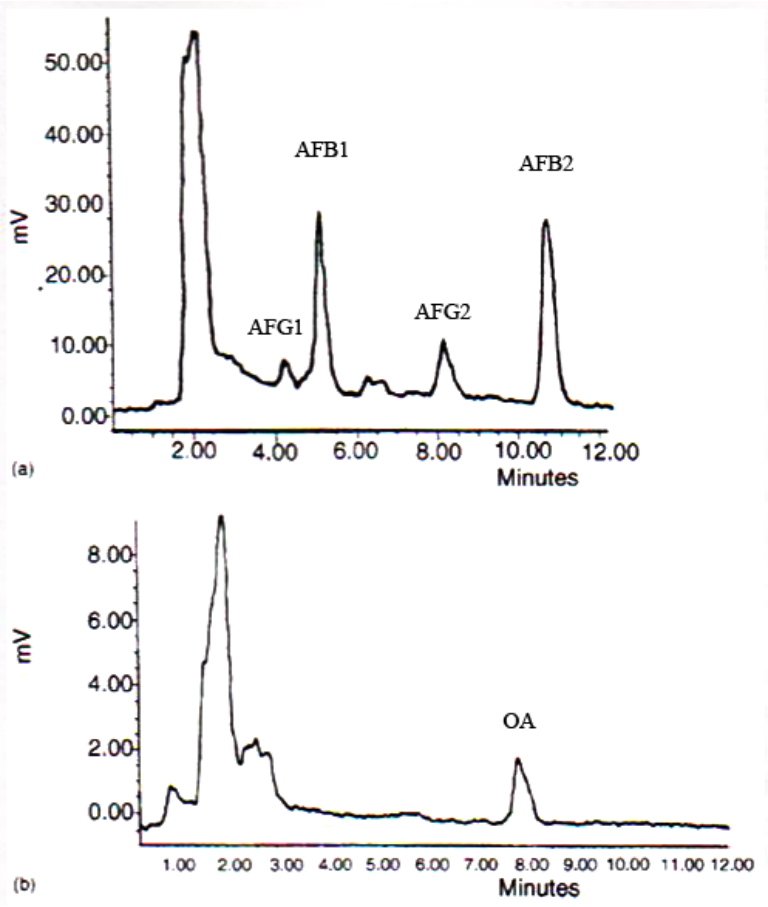


Fig 9. HPLC-fluorescence chromatogram of (a) hem iacetal forms of AFB1, AFG1 and aflaloxins AFB2, AFG2 at 0 25 ng/mL, (b) ochratoxin A (OA) at 0.1 ng/mL.

For aflatoxins the fluorescence detector was set at 360 and 420 nm and the mobile phase consisted of a mixture of deionised water-acetonitrile-methanol (60:25:15, v/v). For ochratoxin A the detector was set at 333 and 470 nm and the mobile phase was acetonitrile-deionised water-glacial acetic acid (49.5:49.5:1, v/v).

The flow rate was 1 mL/min for each mobile phase and the injected volume of working standards and airborne samples was 50 μL. (Fig. 2).

2.5. Extraction aflatoxins BI, B2, Gl, G2 and ochratoxin A procedure

Aflatoxins extractions from the glass fibre filter were carried out with 3 mL of methanol-acetonitrile (50:50, v/v) and, the solutions were kept in an ultrasonic bath for 10 min. The aflatoxins were then processed for derivatisation before the samples being injected into the HPLC.

Ochratoxin A was extracted from another glass fibre filter with 3 mL of methanol-deionised water (80:20, v/v), the solution was kept in an ultrasonic bath for 10 min and immediately injected into the HPLC without derivatisation.

The aflatoxins and ochratoxin A extracts were not filtered before the analysis and the samples were analysed immediately after extraction to prevent solvent evaporation.

2.6. Aflatoxin derivatisation

An aliquot of 500 μL of the aflatoxins solution was dried by evaporation under gentle nitrogen stream. Hemi-acetal forms of AFB 1 and AFG1 (fluorescence active substances) were obtained by addition of a mixture of 50 μL of trifluoroacetic acid and 200 nL of n-hexane at 40 C; the solution was mechanically shaken for 15 min. The solution containing all four aflatoxins in its fluorescent form was subsequently dried by evaporation under gentle nitrogen stream, redissolved with 200 μL acetonitrile-deionised water (30:70, v/v) and it was finally injected in the HPLC system (Fig. 3).

3.3. Results and discussion

To check the quality of the proposed method several analytical parameters were determined.

