Romer Labs

# Guide to Mycotoxins

*[4th Edition]*

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Edited by E.M. Binder & R. Krska

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# ROMER LABS GUIDE TO MYCOTOXINS

*Edited by*

E.M. Binder and R. Krska

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# EDITORIAL

Dear reader,

The present *Romer Labs Guide to Mycotoxins* is a continuation of a successful series of booklets launched in 2000 and edited by John L. Richard. The initial idea of the *Guide,* which consisted of three volumes (Overview on Mycotoxins Vol. 1, Sampling and Sample Preparation for Mycotoxin Analysis Vol. 2 and A Total Quality Assurance Program for Risk Management of Mycotoxins Vol. 3), was to provide a concise and practical package of information to those who are dealing with mycotoxins on a more practical level. The *Romer Labs Guide* was very well received in the agro-food industry and was seen among professionals as a great field tool and practical resource for handling mycotoxin risks.

In this edition of the *Romer Labs Guide to Mycotoxins,* we want to give continuity to the idea of providing a toolset for mycotoxin risk management.

The authors who compiled these expert materials are among the most renowned specialists in their field. We are especially proud to have John L. Richard, the initiator of this series, taking part in this project. John has a long history with Romer Labs and has contributed significantly to the company's outstanding expertise in mycotoxin solutions.

We are also very pleased to have gained Rudolf Krska from the University of Natural Resources and Life Sciences, Vienna (BOKU) to co-author and co-edit this book. The achievements of his research group at the BOKU-department IFA-Tulln in the fields of LC-MS/MS mycotoxin analysis and masked mycotoxins are unrivalled. Prof. Krksa is also an experienced editor, as a member of the editorial board of numerous journals, including *Mycotoxin Research*, *The World Mycotoxin Journal and Food Additives and Contaminants*.

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# PREFACE

Dear reader,

The safety of food and feed has become of increasing concern for consumers, governments and producers as a result of the global marketplace and the rise in public awareness about health and quality. Several highly publicized global incidents related to chemical contaminants in food and feed have also attracted much media attention. Trace levels of chemical contaminants can originate from natural sources such as mycotoxins, which are secondary metabolites produced by fungi on agricultural commodities in the field and during storage under a wide range of climatic conditions. The Food and Agriculture Organisation (FAO) has estimated that 25% of the world's food crops are affected by mycotoxins, including many basic foodstuffs and animal feeds, as well as cash crops, such as coffee, which have high economic value. In fact, the percentage of samples which have been tested positive for mycotoxins in more recent studies is actually much higher due to the availability of ultra-sensitive high-performance analytical instrumentation, especially in modern liquid chromatography-mass spectrometry (LC-MS). More than 300 mycotoxins have been identified so far with widely different chemical structures and differing modes of action - some target the kidney, liver, or the immune system and some are carcinogenic. Common mycotoxins include trichothecenes, such as deoxynivalenol, fumonisins, zearalenone, ochratoxin A and aflatoxins. The potential health risks to animals and humans posed by food- and feed-borne mycotoxin intoxication have been recognized by national and international institutions and organisations such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organisation (WHO) and the FAO, which have addressed this problem by adopting regulatory limits for major mycotoxin classes and selected individual mycotoxins. The need to obey these regulatory limits has prompted the development of specific sampling plans and validated analytical methods for the determination of mycotoxins in various food and feed commodities, which will lead to improved exposure estimates and risk assessment strategies with respect to these toxic secondary metabolites. The *Romer Labs Guide to Mycotoxins* is a timely reflection of the research progress related to the significance, sampling and determination of mycotoxins.

The chemical diversity of mycotoxins and their occurrence in a wide range of agricultural commodities and foods poses a great challenge for sample separation and methods of analysis. In order to deal with the increasing demand for mycotoxin analyses, rapid screening methods for single mycotoxins or whole mycotoxin classes have been developed, which are mainly based on immunochemical techniques. Recently, highly sophisticated multi-analyte LC-MS based methods have become available which enable the simultaneous quantification of up to 320 bacterial and fungal metabolites, allowing for

a comprehensive assessment of the range of mycotoxins humans and animals are exposed to. Despite the enormous progress in mycotoxin analysis, major challenges remain. Among these are the determination of conjugated (masked) mycotoxins, the matrix effects observed when performing LC-MS(/MS) measurements, the lack of certified reference materials and the need for reliable rapid methods, particularly for the simultaneous quantification of mycotoxins in foods and feeds. Sampling, however, is still the major issue in mycotoxin analysis due to the sometimes very heterogeneous distribution of the toxic metabolites in agricultural commodities and products intended for human and animal consumption. The proper selection of a sample from the lot and subsequent steps undertaken to produce a portion for the determination of the mycotoxin of interest is essential for the production of sound analytical data. This is why the issue of sampling, as well as proper sampling plans, are also thoroughly discussed in the *Romer Labs Guide to Mycotoxins.*

We would like to express our gratitude to the distinguished contributors John Richard and Tom Whitaker, who both served for the National Center for Agricultural Utilization Research, ARS, USDA as well as Andrew Slate (formerly ARS). Rainer Schuhmacher from the Department IFA-Tulln of the University of Natural Resources and Life Sciences, Vienna (BOKU), Austria is also greatly acknowledged. Thanks to their efforts, the recent developments in all the areas and hot topics addressed above can be presented in the *Romer Labs Guide to Mycotoxins.*

We hope you enjoy reading this book.

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## CONTENTS

# 1. MYCOTOXINS - AN OVERVIEW

JOHN L. RICHARD

## 1.1. Mycotoxins defined

Mycotoxins are toxic chemical compounds produced by molds. They are not used as "building blocks" for the fungus body but are produced by the fungus for other reasons that are not yet fully understood. Perhaps, they function as insecticides, as many of these metabolites are, they might play a role in fighting against plant defense to the fungus, or maybe they assist the fungus in some way to compete in their ecological niche in nature. There are literally hundreds of mycotoxins, some of them are used as antibiotics and are familiar to us, such as penicillin, others are very dangerous as aflatoxins, one of the most potent carcinogens known, and still others, such as diacetoxyscirpenol, are considerably less well-known.

Mycotoxins have been around for as long as crops have been grown. Mycotoxin contamination and the severity of the mycotoxin problem vary from year to year and also from one geographic region to another. The dimension of the problem is illustrated by Adams and Motarjemi, stating "The FAO has estimated that up to 25% of the world's foods are significantly contaminated with mycotoxins (Original in: Smith JE, Solomons GL, Lewis CW, Anderson JG. Mycotoxins in human nutrition and health. European Commission CG XII, 1994)" (Adams and Motarjemi, 1999). Since mycotoxins were discovered as such and science started to describe and characterize them, there have been repeatedly problems associated with various mycotoxins and in various regions of the world. Many molds contaminate the crop during the growing season and others are seed borne and grow along with the plants, still others infect commodities during storage. Contamination and subsequent mycotoxin production may be influenced by the environmental conditions at specific times during the crop development or storage. For these reasons we really have no control over the formation of the mycotoxins in the field. As stated by Park and Stoloff "Mycotoxin contamination is unavoidable and unpredictable, which makes it a unique challenge to food safety"(Park and Stoloff, 1989). However, genetic and breeding studies may eventually provide for obtaining resistant or non-susceptible varieties of certain commodities for fungal contamination and/or mycotoxin formation.

The only proven way to determine, if grain or foods contain mycotoxins or not, is to test for them. However, one might suspect contamination if certain signs or characteristics appear in the grain. Such signs may be visually discolored kernels, musty odor, lighter weight than usual kernels, blanched kernels, or wrinkled kernels in grain.

The fungus that produced a mycotoxin may be absent from a sample due to a number of reasons because the fungus can be killed. However, the mycotoxin will remain unless it is chemically removed or altered. Processes such as ammoniation for aflatoxins can remove them from a commodity and other mycotoxins may be reduced by cleaning procedures, heating or certain processing steps in the manufacture of foods but they may just slightly change the compound and not completely remove its toxicity. Furthermore, most of these methods might not be allowed by law in some parts of the world.

Obviously, mycotoxins have been around for a long time and we really have learned most about them in the last half of the century. However, despite all our knowledge we still continue to have problems year after year throughout the world, ranging from death in animals, to development of tumors, effects on immunity, reproductive deficiency, staggers or tremorgenic problems, digestive distress, poor feed conversion and weight gains in animals. In certain areas of the world human diseases are attributed to mycotoxins and continue to occur (Propst et al. 2007). For all of these reasons most countries have regulatory levels for the occurrence of mycotoxins in certain commodities (FAO, 2004) or feeds and therefore, require grains and/or other commodities to be tested for those specific regulated mycotoxins.

Some mycotoxins occur more frequently in commodities than others and some are known to occur only in certain commodities. For specific information on a selected mycotoxin, please consult section 1.3 with the description of the most common mycotoxins.

Mycotoxins can either begin or continue to be formed in stored commodities and products if the fungus is still viable and the conditions for mycotoxin formation are appropriate. Of course, the commodity or product supplies the nutrients for the fungus but adequate moisture and appropriate temperature for mycotoxin formation are required as well. Therefore, it is important to store commodities and products at moisture contents below 14% and maintain adequate ventilation to keep the stored material dry. Problems can arise when these materials are stored in areas of high humidity. Additionally, cooling can cause condensation to occur and provide sufficient moisture for fungal production of mycotoxins. Finished products should not be stored for long periods of time as mycotoxin contamination can occur in these products. Thankfully, we can manage storage conditions to avoid mycotoxins, unlike the field situation.

## 1.2. A brief history of mycotoxins and mycotoxicoses

### 1.2.1. ANCIENT HISTORY

Until recent times, the true involvement of “mycotoxins” in the historical events of the world in general were quite conjectural because the events themselves were oftentimes not well-documented and the reference to molds were sometimes quite incidental.

However, historians examining these literary pieces of historical episodes have led us to clues of the probable major involvement of "mildew" and perhaps their metabolites in the outcome of these events. For instance, John Baldwin and John Marr, studying historical mycotoxicology, have noted the similarity of writings of the Dead Sea Scrolls on the need to "destroy a house of mildew" to recent outbreaks of modern day "sick buildings" where the fungus *Stachybotrys atra* has been associated with the airborne hazard causing the sickness of individuals housed therein. Their historical interest included a study of the Ten Plagues of Egypt. They concluded that the tenth plague resulted from the oldest son, his family and livestock consuming contaminated grain and dying because the oldest son was the first allowed to open the granaries and utilize any of the contents. They concurred with Dr. Regina Schoental, who first attributed the tenth plague to mycotoxin-contaminated grain (Schoental, 1980).

### 1.2.2. INFECTION BY FUNGI AND MYCOTOXIN FORMATION

Numerous historical writings included mushroom poisonings along with curious prophylactic and therapeutic measures for avoiding the effects of these well-known toxins. Medical texts, even in recent times, included only brief discussions of mushroom poisoning and ergotism as diseases caused by the ingestion of toxigenic fungi. Infectious diseases of mycotic etiology were quite well known and some scientists in this discipline included descriptions of certain mycoses but included remarks that some aspects of the pathologic picture appeared to be caused by a toxin elaborated into the tissue by the invading fungus as evidenced by this passage from Henrici's book "Molds, Yeasts and Actinomycetes" (1930): "It is not clearly understood how the pathogenic fungi injure the tissues. Although fibrosis and giant cell reactions about some lesions bear a resemblance to a foreign body reaction, the extensive necrosis and suppuration which occur in the center of most lesions cannot be readily explained in this way. Moreover, the experimental lesions produced with freshly isolated and highly virulent strains of some species, as *Aspergillus fumigatus* and *Candida albicans*, are so acute as to suggest that these diseases may be caused by the same mechanisms as those found in bacterial infections" (Henrici, 1930). He goes on to describe lesions in a pigeon caused by experimental inoculation with *A. fumigatus* and concludes: "Such findings naturally suggest that a potent toxin is formed." To make this story more complete, USDA scientists were able to show that *A. fumigatus* is capable of producing gliotoxin, a highly immunosuppressive mycotoxin, during the pathogenic state and subsequently showed that turkeys naturally infected with this organism possessed gliotoxin in their infected lung tissue (Richard and DeBey, 1995). This is only one example whereby a toxin is produced by a pathogenic fungus during the pathogenic state.

### 1.2.3. ERGOTISM - HISTORICAL PERSPECTIVE

Most mycotoxins are considered to cause disease in the recipient following ingestion of contaminated food. Such was the case whereby ergotism eventually gained recognition as a toxic disease although some believe that the Chinese used ergot preparations for medicinal purposes over 5000 years ago and there are some medical applications even nowadays. Originally thought to be caused by some supernatural being, ergotism produced a complex of symptoms and signs of disease having gastrointestinal, nervous, dermal, cardiovascular and reproductive system manifestations. This complex was likely due to the package of varied compounds embedded in the ergots and consumed along with the products made, usually bread, from ergotized grain. Accounts in the Middle Ages included descriptions of "St. Anthony's Fire" which in actuality was ergotism where individuals affected described the sensation of fire shooting from the tips of their appendages. Convulsive and gangrenous ergotism are the two major types of the disease and are likely caused by different classes of the ergot alkaloids. "Bewitchment" is often included in descriptions of the disease and likely was involved in the situation leading to the Salem Witchcraft Trials, Salem, Massachusetts (Caporael, 1976). Hallucinogenic properties are attributed to several of the ergot alkaloids including lysergic acid, one of the major components of the ergots. Witches were thought to be possessed by some evil spirit and were therefore persecuted and later exorcised by religious entities.

### 1.2.4. SECONDARY METABOLISM OF FUNGI

Although there is a vast literature on the formation of so-called "secondary metabolites" of fungi from the fermentation of various substrates, little attention was paid to the potential of any of these compounds being toxic. During the late 1800s and early 1900s fermentation technology advanced with these fungal metabolites being a major focus. Large collections of these metabolites were maintained and individual compounds were characterized and identified chemically. Among the leaders in the fungal fermentation area were the Japanese who were interested in solid state fermentation of substrates as food and many traditional Japanese foods are prepared in this manner. Because of their eventual concern for the safety of these foods the Japanese were some of the early investigators of mycotoxins and contributed to the understanding of the role of mycotoxins in human disease. The work of Dr. Raistrick's group in England added much to the numerous kinds of products attainable from fungal fermentation. He was one of the major contributors to the understanding of secondary metabolism by fungi. Of course, alcoholic fermentation studies led by such people as Embden, Meyerhof, Warburg, Parnas and others contributed much to our present day understanding of the breakdown of sugar into alcohol and other products during fermentation through a complex pathway involving the action of a myriad of enzymes possessed by fungi, especially yeasts.

### 1.2.5. ANTIBIOSIS

Essentially concomitant with the fermentation findings noted above developed the interest in antibiosis by fungal products. De Bary in 1879 noted this phenomenon occurring when two organisms were grown on a substrate and one caused the other to cease growing or otherwise inhibited its progress. This microbial antagonism was further investigated by numerous workers including Duchesne who noted that some fungi inhibited the growth of certain bacteria (Duchesne, 1897; Duckett, 1999). Of course Alexander Fleming's discovery of penicillin, produced by *Penicillium chrysogenum*, was a monument to the entire field of antibiosis (Flemming, 1929) but his discovery remained unnoticed until others in England and the United States demonstrated its potential as a cure for devastating diseases. Work at the then, Northern Regional Research Laboratory in Peoria, Illinois, under the leadership of Dr. Coghill, found that penicillin could be produced in large quantities using submerged culture techniques (Moyer and Coghill, 1946). Their earlier experience in fermentation of and production of gluconic acid proved beneficial in the production of penicillin. An important feature in their success was the finding of a strain of *Penicillum* from a rotten cantaloupe from a Peoria market that was capable of producing abundant quantities of penicillin under their cultural conditions (Raper and Fennell, 1946).

### 1.2.6. TOXICITY OF FUNGAL METABOLITES RECOGNIZED

We now know that the antibiotic industry blossomed into a major industry rather quickly and today volumes of information are available on the plethora of antibiotics from fungi for human and veterinary medical use. During the search for antibiotics an important feature became evident to the investigators and for that reason many proposed antibiotics were placed on the shelf and never made it to the drug store shelves. This feature was that the investigators included studies of the toxicity of antibiotics to animals. The interest in many proposed antibiotics waned because their toxicity precluded their clinical use. This was the first clue to many investigators that fungi indeed could produce toxins that could cause disease in humans and other animals because these toxic antibiotics had been produced by fungi. There was much interest during the early 1900s and into the mid-1900s on the deterioration of grains by fungi. Thus, we began to understand the moisture requirements for fungi to have these deleterious effects on our grains and the potential for toxic problems eventually became a realization.

### 1.2.7. TOXIC FUNGAL PROBLEM IN RUSSIA - EARLY UNDERSTANDINGS

Several of the following events occurred within the same time period of the early 1940s to the late 1950s. The episodes of a lethal disease occurring in Russia during

the early years of the Second World War are reasonably well-documented (Mayer, 1953a; 1953b), and the severity of the disease called "Alimentary Toxic Aleukia" (ATA) was greater than ergotism. The major problem arose when grain was left to overwinter in the fields and harvested the following spring. The apparent growth and toxin production by species of *Fusarium* was abundant and again products from the contaminated grain caused an incredibly vicious disease including signs and symptoms of vomiting, diarrhea, profuse sweating, swelling, necrosis of the oral cavity, hemorrhage from all body orifices, central nervous disorders, pulmonary complications and cardiovascular disturbances. The suspected toxin survived processing of foods from the contaminated grain and efforts were increased to find the causative toxin(s) for this disease. The most common species of fungi isolated from the involved grains were *Fusarium sporotrichioides*, *F. poae* and *Cladosporium epiphyllum*. The search for toxic compounds from these three species yielded some interesting compounds but they were never conclusively found to be the etiologic agent of the disease. Retrospectively, it was found that *F. sporotrichioides* is a significant producer of T-2 toxin and because the experimental results of toxicological studies in animals include most of the manifestations of disease found in the Orenburg district of the former Soviet Union in 1944, T-2 toxin is now conjecturally associated with ATA. In fact, T-2 toxin is formed by strains of the organism isolated from the original outbreak.

### 1.2.8. STACHYBOTRYOTOXICOSIS - HISTORICAL PERSPECTIVE

At the same time that the Russians were dealing with ATA, another serious disease was occurring in both horses and humans. Prior to this time horses were thought to be the only animal susceptible to this disease. This disease had occurred in Russia in the 1930s and caused the death of thousands of horses in a condition referred to in the Russian literature as "massovie zabolivanie" meaning "massive illness." The causative fungus, which was earlier thought to be harmless, was *Stachybotrys atra*. The toxins from this organism produced disease with some similarities to ATA but with a major effect being blood disturbances causing hemorrhage and other changes in blood chemistry. The chemical nature of the toxins for this disease, now known as Stachybotryotoxicosis, is similar to those causing ATA. Because the Russian army moved through the use of horses this outbreak in the 1940s caused severe mobility problems for them as indicated in Khruchev's memoirs. In the United States this condition was occurring sporadically and Forgacs and coworkers found that other animals were affected as well as horses. Forgacs had a significant effect upon the entire area of mycotoxicology because of the results of his efforts in examining some of the outbreaks of Stachybotryotoxicosis as well as a condition called Moldy Corn Toxicosis (Forgacs, 1965). Others had reported these diseases occurring in the United States, but Forgacs and coworkers were convinced of the toxic nature of the outbreaks and began a more systematic approach to looking at the toxic metabolites of the fungi isolated from the substrates involved in these disease outbreaks. His examination of

fungi from problems in poultry, swine, horses and cattle and the strong conviction that toxigenic fungi were the cause for major economic problems in livestock and humans are evident in two publications (Forgacs and Carll, 1962; Forgacs, 1962) of significance to the discipline of mycotoxicology. As far as can be determined, Dr. Forgacs was the first person to use the words mycotoxin and mycotoxicosis.

### 1.2.9. FACIAL ECZEMA - HISTORICAL PERSPECTIVE

A disease of importance that had plagued New Zealand's sheep industry was also determined to be caused by a toxic component of a fungus occurring on rye grasses. This disease was a primary liver disease but the manifestation of the disease was a photosensitization resulting from the inability of the affected animals to eliminate a chlorophyll breakdown product from the blood. This compound absorbed energy from the sun and caused necrosis of skin especially in the non-wooled areas such as the face of the sheep. The disease was called facial eczema and was economically a devastating disease in New Zealand. By the late 1950s it was recognized that this disease was attributed to a toxic entity of the fungus, at that time called *Sporidesmium bakeri* (Thorton, 1959; Hore, 1960). The toxin that was eventually isolated from the spores (conidia) of the fungus from the ryegrass was called sporidesmin (Synge and White, 1959). The fungus now is called *Pithomyces chartarum* and the disease continues to be a problem involving contamination of pasture grasses with this organism in New Zealand.

### 1.2.10. MODERN MYCOTOXICOLOGY - AFLATOXIN DISCOVERED

The advent of modern mycotoxicology was the discovery of aflatoxins in peanut meal incorporated in the feed of several animal species, including turkeys and chickens in England in 1961. Blount described this outbreak quite effectively (Blount, 1961) and initially it was known as Turkey X disease until the etiology was considered as aflatoxins. The establishment of this cause and effect relationship was the initiation of a voluminous literature on aflatoxins and on mycotoxins in general. Furthermore the finding that aflatoxins were the most potent carcinogens known allowed for funding of research into this important area of investigation. Although one Russian paper earlier noted immunosuppression by aflatoxin, a group of scientists at the USDA, ARS, National Animal Disease Center in Ames, Iowa pioneered the demonstration of the variety of immunosuppressive effects of aflatoxins. Their research was oriented toward demonstrating the effects of aflatoxins on acquired resistance in poultry, complement activity in guinea pigs, phagocytosis by rabbit alveolar macrophages, lymphocyte stimulation by mitogens, delayed cutaneous hyper-sensitivity and resistance of animals to infectious disease agents. These findings were of unique importance to the full understanding of the biological effects of the aflatoxins and led ultimately to

a worldwide scientific effort to investigate immunomodulation by many mycotoxins (Richard, 2008).

### 1.2.11. MYCOTOXINS - FROM STORAGE TO FIELD PROBLEM

Another important finding in the history of mycotoxins is that during the early years of the aflatoxin problems and studies, it was considered that mycotoxins were only a problem associated with stored grains and other commodities. However, in the early to mid-1970s, it became apparent that corn could become contaminated with aflatoxin in the field because corn at harvest was often contaminated with this potent mycotoxin (Lillejoj et al., 1976). Now we had a new twist to the mycotoxin problem as it was no longer just a storage phenomenon, we also had a plant pathological problem. Subsequently, investigations surrounded the environmental conditions necessary for the development of aflatoxins in corn, peanuts and cottonseed in the field. These efforts eventually expanded to certain tree nut crops as well because they were sometimes contaminated with aflatoxins at harvest. For some of the mycotoxins we now understand more about the conditions necessary for infection by the fungus and formation of the mycotoxins in the field. In conjunction with these efforts there has been a search for varieties of commodities resistant to the growth of the causative organisms and mycotoxin formation, effects of agronomic practices, biocompetition and other interactive phenomena involved in the occurrence of aflatoxin and other mycotoxins in our commodities. Obviously, the study of mycotoxins and mycotoxicoses rapidly became a multidisciplinary activity involving cooperation among such investigators as the analytical chemist, the microbiologist, the agronomist, the agricultural engineer, the entomologist, the plant pathologist, the crop breeder and geneticist, the veterinary and medical practitioner and the farmer/rancher.