3.1. Selectivity and specificity

The presence of any substance coming from the filter appearing at any of the retention times of ochratoxin A (8 min), aflatoxins AFB1 (5 min), AFB2 (10 min), AFG1 (4 min), AFG2 (8 min) (interfering substances) was ruled out (Fig. 4).

3.2. Linearity

Linearity was assessed using working standards at the following concentrations: 0.04, 0.1, 0.2, 0.4, 1, 2 ng/mL for ochratoxin A and 0.25, 0.5, 1.25, 2.5 ng/mL for each aflatoxin (AFB1, AFB2, AFG1, AFG2). Each concentration was injected three times (injection: 50 μL).

The regression lines calculated using least-squares method were:

AFB1 : $y = (-397708) + (5419351)x$, $r^2 = 0.99990$

AFB2 : $y = (-644297) + (7517284)x$, $r^2 = 0.99999$

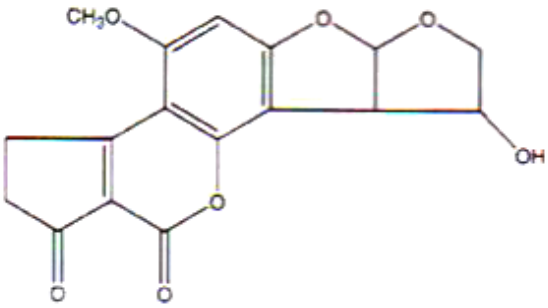
AFG1 : $y = (-113444) + (771223)x$, $r^2 = 0.99830$

AFG2 : $y = (-220695) + (1931764)x$, $r^2 = 0.99990$

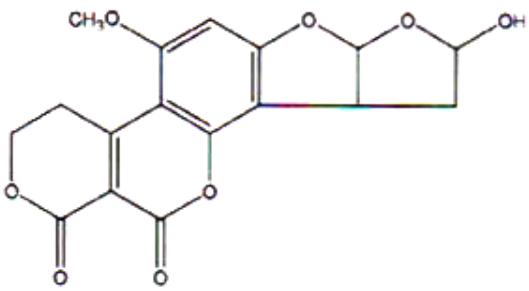
OA : $y = (-578.6) + (420867)x$, $r^2 = 0.99990$

3.3. Limits of detection and quantitation

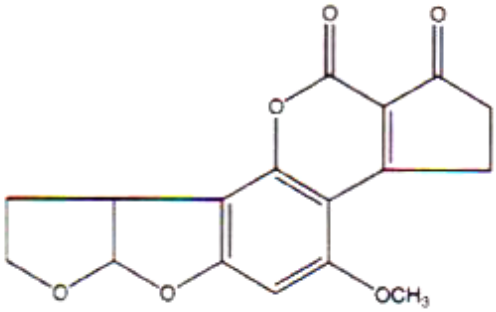
The limits of detection (LODs) were estimated as twice the signal-to-noise ratio (two times s/n). Thus, for the developed method, the above mentioned limits were 0.002 ng for aflatoxins and 0.06 ng for ochratoxin A.



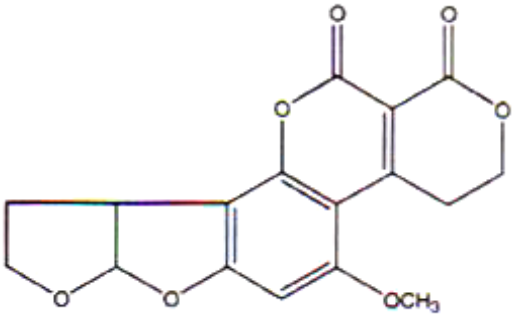
Hemiacetal form of AFB1



Hemiacetal form of AFG1



AFB2



AFG2

Fig 10. Chemical structures of fluorescence active substances

These LODs were similar to those reported in the literature (0.0025 ng AFB1 and AFB2).

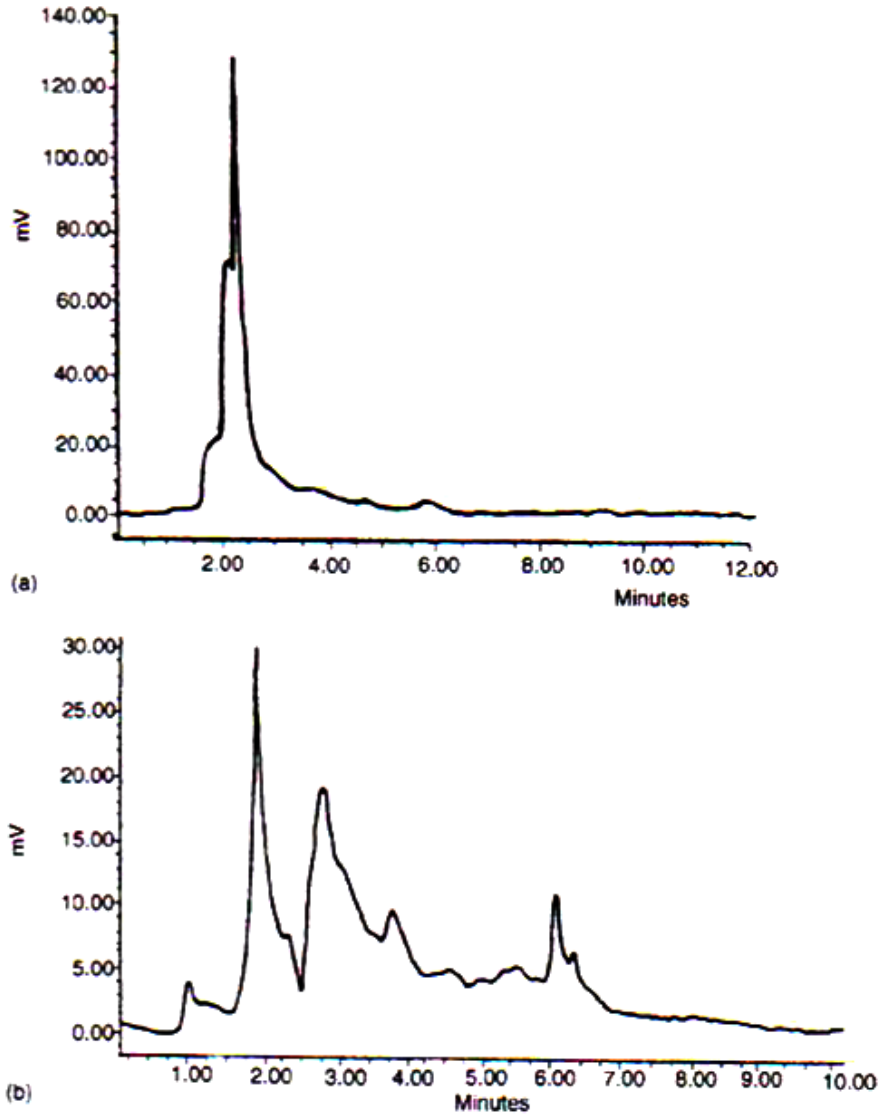


Fig 11. HPLC-fluorescence chromatogram of (a) glass fibre filter blank extracted with methanol-acetonitrile (50:50, v/v), (b) glass fibre filter blank extracted with metbano-dcionised water (80:20, v/v).

As it used be expected, the greater sampled air volume the lower relative detection limit obtained. The results were the following: 1.2 ng/m^3 for OA, 0.4 ng/m^3 for AFB1, AFB2. AFG1, AFG2 for 50 L air samples and 0.04ng/m^3 for OA, 0.013ng/m^3 for AFB1, AFB2, AFG1, AFG2 for 150 L air samples.