### 1.2.12. ANALYSIS - AID TO UNDERSTANDING THE MYCOTOXIN PROBLEM

Along with the discovery of the mycotoxins came an ever increasing need for critical analytical procedures for them. Following the discovery of the aflatoxins, thin layer chromatography was the most often used procedure for analysis, however, rapid expansion of methods became available and high performance liquid chromatography has become the reference method of choice. These techniques rapidly expanded to the analysis of other discovered mycotoxins and secondary metabolites of fungi. As mycotoxins were discovered in a variety of matrices, the need for cleanup procedures to eliminate interfering substances prior to analysis became more necessary for the variety of expanding technologies for the mycotoxins.

### 1.2.13. IN CONCLUSION

To summarize this discussion, the known diseases of humans and other animals that have a mycotoxin etiology are listed below. (This list is adapted from one previously discussed in Richard JL and Thurston JR (eds), Diagosis of Mycotoxicoses, Martinus Nijhoff Publishers, 1986, by Dr. Hesseltine CW ):

- Swine nephropathy in Denmark, Ireland and Sweden and poultry in Denmark
- Trout hepatoma in the United States and Italy
- Facial eczema in sheep in New Zealand
- Ergotism throughout the world
- Salivation factor in cattle and horses consuming fungal-infected legumes
- Dermatosis in humans handling infected celery
- Swine and poultry reproductive problems
- ATA in Russia and cattle deaths due to similar toxins
- Stachybotryotoxicosis in humans and other animal species
- Yellow rice poisoning in humans in Japan
- Vomition and corn rejection by swine
- Onyalai disease in humans in Africa
- Aflatoxicosis of numerous animal species
- Acute mortalities in humans in India and Africa caused by aflatoxin
- Sweet potato poisoning due to mold-damaged sweet potatoes
- Tremorgenic syndromes in swine and dogs
- Lupinosis in sheep in South Africa
- Maltoryzine poisoning in cattle
- Penicillic acid poisoning in swine
- Akakabibyo in the Orient
- Shoshin-Kakke - acute cardiac beriberi in humans
- Leucoencephalomalacia in horses
- Lung edema in swine
- Gliotoxin poisoning in turkeys and camels
- Ochratoxin poisoning in humans

- Fescue foot disease of cattle in the United States
- Hemorrhagic syndrome in poultry caused by aflatoxin
- Certain "sick building" syndromes associated with infestations of *Stachybotrys atra*.

The following are problematic insofar as mycotoxin involvement is discussed:

- Balkan endemic nephropathy
- Reye's syndrome in humans
- Kashin-Beck disease in humans
- Moldy bean disease in horses in Hokkaido
- Diplodiosis in South Africa
- Bermuda grass tremors in cattle in the southern United States
- Cyclopiazonic acid poisoning of livestock and poultry
- Bovine abortion due to corn silage
- Geeldikhop of sheep in South Africa
- Paspalum staggers in the Unites States
- Rubratoxin poisoning in the United States
- Epidemic polyuria in humans in India
- Penitrem intoxication in humans in the United States
- Citrinin poisoning of poultry
- Moldy sweet clover toxicity in cattle
- Hepatitis X in dogs
- Hyperkeratosis in cattle
- Secalonic acid intoxication in animals or humans
- Dendrodochium toxicosis of horses in Russia
- Esophageal cancer of humans in South Africa and China
- Beak lesions of unknown etiology in poultry
- Production problems in dairy cattle associated with corn silage.

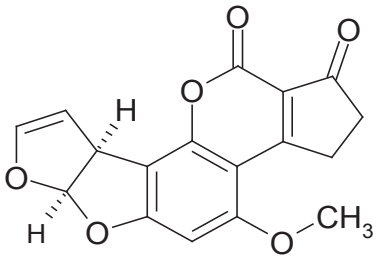
Other examples of diseases possibly can be added to this list, but the reader is given some idea of the magnitude of the mycotoxin problem as historical accounts and scientific evidence would allow. With this awareness and the increasing sensitivity,

specificity and selectivity of testing methods this list will continue to grow. Also, the interaction of the medical community with agricultural specialists in the investigation of outbreaks of potential mycotoxicoses occurring in our food will provide more evidence to further incriminate mycotoxins as significant agents of disease.

## 1.3. Characterization of the most common mycotoxins

Following are brief descriptions of individual mycotoxins. The toxins are presented in alphabetical order, not necessarily in order of importance.

### 1.3.1. AFLATOXINS




| | |
|---|---|
| Name: | **Aflatoxin $B_1$** |
| Formula: | $C_{17}H_{12}O_6$ |
| MW: | 312.3 |
| CAS No.: | 1162-65-8 |
| PubChem: | 14403 |

A group of toxins, consisting primarily of aflatoxin $B_1$, aflatoxin $B_2$, aflatoxin $G_1$, aflatoxin $G_2$ and aflatoxin $M_1$, are named for their respective innate fluorescent properties.

***Production and occurrence***

The major fungus producing aflatoxins is *Aspergillus flavus*. However, another fungus, *Aspergillus parasiticus* and a few other minor species of *Aspergillus* can also produce these toxins. *Aspergillus parasiticus* is especially important in peanuts. Not all strains of a given species are capable of aflatoxin production.

When grain such as corn is growing and there is warm ambient temperature (day >32°C; night >24°C), especially noted during drought conditions, the grain becomes more susceptible to aflatoxin formation. These stressful conditions are more prevalent in hot and dry environments (e.g. the southern United States, but can also occur in the Midwest (Corn Belt)). The organism survives in spores (conidia), which are carried by wind or insects to the growing crop. Any condition that interferes with the integrity of the seed coat allows the organism to gain entry into individual kernels. Insects such as sap beetles carry the organism into the developing ears especially those damaged by corn earworms and European corn borers. The two latter insects

can carry the organism into plants as well. Corn, cottonseed, peanuts and tree nuts are the main crops affected.

Yellow-green spore masses may be visible at sites of kernel damage or may follow an insect feeding path. If heavily damaged kernels are cracked open by hand and examined under a black light (long wave, 365 nm) they may fluoresce bright greenish-yellow (BGYF). This fluorescence is due to a kojic acid derivative formed by the organism that produces aflatoxin and therefore provides only a "presumptive" indication of the presence of aflatoxin and is not to be used as a positive test for aflatoxin. Individual kernels of corn may contain as high as 400,000 ppb (µg/kg) of aflatoxin, therefore, sampling is very important in analysis for levels of contamination in bulk grain lots.

Grains stored under high moisture/humidity (>14%) at warm temperatures (>20 ºC) or/and inadequately dried can potentially become contaminated. Grains must be kept dry, free of damage and free of insects; these conditions can result in mold "hot spots". Initial growth of fungi in grains can form sufficient moisture from metabolism to allow for further growth and mycotoxin formation.

### *Toxicity*

Aflatoxins can cause liver disease in animals, they are also carcinogenic with aflatoxin $B_1$ being the most potent carcinogen (WHO, 2002). Susceptibility varies with breed, species, age, dose, length of exposure and nutritional status. Aflatoxins may cause decreased production (milk, eggs, weight gains, etc.), are immunosuppressive, carcinogenic and mutagenic. Aflatoxins can be present in milk, meat, or eggs if consumed levels are sufficient. Aflatoxin $B_1$ is a human carcinogen and may play a role in the etiology of human liver cancer as speculated by Williams et al. (2004). Ammoniation and some adsorbents will reduce or eliminate the effects of aflatoxins, but can only be applied for animal feedingstuff and in certain countries or within specific states.

### *Regulations*

Of all mycotoxins regulated worldwide, aflatoxin is the most regulated. Many countries might have only legislation with limits for aflatoxins. Following a few examples:

**USA – FDA action levels (FDA, 2000)**

| *Commodity* | *Action level in µg/kg [ppb]* |
|---|---|
| Animal feeds | |
| Corn and peanut products intended for finishing (i.e. feedlot) beef cattle | 300 |
| Cottonseed meal intended for beef, cattle, swine, or poultry (regardless of age or breeding status) | 300 |

| *Commodity* | *Action level in µg/kg [ppb]* |
|---|---|
| Corn and peanut products intended for finishing swine of 100 pounds or greater | 200 |
| Corn and peanut products intended for breeding beef cattle, breeding swine, or mature poultry | 100 |
| Corn, peanut products, and other animal feeds and feed ingredients but excluding cottonseed meal, intended for immature animals | 20 |
| Corn, peanut products, cottonseed meal, and other animal feed ingredients intended for dairy animals, for animal species or uses not specified above, or when the intended use is not known | 20 |
| Brazil nuts | 20 |
| Foods | 20 |
| Peanuts and peanut products | 20 |
| Pistachio nuts | 20 |
| Milk | 0.5 |

**Europe – Legislation for food (EU, 2010b)**

| | *Maximum level in µg/kg [ppb]* | |
|---|---|---|
| | *Aflatoxin $B_1$* | *Sum of $B_1$, $B_2$, $G_1$ and $G_2$* |
| Groundnuts (peanuts) and other oilseeds, to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs, with the exception of:<br>— groundnuts (peanuts) and other oilseeds for crushing for refined vegetable oil production | 8.0 | 15.0 |
| Almonds, pistachios and apricot kernels to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs | 12.0 | 15.0 |

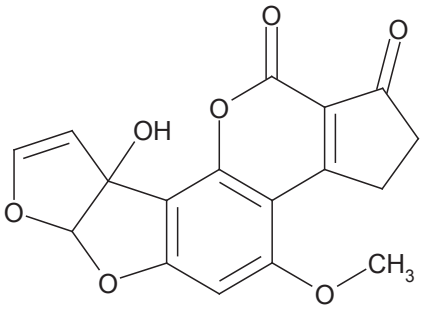
| | *Maximum level in µg/kg [ppb]* | |
|---|---|---|
| | *Aflatoxin $B_1$* | *Sum of $B_1$, $B_2$, $G_1$ and $G_2$,* |
| Hazelnuts and Brazil nuts, to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs | 8.0 | 15.0 |
| Tree nuts, other than the tree nuts listed above, to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs | 5.0 | 10.0 |
| Groundnuts (peanuts) and other oilseeds and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs, with the exception of:<br>— crude vegetable oils destined for refining<br>— refined vegetable oils | 2.0 | 4.0 |
| Almonds, pistachios and apricot kernels, intended for direct human consumption or use as an ingredient in foodstuffs | 8.0 | 10.0 |
| Hazelnuts and Brazil nuts, intended for direct human consumption or use as an ingredient in foodstuffs | 5.0 | 10.0 |
| Tree nuts, other than the tree nuts listed above, and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs | 2.0 | 4.0 |
| Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs | 5.0 | 10.0 |
| Dried fruit and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs | 2.0 | 4.0 |
| All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed above | 2.0 | 4.0 |
| Maize and rice to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs | 5.0 | 10.0 |

| | *Maximum level in µg/kg [ppb]* | |
|---|---|---|
| | *Aflatoxin $B_1$* | *Sum of $B_1$, $B_2$, $G_1$ and $G_2$,* |
| Following species of spices: *Capsicum spp.* (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika) *Piper spp.* (fruits thereof, including white and black pepper) *Myristica fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric) Mixtures of spices containing one or more of the abovementioned spices | 5.0 | 10.0 |
| Processed cereal-based foods and baby foods for infants and young children | 0.10 | |
| Dietary foods for special medical purposes intended specifically for infants | 0.10 | |

**Europe - Legislation for animal feed (EU, 2003)**

| | *Maximum concentration of aflatoxin $B_1$ in mg/kg [ppm] relative to a feedingstuff with a moisture content of 12 %* |
|---|---|
| All feed materials | 0.02 |
| Complete feedingstuffs for cattle, sheep and goats with the exception of: | 0.02 |
| — complete feedingstuffs for dairy animals | 0.005 |
| — complete feedingstuffs for calves and lambs | 0.01 |
| Complete feedingstuffs for pigs and poultry (except young animals) | 0.02 |
| Other complete feedingstuffs | 0.01 |
| Complementary feedingstuffs for cattle, sheep and goats (except complementary feedingstuffs for dairy animals, calves and lambs) | 0.02 |
| Complementary feedingstuffs for pigs and poultry (except young animals) | 0.02 |
| Other complementary feedingstuffs | 0.005 |

## 1.3.2. AFLATOXIN $M_1$




| | |
|---|---|
| Name: | **Aflatoxin $M_1$** |
| Formula: | $C_{17}H_{12}O_7$ |
| MW: | 328.3 |
| CAS No.: | 6795-23-9 |
| PubChem: | 23236 |

This mycotoxin is a hydroxylated metabolite of aflatoxin $B_1$, which was first found present in milk and can be detected in urine as well. It is not produced directly by *Aspergillus flavus* or any other aflatoxin-producing fungus. Therefore, it is not considered as a contaminant of feed grains.

### *Production and occurrence*

Aflatoxin $M_1$ is produced by the consuming animal in its liver, following ingestion of aflatoxin $B_1$. As much as 0.5-5% of aflatoxin $B_1$ will appear in the milk as aflatoxin $M_1$ (Patterson et al., 1980). When dairy cows with different milk yields were given aflatoxin $B_1$ on a daily basis, aflatoxin $M_1$ became detectable in milk 12-24 hours after the first ingestion. However, data obtained from several investigations suggest that 3-6 days of constant daily ingestion of aflatoxin $B_1$ is required before a steady excretion of aflatoxin $M_1$ is achieved. Aflatoxin $M_1$ becomes undetectable 2-4 days after the contaminated diet is withdrawn. Aflatoxin $M_1$ can also be excreted in other animals during lactation. A general "rule of thumb" is that about 1% of aflatoxin $B_1$ in feed will be converted to aflatoxin $M_1$ in milk of a dairy cow.

### *Toxicity*

Aflatoxin $M_1$ is somewhat less potent than aflatoxin $B_1$ in adults. It is the most potent hepatocarcinogen known in the rat and rainbow trout. Acute signs of exposure include vomiting, abdominal pain, pulmonary edema, and fatty infiltration and necrosis of the liver. The carcinogenicity of aflatoxin $M_1$ has been shown in studies with rainbow trout (Canton et al., 1975) and rats (Cullen et al. 1987). Unfortunately, processing of milk and dairy products in ways that are common to the dairy industry do not lead to a significant degradation of aflatoxin $M_1$ and therefore can occur in such products as cheese. Because the young of any species is more susceptible to aflatoxin than adults, aflatoxin $M_1$ becomes of concern through the young consuming milk.

***Regulations***

Similar to the other aflatoxins, aflatoxin $M_1$ regulation is found in most regulations. Following a few examples:

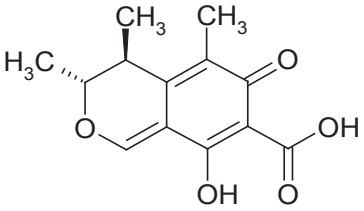
**USA – FDA action levels (FDA, 2000)**

| *Commodity* | *Action level in µg/kg [ppb]* |
|---|---|
| Milk | 0.5 |

**Europe (EU, 2010b)**

| | *Maximum level in µg/kg [ppb]* |
|---|---|
| | *Aflatoxin $M_1$* |
| Raw milk, heat-treated milk and milk for the manufacture of milk-based products | 0.050 |
| Infant formulae and follow-on formulae, including infant milk and follow-on milk | 0.025 |
| Dietary foods for special medical purposes intended specifically for infants | 0.025 |

1.3.3. CITRININ



Name: **Citrinin**

Formula: $C_{13}H_{14}O_5$
MW: 250.3
CAS No.: 518-75-2
PubChem: 219203

This is a yellow compound that is a phenol derivative. It is a lemon-yellow color when found on thin layer chromatograms viewed under visible light. The crystalline pure citrinin is also yellow.

***Production and occurrence***

The name of the compound came from the organism *Penicillium citrinum* from which this mycotoxin was first isolated. Since that time the compound has been shown to

be produced by several other *Penicillium* species and also by a select few species of *Aspergillus*.

Citrinin has been isolated from its natural occurrence in cereal grains such as wheat, barley, oats, rice and corn. Probably the major characteristic of its occurrence is that it often co-occurs with ochratoxin A in the cereals and most isolates of fungi that produce citrinin also produce ochratoxin A. The conditions under which citrinin occurs in the field are presumed to be similar to that for ochratoxin and levels have been found in cereal grains as high as 80 ppm. Unfortunately, little is known regarding the field occurrence of either ochratoxin or citrinin and therefore they are considered as storage problems in grains, although ochratoxin is known to occur in certain crops at harvest such as grapes but this is usually the result of production by some of the "black" aspergilli such as *A. carbonarius*.

Again, grains with any visible presence of mold should be suspect and especially if the fungi are identified and found to be species that are capable of citrinin production. Musty smelling grain should be suspect of any mycotoxins but only testing for the specific mycotoxins can be absolute proof.

It is likely that most of the citrinin in grains occurs during storage, at least until we gain further insight into the field occurrence. Therefore, grain should be adequately stored, kept dry and at <14% moisture and insects damage should be avoided or kept to a minimum. Maintaining the integrity of the seed coat and avoiding favorable moisture for fungal growth can keep mycotoxins from forming during storage.

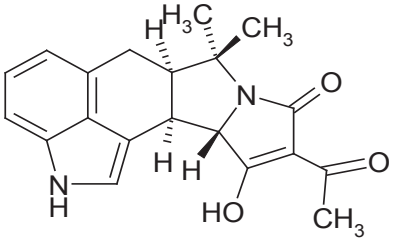
### *Toxicity*

Toxicity concerns for citrinin appear to be aimed toward poultry with the effects primarily on the kidney of these species. Regarding its relative toxicity, citrinin appears to be considerably less toxic to poultry than oosporein or ochratoxin A, two other important nephrotoxic mycotoxins. High levels of citrinin may affect the liver in addition to the kidney. Citrinin produces necrosis of the distal tubule epithelium in the kidney and is a pH dependent tautomer. As citrinin it is neutral but when it is excreted in the alkaline urine it becomes a phenol which is rapidly reabsorbed by the kidney where it tautomerizes back to citrinin. In poultry common symptoms of toxicity by citrinin includes increased water consumption and diarrhea. These symptoms have been caused by levels as low as 130 and 260 ppm dietary citrinin. The diarrhea appears to be caused by the increased urine excretion due to altered function and degenerative processes of the renal tubules and not due to gastrointestinal disturbances. It is highly likely that when citrinin and ochratoxin occur in combination in grain and then fed to animals, there can be an exacerbation of the effects because of the similarity of the effects of both toxins. Any search for either toxin should include the other as well.

### *Regulations*

No regulations are present for citrinin in commodities or any other product.

## 1.3.4. CYCLOPIAZONIC ACID



| | |
|---|---|
| Name: | **Cyclopiazonic Acid** |
| Formula: | $C_{20}H_{20}N_2O_3$ |
| MW: | 336.4 |
| CAS No.: | 18172-33-3 |
| PubChem: | 65261 |

It is a toxic compound (an indole tetramic acid) produced by several fungi. They are not fluorescent and must be visualized on thin layer chromatograms by spray reagents.

***Production and occurrence***

The compound was originally described from *Penicillium cyclopium*, thus the name. Other organisms include *P. patulum*, *P. viridicatum*, *P. puberulum*, *P. crustosum*, *P. camembertii*, *Aspergillus versicolor*, *A. oryzae*, *A. tamarii* and *A. flavus*. The latter may be of considerable significance as many isolates produce this compound.

Cyclopiazonic acid was first described from stored corn in Iowa as well as from corn taken directly from the field (Gallagher et al., 1978). Therefore, the compound is formed in the field, at least in corn, and likely the conditions favoring aflatoxin formation in this crop are similar for cyclopiazonic acid (i.e., high temperatures and drought stress). In fact, it appears that cyclopiazonic acid is produced in preference to aflatoxin at higher temperatures in isolates of *Aspergillus flavus* that produce both compounds. This compound has also been found in peanuts, sunflower seeds (screenings), kodo millet and cheese likely as naturally occurring. The exact conditions necessary for their occurrence in these commodities or products is not known. Some of the isolates of the producing organisms are used in fermented foods so selection must be made to use isolates that do not produce cyclopiazonic acid.

Most of the commodities on which cyclopiazonic acid occurred had evidence of mold damage but where *A. flavus* is the producing organism there may be little or no evidence of the presence of the mold. The organisms involved with producing this compound are important as storage fungi growing saprophytically on grain or they may be important plant pathogens.

As mentioned most of the fungi are likely involved in contaminating commodities while in storage but certainly field contamination occurs as well for those commodities noted above. Again, grain should be stored below 14% moisture and kept this way to avoid fungal growth and toxin production. Condensation should be avoided to control mold growth as once initiated the fungus can create sufficient moisture of metabolism to allow for further growth and mycotoxin formation.

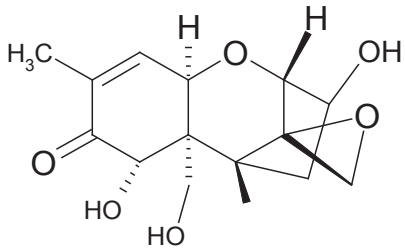
***Toxicity***

Cyclopiazonic acid may be important as a single mycotoxin or as a co-contaminant as some isolates of fungi may produce other mycotoxins simultaneously. In fact, the original outbreak of aflatoxin poisoning likely included cyclopiazonic acid in the toxic episode in poultry. Cyclopiazonic acid accumulates in skeletal muscle of selected animals and human exposure may occur through ingestion on contaminated muscle tissue. It has been shown to be toxic in several animal species including swine, chickens, turkeys, guinea pigs, rats and dogs (Purchase, 1971). Toxic evidence in animals, depending upon the species, includes gastrointestinal changes of necrosis and inflammation, hepatitis, kidney lesions and in coordination due to effects on muscle tissue (Norred, 1990). The importance of this compound in immunosuppression has been studied with little significance on this system. Just recently, a number of publications shed more light on the effects of cyclopiazonic acid in turkeys (Abbas, 2011; Miller et al., 2011a; Miller et al., 2011b; Miller et al., 2011c).

***Regulations***

No regulations are present for cyclopiazonic acid in commodities or any other product.