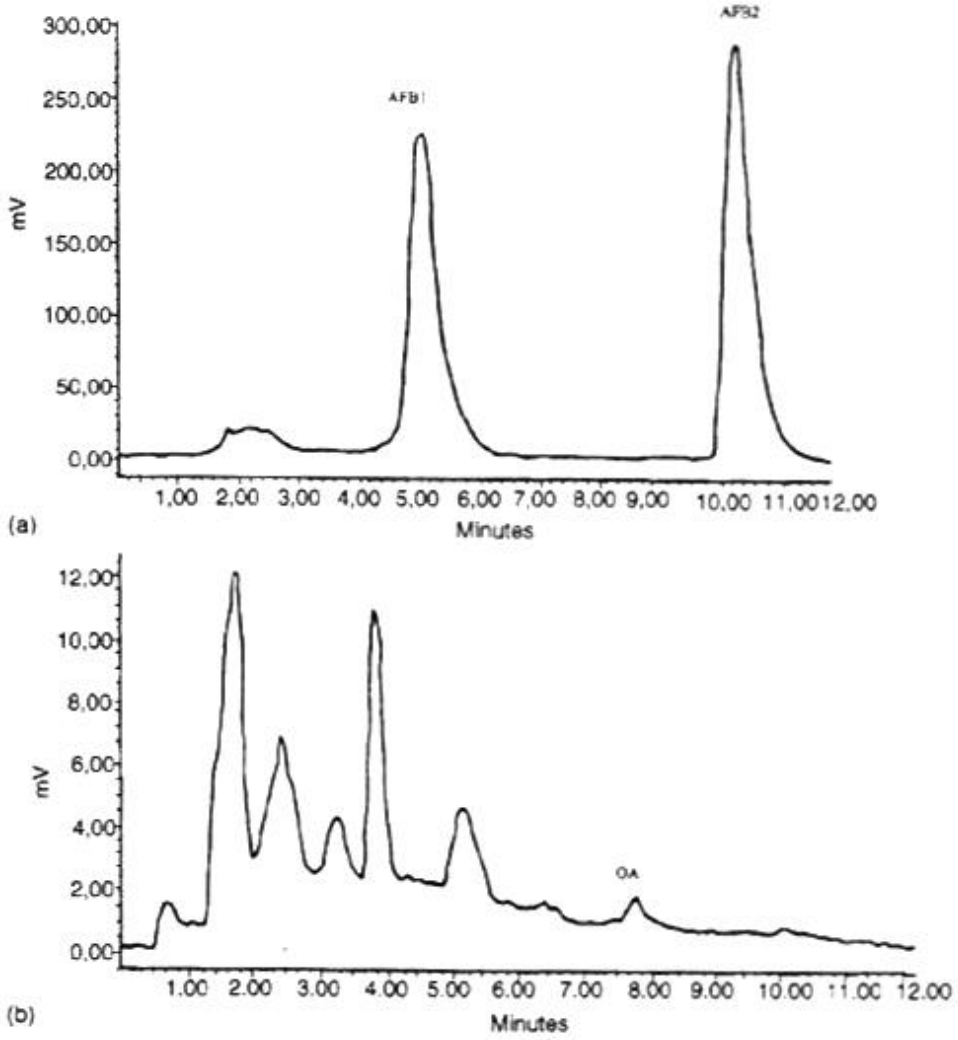


Fig 12. HPLC-fluorescence chromatogram of (a) a glass fibre filter sample spiked with 2,5ng/mL of AFB1 and AFBC, (b) a glass filter sample spiked with 0.04 ng/mL of OA.

Table 1

Recovery percentages and precision (R SD.) of aflatoxins

Anatoxins	Concentratior spiked (ng/mL)		
	0.1	0.5	2,5
AFB1	85.0 (2.7)	98.0 (2.8)	88.8 (1.2)
AFB2	98.3 (5.8)	82.0 (3.4)	86.4 (2.0)

R.S.D values (%) in parentheses.

Table 2

Recovery percentages and precision (R.S.D.) of ochratoxin A (OA)

Ochratoxin A (OA)	Concentrator spiked (ng/mL)		
	0.04	0.2	1
	80.3 (5.5)	96.8 (1.7)	103(5.0)

R.S.D. values (%) in parentheses.

The limit of quantitation (LOQ) is the lowest concentration of ochratoxin A and aflatoxins that can be measured with an approved precision (relative standard derivation, R.S.D. <7%). Thus, the LOQ for ochratoxin A and aflatoxins using 150 L air samples was estimated as 0.8 ng/m^3 for ochratoxin A, 0.13 ng/m^3 for AFB1, AFB2 and 0.33 ng/m^3 for AFG1 and AFG2.

3.3.4. Extraction recoveries and intra assay-precision

After 18 fibre filters were spiked with working standard solutions, extraction recoveries and intra assay-precision of ochratoxin A, aflatoxin B1, and aflatoxin B2 were calculated by comparison of peak areas of the compounds from fibre filters with those from working standards solutions at the intended final concentrations. The intended final concentration of the 18 glass fibre filters were 0.04,0.2 and 1 ng/mL of ochratoxin A, and 0.1, 0.5 and 2.5 ng/mL of AFB1 and AFB2 (Fig 5), respectively, on three separate runs.

The recovery percentages obtained for aflatoxins AFB1, AFB2, were 85, 98 and 88.8% for AFB1 and 98, 82.5 and 86.4% for AFB2 for glass fibre filter spiked with 0.1.0.5 and 2.5 ng/mL aflatoxins B1, B2. The percentages for ochratoxin A (OA) were 80.30, 96.80 and 103% for a glass fibre filter spiked with 0.04, 0.2 and 1 ng/mL of ochratoxin A. Results are represented in Tables 1 and 2.

To evaluate the precision of the method, glass fibre filter samples spiked with 0.1, 0.5 and 2.5 ng/mL of AFB1 and AFB2 were analysed three times at each concentration on three separates runs. Similarly, samples spiked with 0.04, 0.2 and 1 ng/mL of OA were analysed six times at each concentration on three separates

runs. Precision of the developed method was expressed as relative standard derivation. R.S.D. (Tables 1 and 2).

3.3.5. Analysis of the airborne particulate from a coffee factory

The optimised method was applied to assess the likely occurrence of airborne aflatoxins (AFB 1, AFB2. AFG1. AFG2) and ochratoxin A (OA) in a coffee factory during the performance of different work activities. Over 2 days, 11 samples of airborne dust were collected. As it has already been mentioned, the volume of air sampled was in accordance with the operations carried out at the specific workplace. All the results show that the concentration of aflatoxins and ochratoxin A (OA) during the performance of the activity in the coffee factory were less than the LOD of the here proposed method.

CONCLUSION

Mycotoxin analysis in food commodities continues to represent a challenge to analytical chemists. In recent years, the implementation of lower regulatory levels in the European Union has necessitated the development of methods with ever lower limits of detection and their validation by international inter laboratory collaborative study with the purpose of setting official methods. The necessity of wider import controls has also encouraged the move to multi-toxin methods using LC-MS for quantification and analyte confirmation. The greater awareness of the mycotoxin problem has also led to an increased array of rapid screening methods that can be used for testing and control on a frequent monitoring basis and which can be incorporated into HACCP (hazard analysis and critical control point) plans.

The filamentous fungi and the mycotoxins they produce have existed for all recorded history. It may thus be supposed that the determination of mycotoxins in human food will remain a necessity for a long time in the future and that general advances in analytical science will be reflected in similar advances in mycotoxin analysis.

1. We have studied several methods of chromatographic analysis.
2. Was developed a standard method HPLC analysis to determine low molecular weight mycotoxins.
3. Was researched the aflotoxins (G1, G2, B1, B2) that contaminate maize.

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LIST OF PUBLISHED WORKS

ЎЗБЕКИСТОН РЕСПУБЛИКАСИ ОЛИЙ ВА ЎРТА МАХСУС ТАЪЛИМ ВАЗИРЛИГИ

ТОШКЕНТ КИМЁ-ТЕХНОЛОГИЯ ИНСТИТУТИ

ТЕХНИК ВА ИЖТИМОИЙ-ИҚТИСОДИЙ ФАНЛАР СОҲАЛАРИНИНГ МУҲИМ МАСАЛАЛАРИ

Республика Олий ўқув юртлараро илмий ишлар тўплами

АКТУАЛЬНЫЕ ВОПРОСЫ В ОБЛАСТИ ТЕХНИЧЕСКИХ И СОЦИАЛЬНО-ЭКОНОМИЧЕСКИХ НАУК

Республиканский межвузовский сборник научных трудов

ЧАСТЬ I

Тошкент 2017

ASPERGILLUS AVLODIGA MANSUB ZAMBURUG'LAR: MIKOTOKSINLAR SINTEZI VA TARQALISHI

Do'stmuxamedov I.
Toshkent kimyo-texnologiya institute

Saprofit tuproq zamburug'i Aspergillus fumigatus keng tarqalgan oziq-ovqat va hayvon ozuqalarining kontaminanti hisoblanadi, u odam va hayvon organizmida mikoz va mikotoksikoz qo'zg'atuvchi hisoblanadi.

A.fumigatus keng doiradagi biologik faol birikmalarni sintez qiladi, ular alkaloidlar, tremorgen mikotoksinlar, antibiotiklar, gidrolitik fermentlar. Zamburug' tomonidan sintez qilinuvchi toksik birikmalar aspergillofumitoksikozni keltirib chiqaradi, bu esa asab tizimiga ta'sir qiladi va qishloq xo'jaligi hayvonlarida og'ir alimentar toksikozlar keltirib chiqaradi. A.fumigatus turli oziq-ovqatlarda, araxis, mevalarda, sharbatlarda, go'sht maxsulotlarida aniqlanadi.

Aspergillus Mich avlodiga mansub zamburug'lar yuqori metabolik faollikka va moslashuvchanlik xususiyatiga ega. Ushbu zamburug'lar keng va ko'plab turli substratlarda tarqalgan, biroq ularning boshlang'ich yashash joylari tuproq hisoblanadi, asosan issiq iqlimga ega hududlarda tarqalgan bo'ladi.