## 1.3.5. DEOXYNIVALENOL




Name: **Deoxynivalenol (DON)**

Formula: $C_{15}H_{20}O_6$
MW: 296.3
CAS No.: 51481-10-8
PubChem: 40024

Deoxynivalenol is also known as vomitoxin or DON. It may co-exist with other toxins produced by the same organism that produces this toxin, especially zearalenone.

***Production and occurrence***

*Fusarium graminearum* is the principal DON-producing fungus in grains but *Fusarium culmorum* is often involved as well especially in certain geographical areas of the world.

Corn and small grains such as wheat, oats and barley are the major crops affected. The organism survives on old infected residue left on the field from the previous season, providing an inoculum source for the new crop. The organism does well in cool, moist conditions with contamination of the crop occurring when spores (conidia)

of the organism are windblown to the silks of the corn and in small grain to the anthers (male portions of the flower) which emerge outside the floret during what is called anthesis. The fungus penetrates the host ear or floret and produces the disease and DON. In wheat, it appears that DON production is necessary for the organism to produce the disease.

In corn the "ear rot" produced by *F. graminearum* may appear as purple to pink stained kernels with visible pink mold growth over the affected areas of the ear. Sometimes the growth of the fungus will appear through the husk as pink growth and staining and the entire ear will be affected. Wheat heads may appear prematurely ripe and the ripe kernels will have a blanched appearance (tombstone kernels) and may have pink stain present from the fungus. This is not quite as evident in barley kernels, but oat kernels will have pink staining as well. The disease in wheat is called head blight, scab or pink scab.

Storage under good conditions (<14% moisture) will minimize further elaboration of the toxin by these toxigenic fungi. Conditions favorable to mold growth should be avoided as well as insect pests and moisture. Generally, storage is not considered a problem for DON contaminated wheat and corn that has matured and been stored at moisture percentages below 14%.

### *Toxicity*

Swine are the animals most usually affected by this toxin and exhibit reduced intake of contaminated grain, if they do eat it, they may vomit. Levels above 1 ppm are considered potentially harmful to these animals. Pet foods prepared with wheat contaminated with this toxin have been involved in acute toxicities. DON is a known immunosuppressant and may cause kidney problems. Humans are thought to exhibit a similar vomition syndrome when consuming DON-contaminated grain. DON does not appear to carry over into tissues or fluids of animals consuming toxic levels. Baking and malting are adversely affected by contaminated wheat and barley.

### *Regulations*

While DON is equally regulated for food as aflatoxin in the European Union but has only recommended maximum levels for feed, in the US only advisory levels exist.

**USA – FDA advisory levels (FDA, 2010)**

- 1 ppm DON on finished wheat products, e.g. flour, bran, and germ that may potentially be consumed by humans. FDA is not stating an advisory level for wheat intended for milling because normal manufacturing practices and additional technology available to millers can substantially reduce DON levels in the finished wheat product from those found in the original raw wheat. Because there is significant variability in manufacturing processes, an advisory level for raw wheat is not practical.

- 10 ppm DON on grains and grain by-products (on an 88% dry matter basis) and 30 ppm in distillers grains, brewers grains, and gluten feeds and gluten meals derived from grains (on an 88% dry matter basis) destined for ruminating beef and feedlot cattle older than 4 months and ruminating dairy cattle older than 4 months, with the added recommendations that the total ration for ruminating beef and feedlot cattle older than 4 months not exceed 10 ppm DON, and the total ration for ruminating dairy cattle older than 4 months not exceed 5 ppm DON. For chickens, 10 ppm DON on grains and grain by-products with the added recommendation that these ingredients not exceed 50% of the diet of chickens.
- 5 ppm DON on grains and grain by-products destined for swine with the added recommendation that these ingredients not exceed 20% of their diet.
- 5 ppm DON on grains and grain by-products destined for all other animals with the added recommendation that these ingredients not exceed 40% of their diet.

**Europe – Legislation for food (EU, 2006b; 2007)**

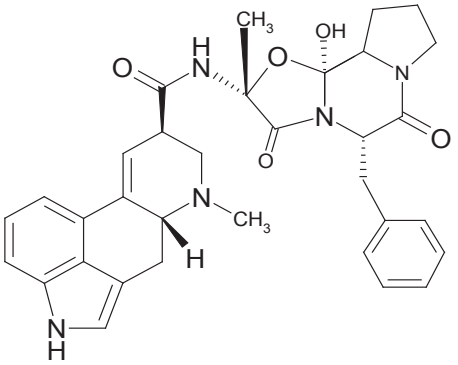
| | *Maximum levels in µg/kg [ppb]* |
|---|---|
| | *Deoxynivalenol* |
| Unprocessed cereals other than durum wheat, oats and maize | 1250 |
| Unprocessed durum wheat and oats | 1750 |
| Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling | 1750 |
| Cereals intended for direct human consumption, cereal flour (including maize flour, maize meal and maize grits), bran as end product marketed for direct human consumption and germ, with the exception of processed cereal-based foods and baby foods for infants and young children | 750 |
| Pasta (dry) | 750 |
| Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals | 500 |
| Processed cereal-based foods and baby foods for infants and young children | 200 |
| Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10 | 750 |

| | *Maximum levels in µg/kg [ppb]* |
|---|---|
| | *Deoxynivalenol* |
| Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10 | 1250 |

**Europe - Recommendation for feed (EU, 2006a)**

| | *Guidance value in mg/kg [ppm] deoxynivalenol relative to a feedingstuff with a moisture content of 12 %* |
|---|---|
| Feed materials | |
| — Cereals and cereal products with the exception of maize by-products | 8 |
| — Maize by-products | 12 |
| Complementary and complete feedingstuffs with the exception of: | 5 |
| — Complementary and complete feedingstuffs for pigs | 0.9 |
| — Complementary and complete feedingstuffs for calves (< 4 months), lambs and kids | 2 |

1.3.6. ERGOT




Name: **Ergotamine**

Formula: $C_{33}H_{35}N_5O_5$
MW: 581.7
CAS No.: 113-15-5
PubChem: 3251

Ergot alkaloids are a large group of compounds produced by fungi that attack a wide variety of grass species, including small grains, during the growing season. These compounds are chemically divided into the clavine alkaloids, lysergic acids, simple lysergic acid amides and peptide alkaloids. Two common alkaloids examined for in ergot are ergotamine and ergovaline.

### *Production and occurrence*

The major ergot fungus is *Claviceps* which produces sclerotia in several grass species with *C. purpurea* being the most commonly found species. However, *C. fusiformis* has produced ergot in pearl millet. *C. paspali* has been associated with problems in Dallis grass poisonings. Ergot does occur in sorghum and is caused by the organism *Sphacelia sorghi*. While other fungi are also capable of producing ergot alkaloid these are the major species that are producers of ergot in grain.

The entire life cycle of the organism *Claviceps* is quite complex but for simplicity, this organism and the other fungi mentioned above replace the developing ovaries of the developing seed with hard masses of fungal tissue called sclerotia (sometimes called "Ergots"). The sclerotia are brown to purple-black in color and contain the ergot alkaloids. The fungus gains entry into the host plant from ergots that have been in the soil. The infecting fungal elements are assisted by wind and splashing rain in gaining access to the host plant where the florets are invaded with subsequent development of sclerotia. The fungus uses nutrients from the plant for development of the ergots and biosynthesis of the ergot alkaloids. The ergots are harvested with the grain and if not eliminated by screening or some other process they can end up in feed or food made from the contaminated grain. Ergot is not a storage initiated problem but the ergots can be present in stored grains resulting from harvesting of ergots along with the grain.

### *Toxicity*

Ergotism is one of the oldest known mycotoxicoses with ancient records of its occurrence. One of the most publicized events was the human epidemics produced by ergot in the Middle Ages known as St. Anthony's fire with symptoms of gangrene, central nervous and gastrointestinal effects. Animals are affected similarly to what has been observed in humans. In swine agalactia has been attributed to ergot alkaloids. The loss of ears and other appendages is a common effect of ergot in animals. Two types of ergotism have been described; gangrenous and convulsive. The differences may be due to the different kinds of alkaloids present in the ergot as variations in amount and kinds of alkaloids can occur in the ergot (sclerotia). Recent outbreaks have occurred in Ethiopia (1978) where gangrene and loss of limbs occurred and in India (1975) where the effects were more of the nervous type symptoms of giddiness, drowsiness, nausea and vomiting. Ergometrine was the alkaloid found in the Ethiopian sclerotia and in India the clavine alkaloids agroclavine, elymoclavine, chanoclavine, penniclavine and setoclavine were found. The ergots produced in these two outbreaks were caused by different species

of *Claviceps*. Because some of the ergot alkaloids are vasoconstrictive and have other beneficial pharmacological properties, they have been used therapeutically. In the United States, most of the widely grown tall fescue posesses an endophytic fungus called *Neotyphodium coenophialum*. This endophyte produces ergovaline, an ergopeptine, which can, if consumed levels are sufficient, produce ergot-like toxicosis in animals grazed on pastures containing the fescue grass (CAST, 2003).

***Regulations***

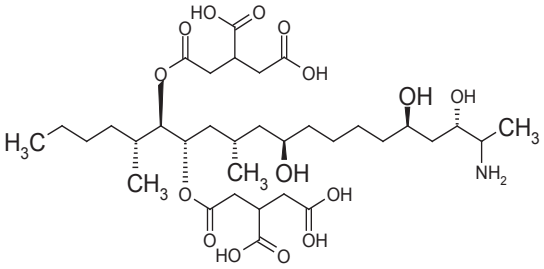
**USA**

There are no regulatory actions for ergot in grain, but the GIPSA classifies grain containing 0.05% or more ergots as "ergoty".

**Europe**

Also in Europe, while there is discussion on establishing legal limits, so far no regulation exists. However, the European Food Safety Agency (EFSA, 2005) addressed the question stating their opinion as base for possible future legislation.

## 1.3.7. FUMONISINS



| | |
|---|---|
| Name: | **Fumonisin $B_1$** |
| Formula: | $C_{34}H_{59}NO_{15}$ |
| MW: | 721.8 |
| CAS No.: | 116355-83-0 |
| PubChem: | 3431 |

Fumonisins are a group of toxins, primarily, $FB_1$, $FB_2$, $FB_3$. They are not fluorescent and were discovered in 1988, in South Africa (Gelderblom et al., 1988; Marasas, 2001)

***Production and occurrence***

There are two major producers, *Fusarium verticillioides* and *Fusarium proliferatum*, however other closely related species are capable of producing these toxins but are less important in grains. All strains are considerably variable in toxin producing ability.

Corn is the major commodity affected by this group of toxins, although a few occurrences have been reported in rice and sorghum. Fumonisins have been reported in barley but this awaits confirmation in further samples. The exact conditions for

disease are not known but drought stress followed by warm, wet weather during flowering seems to be important. Insect damage to maturing corn ears allows for environmentally present strains of the organism to enter the ear and kernels. Wet weather just prior to harvest may exacerbate the contamination with fumonisins in corn. However, the organism is present in virtually every seed and is present in the corn plant throughout its growth and therefore is present in the ears and kernels. Sometimes there is considerable amount of fumonisins present in symptomless kernels of corn.

As mentioned, some corn kernels may have no evidence of infection as the organism is internal and capable of toxin production. Other corn may demonstrate "pink kernel rot" with a closely adhering organism on the kernels. Sometimes the kernels will be covered with white fungal growth instead of pink. Those kernels with insect or bird damage or broken kernels will often contain the highest levels of toxin. Thus, corn screenings will contain the highest levels of toxins and are often found to be the cause of animal toxicoses. In rice, fumonisins have been found to be present where sheath rot disease is present.

Grains should be harvested without kernel damage, screened and dried to a level of moisture suitable for storage (<14%). Conditions favorable to mold growth likely will cause the further formation of fumonisins in storage. Grains should be kept free of additional moisture or insects. At this time, not much information is available for storage of fumonisin-contaminated corn, but cleaning can considerably reduce the concentration levels in corn.

### *Toxicity*

A major disease of horses that includes a softening of the white matter in the brains (leukoencephalomalacia) is caused by the fumonisins (Marasas et al., 1988). Swine lung edema is also produced by the fumonisins (Harrison et al., 1990; Ross et al., 1990). Other diseases such as liver disease and tumors have been noted in rodents (Voss et al., 2001). The fumonisins are tumor promoters and one study demonstrated total carcinogenesis, which has been confirmed in a two year study by the FDA. It is not known whether the fumonisins are truly involved in causing esophageal tumors in certain human populations. Fumonisins remain as suspect entities in neural tube defects in humans in certain regions of the world and is a current area of investigation (Marasas et al., 2004). Regardless of the other effects on animals, the liver is often involved in the toxicity. There is no carryover of fumonisins into milk in cattle and there appears to be little absorption of toxin in tissues but what little is rapidly taken up is rapidly eliminated.

### *Regulations*

**USA – FDA guidance levels (FDA, 2001)**

For human foods:

| *Product* | *Total fumonisins ($FB_1$+$FB_2$+$FB_3$) in mg/kg [ppm]* |
|---|---|
| Degermed dry milled corn products (e.g. flaking grits, corn grits, corn meal, corn flour with fat content of < 2.25%, dry weight basis) | 5 |
| Whole or partially degermed dry milled corn products (e.g. flaking grits, corn grits, corn meal, corn flour with fat content of > 2.25 %, dry weight basis) | 4 |
| Dry milled corn bran | 4 |
| Cleaned corn intended for masa production | 4 |
| Cleaned corn intended for popcorn | 3 |

For animal feeds:

| *Corn and corn by-products intended for:* | *Total fumonisins ($FB_1$+$FB_2$+$FB_3$) in mg/kg [ppm]* |
|---|---|
| Equids and rabbits | 5 (no more than 20% of diet) |
| Swine and catfish | 20 (no more than 50% of diet) |
| Breeding ruminants, breeding poultry and breeding mink | 30 (no more than 50% of diet) |
| Ruminants > 3 months old being raised for slaughter and mink being raised for pelt production | 60 (no more than 60% of diet) |
| Poultry being raised for slaughter | 100 (no more than 50% of diet) |
| All other species or classes of livestock and pet animals | 10 (no more than 50% of diet) |

**Europe – Legislation for food (EU, 2006b; 2007)**

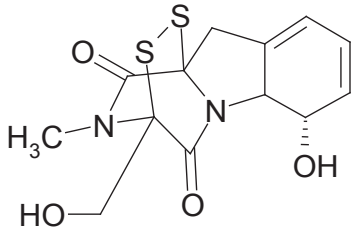
| | *Maximum level [µg/kg] sum of fumonisin $B_1$ and $B_2$* |
|---|---|
| Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling | 4000 |

| | *Maximum level [µg/kg] sum of fumonisin $B_1$ and $B_2$* |
|---|---|
| Maize intended for direct human consumption, maize-based foods for direct human consumption, with the exception of maize-based breakfast cereals, maize-based snacks and processed maize-based foods and baby foods for infants and young children | 1000 |
| Maize-based breakfast cereals and maize-based snacks | 800 |
| Processed maize-based foods and baby foods for infants and young children | 200 |
| Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10 | 1400 |
| Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10 | 2000 |

**Europe - Recommendation for feed (EU, 2006b)**

| | *Guidance value in mg/kg [ppm] fumonisin $B_1 + B_2$ relative to a feedingstuff with a moisture content of 12 %* |
|---|---|
| Feed materials | |
| — maize and maize products 60 | 60 |
| Complementary and complete feedingstuffs for: | |
| — pigs, horses (*Equidae*), rabbits and pet animals | 5 |
| — fish | 10 |
| — poultry, calves (< 4 months), lambs and kids | 20 |
| — adult ruminants (> 4 months) and mink 50 | 50 |

## 1.3.8. GLIOTOXIN




Name: **Gliotoxin**

Formula: $C_{13}H_{14}N_2O_4S_2$
MW: 326.4
CAS No.: 67-99-2
PubChem: 6223

This is an unusual, highly immunosuppressive mycotoxin that belongs to a class of fungal metabolites called epipolythiodioxopiperazines. They are characterized by a disulfide group that connects across the top of the molecule. Gliotoxin was originally described because of its antifungal and other antibiotic properties.

***Production and occurrence***

Gliotoxin is produced by a wide variety of fungi including the common skin inhabitant and opportunistic pathogen, *Candida albicans*. Of considerable interest is that *Aspergillus fumigatus*, an agent of respiratory disease in humans and other animals, especially poultry, produces this mycotoxin. Several *Penicillium* species and a couple of *Gliocladium* species also produce gliotoxin.

The only known case in which gliotoxin was found to contaminate feed was a situation where camels ingested gliotoxin-contaminated hay and became intoxicated (Gareis and Werndery, 1994). Otherwise, gliotoxin has been associated only with the infectious agents, *Aspergillus fumigatus* and *Candida albicans*. In the latter case the human patients with *Candida albicans* induced vaginitis had gliotoxin in vaginal secretions (Shah et al., 1995). However, gliotoxin was found in necrotic bovine udder tissue infected with *A. fumigatus* (Bauer et al., 1989) and in peritoneal lavages from mice inoculated with this organism (Eichner et al., 1988). Of considerable importance is that gliotoxin was found in infected tissues of turkeys experimentally infected with this same organism (Richard and DeBey, 1995a). Subsequently, gliotoxin was found to occur naturally in turkeys with avian aspergillosis caused by *A. fumigatus* (Richard and DeBey, 1995b; Richard et al., 1996). Of interest is that this highly immunosuppressive compound may be produced in the pathogenic state in humans as the organism, *A. fumigatus*, is a respiratory pathogen in compromised individuals especially AIDS patients. It is unknown whether this compound functions as a virulence factor for this fungal pathogen.

***Toxicity***

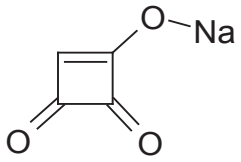
This compound was first isolated because of its antibiotic potential but its toxicity precluded its use clinically. Later, gliotoxin was found to be immunosuppressive when it inhibited phagocytosis by macrophages in tissue culture. Since then other immunosuppressive

activities have been attributed to this mycotoxin. Turkey lymphocytes in culture were inhibited from being stimulated by phytohemagglutinin with levels of gliotoxin as low as 10 ng/ml. Oral doses of gliotoxin of 7.5 mg/kg body weight killed all 1-day-old turkey poults within 24 hr (Richard et al., 1994). Only one of eight poults given 5 mg gliotoxin/kg body weight died in the same time period. Turkeys appear to be more sensitive to gliotoxin than rats, mice, rabbits and hamsters. Although gliotoxin was found to occur naturally in hay and caused intoxication in camels, this route of exposure has not been significant regarding the toxicity of gliotoxin. However, its role in avian aspergillosis and possibly human candidosis and mammalian aspergillosis may be important. Its involvement in the pathogenesis of these diseases is strengthened by the fact that this mycotoxin is produced in the infected tissue of the host and has been found to do so in natural infections caused by *A. fumigatus* in turkeys and *C. albicans* in humans.

***Regulations***

No regulations are present for gliotoxin in commodities or any other product.

## 1.3.9. MONILIFORMIN




| | |
|---|---|
| Name: | **Moniliformin** |
| Formula: | $C_4HNaO_3$ |
| MW: | 120.0 |
| CAS No.: | 71376-34-6 |
| PubChem: | 40452 |

This is an unusual chemical produced by several species of *Fusarium*, which is quite lethal to chickens. It is a plant growth regulator and is phytotoxic as well.

***Production and occurrence***

Moniliformin was first isolated from a culture of *Fusarium moniliforme* (Steyn et al., 1978) which was actually misidentified and should have been identified as *F. proliferatum*. Taxonomic relationships within this group have only recently been clarified. Therefore, we now know that *F. moniliforme* isolates rarely, if at all, produce moniliformin. Several other species of *Fusarium* produce moniformin, many of which are known plant pathogens in cereal grains.

Samples of oats, wheat, corn rye and triticale have been shown to be contaminated with moniliformin. The exact conditions favoring production of moniliformin are unknown but one would suspect that conditions such as cool, wet weather may favor *Fusarium* contamination of grain in the field, especially if these conditions are present at the time the plants are flowering. However, any condition that produces stress on the plant, such as corn, may be appropriate for the production of moniliformin as well. This compound often

occurs in fumonisin-contaminated corn as both compounds are produced by isolates of *F. proliferatum* on this commodity. Insect damage may also provide for a portal of entry of the fungus to the host plant.

As with most *Fusaria*, corn kernels may or may not have visible evidence of fungus as the infection may be internal with no visible presence on the exterior. However, some grains will show a whitish to pink discoloration from the mold growth. Anything that disrupts the integrity of the seed coat should cause an awareness of the potential for the presence of fungi and mycotoxins.

Again, grains should be harvested without kernel damage, screened to avoid broken material and dried to an acceptable level of moisture (<14%). Conditions that favor mold growth will likely increase the level of moniliformin in stored grains. Insects should also be avoided in stored grain for the same reason. Very little specific information on the occurrence of moniliformin in stored commodities is available.

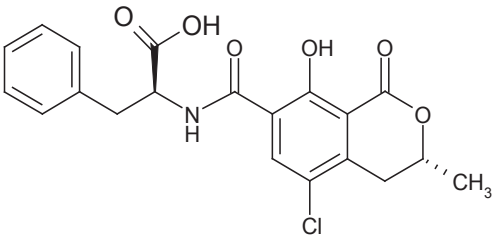
***Toxicity***

The information available on the toxicity of moniliformin is from experimental studies as there are no know outbreaks of moniliformin toxicity in animals including humans. Moniliformin has been tested mostly in poultry and it is quite toxic to one-day-old chicks with an $LD_{50}$ of 5.4 mg moniliformin/kg body weight (Burmeister et al., 1974). The exact mode of action of this toxin is not known and birds given dietary levels often die without lesions. Clinical signs of toxicity include depression, ataxia and weakness with labored respiration especially terminally. Like many other mycotoxins, weight gains in animals were notably depressed by moniliformin. Much of the work done with this mycotoxin was with feeding studies using culture material which contained other mycotoxins produced by the cultured fungus. Therefore, the importance of this mycotoxin may be related to its co-occurrence with other mycotoxins such as fumonisins, fuaric acid and fusarins. The toxicity of this mycotoxin was more toxic in broilers than fumonisins and the toxicities of the two mycotoxins were additive.

***Regulations***

No regulations are present for moniliformin in commodities or any other product.

1.3.10. OCHRATOXIN




| | |
|---|---|
| Name: | **Ochratoxin A** |
| Formula: | $C_{20}H_{18}ClNO_6$ |
| MW: | 403.8 |
| CAS No.: | 303-47-9 |
| PubChem: | 442530 |

This mycotoxin is often referred to as ochratoxin A. Although ochratoxin B exists, it is of limited importance in diseases. Ochratoxin A is an innately fluorescent compound and detection during analysis is usually based on this property.