Ushbu avlodning bir necha turlari shartli patogen mikroorganizmlar va bir qator toksik moddalar produsentlari hisoblanadi. Ushbu zamburug'larning eng keng tarqalgan mikotoksinlari aflatoksinlar (A.flavus, A.parasiticus), oxratoksinlar (A.ochraeus) va sterigmatosistin (A.versicolor, A.nidulans) hisoblanadi. Ushbu sanab o'tilgan oziq-ovqatlar va yemlarning tabiiy kontaminantlari hisoblanuvchi toksinlar va ularning produsentlarining o'rganilganligi atrof muhitni himoya qilish, odamlar salomatligi va hayvonlar salomatligini himoya qilish bilan bog'liqdir. Aspergillus avlodiga mansub zamburug'lar kamdan-kam alkaloidlar bilan yodga olinadi, ularning ko'pchiligi odam va hayvonlar organizmiga toksik ta'sir ko'rsatadi va substratlarda to'planib qolish xususiyatiga ega. Shu sababli ushbu metabolitlarni tadqiq qilish va Aspergillus avlodiga mansub zamburug'larning izolyatlari va turlarning sintetik xususiyatlarini aniqlash muhim ahamiyatga ega.

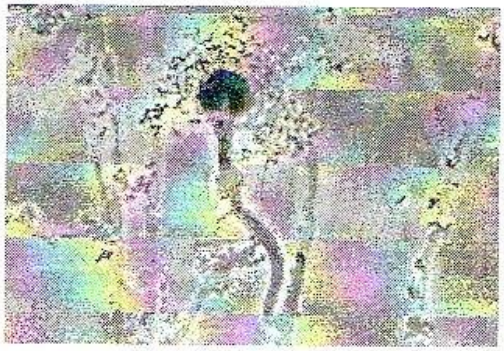
Tuproq tarkibidagi aspergillalarni o'rganish jarayonida Aspergillus avlodiga tegishli bir nechta izolyatlardan tashkil topgan, kolleksiyasi yaratildi: A.niger, A.phoenicis, A. fumigatus, A. flavus, A. versicolor, A. ustus, A. clavatus, A. ochraceus.

Tadqiq qilinayotgan kulturalarning ikkilamchi metabolitlarni tahlil qilishda uchta indol tutuvchi alkaloidlar guruhiga asosiy e'tibor qaratildi: klavin alkaloidlari, alfa-siklopiazon kislota (SPK) va diketopiperazin alkaloidlari.

Barcha o'rganilgan kulturalar orasida klavin alkaloidlari A.fumigatusning 13 izolyatlar orasidan 6 tasida aniqlandi, bu 46%ni tashkil qiladi. Ushbu guruh fumigaklavin B. deb aniqlangan bitta metabolit bilan ko'rsatildi. Boshqa aspergilla turlari klavin alkaloidlari sintezlamadi.SPKning toksikligi tufayli o'ziga katta e'tibor tortmoqda, va oxirgi tadqiqotlar shuni ko'rsatdiki, Aspergillus va Penicillium avlodlariga mnsub zamburug'larining keng tarqalganligini ko'rsatdi. Ushbu mikotoksin o'rganilgan izolyatlarning 26.5% da aniqlandi, bular: A.fumigatus, A.flavus, A.versicolor, A.phoenicis, A.clayatus. Ushbu tuproq izolyatlarning metabolitlari ham shimoliy ham janubiy hududlarda aniqlangan.Indol tutuvchi diketopiperazin alkaloidlari Aspergillus avlodiga mansub zamburug'larida keng tarqalgan. Aniqlangan metabolitlarning ushbu sinfi to'rt turdagi – A.flavus. A.fumigatus, A.clavatus, A.ochraceus izolyatlarida aniqlangan.

SPK qatorida A.flavus turi vakillari 2 metabolit xos hisoblanadi: N-metilfenilalaniltriptofanildiketopiperazin va uning dimeri ditriptofenalin. Ditriptofenalin o'rganilgan 19 izolyatning 14 tasida aniqlandi, N- metilfenilalaniltriptofanildiketopiperazin bo'lsa faqatgina 5 izolyatda aniqlandi.

Tuproqdan ajratilgan aspergillus fumigatusnin petri likobchasida o'stirilgan koloniyalari



Aspergillus fumigatus

SPK va fumigaklavin B. dan boshqa A.fumigatosning boshqa turlarini o'rganilganda, ular yana beshta indol tutuvchi diketopiperazin alkaloidlari sintez qilishi aniqlandi, ular A.fumigatusnga xos metabolitlar va bitta biosintetik zanjir bilan bog'langan, brevianamid F (proliltriptofanildiketopiperazin), fumitremorgin S va verrukulogen deb aniqlandi. Ikki minor metabolitlar dimetilallil radikal tutgan brevianamid F.ning hosilalarini o'zida aks ettirdi.
A.flavusning ko'plab izolyatlari va A.fumigatusning barcha izolyatlari u yoki bu indol tutuvchi alkaloidlar sintez qilishi aniqlandi, ularning ko'pchiligi havfli mikotoksinlarga kiradi: SPK – nefrotoksin va mutagen, verrukulogen va fumitremorgin S – tremorgen toksinlar guruhi. Aspergillalarning ushbu turlari oziq-ovqat va hayvon ozuqalarining eng havfli kontaminantlari hisoblanadi.

9 turdagi A.ochraceusning to'rttasida va A.clavatusning bitta izolyatida Brevianamid F aniqlandi. A.ustus va A.nigerdan boshqa Aspergillus avlodiga mansub turlar alkaloid tabiatga ega indol tutuvchi mikotoksinlarning faol produsentlari hisoblanishi aniqlandi: SPK, klavin va diketopiperazin alkaloidlar.

Adabiyotlar

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AFLOTOKSINLARNI YUQORI SAMARALI SUYUQLIK XROMOTOGRAFIYASIDA TAHLIL QILISH

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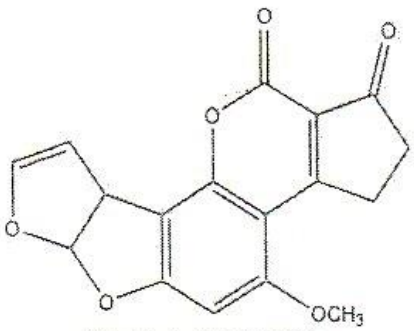
Mikotoksinlar – mikroskopik mog'or zamburug'larining yashash jarayonida ishlab chiqaruvchi ikkilamchi metabolitlari hisoblanadi – ular o'simlik, hayvon ozuqa maxsulotlarini ifloslantiradi, va inson hamda chorva hayvonlari salomatligiga jiddiy zarar yetkazishi mumkin. Oziq-ovqat maxsulotlarida ko'pincha mikotoksinlarning bir necha turi uchraydi, shu sababli ularning toksik ta'siri bir necha bor oshadi. Barcha mikotoksinlar bir necha sinflarga bo'linadi va o'zining xususiyatlari va tuzilishiga ko'ra farqlanadi, bu esa ularni aniqlashni qiyinlashtiradi.

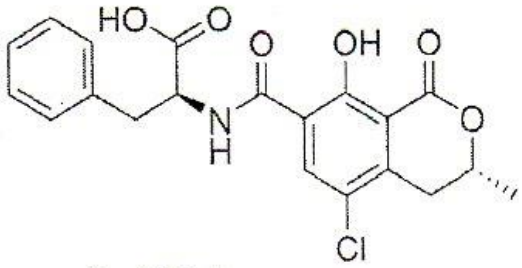
Hozirgi kunda 240 dan ortiq mog'or zamburug'lari ma'lum, ularning 100 ga yaqin turi toksik birikmalarni sintez qiladi, bu birikmalar odam va hayvon organizmida alimentar mikotoksikozlarni keltirib chiqaradi.

Mikotoksinlar orasida aflotoksinlar o'zining toksikligi va kanserogenligi, hamda keng tarqalganligi bilan ajralib turadi. Aflotoksinlar – Aspergillus flavus va Aspergillus parasiticus zamburug'lari ishlab chiqaradigan mikotoksinlar hisoblanadi. Ular kakao, kofe, mevalar, araxis, makkajo'xori va boshqa g'alla, moyli kulturalarning ifloslantiruvchilari hisoblanadi; kuchli gepatokanserogen ta'sir bilan ifodalanadi.

Aflotoksinlar – oq rangli kristall moddalar bo'lib, nisbatan issiqlikka chidamli, hamda oziq-ovqat maxsulotlarini pasterizatsiya jarayoni va oshxonada issiqlik bilan ishlov berish jarayonida buzilmaydi. Aflotoksinlar quyosh nuri va UB nurlar, shuningdek ishqor va oksidovchi moddalar ta'sirida parchalanadi. Ular dimetilsulfoksidda, asetonitrilda, spirtlarda, xloroformda va metilen xlorida yaxshi eriydi, suvda o'rtacha eriydi (o'rtacha 1dm^2 20mg), hamda geksanda, dietil efirda va petroley efirida deyarli erimaydi.