### *Production and occurrence*

The primary producers of ochratoxin are *Aspergillus ochraceus* and *Penicillium verrucosum*. Other fungi such as *Aspergillus niger* and *Aspergillus carbonarius* may be important in some commodities or geographic areas.

Little is known of the conditions necessary for involvement of the producing fungi in grains and other commodities during development in the field. Therefore, ochratoxin has been regarded as being produced most likely in storage under conditions that would favor mold growth (adequate moisture/humidity and temperature).

Because of the diverse commodities on which the producing organisms and ochratoxin are found, the description of such is difficult. However, visible mold from the major species producing ochratoxin which vary from yellowish tan with *A. ochraceus* to blue-green with *Penicillium* species and black with *A. niger* or *A. carbonarius*. Visible mold may not be present for ochratoxin to occur in grains and other commodities. Grain that has a "musty" odor should be suspect for mycotoxins and ochratoxin would be included in the suspect list. Any time the integrity of the seed coat of grain has been compromised such as stress cracks and broken kernels, there is potential for invasion by the ochratoxin-producing fungi. Appropriate sampling for analysis is important as "hot spots" can occur in storage for the growth and ochratoxin production by these fungi.

As mentioned above, this is likely the major way that commodities become contaminated with ochratoxin. Grains stored under high moisture/humidity (>14%) at warm temperatures (>20°C) and/or inadequately dried potentially can become contaminated. Damage to the grain by mechanical means, physical means or insects can provide a portal of entry for the fungus. Initial growth of fungi in grains can form sufficient moisture from metabolism to allow for further growth and mycotoxin formation.

### *Toxicity*

Ochratoxin is primarily a kidney toxin but if the concentration is sufficiently high there can be damage to the liver as well (Pfohl-Leszkowic and Manderville, 2007). Ochratoxin is a carcinogen in rats and mice and is suspect as the causative agent of a human disease, Balkan Endemic Nephropathy, that affects the kidneys and often tumors are associated with the disease (Wolstenholme et al., 1967). The toxin may be still present in products made from grain and the human population is exposed in this manner. A significant impact of ochratoxin is that it occurs in such a wide variety of commodities such as raisins, barley, soy products and coffee in amounts that may be relatively low. However, the levels may accumulate in the body of either humans

or animals consuming contaminated food because ochratoxin is often not rapidly removed from the body and significant amounts may accumulate in the blood and other selected tissues. Ochratoxin produces necrosis of the proximal tubule epithelium and then is released whereby it is reabsorbed by albumin and continues to be circulated via the bloodstream. The awareness of the occurrence of ochratoxin in this wide variety of commodities has been possible through increased sensitivity of the methods for the analysis of ochratoxin. Of significance is the finding of high levels of ochratoxin in house dust (Richard et al., 1999) and could be an important entity in inhalation toxicology in humans and other animals as this compound apparently is absorbed efficiently by respiratory epithelium.

***Regulations***

Within the US, no ochratoxin regulation exists, but ochratoxin A is regulated in the European Union.

**Europe – Legislation for food (EU, 2006b; EU, 2010a)**

| | *Maximum levels in µg/kg [ppb]* |
|---|---|
| | *Ochratoxin A* |
| Unprocessed cereals | 5.0 |
| All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption with the exception of foodstuffs for infants and young children | 3.0 |
| Dried vine fruit (currants, raisins and sultanas) | 10.0 |
| Roasted coffee beans and ground roasted coffee, excluding soluble coffee | 5.0 |
| Soluble coffee (instant coffee) | 10.0 |
| Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15 % vol) and fruit wine | 2.0 |
| Aromatised wine, aromatised wine-based drinks and aromatized wine-product cocktails | 2.0 |
| Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted, intended for direct human consumption | 2.0 |

| | *Maximum levels in µg/kg [ppb]* |
|---|---|
| | *Ochratoxin A* |
| Processed cereal-based foods and baby foods for infants and young children | 0.5 |
| Dietary foods for special medical purposes intended specifically for infants | 0.5 |
| Spices<br>*Capsicum* spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika), *Piper* spp. (fruits thereof, including white and black pepper), *Myristica fragrans* (nutmeg), *Zingiber officinale* (ginger), *Curcuma longa* (turmeric), Mixtures of spices containing one or more of the abovementioned spices | 30 (until 30.6.2012)<br>15 (as from 1.7.2012) |
| Liquorice (*Glycyrrhiza glabra*, *Glycyrrhiza inflate* and other species)<br>Liquorice root, ingredient for herbalinfusion<br>Liquorice extract, for use in food in particular beverages and confectionary | 20<br>80 |

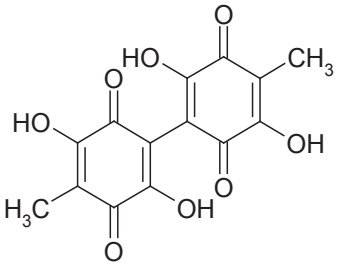
**Europe - Recommendation for feed (EU, 2006a)**

| | *Guidance value in mg/kg [ppm] ochratoxin A relative to a feedingstuff with a moisture content of 12 %* |
|---|---|
| Feed materials | |
| — Cereals and cereal products | 0.25 |
| Complementary and complete feedingstuffs | |
| — Complementary and complete feedingstuffs for pigs | 0.05 |
| — Complementary and complete feedingstuffs for poultry | 0.1 |

### 1.3.11. OOSPOREIN

It is a mycotoxin belonging to a class of pigment compounds known as quinones and is produced by several genera of fungi that occur on cereal grains and other substrates.

The natural occurrence of oosporein has not been sufficiently studied. When isolated in fairly pure form using thin layer chromatographic conditions and viewed under visible light the compound is red on the silica gel plate. Pure crystalline material is red also.




| | |
|---|---|
| Name: | **Oosporein** |
| Formula: | $C_{14}H_{10}O_8$ |
| MW: | 306.2 |
| CAS No.: | 475-54-7 |
| PubChem: | 5359404 |

***Production and occurrence***

The original description of this mycotoxin was from its production by the fungus, *Oospora colorans*, but since the original isolation it has been reported to be produced by fungi such as *Acremonium*, *Chaetomium*, *Penicillium* and *Beauveria*.

The conditions that might allow for the formation of oosporein in cereal grains is not known and, as stated above, little is known of the natural occurrence of this compound. However, several of the fungi capable of producing oosporein have been isolated from grains and laboratory studies have shown that such grains can support formation of the compound. Corn contaminated with *Chaetomium trilaterale* contained 300 ppm of oosporein but under, more or less, ideal conditions for production. More information is needed as to whether oosporein occurs naturally in a variety of grains or other crops. Concern for the occurrence in crops usually exists only when a problem similar to the type of toxicity caused by oosporein occurs in animals. For a long time, analytical methods were inadequate to find this mycotoxin in mixed feeds and other complex matrices.

Again, no information is available regarding the importance of the initiation of oosporein production in stored grains. However, one would expect that conditions that favor the growth of molds in grains would also be applicable to the fungi that produce oosporein. Therefore, storing grain at <14% moisture and keeping it in that state is important to keep oosporein from being formed in storage. Oosporein has been found naturally occurring in poultry rations (Ross et al., 1989).

***Toxicity***

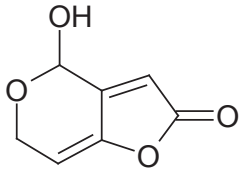
The information on the toxicity of oosporein is from experimental studies in animals, most of which have been conducted in poultry (Richard et al., 1974). In most studies the levels of dietary ooporein were quite high to get the conditions typical of oosporein toxicity. Oosporein is primarily a nephrotoxic compound affecting the renal tubules

of the kidney causing a malfunction of the tubules in the elimination of fluids. This condition then causes the precipitation of urates (uric acids) in the kidneys and on the serosal surfaces of several body organs and in severe cases may occur within the liver and spleen. Similarly, muscle tissue and tendons and joints may be involved in severe cases. The general condition produced by oosporein in poultry is known as visceral gout (Pegram and Wyatt, 1981). This condition may have several different causes in poultry but oosporein may be an important cause and should be suspect in cases of visceral gout. Although high levels of oosporein were required to produce these lesions in poultry it is unknown whether these levels are meaningful since the levels of occurrence in grains are not known.

***Regulations***

No regulations are present for oosporein in commodities or any other product.

1.3.12. PATULIN




| | |
|---|---|
| Name: | **Patulin** |
| Formula: | $C_7H_6O_4$ |
| MW: | 154.1 |
| CAS No.: | 149-29-1 |
| PubChem: | 4696 |

The molds responsible for patulin production are common inhabitants of our environment. Patulin is a mycotoxin included in a group of compounds commonly known as toxic lactones. Patulin is a cyclic compound that is not fluorescent.

***Production and occurrence***

Patulin is produced by several fungi, most of which belong to the genera *Aspergillus* and *Penicillium*. Patulin actually gets its name from the mold *Penicillium patulinum*. Since 1986, additional genera have been added to the potential list of patulin producers.

Patulin contamination is primarily associated with damaged and rotting fruits and fruit juices made from poor quality fruits. Patulin producing molds are found on such fruits as peaches, pears, grapes and especially apples. Recent reports indicate that patulin can be found in some vegetables. By far the most common site of occurrence of patulin is in apples.

Patulin is particularly associated with apples exhibiting "brown rot" or other rotting characteristics. Any fruit with visible signs of rotting, decay or mold growth can be suspect and containing patulin (Frank, 1977).

Fruits stored under conditions that promote bruising and rotting increase the probability of patulin formation. Patulin is very stable in apple juice and grape juice.

In many foodstuffs, sucrose actually protects patulin from degradation during heat treatment.

***Toxicity***

While patulin may be an important mycotoxin in problems associated with silage, confirmation of such has not be established but should be considered when investigating silage problems in especially dairy cattle. Initial studies of patulin indicated that it had antibiotic properties against certain bacteria. Further studies indicated, however, that the patulin was too toxic for use in humans. While some animal studies suggest a carcinogenic potential of patulin (Becci et al., 1981) by IARC. Symptoms of patulin include hemorrhaging in the digestive tract in cattle.

***Regulations***

**USA – FDA action levels (FDA, 2001)**

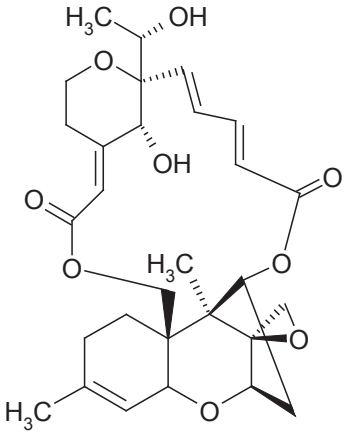
FDA has established an action level for patulin in apple juice of 50 micrograms per kilogram (50 parts per billion) as determined on single strength apple juice or reconstituted single strength apple juice

**Europe – Legislation for food (EU, 2006b)**

| | *Maximum levels [µg/kg] patulin* |
|---|---|
| Fruit juices, concentrated fruit juices as reconstituted and fruit nectars | 50 |
| Spirit drinks (15), cider and other fermented drinks derived from apples or containing apple juice | 50 |
| Solid apple products, including apple compote, apple puree intended for direct consumption with the exception of foodstuffs for infants and young children | 25 |
| Apple juice and solid apple products, including apple compote and apple puree, for infants and young children and labeled and sold as such | 10.0 |
| Baby foods other than processed cereal-based foods for infants and young children | 10.0 |

## 1.3.13. STACHYBOTRYS TOXINS

As the name implies, these are simply toxins produced by a fungus known as *Stachybotrys chartarum* (syn. *S. atra* or *alternans*). The most notable of these toxins are called satratoxins and occur in conidia of the producing fungus.




Name: **Satratoxin H**

Formula: $C_{29}H_{36}O_9$
MW: 528.6
CAS No.: 53126-64-0
PubChem: 5477707

***Production and occurrence***

*S. chartarum* is the only organism that apparently produces the satratoxins (Jarvis et al., 1998). The greenish-black organism is cellulolytic, saprophytic and requires significant moisture to grow and produce the toxin laden conidia.

The satratoxins present in the conidia occur under conditions favoring the growth of the fungus. The latter grows abundantly when moist cellulolytic materials such as wallboard, wallpaper, straw or other cellulose material are available and produces copious amounts of conidia. The conidia are often not viable when found individually in air samples and therefore are usually not described as being found in sampling for viable particles. However, the dead conidia can still retain toxigenic and allergenic components. When the conidia are originally formed by the fungus they are produced in a slimy matrix and are not easily air-borne at this time. However, following some desiccation they become air-borne when disturbed. This fungus is not a plant pathogen and occurs on the materials mentioned above after they have become wet by rain, floods or other means of water intrusion into the cellulose components. Following these conditions the growth of the fungus may appear as a black tar-like substance on the substrate that smears when touched. One should not consider that any black mold is *Stachybotrys* but a competent mycologist should be sought for identification of the fungus. Materials that have been heavily damaged by this fungus should be removed but bleach solution should be used to drench the affected area prior to removal. Bleach solution appears to kill the fungus but its ability to neutralize the toxin is questionable. Contamination of heating and air conditioning duct work should be avoided or prevented. If contamination occurs a thorough cleaning should be conducted. Protective gear should be worn by those conducting cleaning operations.

***Toxicity***

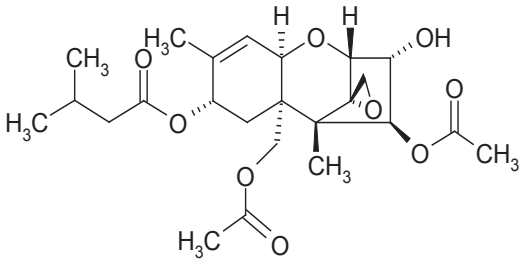
*Stachybotrys* toxins have been involved in intoxications for many years, perhaps even in ancient times, and have had historical significance in Russia whereby horses were notably affected by contaminated bedding and feed material. Most affected animals exhibited necrotic lesions where they had contacted the contaminated material, especially around the mouth (Moseliani, 1940). Gastrointestinal disturbances were noted and hemorrhage was a common finding. This condition was later found to affect humans as well and vivid descriptions of this devastating disease are present in the literature. Aside from exposure by contact and ingestion of contaminated material, air-borne exposure is considered important in modern times. Several outbreaks of disease appear to involve the toxin from *Stachybotrys* and include hemorrhagic lung or respiratory problems especially in young infants, however adults may be affected as well and the problem appears to be exacerbated by smoking. Manifestations of such air-borne exposure include bleeding from the nose with coughing up blood and respiratory congestion. The toxins cause a weakening of the blood vessels and bleeding results. The major problem with this entire mycotoxin-fungus-disease relationship is that the true culprits involved in outbreaks have not been identified except that *Stachybotrys* appears to be involved and the most likely known mycotoxins are the satratoxins. However, other toxins may be produced by this and other organisms, as well as perhaps some antigenic stimuli, to cause the syndromes presented in outbreaks. Because of the myriad of mycotoxins produced by *Stachybotrys* it is difficult to determine which of the mycotoxins may be proximal entities in the intoxications described and of those likely involved it is not known as to the concentration of them that are important in the intoxications. Furthermore, many of the manifestations of disease associated with this organism appear to have some elements of allergic phenomena as well. So, therefore, determining the kind and concentration of these mycotoxins in a sample becomes problematic insofar as cause and effect. Thus, the important aspect is to remove individuals from environments contaminated with *Stachybotrys* and then eliminate the fungus or destroy any contaminated food.

***Regulations***

No regulations are present for *Stachybotrys* in commodities or any other product.

## 1.3.14. T-2 TOXIN

This mycotoxin is a member of the fungal metabolites known as the trichothecenes. The trichothecenes are divided into two groups: macrocyclic and non-macrocyclic. T-2 toxin is in the non-macrocyclic group; this group is also subdivided into the Type-A and Type-B trichothecences. The Type-A trichothecenes include T-2 toxin, HT-2 toxin and diacetoxyscirpenol (DAS) as mycotoxin components.



| | |
|---|---|
| Name: | **T-2 Toxin** |
| Formula: | $C_{24}H_{34}O_9$ |
| MW: | 466.5 |
| CAS No.: | 21259-20-1 |
| PubChem: | 11969549 |

***Production and occurrence***

*Fusarium sporotrichioides* is the principle fungus responsible for the production of T-2 toxin. Some strains of this fungus also produce DAS and HT-2 toxin, however DAS is the least common of the three toxins. Corn, wheat, barley, oats, rice, rye and other crops have been reported to contain the T-2 toxin. Natural occurrence has been reported in Asia, Africa, South America, Europe and North America. Natural levels range from near zero to 10 ppm with a few exceptions showing levels of 15-40 ppm. The toxin production is greatest with increased humidity and temperatures of 6-24°C. Storage of commodities below 14% moisture will minimize further fungal growth and production of the T-2 toxin. In addition grains kept free of insect damage and dried prior to storage may decrease the effects of further contamination. Poultry feed and certain other food products are most commonly contaminated. In the United States T-2 toxin is infrequently found and, if found, likely results from inadequate storage of products.

The *Fusarium* mold on corn primarily appears white, in some instances the mold can also appear pink to reddish, often beginning at the tip of the ear. Occasional blue-black specks will be found on the husk and ear shank to indicate mold contamination.

***Toxicity***

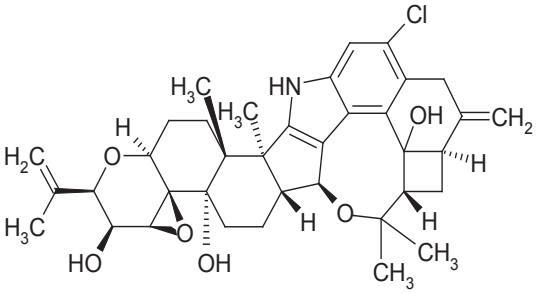
The major attribute of the T-2 toxin and other trichothecenes is that they inhibit protein synthesis which is followed by a secondary disruption of DNA and RNA synthesis (Ueno, 1984). It affects the actively dividing cells such as those lining the gastrointestinal tract, skin, lymphoid and erythroid cells. It can decrease antibody levels, immunoglobulins and certain other humoral factors. The effects include weight loss or poor weight gain, bloody diarrhea, dermal necrosis or beak lesions, hemorrhage and decreased production (weight gain, eggs, milk, etc.). The Type-A trichothecenes are more toxic to poultry species than the Type-B trichothecenes. Yellow caseous plaques, occurring at the margin of the beak, mucosa of the hard palate, angle of the mouth and tongue, characterize typical oral lesions. Severity of the lesions will increase with prolonged feeding and increased dietary levels. Beak or oral lesions can occur at dietary levels of 4 mg/kg after 1 week, 0.4 mg/kg after 7 weeks, and with 1-4

mg/kg, beak or oral lesions occurred in addition to decreased weight and feed intake after 3 weeks (Richard, 2007). There is also a synergism between aflatoxin and T-2 toxin discussed (Huff et al., 1988).

***Regulations***

Within the U.S., no regulation for T-2 toxin exists, but a regulation for T-2 toxin, as sum of T-2 and HT-2 toxin, is pending in Europe. Regulation EC/1881/2006 (EU, 2006b) already lists "Unprocessed cereals and cereal products" for a future maximum level, but gives no numbers yet. In a footnote, the following statement was made: "The maximum level applies to unprocessed cereals placed on the market for first-stage processing. 'First-stage processing' shall mean any physical or thermal treatment, other than drying, of or on the grain. Cleaning, sorting and drying procedures are not considered to be 'first-stage processing' insofar no physical action is exerted on the grain kernel itself and the whole grain remains intact after cleaning and sorting. In integrated production and processing systems, the maximum level applies to the unprocessed cereals in case they are intended for first-stage processing."

## 1.3.15. TREMORGENS




| | |
|---|---|
| Name: | **Penitrem A** |
| Formula: | $C_{37}H_{44}ClNO_6$ |
| MW: | 634.2 |
| CAS No.: | 12627-35-9 |
| PubChem: | 337313 |

Any one of a large group of mycotoxins capable of producing tremors in naturally intoxicated or experimentally dosed animals. They include: paspalitrems, paxilline, aflatrems, lolitrems, paspalinine, penitrems, verruculogen, fumitremorgens, territrems and janthitrems. Most notable are the penitrems (particularly penitrem A), paspalinine and paspalitrems which have been involved in or closely implicated in natural intoxications. While others have been found in feed causing disease, a cause and effect relationship has not been fully established.

***Production and occurrence***

Most of the fungi producing these compounds are of the genus *Penicillium* with *P. crustosum* being most notable for its production of penitrems. Some *Aspergillus spp.*

are producers of aflatrem, verruculogen and fumitremorgens and at least one *Claviceps sp.* (*C. paspali*) appears to produce tremorgens (paspalinine and palpalitrems).

In the case of *Claviceps sp.* in certain grasses such a Dallis grass, the fungus replaces the ovary in developing flowers and sclerotia (masses of fungal tissue) replace the seed or grains at maturity. Vectors such as insects and animals are likely more important in disseminating the fungus than are weather conditions. The lolitrems are considered to be produced by an endophytic fungus in ryegrass in select areas of the world (New Zealand, especially). The penitrems have been involved in the saprophytic contamination of material such as cream cheese, bread, beer and walnuts. Again, the fumitremorgens, aflatrems and verruculogen are likely the result of the producing fungi growing on any number of dead plant materials or other products. The contact of plant material with soil could be important in the occurrence of some of these mycotoxins in commodities.

In most cases of tremorgenic episodes there is evidence of mold growth on the commodity or product or there is presence of the brownish to blackish sclerotia of the fungus among the grass grains. However, ryegrasses contaminated with lolitrems have no symptoms of contamination with the organism but signs of the organism can be evident microscopically in the ryegrass.

Other than *Claviceps* contamination of grasses and the occurrence of lolitrems in ryegrass, most of the tremorgens would appear to be important in storage of commodities or products. In some cases, as mentioned above, the contamination may be the result of the crop contacting the soil allowing for the fungus to contaminate the grain and allow for tremorgen production. This phenomenon occurs primarily in mature crops. Inadequate storage of materials usually is the cause for contamination. In the case of walnuts, dogs consuming walnuts usually occurs after the walnuts have overwintered on the ground and became contaminated with *Penicillium crustosum*.

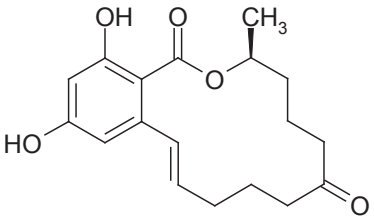
### *Toxicity*

The tremorgens appear to be neurotoxic and cause only chemical lesions in the central nervous system. Dogs appear to seek out materials contaminated with *P. crustosum* and become ataxic and develop full body tremors with intermittent extensor rigidity. Anesthesia during the episode allows for relaxation of the affected no apparent residual effects exist. Animals that are affected aren't able to eat or drink and dehydration is likely without adequate therapy. Diseases known or likely caused by tremorgens are: ryegrass staggers (Gallagher et al., 1981), bermudagrass tremors, corn staggers, penitrem toxicosis of dogs (Hayes et al., 1976) and humans, paspalum staggers (Cole et al., 1977) and some miscellaneous tremorgenic intoxications (Selala et al., 1991). Tremorgenic intoxications occur worldwide.

### *Regulations*

No regulations are present for tremorgens in commodities or any other product.

## 1.3.16. ZEARALENONE




| | |
|---|---|
| Name: | **Zearalenone** |
| Formula: | $C_{18}H_{22}O_5$ |
| MW: | 318.4 |
| CAS No.: | 17924-92-4 |
| PubChem: | 5281576 |

This compound is chemically a phenolic resorcyclic acid lactone that is primarily an estrogenic fungal metabolite. It is observed on thin layer chromatographic plates under short wavelength ultraviolet light as a greenish fluorescent compound. This compound may be produced in concert with deoxynivalenol by certain isolates of the fungus.

***Production and occurrence***

The major species of fungus responsible for producing this mycotoxin is *Fusarium graminearum*. In some of the older literature this organism is called *F. roseum*. Grain infected with this organism often will have a pink color because of a pigment that may be simultaneously produced with the zearalenone.

Most often the compound is found in corn, however, it is found also in other important crops such as wheat, barley, sorghum and rye throughout various countries of the world. In wheat the conditions for the occurrence of zearalenone would be essentially the same as for the occurrence of deoxynivalenol as the organism gains entry into the host plant in the same manner. Generally, the *Fusarium* species grow in moist cool conditions and similarly invade crops under these more favorable conditions. As noted above, the same organism produces both of these compounds. This same organism is capable of producing both compounds in corn. The finding of aflatoxin co-occurring with zearalenone and deoxynivalenol would imply that infection was established by two different fungi, *Aspergillus flavus* in the case of aflatoxin and *F. graminearum* in the case of the latter two mycotoxins. In wheat, sorghum and corn, it is well-established that zearalenone occurs in preharvest grain but in other commodities the surveys are insufficient to determine if the zearalenone occurred pre- or postharvest. Variations in the incidence of zearalenone occur with different crop years, cereal crop and perhaps geographical areas.

As with other fungi, to avoid growth of *F. graminearum* in grains during storage the moisture level should be <14%. Perhaps, zearalenone can be produced in relatively cool conditions compared to some other mycotoxins but it is likely that most grains mentioned above can become contaminated with zearalenone during storage and levels that were present in the grain preharvest may increase if the grain is not sufficiently dried and stored.

### *Toxicity*

The most notable effect of zearalenone is that it causes precocious development of mammae and other estrogenic effects in young gilts as well as prepucial enlargement in young barrows. Swine appear to be the animals most significantly affected and are considerably more sensitive than rodents. Weak piglets and small litter size have been attributed to the effects of zearalenone when fed to sows during gestation. Levels of 0.5 to 1.0 ppm of dietary zearalenone have been associated with the latter effects while hyperestrogenism in swine was associated with dietary levels of 1.5 to 5.0 ppm. Twelve ppm zearalenone was found in sorghum that was involved in bovine abortion. Zearalenone appears to bind to estrogen receptors and can result in hormonal changes. Zearalenone does not appear to be involved in mortalities because of its high oral LD50. Interestingly, zearalenone or its metabolites have been suspected to cause precocious pubertal changes in young children in Puerto Rico. The occurrence of this phenomenon in other countries needs confirmation as to the causation. Of note is that the metabolite of zearalenone known as α-zearalenol, is actually more estrogenic than is the parent compound (Richardson et al., 1985).

### *Regulations*

Within the US, no regulation for zearalenone exists. The European Union has a regulation for food and recommendations for feedstuffs.

**Europe (EU, 2007)**

| | *Maximum levels in µg/kg [ppb]* |
|---|---|
| | *Zearalenone* |
| Unprocessed cereals other than maize | 100 |
| Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling | 350 |
| Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of the products listed below | 75 |
| Refined maize oil | 400 |
| Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereals | 50 |
| Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals | 100 |

| | *Maximum levels in µg/kg [ppb]* |
|---|---|
| | *Zearalenone* |
| Processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children | 20 |
| Processed maize-based foods for infants and young children | 20 |
| Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10 | 200 |
| Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10 | 300 |

**Europe - Recommendation for feed (EU, 2006a)**

| | *Guidance value in mg/kg [ppm] deoxynivalenol relative to a feedingstuff with a moisture content of 12 %* |
|---|---|
| Feed materials | |
| — Cereals and cereal products with the exception of maize by-products | 2 |
| — Maize by-products | 3 |
| Complementary and complete feedingstuffs | |
| — Complementary and complete feedingstuffs for piglets and gilts (young sows) | 0.1 |
| — Complementary and complete feedingstuffs for sows and fattening pigs | 0.25 |
| — Complementary and complete feedingstuffs for calves, dairy cattle, sheep (including lamb) and goats (including kids) | 0.5 |

## 1.4. Relevant mycotoxins for common grains

| *Potentially affected commodities* | *Major mycotoxins* | *Conducive weather conditions* |
|---|---|---|
| Barley | Citrinin | Poor storage |
| | Deoxynivalenol | Cool, moist weather |
| | Ochratoxin | Poor storage |
| | T-2 toxin | Cool, humid weather; poor storage |
| | Zearalenone | Cool, moist weather |
| Corn | Aflatoxin | High temperature and drought |
| | Citrinin | Poor storage |
| | Cyclopiazonic acid | High temperature and drought |
| | Deoxynivalenol | Cool, moist weather |
| | Fumonisin | Drought followed by warm, wet weather |
| | T-2 toxin | Cool, humid weather; poor storage |
| | Zearalenone | Cool, moist weather |
| Millet | Cyclopiazonic acid | High temperature and drought |
| | Ergot | Varied conditions |
| Oats | Citrinin | Poor storage |
| | Deoxynivalenol | Cool, moist weather |
| | Moniliformin | Cool, moist weather |
| | T-2 toxin | Cool, humid weather; poor storage |
| Rice | Citrinin | Poor storage |
| | Fumonisin | Drought followed by warm, wet weather |

| *Potentially affected commodities* | *Major mycotoxins* | *Conducive weather conditions* |
|---|---|---|
| | T-2 toxin | Cool, humid weather; poor storage |
| Sorghum | Ergot | Varied conditions |
| | Tremorgens | Poor storage |
| | Zearalenone | Cool, moist weather |
| Wheat | Citrinin | Poor storage |
| | Deoxynivalenol | Cool, moist weather |
| | Moniliformin | Cool, wet weather |
| | T-2 toxin | Cool, humid weather; poor storage |
| | Zearalenone | Cool, moist weather |

## 1.5. Relevant mycotoxins for various commodities

| *Potentially affected commodities* | *Major mycotoxins* | *Conducive weather conditions* |
|---|---|---|
| Coffee | Ochratoxin | Poor storage |
| Cottonseeds | Aflatoxin | High temperature and drought |
| Fruits | Ochratoxin | Poor storage |
| | Patulin | Damaged, rotten fruits |
| Grasses | Ergot | Varied conditions |
| | Tremorgens | Poor storage |
| | T-2 toxin | Cool, humid weather; poor storage |
| Peanuts | Aflatoxins | High temperature and drought |
| | Cyclopiazonic acid | High temperature and drought |
| Soy products | Ochratoxin | Poor storage |

| *Potentially affected commodities* | *Major mycotoxins* | *Conducive weather conditions* |
|---|---|---|
| Tree nuts | Aflatoxins | High temperature and drought |
| | Tremorgens | Poor storage |

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# 2. SAMPLING AND SAMPLE PREPARATION FOR MYCOTOXIN ANALYSIS

THOMAS B. WHITAKER AND ANDREW B. SLATE

## 2.1. Introduction

Mycotoxins are toxic and/or carcinogenic compounds produced by various fungal species that grow on various agricultural commodities (Cillen and Newberne, 1994).

Commodities can be contaminated either in the field or in storage. Pre and post harvest strategies to prevent crop contamination include: yearly crop rotations, irrigation in hot and dry weather, use of pesticides to reduce the insect population, drying crops to a safe moisture level, and providing protective storage (Phillips, Clement, and Park, 1994). Because mycotoxins are toxic in humans and other animals and carcinogenic in some animals, many countries regulate the maximum level that can occur in foods and feeds. Most regulations are concerned with controlling aflatoxin because it is considered the most toxic and carcinogenic of the naturally occurring mycotoxins. A recent FAO/WHO survey indicated that almost 100 countries regulate aflatoxin in foods and feeds (FAO, 1995). However, maximum levels differ widely from country to country because of a lack of agreement on what constitutes a safe maximum level for humans.

It is important to be able to detect and quantify the mycotoxin concentration in foods and feeds destined for human and animal (human) consumption. Nonetheless, analyzing samples for the occurrence of mycotoxins is not a simple task. A sampling procedure is a multistage process and consists of three distinct phases: sampling, sample preparation and analysis (Cheli et al., 2009).

In research, quality assurance, and regulatory activities, correct decisions concerning the fate of commercial lots can only be made if the mycotoxin concentration in the lot can be determined with a high degree of accuracy and precision. The mycotoxin concentration of a bulk lot is usually estimated by measuring the mycotoxin concentration in a small portion of the lot or a sample taken from the lot (Figure 2.1).

The mycotoxin concentration in the bulk lot is assumed to be the same as the measured mycotoxin concentration in the sample. Then based on the measured sample concentration, some decision is made about the edible quality of the bulk lot or the effect of a treatment or a process on reducing aflatoxin in the lot. For example, in a regulatory environment, decisions will be made to classify the lot as acceptable or unacceptable based upon a comparison of the measured sample concentration to a legal limit or maximum level. If the sample concentration does not accurately reflect

the lot concentration, then the lot may be misclassified and there may be undesirable economic and/or health consequences. Fortunately, sampling plans can be designed to minimize the misclassification of lots and reduce the undesirable consequences associated with regulatory decisions about the fate of bulk lots.

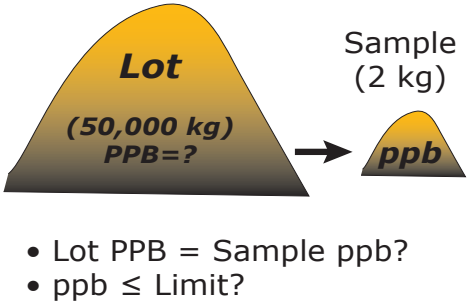



**Figure 2.1** - Lot mycotoxin concentration is assumed to equal the measure mycotoxin concentration in a small sample.

## 2.2. Definition of sampling plan

A mycotoxin-sampling plan is defined by a mycotoxin test procedure and a defined accept/reject limit. A mycotoxin-test procedure is a multi-stage process (Figure 2.2) and generally consists of three steps: sampling, sample preparation, and analysis (quantification).

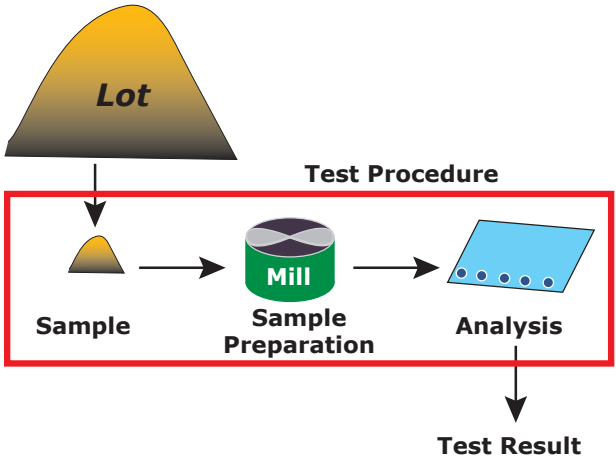



**Figure 2.2** - A mycotoxin-test procedure usually consists of a sampling, sample preparation and analytical step.

The sampling step specifies how the sample will be selected or taken from the bulk lot and the size of the sample. For granular products, the sample preparation step is also a two-part process where the sample is ground in a mill to reduce particle size and a subsample is removed from the comminuted sample. Finally in the analytical step, the mycotoxin is solvent extracted from the comminuted subsample and quantified using approved procedures.

The measured mycotoxin concentration in the sample is used to estimate the true mycotoxin concentration in the bulk lot or compared to a defined accept/reject limit that is usually equal to a maximum level or regulatory limit. Comparing the measured concentration to an accept/reject limit is often called acceptance sampling because the measured concentration value is not as important as whether the measured concentration (and thus the lot concentration) is above or below the maximum level. In quality assurance and research activities, a precise and accurate estimate of the true lot mycotoxin concentration becomes important.

## 2.3. Uncertainty

There is always some level of uncertainty associated with a sampling plan. Because of the uncertainty associated with a mycotoxin-sampling plan, the true mycotoxin concentration of a bulk lot can't be determined with 100 % certainty; nor can all lots be correctly classified into good and bad categories (based upon some maximum level) with 100 % accuracy. Accuracy and precision are two types of uncertainties associated with a sampling plan (Cochran and Cox, 1957).

### 2.3.1. ACCURACY

Accuracy is defined as the closeness of measured values to the true value. Another term associated with accuracy is "bias". A bias is some influence that makes the measured values deviate from the true value in a consistent manner on the average. Using target practice as an example, the center of the target is analogous to the true value and holes in the target represent the measured values. Figure 2.3 shows that the rifle used on the left is not as accurate as the rifle used on the right where the average of the cluster of shots is around the center of the target.

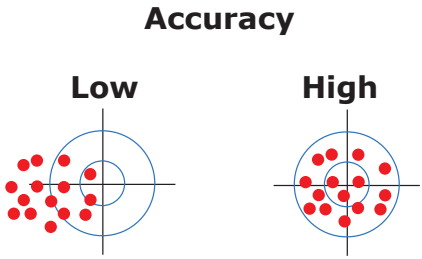



**Figure 2.3** - Examples of low and high accuracy using target practice as an example.

Mathematically, accuracy (A) is the difference between the true value (U) and the average of the n measured values (Xi).

$$A = U - [SUM (Xi)/n] \quad (1)$$

Accuracy is associated with a bias. Biases have the potential to occur in the sample selection process, sample preparation, and in the quantification steps of the test procedure. Biases should be the easiest to control and reduce to acceptable levels, but methods to reduce bias are difficult to evaluate because of the difficulty in knowing the true mycotoxin concentration of the lot. Sample selection, sample preparation equipment, and analytical methods are continuously performance tested to minimize any biases.

### 2.3.2. PRECISION

Precision is defined as the closeness of measured values to each other. Another term for precision is variability. The definition of precision makes no mention about how close the measured values are to the true value. Using target practice to illustrate precision, the closeness of the holes to each other is a measure of precision (Figure 2.4).

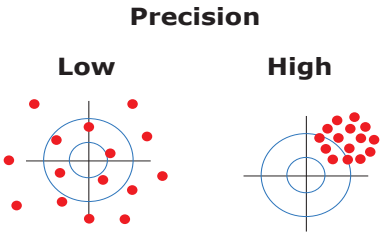



**Figure 2.4** - Examples of low and high precision using target practice as an example.

Three statistical measures of variability, variance (V), standard deviation (S), or coefficient of variation (CV) can be used as a measure of precision (P).

$$V = [\sum_i (xi-m)^2/(n-1)] \text{ for I} = 1,2,\ldots,n \quad (2)$$

$$S = \text{square root (V)} \quad (3)$$

The CV, expressed as a percent,

$$CV = 100 * (S/m) \quad (4)$$

Where $x_i$ is the measured value and m is the mean of the n xi values. Precision is associated with variability, which can occur with each step of the mycotoxin test procedure.

When describing the uncertainty of a process, one must consider the various combinations of accuracy and precision that may occur. As shown in Figure 2.5, there are four extreme combinations of accuracy and precision: low precision and low accuracy, low precision and high accuracy, high precision and low accuracy, and high precision and high accuracy.

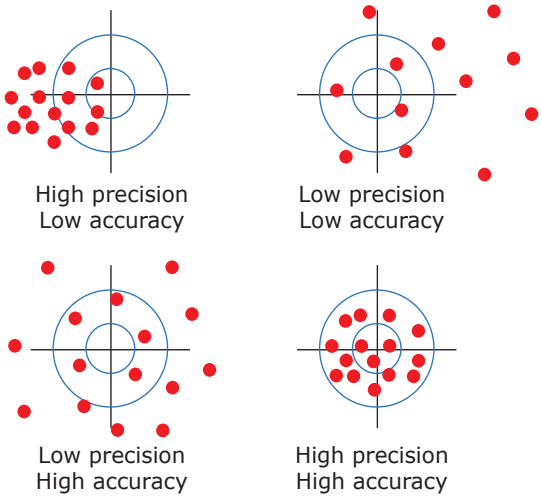



**Figure 2.5** - The four extreme combinations of uncertainty that can occur with a sampling plan.

The worst possible situation is to have a process with low precision and low accuracy. The best possible situation is to have a process that has both high precision and high accuracy. The goal associated with detecting a mycotoxin in a bulk shipment is to design a mycotoxin test procedure or sampling plan that has both high precision and high accuracy.

## 2.4. Sample selection

Procedures used to take a sample from a bulk lot are extremely important. Every individual item in the lot should have an equal chance of being chosen (called random sampling). Biases are introduced by sample selection methods if equipment and procedures used to select the sample prohibit or reduce the chances of any item in the lot from being chosen. Examples of bias in the sample selection process, shown in Figure 2.6, are the use of a sampling probe that doesn't allow larger particles into the probe, a probe that doesn't reach every location in the shipment, and use of a single probing point in a poorly mixed lot.

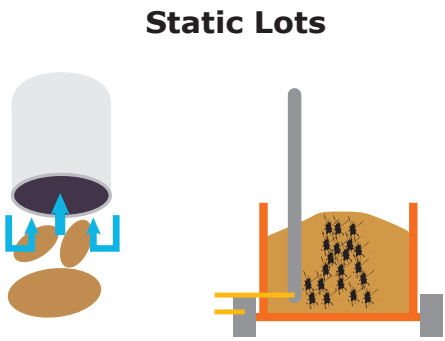



**Figure 2.6** - Different types of biases associated with selecting samples from bulk lots. (1) Particles larger than probe opening; (2) Some particles in the lot cannot be reached; (3) Using a single probing point with an unmixed lot.

If the lot has been blended thoroughly from the various material handling operations, then the contaminated particles are assumed to be distributed uniformly throughout the lot (Williams, 1991). In this situation, it is probably not too important from what location in the lot the sample is drawn. However, if the lot is contaminated because of moisture leaks that cause high moisture clumps or for other localized reasons, then the mycotoxin-contaminated particles may be located in isolated pockets in the lot (Shotwell, et al., 1975). If the sample is drawn from a single location, the contaminated particles may be missed or too many contaminated particles may be collected (Figure 2.7).

Because contaminated particles may not be distributed uniformly throughout the lot, the sample should be an accumulation of many small incremental samples taken from many different locations throughout the lot (Bauwin and Ryan, 1982; Hurburgh and Bern, 1983). FAO/WHO recommends that each incremental portion be about 200 g and one incremental portion be taken for every 200 kg of product (FAO, 2001). The accumulation of many small incremental portions is called a bulk or aggregate sample. If the bulk sample is larger than desired, the bulk sample should be blended and subdivided until the desired sample size is achieved (Figure 2.8).

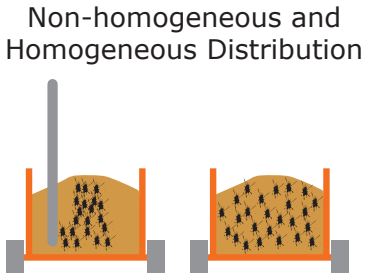



**Figure 2.7** - Extreme spatial distributions among contaminated particles in a bulk lot.

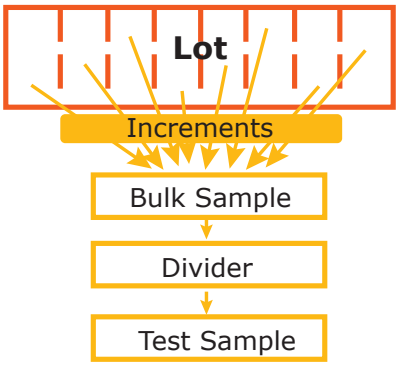



**Figure 2.8** - A test sample is removed from a bulk sample. A bulk sample is the accumulation of many small incremental portions taken from many different locations in the lot.

The smallest sample size that is subdivided from the bulk sample and comminuted in a grinder in the sample preparation step is called the test or laboratory sample. It is generally more difficult to obtain a representative (lack of bias) test sample from a lot at rest (static lot) than from a moving stream of the product (dynamic lot) as the lot is moved from one location to another. Sample selection methods differ depending on whether the lot is static or dynamic.

### 2.4.1. STATIC LOTS

Examples of static lots are commodities contained in storage bins, railcars, trucks, or many small containers such as sacks. When drawing a sample from a bulk container, a probing pattern should be developed so that product can be collected from different locations in the lot. An example of several probing patterns used by the USDA to collect samples from peanut lots is shown in Figure 2.9 (USDA, 1975; Parke et al., 1982; Whitaker and Dowell, 1995).

The sampling probe should be long enough to reach the bottom of the container when possible. Attempts should be made use a sampling rate similar to the 200 g per 200 kg mentioned above. However, it may not be possible to achieve the suggested sampling rate because of the design of the sampling equipment, size of the individual containers, and the size of the lot. As an example, a test sample (TSS) of 5,000 g is to be taken from a lot (LS) of 25,000 kg. The preferred increment size (ISS) is 200 g. The minimum number of increments needed to provide a test sample of 5,000 g is TSS/ISS or 25 incremental portions of 200 g each. If a total of 25 incremental portions are to be taken from the lot of 25,000 kg, then an increment is taken for every 1,000 kg (25,000/25) of lot (taking an increment every 1,000 kg of lot is larger than the recommended 200 kg of lot). If a 200 g incremental portion is taken every

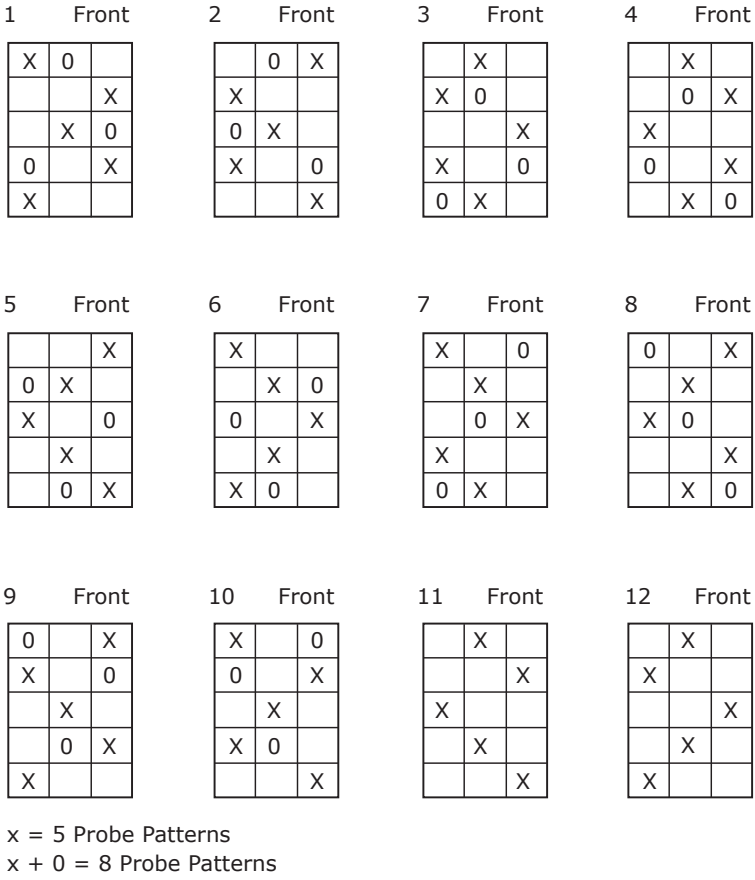



**Figure 2.9** - Example of several five- and eight-probe patterns used by the U. S. Department of Agriculture to sample large peanut containers (trucks and wagons) for grade.