Aflotoksin B1 (AFB1)



Oxratoksin A

Aflotoksinning 4 turi mavjud AFB1, AFB2, AFG1, AFG2. Ularning biosintezi haroratga va muhitning namlik sharoitiga bog'liq bo'ladi. YuSSX oziq-ovqat tarkibidagi aflotoksinlarni aniqlashning umumiy usuli hisoblanadi.

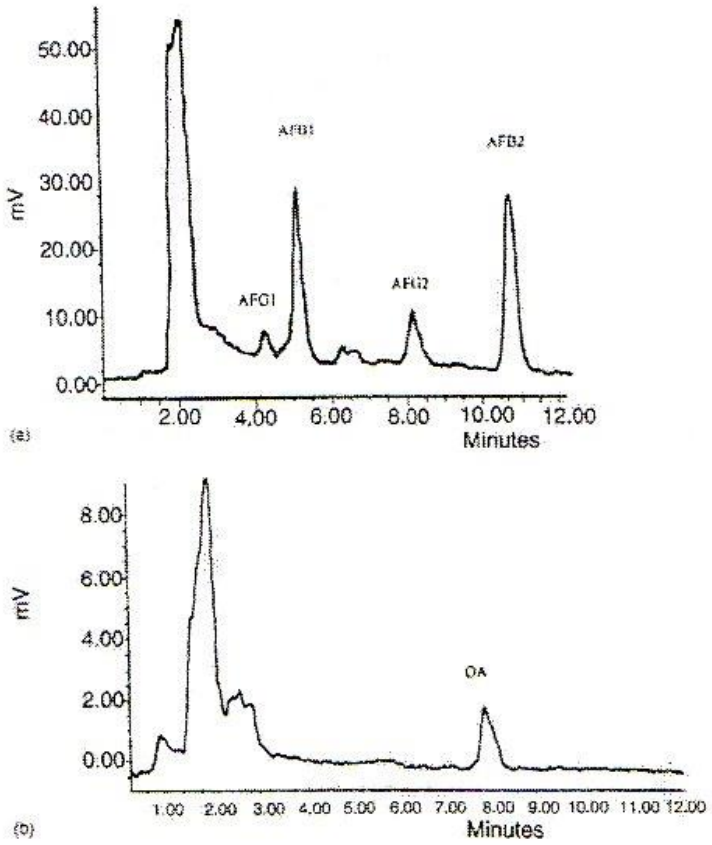
O'tkazilgan tajribada aflotoksinlarni yuqori samarali suyuqlik xromotografiyasida tahlil qilindi. Tajribani o'tkazish uchun laboratoriyada quyidagi ishlar amalga oshirildi.

Har bir aflotoksin turidan 0.25 ng/mL, 0.5 ng/mL, 1.25 ng/mL, 2.5 ng/mL (AFB1, AFB2, AFG1,AFG2) standart ishchi eritmalari olindi va tayyorlangan standart eritmalarni methanol-asetonitril (50:50 v/v) bilan aralashtirib tayyorlandi. Oxratoksinning (OA) 0.04 ng/mL, 0.2 ng/mL, 2 ng/mL standart ishchi eritmalari olindi va tayyorlangan standart eritmalarni methanol-dionizatsiyalangan suv (50:50 v/v) bilan aralashtirib tayyorlandi.

Tajriba flouressensiya detektori mavjud, izokratik quvur bilan jihozlangan, avtosamplerli suyuqlik xromotografiyasida o'tkazildi.

Aflotoksinlarni tahlili uchun flouressensiya detektori 360 va 420 nm ga sozlandi va qo'zg'aluvchi faza dionizatsiyalangan suv-asetonitril-metanol (60:25:15 v/v) hajmda aralashmasi konsistensiyasida kiritildi. Oxratoksinlarni (OA) uchun flouressensiya detektori 333 va 470 nm ga sozlandi va qo'zg'aluvchi faza asetonitril-dionizatsiyalangan suv-sovuq sirka kislotasi (49.5:49.5:1 v/v) hajmda aralashmasi konsistensiyasida kiritildi.

Har bir qo'zg'aluvchi faza uchun oqim tezligi 1 mL/min tashkil qildi va standart ishchi eritmalar va havo orqali kiritiluvchi namunalar hajmi 50 µL ni tashkil qildi.



AFB1, AFG1 aflotoksinlarning yarimatsetal shakllari va AFB2, AFG2 aflotoksinlarning 0.25 ng/mL (a), oxratoksinning 0.1 ng/mLda (b), YuSSX-flouressensiyasi.

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