200 kg of lot, then a total of 125 (25,000/200) increments of 200 g each will be taken and the bulk sample size is 25,000 g or five times bigger than the needed test sample size of 5,000 g. The 25,000 g bulk sample has to be subdivided to obtain the 5,000 g test sample. A flow diagram showing the interactions between all the variables is shown in Figure 2.10.

Given: BSS = Bulk Sample Size
TSS = Test Sample Size
ISS = Increment Size

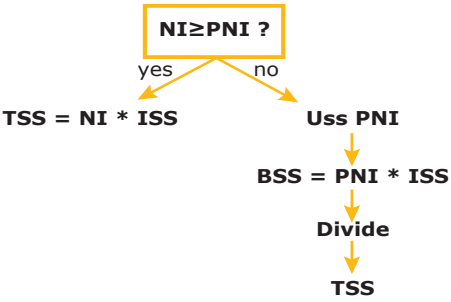



**Figure 2.10** - Interaction between lot size, increment size, and test sample size.

LS = Lot Size
ILS = Incremental Lot Size

Then: Number Increments (NI)
NI = TSS / ISS
Preferred # Increments (PNI)
PNI = LS / ILS

When sampling a static lot in separate containers such as sacks or retail containers, the sample should be taken from many containers dispersed throughout the lot. When storing sacks in a storage facility, access lanes should be constructed in order to allow access to sacks at interior locations. The recommended number of containers sampled can vary from one in four in small lots (less than 20 metric tons) to the square root of the total number of containers for large (greater than 20 metric tons) lots (FAO, 2001).

If the lot is in a container where access is limited, the sample should be drawn when the product is either being removed from or being placed into the container. If the accumulated bulk sample is larger than required, the bulk sample should be thoroughly blended and reduced to the required test sample size using a suitable divider that randomly removes a test sample from the bulk sample.

### 2.4.2. DYNAMIC LOTS

True random sampling can be more nearly achieved when selecting a bulk sample from a moving stream as the product is transferred (i.e. conveyor belt) from one location to another. When sampling from a moving stream, small increments of product should be taken along the entire length of the moving stream (Figure 2.11); small increments of product should be taken across the entire cross section of the moving stream; composite all the increments of product to obtain a bulk sample; if

the bulk sample is larger than required, then blend and subdivide the bulk sample to obtain the desired size test sample.

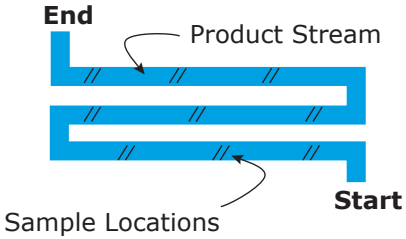



**Figure 2.11** - Sample selection from a moving stream of product should be the accumulation of many small incremental portions taken from the beginning to the end of the product stream.

Automatic sampling equipment such as cross-cut samplers (Figure 2.12) are commercially available with timers that automatically pass a diverter cup through the moving stream at predetermined and uniform intervals. When automatic equipment is not available, a person can be assigned to manually pass a cup though the stream at periodic intervals to collect the bulk sample. Whether using automatic or manual methods, small increments of product should be collected and composited at frequent and uniform intervals throughout the entire time product flows past the sampling point.

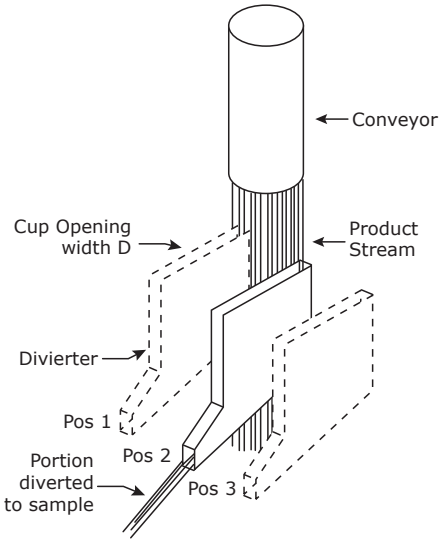



**Figure 2.12** - The automatic sampler cup should move at a constant velocity and cut through the entire stream of product.

Cross-cut samplers should be installed in the following manner: (a) the plane of the opening of the sampling cup should be perpendicular to the direction of flow; (b) the

sampling cup should pass through the entire cross sectional area of the stream; and (c) the opening of the sampling cup should be wide enough to accept all items of interest in the lot. As a general rule, the width of the sampling cup opening should be two to three times the largest dimensions of the items in the lot.

The size of the bulk sample, S in kg, taken from a lot by a cross cut sampler is

$$S = (D)(L) / (T)(V), \quad (5)$$

Where D is the width of the sampling cup opening in cm, L is the lot size in kg, T is interval or time between cup movement through the stream in seconds, and V is cup velocity in cm/sec.

Equation (4) can also be used to compute other terms of interest such as the time between cuts, T. For example, the required time, T, between cuts of the sampling cup to obtain a 10 kg sample from a 30,000 kg lot where the sampling cup width is 5.08 cm (2 inches), and the cup velocity through the stream 30 cm/sec. Solving for T in equation 5,

$$T = (D)(L) / (S)(V)$$

$$T = (5.08 \text{ cm} \times 30{,}000 \text{ kg})/(10 \text{ kg} \times 30 \text{ cm/sec}) = 508 \text{ sec}$$

If the lot is moving at 1000 kg per minute, the entire lot will pass through the sampler in 30 minutes and only three or four cuts will be made by the cup through the lot. This may be considered too infrequent, because too much product passes the sampling point between the times the cup cuts through the stream. The interaction among the variables in equation 5 needs to be fully understood in terms of the amount of sample accumulated and the frequency of cuts through the product.

### 2.4.3. BULK VERSUS TEST SAMPLE

Because contaminated particles may not be uniformly dispersed throughout the lot, many incremental portions are taken from many different locations throughout the lot and accumulated to form a bulk sample. As a result, the bulk sample is usually larger than the desired test sample size used to estimate the lot mycotoxin concentration. For granular material, the test sample is the smallest sample of granular product ground in a mill in the sample preparation step. For finely ground materials (corn flour) or liquids (milk), the test sample is the smallest sample used in the analytical step to quantify the mycotoxin. When the bulk sample is larger than the test sample, mechanical dividers such as a Boerner or riffle divider should be used to remove the desired test sample from the bulk sample. Mechanical dividers are considered to produce random divisions (Parker et al., 1982); therefore, the bulk sample doesn't have to be blended

before the test sample is removed. However, if the test sample is to be removed from the bulk sample using quartering or a manual device such as a cup or scoop, then the bulk sample should be blended before the test sample is removed.

If the test sample is a granular product such as shelled corn or nuts, then the test sample should not be further reduced in size before grinding the sample in the sample preparation step. As the test sample becomes smaller, the uncertainty (precision) associated with estimating the true lot mycotoxin concentration becomes greater. As will be shown later, the size of the test sample put through the grinder should be as large as possible. Recommended sample sizes for various commodities are shown in Table 2.1.

**Table 2.1 - Product sample sizes used by the United States Food and Drug Administration.**

| *Product* | *Description* | *Package Type* | *Lot Size* | *Number of Sample Units* | *Unit Size* | *Sample (lbs.)* |
|---|---|---|---|---|---|---|
| Peanut butter | smooth | consumer & bulk | | 24 | 0.5 | 12 |
| | | | | | 1 | 12 |
| Peanuts | crunchy butter, raw, roasted, ground topping | consumer & bulk | | 12 | 1 | 48 |
| Tree nuts | inshell, shelled slices or flour paste | consumer & bulk | | 48 | 1 | 10 |
| | | | | | 1 | 50 |
| | | | | | 1 | 12 |
| Brazil nuts | inshell in import status | bulk | <200 bags | 20 | 1 | 20 |
| | | | 201-800 bags | 40 | 1 | 40 |
| | | | 801-2,000 bags | 60 | 1 | 60 |
| Pistachio nuts | inshell in import status | bulk | 75,000 lbs | 20% of units | 1 | 50 lbs. |
| | | | <75,000 lbs | 20% of units | | 25 lbs. |
| Corn | shelled, meal, flour, grits | consumer & bulk | | 10 | 1 | 10 |
| Cotton seed | | bulk | | 15 | 4 | 60 |
| Oilseeds meals | peanut, cottonseed | bulk | | 20 | 1 | 20 |
| Edible seeds | pumkin, melon, sesame, etc. | bulk | | 50 | 1 | 50 |

| *Product* | *Description* | *Package Type* | *Lot Size* | *Number of Sample Units* | *Unit Size* | *Sample (lbs.)* |
|---|---|---|---|---|---|---|
| Ginger root | dried, whole ground | consumer & bulk | "n" units | √n<br>10 | 10 x<br>0.06 | 15<br>10 |
| Milk | whole, low fat, skim | consumer & bulk | | 10 | 1 | 10<br>10 |
| Small grains | sorghum, wheat, barley, etc. | bulk | | 10 | 1 | 10 |
| Dried fruit | i.e. figs | consumer & bulk | | 50 | 1 | 50 |
| Mixtures | commodity particles large<br>commodity particles finely ground | consumer & bulk | | 50<br>10 | 1<br>1 | 50<br>10 |

## 2.5. Sample preparation

Once a sample has been taken from the lot, the test sample must be prepared for mycotoxin quantification. Since it is not practical to extract the mycotoxin from a large test sample, the mycotoxin is usually extracted from a much smaller portion of product (subsample or test portion) taken from the test sample. If the commodity is a granular product such as shelled corn, it is essential that the entire test sample be comminuted in a suitable mill before a subsample is removed from the test sample (Dickens and Whitaker, 1982; Campbell, et al., 1986). Removing a subsample of whole seed from the test sample before the comminuting process would eliminate the benefits associated with the larger size test sample of granular product. After the test sample has been comminuted, a subsample is removed from the comminuted test sample for mycotoxin extraction (Figure 2.13).

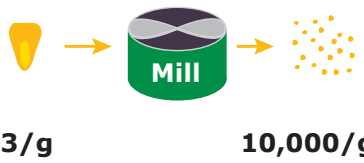



**Figure 2.13** - A test sample of granular product should be ground in a mill to reduce particle size.

Grinders should be used that reduce the particle size of the seed in the test sample to the smallest size possible. Grinders that produce small particles, provide a more homogeneous test sample (Figure 2.13). As a result the mycotoxin concentration of the subsample will more nearly reflect the true mycotoxin concentration of the test sample. Some grinders such as the Romer Series II mill (Malone, 2000) and the USDA peanut mill (Dickens and Satterwhite, 1969) are designed to automatically provide a subsample during the grinding process. If the mill doesn't provide a subsample, the subsample can be obtained using a riffle divider. If the subsample is obtained using a manual device such as a scoop, blend the comminuted test sample before scooping out a subsample.

Normally, there will be no sample preparation step associated with samples of non-granular products such as liquids (milk) or paste (peanut butter). A small portion of the sample may have to be removed for mycotoxin analysis because the entire sample cannot be analyzed. However, it is important to blend or mix liquid samples and paste samples before removing a small portion for mycotoxin analysis.

Subsample sizes vary, but usually are on the order of 25 to 1000 grams depending on particle size. The smaller the particle size, the smaller the subsample size can be without increasing error or uncertainty.

## 2.6. Analytical quantification

Once the subsample is removed from the ground test sample, the mycotoxin is extracted by blending a solvent with the comminuted subsample. Before the mycotoxin can be quantified in the solvent extract, analytical methods usually consist of several steps related to removing interfering compounds (ie. oils) and concentrating the mycotoxins for quantification. These steps may include centrifugation, filtration, drying, and dilution (Steyn et al., 1991). Several types of analytical methods used to quantify mycotoxins extracted from the subsample are thin layer chromatography, ELISA methods that use antibody technology, and high performance liquid chromatography.

There are several possible sources of biases associated with analytical methods. Less than 100% of the mycotoxin may be extracted from the subsample by the solvents; compounds other than mycotoxins may be extracted into the solvent and mistakenly quantified as a mycotoxin; mycotoxin standards used in quantification may not be exact; and instruments to measure the mycotoxin may not be correctly calibrated. Because of these possible biases, organizations such as the Association of Official Analytical Chemists (AOAC) evaluate the performance of analytical methods using collaborative studies.

## 2.7. Accept/reject limit

Once the mycotoxin concentration is quantified, the sample value is used to estimate the true lot concentration or is compared to an accept/reject limit (ARL). The ARL

is a predefined threshold value, usually equal to a legal limit used in regulatory applications. If the sample mycotoxin value is less than or equal to the ARL, the lot is accepted. Otherwise the lot is rejected. When lots are inspected by regulatory agencies, the ARL is usually set equal to the legal limit. However, manufacturers of consumer-ready products will often use an ARL less than the legal limit to reduce the chances that consumer-ready products will be found by regulatory agencies with mycotoxin concentrations above the legal limit. Often private industry will use an ARL that is about half the legal limit.

Many countries agree on the need to establish legal limits, but often disagree on the value of the limit. A survey by FAO in 2003 (FAO, 2003) showed that some countries have aflatoxin legal limits based upon B1 only and some countries use total (B1+B2+G1+G2) aflatoxins and these regulatory limits vary widely. As a result, Codex has a program to harmonize mycotoxin maximum levels and sampling plans for products in the international trade. For example, the CODEX Committee on Contaminants in Foods (CCCF) has established a standard aflatoxin limit for peanuts destined for further processing at 15 ng/g total aflatoxins (FAO, 2001). This limit doesn't infringe on any nations internal limits.

## 2.8. Random variation

Even when using accepted sampling, sample preparation, and analytical procedures (Campbell et al., 1986; AOAC, 1990; Nesheim, 1979), there are errors (the term error will be used to denote variability) associated with each of the above steps of the mycotoxin test procedure (Whitaker et al., 1974). Because of these errors, the true mycotoxin concentration in the lot cannot be determined with 100 percent certainty by measuring the mycotoxin concentration in a test sample taken from the lot. For example, 10 replicated aflatoxin test results from each of six contaminated shelled peanut lots are shown in Table 2.2 (Whitaker et al., 1972). For each test result in the table, the mycotoxin test procedure consisted of (a) comminuting a 5.45kg test sample of peanut kernels in a USDA subsampling mill developed by the U.S. Department of Agriculture (Dickens and Satterwhite, 1969), (b) removing a 280-g subsample from the comminuted test sample, (c) solvent extracting aflatoxins from a 280-g subsample as described by AOAC Method II (AOAC, 1990), and (d) quantifying the aflatoxins densitometrically using thin layer chromatography (TLC). The 10 aflatoxin test results from each lot are ranked from low to high to demonstrate several important characteristics about replicated aflatoxin test results taken from the same contaminated lot.

**Table 2.2 Distribution of aflatoxin test results for ten 5.4 kg samples from each of six lots of shelled peanuts[a,b]**

| *Lot Number* | *Sample Test Result (ng/g)* | | | | | | | | | | *Mean (ppb)* | *SD[c] (ppb)* | *CV[d] (%)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 2 | 4 | 8 | 14 | 28 | 43 | 10 | 15 | 150 |
| 2 | 0 | 0 | 0 | 0 | 3 | 13 | 19 | 41 | 43 | 69 | 19 | 24 | 126 |
| 3 | 0 | 6 | 6 | 8 | 10 | 50 | 60 | 62 | 66 | 130 | 40 | 42 | 105 |
| 4 | 5 | 12 | 56 | 66 | 70 | 92 | 98 | 132 | 141 | 164 | 84 | 53 | 63 |
| 5 | 18 | 50 | 53 | 72 | 82 | 108 | 112 | 127 | 182 | 191 | 100 | 56 | 56 |
| 6 | 29 | 37 | 41 | 71 | 95 | 117 | 168 | 174 | 183 | 197 | 111 | 66 | 59 |

[a] Reference Whitaker et al. 1972.

[b] Aflatoxin test results are order by aflatoxin concentration, [ng/g].

[c] SD = Standard Deviation

[d] CV = Coefficient of Variation = SD*100/mean

First, the wide range among replicated sample results from the same lot reflects the large variability associated with estimating the true mycotoxin content of a bulk lot. In Table 2.2, the variability is described by the standard deviation (SD) and the coefficients of variation (CV). The maximum sample result can be four to five times the lot concentration (the average of the 10 sample results is the best estimate of the lot concentration). Secondly, the amount of variation among the 10 sample results appears to be a function of the lot concentration. As the lot concentration increases, the standard deviation among sample results increases, but the standard deviation relative to the lot mean, as measured by the CV, decreases. Thirdly, the distribution of the 10 sample results for each lot in Table 2.2 is not always symmetrical about the lot concentration. The distributions are positively skewed, meaning that more than half of the sample results are below the true lot concentration. However, the distribution of sample test results becomes more symmetrical as the lot concentration increases. This skewness can be observed by counting the number of aflatoxin test results above and below the lot concentration in Table 2.2 (average of the 10 sample test results). If a single sample is tested from a contaminated lot, there is more than a 50% chance that the sample test result will be lower than the true lot concentration. While it can't be shown in Table 2.2, the skewness is greater for small sample sizes and the distribution becomes more symmetrical as sample size increases (Whitaker et al., 1972). The above characteristics described by Table 2.2 for aflatoxin in peanuts are also generally found for other mycotoxins and other commodities (Dickens et al., 1979; Whitaker et al., 1993; Whitaker et al., 1998).

The sources of the variability among mycotoxin test results in Table 2.2 are associated with each step of the mycotoxin test procedure (Figure 2.2). The sampling, sample preparation, and analytical steps of the mycotoxin test procedure each contribute to the total variability observed among mycotoxin test results shown in Table 2.2 As shown in Figure 2.14, the total error or variability is the sum of the sampling, sample preparation, and analytical variability.

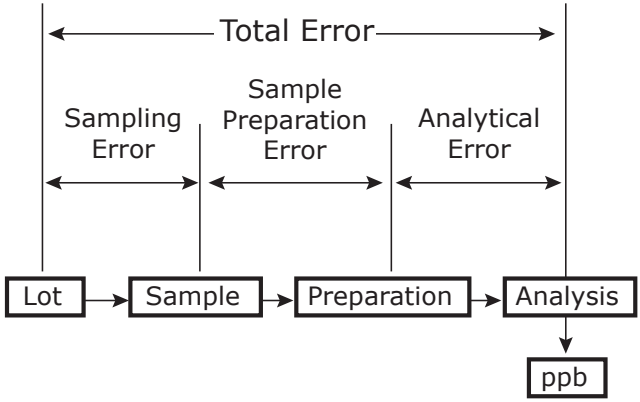



**Figure 2.14** - Total error of the mycotoxin-test procedure is the sum of sampling, sample preparation, and analytical errors.

Among the statistical measures of variability shown in equations 2 to 4, only the variance is additive. Therefore, it is assumed that the total variance (VT) associated with a mycotoxin test procedure is the sum of the sampling (VS), sample preparation (VSS), and analytical variances (VA).

$$VT = VS + VSS + VA \quad (6)$$

Reasons why each step of the mycotoxin test procedure contributes to the overall variability are discussed below. An example of the magnitude of the contribution each step contributes to the total variability is also shown when testing shelled corn (maize) for aflatoxin.

### 2.8.1. SAMPLING VARIABILITY

Studies by researchers on a wide variety of agricultural products (peanuts, cottonseed, shelled corn and pistachio nuts) indicate that, especially for small sample sizes, the sampling step is usually the largest source of variability associated with the mycotoxin test procedure (Dickens et al., 1979). Even when using accepted sample selection equipment and random sample selection procedures, sampling error is large because of the extreme mycotoxin distribution among contaminated particles within a lot.

Studies by researchers on a wide variety of agricultural products such as peanuts and shelled corn (Johansson et al., 2000a; Cucullu et al., 1986; Cucullu et al., 1977) indicate that a very small percentage (0.1%) of the kernels in the lot is contaminated and the concentration on a single kernel may be extremely high. Cucullu et al. (1986) reported aflatoxin concentrations in excess of 1,000,000 ng/g (parts per billion, ppb for individual peanut kernels and 5,000,000 ng/g for cottonseed. Shotwell et al., 1974) reported finding over 400,000 ng/g of aflatoxin in a corn kernel.

Because of this extreme range in aflatoxin concentrations among a few contaminated kernels in a lot, variation among replicated sample test results tends to be large. As an example, the sampling variance, VS, associated with testing shelled corn was estimated empirically (Johansson et al., 2000a) and is shown in equation 7 for any sample size, ns.

$$VS = (12.95/ns)\, M^{0.98} \qquad (7)$$

Where M is the aflatoxin concentration in the lot in nanograms of total aflatoxins per g of corn (ng/g) or parts per billion (ppb), ns is the mass of shelled corn in the sample in kg (kernel count per gram was 3.0). From equation 7 one can see that the sampling variance is a function of the lot aflatoxin concentration M and sample size ns. The sampling variance among replicated 0.91 kg (2 lb) samples taken from a lot of shelled corn at 20 ppb is 268.1. The coefficient of variation is 81.8%.

Researchers have developed equations to describe the sampling variance for several commodities and mycotoxins (Whitaker et al., 1974; Whitaker et al., 1993; Whitaker et al., 1998; Johansson et al., 2000a). The equations are specific for the type mycotoxin and the type product studied, but generally show that sampling variance increases with an increase in concentration, and decreases with an increase in sample size.

### 2.8.2. SAMPLE PREPARATION VARIABILITY

Once the test sample has been taken from the lot, the sample must be prepared for mycotoxin quantification. Since it is not practical to extract the mycotoxin from a large test sample, the test sample is comminuted in a mill and the mycotoxin is extracted from a small subsample taken from the comminuted test sample. If the commodity is a granular product such as shelled corn, it is essential that the entire test sample be comminuted in a suitable mill before a subsample is removed from the test sample (Campbell et al., 1986). Removing a subsample of whole seed from the test sample before the comminuting process is a sample size reduction process and eliminates the benefits associated with the larger size sample of granular product. After the sample has been comminuted in a mill to reduce particle size, a subsample is removed for mycotoxin extraction. It is assumed that the mycotoxin distribution among contaminated particles in the comminuted sample is similar to the distribution among contaminated kernels found in the lot. As a result, there is also variability

among replicated subsamples taken from the same test sample. However, the sample preparation variance is not as large as the sampling variance due to the large number of comminuted particles in the subsample. An example of sample preparation variance for aflatoxin and shelled corn, VSS, is shown below in equation 8 for any subsample size nss (Johansson et al., 2000a).

$$VSS = (62.70/nss)\ M^{1.27} \quad (8)$$

Where M is the aflatoxin concentration in the test sample in ppb, nss is the mass of shelled corn in the subsample in grams. The variance in equation 8 also reflects the use of a Romer Series II mill that produces a particle size where most of the particles will pass through a number 20 screen. From equation 8, it can be seen that the sample preparation variance is also a function of the aflatoxin concentration in the sample and the subsample size. The sample preparation variance associated with a 50 g subsample taken from a sample at 20 ppb is 56.3 and the CV is 37.5%.

Researchers have developed equations to describe the sample preparation variance for several commodities, type mills, and mycotoxins (Whitaker et al., 1993; Whitaker et al., 1998; Johansson et al., 2000a). The equations are specific for the type mycotoxin, type mill (particle size), and the type product used in the study. The type mill affects the particle size distribution. If the average particle size decreases (number of particles per unit mass increases), then the subsampling variances for a given size subsample decreases.

### 2.8.3. ANALYTICAL VARIABILITY

Once the subsample is removed from the comminuted test sample, the mycotoxin is solvent extracted. Analytical methods usually involve several steps such as solvent extraction, centrifugation, drying, dilution, and quantification (Steyn et al., 1991). As a result, there can be considerable variation among replicated analyses on the same subsample extract. The analytical variance, VAh, associated with high performance liquid chromatography (HPLC) techniques used to measure aflatoxin in shelled corn is given by equation 9 (Whitaker et al., 1996) for any number of aliquots, na.

$$VAh = (0.143/na)\ M^{1.16} \quad (9)$$

Where M is the aflatoxin concentration in the subsample in ppb, na is the number of aliquots quantified by HPLC methods. The analytical variance and CV associated with using HPLC to measure aflatoxin in a comminuted subsample of corn at 20 ng/g, is 4.6 and 10.7%, respectively.

High performance liquid chromatography tends to have less variability than other analytical technologies such as thin layer chromatography (TLC) and immunoassay (ELISA) methods (Whitaker et al., 1996). Using precision estimates from collaborative

studies, the analytical variances associated with TLC (VAt) and ELISA (VAe) methods to measure aflatoxin in corn are shown in equations 10 and 11, respectively.

$$VAt = (0.316/na)\ M^{1.744} \quad (10)$$

$$VAe = (0.631/na)\ M^{1.293} \quad (11)$$

The coefficients of variation associated with measuring aflatoxin in a corn subsample at 20 ppb with the TLC and ELISA methods are 38.3 and 27.5%, respectively. The variability associated with HPLC, 10.7%, (equation 9) is lower than either TLC or ELISA.

All of the analytical variance information described above reflects results from single laboratories and do not reflect among-laboratory variances. As a result, some laboratories may have higher or lower variances than those reported in equations 9, 10, and 11. Among laboratory variance is about double the within-laboratory variance (Whitaker et al., 1996).

## 2.8.4. TOTAL VARIABILITY

As shown in Figure 2.14 and equation 6, the total variability, VT, (using variance as the statistical measure of variability) associated with a mycotoxin test procedure is equal to the sum of the sampling (VS), sample preparation (VSS), and analytical (VA) variances associated with each step of the mycotoxin test procedure. The total variability associated with testing shelled corn for aflatoxin, grinding the test sample in a Romer Series II mill, and quantifying aflatoxin by immunoassay is the sum of equations 7, 8, and 11.

$$VT = (12.95/ns)\ M^{0.98} + (62.70/nss)\ M^{1.27} + (0.631/na)\ M^{1.293} \quad (12)$$

Using equation 12, the total, sampling, sample preparation, and analytical variances associated with testing a shelled corn over a range of lot concentrations (M) when using a 0.91 kg sample (ns), grinding the test sample in a Romer Series II mill, taking a 50 g subsample (nss) from a comminuted sample, and quantifying aflatoxin in one aliquot (na) by immunoassay methods are shown in Figure 2.15.

When sampling a shipment of shelled corn at 20 ppb, the magnitude of the variance associated with each step of the above aflatoxin test procedure (equation 12) is shown below in equation 13.

$$VT = 268.1 + 56.3 + 30.4 = 354.8 \quad (13)$$

As shown in Table 2.3, the sampling, subsampling, and analytical variances account for 75.6, 15.9, and 8.5 percent of the total mycotoxin testing variance, respectively.

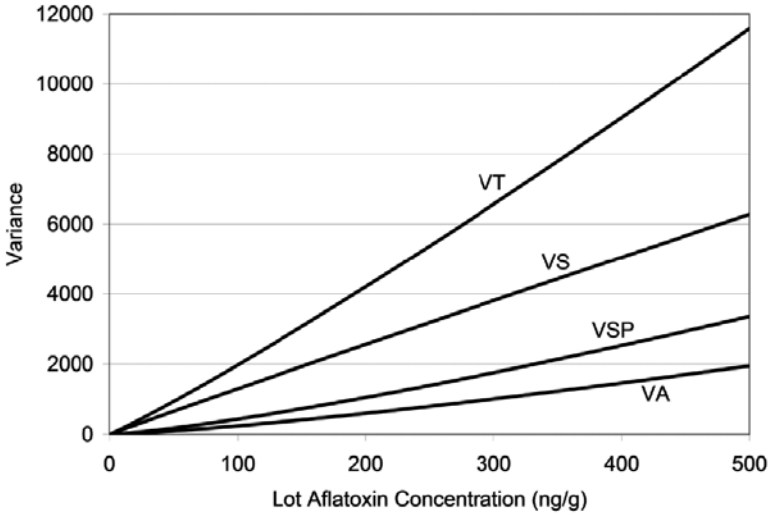



**Figure 2.15** - Variability of each step of the aflatoxin-test procedure, as measured by the variance (V), increases with aflatoxin concentration. The total variance, VT, is the sum of sampling variance, VS, sample preparation variance, VSS, and analytical variance, VA.

**Table 2.3 - The variability, as measured by the variance, associated with 0.91 kg sample, Romer Series II mill to grind the sample, a 50 g subsample, measuring aflatoxin in 1 aliquot (al) by immunoassay (Imm) analytical methods to measure aflatoxin in shelled corn at 20 ppb. Sampling, sample preparation, and analysis errors accounts for about 75.5, 15.9, and 8.6 % of the total error, respectively.**

| *Lot Shelled Corn at 20 ppb Aflatoxin* | | |
|---|---|---|
| *Test Procedure* | *Variance* | *Ratio (%)* |
| 0.91 kg | 268.1 | 75.5 |
| Romer, 50 g | 56.3 | 15.9 |
| Imm, 1 al. | 30.4 | 8.6 |
| Total | 354.8 | 100.0 |

As the above example demonstrates, the sampling step accounts for most of the variability (uncertainty) associated with the total variability of a mycotoxin test procedure because of the extreme aflatoxin distribution among contaminated seed in a lot. For shelled corn, it is estimated that only 6 kernels in 10,000 are contaminated in a lot at 20 ppb (Johansson et al., 2000b). Because of this extreme mycotoxin

distribution among seed in a contaminated lot, it is easy to miss the contaminated seed with a small sample and underestimate the true lot concentration. On the other hand, if the test sample contains one or more highly contaminated seeds, then the test sample will over-estimate the true mycotoxin contamination in the lot. Even using proper sample selection techniques, the variation among test sample concentrations is large due to the mycotoxin distribution described above.

## 2.9. Reducing variability of a mycotoxin test procedure

The only way to achieve a more precise estimate of the true lot concentration is to reduce the total variability of the test procedure. The total variability of the test procedure can be reduced by reducing the variability associated with each step of the mycotoxin test procedure. Increasing the size of the sample can reduce the sampling variability. The sample preparation variability can be reduced either by increasing the size of the subsample and/or by increasing the degree of comminuting (increasing the number of particles per unit mass in the subsample). The analytical variance can be reduced by either increasing the number of aliquots quantified by the analytical method and/or using a more precise quantification method (i.e. using HPLC instead of TLC). If the variability associated with one or more of these steps can be reduced, then the total variability associated with a mycotoxin test result can be reduced (equation 6).

Decreasing the total variability (improving precision) associated with a mycotoxin test procedure will decrease the range of possible aflatoxin test results when replicated tests are made on the same lot. The range of mycotoxin test results associated with any size sample and subsample, and number of analyses about the lot concentration M can be estimated from the total variance, VT, or standard deviation, SD, (square root of the total variance) associated with the mycotoxin test procedure. Approximately ninety five percent of all test results will fall between a low of (M - 1.96*SD) and a high of (M + 1.96*SD).

As an example, when sampling a lot of shelled corn at 20 ppb using a 0.91 kg sample (ns), grinding the test sample in a Romer Series II mill, taking a 50-g subsample (nss) from a comminuted sample, and quantifying aflatoxin in one aliquot (na) by immunoassay method, equation 13 shows that the total variance and standard deviation are 354.8 and 18.8, respectively. The range of aflatoxin test results should fall between 20 +/- (1.96*18.8) or 20 +/- 37 or 0 and 57 ppb (Table 2.4).

The calculated range of aflatoxin test results is only valid for a normal distribution where test results are symmetrical about the mean. The distribution among aflatoxin test results is usually skewed, but will approach a symmetrical distribution as sample size becomes large.

### 2.9.1. SAMPLE SIZE

The effect of increasing sample size on reducing the total variability and the range of mycotoxin test results when testing a contaminated lot of shelled corn at 20 ppb aflatoxin is shown in Table 2.4 when increasing sample size from 0.91 to 4.54 kg.

**Table 2.4 - Effect of increasing sample size on reducing the sampling variability.**

| *Sample Size Effect – Shelled Corn at 20 ppb* | | | |
|---|---|---|---|
| *Test* | *Variance* | *Test* | *Variance* |
| 0.91 kg | 266.5 | 4.54 kg | 53.3 |
| Romer, 50 g | 56.3 | Romber, 50 g | 56.3 |
| TLC, 1 al. | 27.9 | TLC, 1 al. | 27.9 |
| Total | 350.7 | Total | 137.5 |
| Range 20 +/- 37 | | Range 20 +/- 23 | |

Increasing sample size by a factor of five from 0.91 to 4.54 kg will cut the sampling variance in equation 13 by a factor of five from 266.3 to 53.3. The total variance is reduced from 350.7 to 137.5.

$$VT = 53.3 + 56.3 + 27.9 = 137.5 \quad (14)$$

The range of aflatoxin test results is reduced from 20 +/- 37 to 20 +/- 23 ppb as sample size is increased from 0.91 to 4.54 kg, respectively.

### 2.9.2. SUBSAMPLE SIZE

The effect of increasing subsample size from 50 to 100 g on reducing the sample preparation variance is shown in Table 2.5.

**Table 2.5 - Effect of increasing subsample size on reducing sample preparation variability.**

| *Subsample Size – Shelled Corn at 20 ppb* | | | |
|---|---|---|---|
| *Test* | *Variance* | *Test* | *Variance* |
| 0.91 kg | 266.5 | 0.91 kg | 266.5 |
| Romer, 50 g | 56.3 | Romer, 50 g | 28.2 |
| TLC, 1 al. | 27.9 | TLC, 1 al. | 27.9 |
| Total | 350.7 | Total | 322.6 |
| Range 20 +/- 37 | | Range 20 +/- 36 | |

The sample preparation variance is cut in half and is reduced from 56.3 to 28.2. The total variance is reduced from 350.7 to 322.6. The range of aflatoxin test results is reduced from 20 +/- 37 to 20 +/- 36.

### 2.9.3. NUMBER OF ALIQUOTS QUANTIFIED

The effect of increasing number of aliquots quantified in the analytical step from 1 to 2 on reducing the analytical variance for immunoassay type method is shown in table 2.6.

**Table 2.6 - Effect of increasing number of aliquots quantified for aflatoxin on reducing analytical variability.**

| *Aliquot Effect – Shelled Corn at 20 ppb* | | | |
|---|---|---|---|
| *Test* | *Variance* | *Test* | *Variance* |
| 0.91 kg | 266.5 | 0.91 kg | 266.5 |
| Romer, 50 g | 56.3 | Romer, 50 g | 56.3 |
| TLC, 1 al. | 27.9 | TLC, 1 al. | 14.0 |
| Total | 350.7 | Total | 336.8 |
| Range 20 +/- 37 | | Range 20 +/- 37 | |

The analytical variance is cut in half and is reduced from 27.9 to 14.0. The total variance is reduced from 350.7 to 336.8. The reduction is so small that the range of aflatoxin test results is not significantly affected.

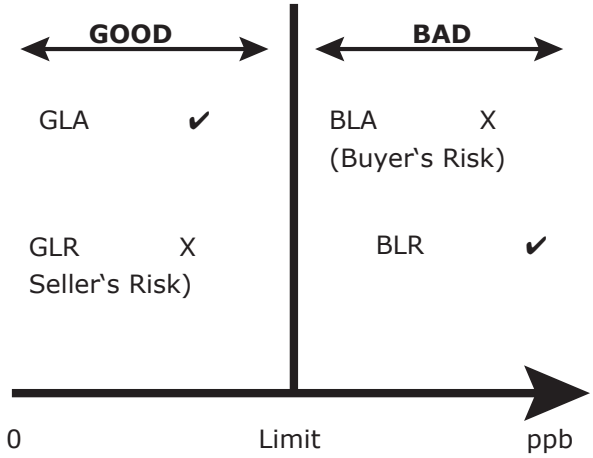
There are different costs associated with reducing the variability of each step of a mycotoxin test procedure. One needs to try and maximize the variance reduction for a given cost. Increasing sample size is usually the best use of resources when reducing the total variability of mycotoxin test results.

## 2.10. Designing mycotoxin sampling plans

Because of the variability among mycotoxin test results, two types of mistakes are associated with any mycotoxin-sampling plan. First, good lots (lots with a concentration less than or equal to the legal limit) will test bad and be rejected by the sampling plan. This type of mistake is often called the seller's risk (false positives) since these lots will be rejected at an unnecessary cost to the seller of the product. Secondly, bad lots (lots with a concentration greater than the legal limit) will test good and be accepted by the sampling program. This type of mistake is called the buyer's

risk (false negatives) since contaminated lots will be processed into feed or food causing possible health problems and/or economic loss to the buyer of the product. In order to maintain an effective regulatory and/or quality control program, the above two risks associated with a sample design must be evaluated (Table 2.7). Based upon these evaluations, the costs and benefits (benefits refers to removal of mycotoxin contaminated lots) associated with a sampling program needs to be evaluated.

**Table 2.7 - Four possible outcomes when classifying lots as good or bad. Good lots rejected (GLR) and bad lots accepted (BLA) are incorrect decisions. Good lots accepted (GLA) and bad lots rejected (BLR) are correct decisions.**




A lot is termed bad when the sample test result X is above some predefined accept/reject limit Xc and the lot is termed good when X is less than or equal to Xc. While Xc is usually equal to the legal limit Mc, Xc can be greater than or less than Mc. For a given sample design, lots with a mycotoxin concentration M will be accepted with a certain probability P(M)=prob(X< Xc |M) by the sampling plan. A plot of P(M) versus the lot concentration M is called an operating characteristic (OC) curve. Figure 2.16 depicts the general shape of an OC curve.

As M approaches 0, P(M) approaches 1 or 100%, and as M becomes large, P(M) approaches zero. Lots with little to no contamination (M=0) are accepted by the sampling plan 100% of the time; lots with very high levels of contamination (M= large) are never accepted (rejected 100% of the time) by the sampling plan; lots with contamination levels near the accept/reject limit are accepted by the sampling plan less than 100% of the time. The shape of the OC curve is uniquely defined for a particular sampling plan design with designated values of sample size, degree of comminution, subsample size, type analytical method, and number of analyses, and the accept/reject limit Xc.

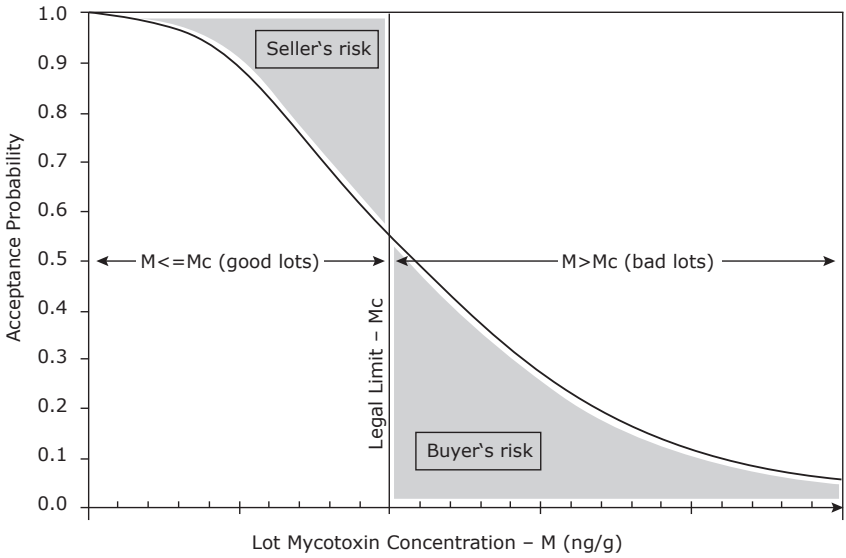



**Figure 2.16** - General shape of an operating characteristic (OC) curve. The shape of the OC curve is unique for an aflatoxin test procedure and indicates the magnitude of the buyer's and seller's risks.

## 2.10.1. CALCULATION OF ACCEPTANCE PROBABILITY

The acceptance probability P(M) associated with sampling a commodity for a mycotoxin can be computed if the distribution among replicated sample test results can be described and if the appropriate variance relationships are known for a mycotoxin test procedure. Several skewed distributions such as the negative binomial and compound gamma have been shown to adequately describe the observed mycotoxin distribution of sample test results for several commodities and several mycotoxins. An example of the probability of accepting and rejecting shelled corn lots over a range of lot concentration for the sampling plan where ns = 4.54 kg, Romer Series II mill, nss = 50 g, immunoassay analytical method, na = 1 aliquot, and accept/reject limit Xc = 20 ppb is shown in Table 2.8.

Table 2.8 shows that most of the lots below 10 ppb are accepted by the sampling plan and most of the lots above 60 ppb are rejected by the sampling plan. For example, 95% and 2 % of the lots at 10 and 60 ppb are accepted by the sampling plan, respectively. As lot concentration M increases, the percentage lots accepted by the sampling plan decreases. The acceptance probabilities in Table 2.8 are plotted in Figure 2.17 and a smooth curve forced through the points.

**Table 2.8 - Probability of accepting and rejecting lots of shelled corn over a range of lot aflatoxin concentrations for a sampling plan that uses 4.54 kg sample size, Romer Series II mill, 50 g subsample, ELISA method, 1 aliquot, and accept/reject limit of 20 ppb.**

| *Lot Concentration Lot M [ppb]* | *Probability of Accepting Lot at M* $P(X< Xc\|M)$ | *Probability of Rejecting* $1 - P(X< Xc\|M)$ |
|---|---|---|
| 0 | 1.000 | 0.000 |
| 5 | 0.983 | 0.017 |
| 10 | 0.887 | 0.113 |
| 15 | 0.726 | 0.274 |
| 20 | 0.551 | 0.449 |
| 25 | 0.396 | 0.604 |
| 30 | 0.274 | 0.726 |
| 35 | 0.185 | 0.815 |
| 40 | 0.122 | 0.878 |
| 45 | 0.079 | 0.921 |
| 50 | 0.051 | 0.949 |
| 55 | 0.032 | 0.968 |
| 60 | 0.020 | 0.980 |

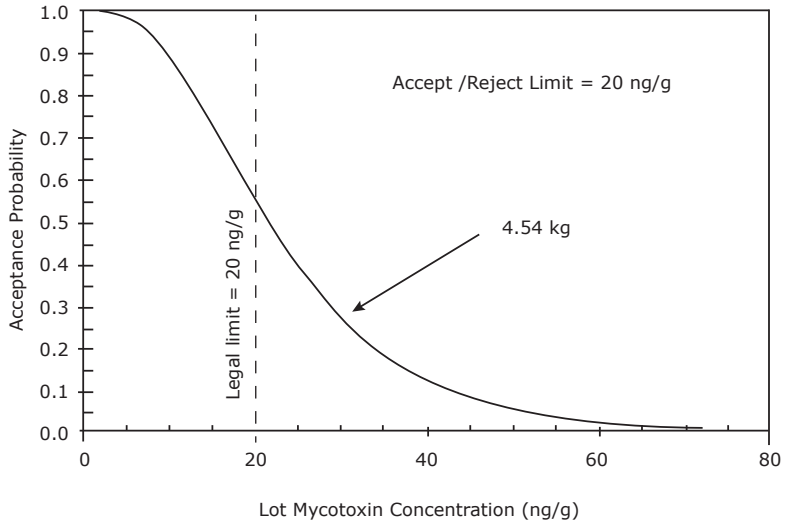



**Figure 2.17** - Operating characteristic curve for a sampling plan that uses a 4.54 kg sample, Romer Series II mill, 50 g subsample, ELISA method, 1 aliquot, and a 20 ppb accept/reject limit to detect aflatoxin in shelled corn.

For a given sampling plan, the OC curve indicates the magnitudes of the buyer's and seller's risk. When Mc is defined as the legal limit or the maximum lot concentration acceptable, lots with M > Mc are bad and lots with M <= Mc are good. In Figure 2.17, the area under the OC curve for M > Mc represents the buyer's risk (bad lots accepted) while the area above the OC curve for M < Mc represents the seller's risk (good lots rejected) for a particular sampling plan. Using the example in Table 2.8, if lots at 20 ppb or less are considered good lots and lots greater than 20 ppb are consider bad lots, then lots rejected below 20 ppb are considered a measure of the seller's risk (good lots rejected) and the lots accepted above 20 ppb are considered the buyer's risk (bad lots rejected).

Because the shape of the OC curve is uniquely defined by the sample size, degree of comminution, subsample size, the number of analyses and the accept/reject limit, these parameters can be used to reduce the buyer's and seller's risks associated with a sampling plan.

## 2.10.2. SAMPLE SIZE EFFECT ON RISKS

The effect of increasing sample size on the shape of the OC curve when testing shelled corn lots for aflatoxin is shown in Figure 2.18 where the accept/reject limit is equal to the legal limit of 20 ppb. As sample size increases from 0.91 to 9.07 kg, the slope of the OC curve about legal limit increases forcing the two areas associated with each risk to decrease. As a result, increasing the size sample decreases both the buyer's and seller's risks. The same effect can be obtained by increasing either the degree of sample comminution, subsample size or number of analyses.

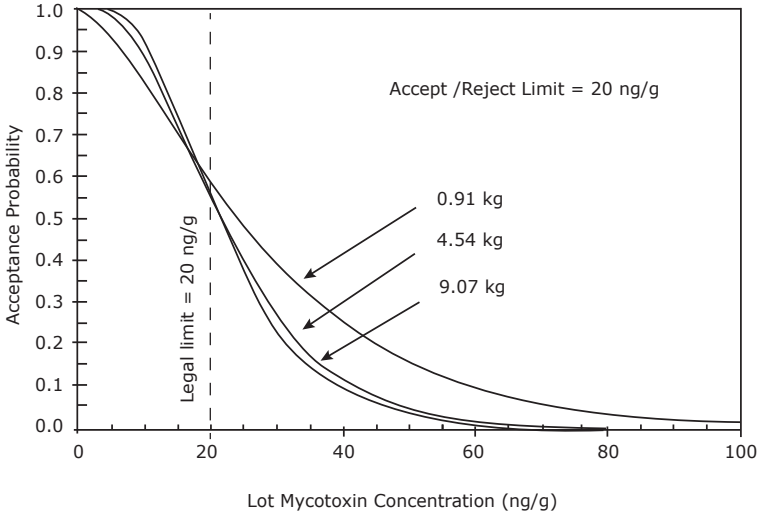



**Figure 2.18** - Three operating characteristic (OC) curves describing the performance of aflatoxin sampling plans to detect aflatoxin in shelled corn. The three OC curves show that increasing sample size reduces both buyer's and seller's risks.

## 2.10.3. ACCEPT/REJECT LIMIT EFFECTS ON RISKS

The effect of changing the accept/reject limit, relative to the legal limit, on the two risks when testing shelled corn lots for aflatoxin is shown in Figure 2.19. If the legal limit is assumed to be 20 parts per billion (ppb), then changing Xc to a value less than 20 ppb shifts the OC curve to the left. Compared to the sampling plan where Xc = 20, the buyer's risk decreases, but the seller's risk increases. If Xc becomes larger than 20, the OC curve shifts to the right. As a result, the seller's risk decreases but the buyer's risk increases. Changing the accept/reject limit relative to the legal limit can reduce only one of the two risks, because reducing one risk will automatically increase the other risk.

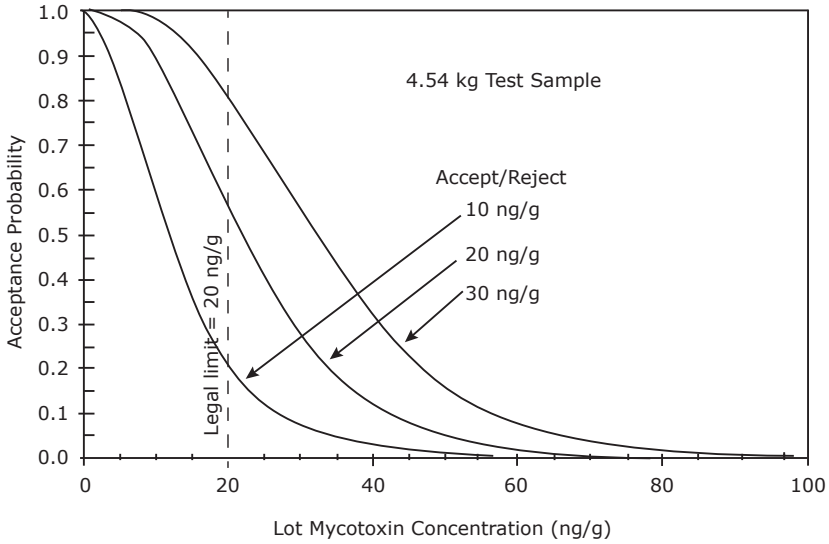



**Figure 2.19** - Three operating characteristic curves for three accept/reject limits on the performance of sampling plans to detect aflatoxin in shelled corn. If the accept/reject limit (10ppb) is less than the legal limit (20 ppb), the seller's risk increases and the buyer's risk decreases. If the accept/reject (30 ppb) is greater than the legal limit (20 ppb), the seller's risk decreases and the buyer's risk increases.

## 2.10.4. MULTIPLE SAMPLES

Increasing the number of samples of a given size taken from a contaminated lot can reduce the risks associated with classifying lots. If the mycotoxin among all samples is averaged, the effect is the same as that described in section 2.10.2 (Figure 2.18) for the effect of increasing sample size. However, if all multiple sample test results are required to test less than some accept/reject limit, the effect is the same as shown in section 2.10.3 (Figure 2.19) for changing the accept/reject limit relative to the legal limit. Three sampling plans showing the effect of requiring either one, two, or three 4.54 kg samples to all test less than or equal to 20 ppb is shown in Figure 2.20.

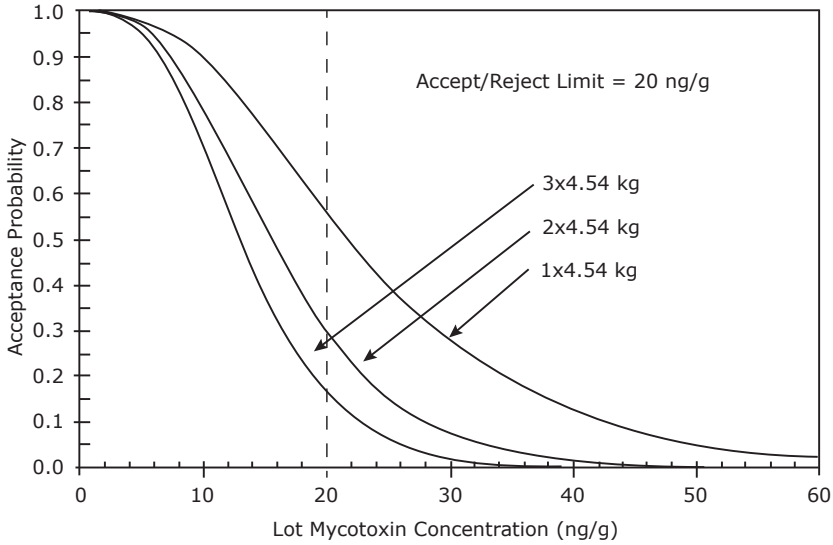



**Figure 2.20.** Three operating characteristic curves showing the effect of requiring either one, two, or three 4.54 kg samples of shelled corn to all test less than or equal to the accept/reject limit of 20 ppb total aflatoxin to accept the lot.

As the number of samples required to test less than or equal to the accept/reject limit increases, the OC curve shifts to the left reducing the buyer's risk but increasing the seller's risk. The result is similar to reducing the accept/reject limit. This type of sampling plan is often used late in the marketing system on finished product destined for animals or humans have little chance of product containing mycotoxin above the legal limit. The buyer is placing most of the risk on the seller.

## 2.11. Conclusions

Because of the uncertainties (biases and variability) associated with a mycotoxin test procedure, it is difficult to determine with 100 % certainty the true concentration of a bulk lot. Even when the sample is correctly selected (no biases), there will be variability associated with the mycotoxin test procedure. The variance associated with a mycotoxin test procedure is the sum of sampling, sample preparation, and analytical variances. For small sample sizes, sampling is usually the largest source of variability. Increasing sample size, the degree of sample comminution, subsample size, and the number of aliquots quantified can reduce the variability associated with a mycotoxin test procedure. Reducing variability of the mycotoxin test procedure will reduce the number of lots misclassified by the sampling plan.

Methods have been developed to predict the seller's and buyer's risks, the total number of lots accepted and rejected, the amount of mycotoxin in the accepted and

rejected lots, and the costs associated with a mycotoxin inspection programs for several commodities (FAO, 1993; Whitaker and Dickens, 1979; Johansson et al., 2000c; Whitaker et al., 1995). These methods have been used by the UDSA/AMS and the peanut industry to design aflatoxin-testing programs for shelled peanuts (Whitaker et al., 1995) and by the FAO (FAO, 1993) to design the aflatoxin-testing plan for raw shelled peanuts.

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# 3. MYCOTOXIN ANALYSIS

RUDOLF KRSKA AND RAINER SCHUHMACHER

## 3.1. Introduction

The safety of food and feed has become of increasing concern for consumers, governments and producers as a result of the global marketplace where foods and feeds are produced and distributed throughout the world, as well as because of a rise in public awareness about health and quality. Several highly publicized global incidents related to chemical contaminants in food have also attracted much media attention (Krska et al., 2012). Trace levels of chemical contaminants in foods can originate from natural sources such as mycotoxins (Murphy et al., 2006; Reddy et al., 2010), natural toxins produced by fungi and phycotoxins (Mayer, 2009; Aráoz et al., 2010), natural toxins in food produced by algae.

It is essential to analyse food and feed samples using sensitive, fast and accurate analytical methods to provide information about the levels of mycotoxins in foods and feeds and to support food safety standard setting activities. This requirement, in conjunction with the rising number of sample matrices and analytes of interest, has led to the development of both rapid screening methods for various analytes, mostly based on immunochemical techniques, and of highly sophisticated multi-analyte methods based on liquid chromatography coupled with multiple-stage mass spectrometry (LC-MSn) to allow identification and simultaneous determination of a wide range of secondary fungal contaminants (Krska et al., 2012; Krska et al., 2005).

However, the chemical diversity of the mycotoxins and the wide range of agricultural commodities and foods available pose a challenge in method development. Concentration levels in food and mixed feed may also vary considerably. Until recently, most of the analytical tools developed targeted single classes and specific substrates and comprise extraction and clean-up steps to reduce or eliminate unwanted matrix components as well as the enrichment of sample extracts. Rapid, mostly immunoanalytical screening tests and a number of new techniques such as biosensors are emerging rapidly, in addition to validated official analytical methods based on chromatographic principles (Krska et al., 2007). Several national authorities maintain an active research program to study the distribution and sources of mycotoxins in cereals and food and conduct major national surveys (Krska et al., 2012).

This chapter is dedicated to the determination of mycotoxins and covers proper extraction and clean-up procedures, separation and detection techniques including rapid strip tests and puts a special emphasis on LC-MS/MS multi-toxin methods. Recent developments in the determination of mycotoxins in foods have been reviewed

by Shephard et al. (2010), who covered aflatoxins, *Alternaria* toxins, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone. A recent review of methodologies for mycotoxins by Maragos and Busman (2010) emphasizes the need for methods with increased throughput.

## 3.2. Extraction

Analytical methods based on chromatography (Ahuja, 1999) or immunoassays usually require solvent extraction to liberate the mycotoxin from the sample matrix, and the subsequent clean-up of the extract in order to reduce matrix effects (Krska et al., 2008). Various combinations of solvents, sometimes with the addition of modifiers (e.g. acids, bases, etc.), are used for extraction depending on the physicochemical properties of the mycotoxins, the sample matrix and the type of clean-up used afterwards (Zöllner et al., 2006). Extraction can be performed when two immiscible liquid phases or a solid and a liquid phase are present. Depending on the conditions, the mycotoxin -- but also substances with similar properties -- will migrate into the extraction solvent until equilibrium is reached. As a result, the desired compounds can be concentrated in a solvent and interferences can already partly be eliminated. This is essential when dealing with food and feed commodities, which usually are complex matrix systems with numerous compounds present.

Liquid-solid extraction is one of the fundamental operations in mycotoxin analysis. This type of extraction is performed if the sample is available in a solid form, as e.g. cereals including maize, and most other foods and agricultural products. If solid samples are not available, freeze-drying or dehydration can be an alternative for making the sample easier to handle. The purpose of the extraction step is to dissolve the mycotoxins of interest quantitatively in an ideal way (De Levie, 1997) in the solvent mixture, with as few additional, unwanted compounds as possible to avoid interferences. Hence, the choice of an appropriate extraction solvent is another crucial task to consider. Polar mycotoxins, such as nivalenol (Krska et al., 2001), favour polar solvents and also the pH plays a key role during extraction because it determines the species in which the analyte is present in. Consideration of pH is necessary, especially for mycotoxins with acidic and/or basic functional groups, such as moniliformin and fumonisins which may carry several carboxylic functions. The eluotropic list of solvents is a useful tool as the solvents are sorted according to their polarity, starting with the lowest. Not only is there the criterion that the extraction solvent shall extract the analyte quantitatively, it should easily be recoverable, non-toxic and non-flammable. Other aspects may be important as well depending on the conditions, such as volatility which makes it easier to reduce the volume after extraction, which is an often required preconcentration step during clean-up procedures.

If the solvent shall remain in the final sample extract and is thus to be used for chromatography with a subsequent UV detection, no absorbance of the solvent at the

analytical wavelength of the analyte´s absorption is a prerequisite.The most common procedures used for extraction are shaking and blending. In addition, mechanical tube rollers or gentle end over end mixing are widely used. By plotting the transfer of the analyte into the solvent against time, a compromise between the ideal time (leading to quantitative extraction) and what is practical can be achieved. Trenholm et al. (1985) revealed that naturally contaminated samples need longer extraction times than spiked samples. It is, therefore, recommended that the extraction procedure is evaluated by using samples that match, as closely as possible, the contaminations under real conditions. In the presence of ion-pairing agents, also ionisable mycotoxins, such as moniliformin can be extracted into organic solvents as neutral ion pairs. Hence, a suitable buffer, which controls the pH value is required.

For the extraction of mycotoxins from food and feed, including grains and grain-based products, mostly organic solvent/water mixtures are used such as methanol/water and acetonitrile/water. Analytical methods based on chromatography or immunoassays usually require solvent extraction to liberate the mycotoxin from the sample matrix, and the subsequent clean-up of the extract to reduce matrix effects. Various combinations of solvents, sometimes with the addition of modifiers (e.g. acids, bases, etc.), are used for extraction, depending on the physicochemical properties of the mycotoxins, the sample matrix and the type of clean-up used afterwards (Krska et al., 2008; Zöllner and Mayer-Helm, 2006).

## 3.3. Clean-up

Following extraction, the resulting raw extract is usually processed further to remove unwanted substances and often concentrated to make determination of toxins at the lowest concentrations possible. It is the main objective in mycotoxin clean-up procedures to remove interferences in order to make a chromatographic separation and subsequent detection and identification as unambiguous as possible (Krska, 1998; Ahuja, 1992). Matrix components such as lipids, carbohydrates and peptides that are usually present in the raw extract make an additional purification step necessary prior to the ultimate separation and detection step.

### 3.3.1. LIQUID-LIQUID SEPARATION

Liquid-liquid partitioning is a well-known and well-established clean-up technique. It is based on the partition between immiscible solvents, one of which contains the analyte. The analyte then migrates into the other phase until an equilibrium has been reached. This step can be performed several times with fresh solvent in order to extract the analyte quantitatively. After the partitioning stage, rotary evaporation is usually performed to reduce the amount of solvent and pre-concentrate the analyte. This technique is simple and easy to perform with standard laboratory equipment and

often still forms part of official methods. It is, however, used less frequently nowadays because it is labour intensive and large volumes of (sometimes chlorinated) solvents are required. Liquid-liquid partitioning is a batch method and can not be automated. Therefore, the method is now often replaced by less labour intensive techniques such as Solid Phase Extraction (SPE).

### 3.3.2. SOLID PHASE EXTRACTION

Solid phase extraction (SPE) can be performed on- or off-line. A typical SPE sequence starts with the conditioning of the column (i.e. activating it with solvent). The aqueous sample is then applied and the analyte is trapped together with the matrix. Most of the interferences are removed by a rinsing step, with the analyte staying on the column. Consequently, the analyte is eluted and a further preconcentration step is employed evaporating excess solvent, e.g. with nitrogen. Vacuum manifolds enable the simultaneous preparation of large batches with up to e.g. 96 samples. SPE methods have been developed for a number of mycotoxins as a convenient alternative to liquid-liquid separation. Analytes include A and B trichothecenes, zearalenone (ZEN), ochratoxin A (OTA) and fumonisins (Langseth and Rundberget, 1998).

C8 and C18 bonded silica columns are the most frequently used as they are pressure resistant and give reproducible results. SPE does not have any significant drawbacks compared to conventional liquid-liquid separation, especially in combination with subsequent liquid chromatographic methods, but the advantages include the consumption of less solvent and the possibility of automation. Less time is needed and percolation of samples in the field is possible.

The MycoSep® multifunctional SPE clean-up columns (Romer, 1986) consist of adsorbents, which are packed in a plastic tube. A rubber flange, a porous frit and a one-way valve on the lower end ensure that the extract is forced through the packing material, when the column is inserted into the culture tube. On top of the plastic tube, the purified extract appears within seconds. No additional rinsing steps are required and almost all interfering substances are retained on the column, while the analyte does not show an affinity to the packing material. Columns are usually suitable for one analyte only and are available for a range of mycotoxins such as aflatoxins, deoxynivalenol (DON) and patulin (Trucksess et al., 1995). MycoSep® multifunctional have also been successfully employed in a multi-analyte-LC-MS/MS method for the simultaneous determination of the *Fusarium* mycotoxins nivalenol, deoxynivalenol, fusarenon-X, 3-acetyl-deoxynivalenol, the sum of 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol, diacetoxy-scirpenol, HT-2 toxin, T-2 toxin and zearalenone in maize (Berthiller et al., 2005).

Anionic compounds can be isolated on SAX (strong anion exchange) bonded SPE-silica columns. Ion exchange mechanisms are employed if the analyte can be made present as an ion (e.g. moniliformin, MON). The retention is based on the electrostatic attraction of a charged functional group of the analyte to the charged group on the silica surface of the column. Other species from matrix components of the same charge

may interfere with the adsorption and reduce the selectivity. A solution with high ionic strength can be used for elution because of its higher affinity to the sorbent. Ion exchange columns are well suited for the clean-up of samples containing moniliformin. SAX columns are used to isolate strong anionic (very low pKa, <1) or weak anionic (low pKa, >2) compounds (mostly strong or weak acids) and should be conditioned with the sample solvent. It is important to note that the packing should not dry up between conditioning and sample addition and that columns can be used again several times, after regeneration. SAX columns are used for the determination of mycotoxins such as ochratoxin A and fumonisins.

### 3.3.3. THE QUECHERS APPROACH

Another option, only recently introduced in multi-mycotoxin analysis, is the use of the various modifications of the QuEChERS approach (Quick, Easy, Cheap, Effective, Rugged and Safe approach), currently widely used in multi-pesticide analysis, for very fast extraction and purification. The key principle is the partitioning of an acetonitrile-water mixture induced by addition of inorganic salts. While the analytes are transferred into an organic phase, more polar matrix impurities are left in an aqueous layer (Anastassiades et al., 2003). There is an increase in the number of publications on mycotoxin determination using the QuEChERS method (Shephard et al., 2011). The QuEChERS-like method was found to be easy to handle and allows high sample throughput. Up to now, the QuEChERS approach has been successfully implemented, e.g. in the extraction of nivalenol from cereals (Desmarchelier et al., 2010; Zachariasova et al., 2010), cereal-based products (Cunha and Fernandes, 2010; Sospedra et al., 2010), and silage (Rasmussen et al., 2010).

### 3.3.4. IMMUNOAFFINITY COLUMNS

Immunoaffinity columns (IACs) for clean-up purposes have become increasingly popular in recent years because they offer high selectivity (Visconti et al., 1998) ) and thus facilitate highly pure cleaned-up sample extracts. They are easy to use for the purification of samples that are contaminated with different mycotoxins. The analyte molecules (i.e. the mycotoxin) are bound selectively to the antibodies on the column after a preconditioning stage. The extract has to be applied with a limited proportion of organic solvent to prevent denaturation of the bound mycotoxin specific antibodies. As matrix components do not interact with the antibodies, a rinsing step removes most of the possible interferences. In the final elution stage, the toxin is eluted by antibody denaturation through the application of usually pure organic solvents. The columns generally feature a higher recovery than standard liquid-liquid partitioning. Single analyte columns include those for aflatoxins, zearalenone, ochratoxin A, fumonisins and deoxynivalenol (DON). The succesful use of immunoaffinity columns in combination

with subsequent LC-MS determination has recently been demonstrated within an interlaboratory comparison study for the determination of fumonisins B1 and B2 in corn (Senyuva et al., 2010). Immunoaffinity columns have also become available for the simultaneous determination of aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, zearalenone, T-2 and HT-2 toxins (Lattanzio, 2007). The fact that columns are typically only used once and their relative high costs are major disadvantages.

### 3.3.5. OTHER TECHNIQUES

A number of alternative clean-up techniques for mycotoxins have been described in the literature: a microwave-assisted extraction (MAE) method has been developed by Pallaroni et al. for zearalenone (ZEN) in wheat and corn and subsequent determination by LC-MS with an atmospheric pressure chemical ionization interface (APCI). The extraction was performed with 1:1 (v/v) methanol-acetonitrile at 80 Deg C for 5 min. The extraction and clean-up is performed in one step (Pallaroni et al., 2002).

*Supercritical fluid extraction* (SFE) is another possibility to reduce the number of sample preparation steps. An SFE method has been published by Krska for DON, where the extraction and clean-up are performed with supercritical $CO_2$, which is non-toxic, non-flammable and chemically inert. An extraction thimble is filled with sample and a modifier (e.g. methanol) is added to the extraction solvent. The analyte is trapped in an SPE silica trap and detected by HPLC-DAD. Obtained recoveries for DON in wheat flour were, however, only the range of 53%.

*Summary of typical clean-up procedures for various mycotoxins:*

Aflatoxins: IAC, SPE

Type-A Trichothecenes: SPE, Mycosep columns

Type-B Trichothecenes: liquid-liquid separation, IAC (DON), SPE, Mycosep

Zearalenone: liquid-liquid separation, IAC, SPE, Mycosep

Moniliformin: Ion exchange column

Beauvericin: Liquid-liquid separation, SPE, Mycosep

Ochratoxin A: IAC, SPE, Ion exchange column

Fumonisins: IAC, SAX, SPE

Patulin: liquid-liquid separation, Mycosep, SPE

## 3.4. Rapid strip tests

In the last decade, rapid immuno-assay based tests have been used increasingly in the food and feed sector, where applications range from the screening for foodborne pathogens, drug residues and mycotoxins, to allergens and recently genetically modified organisms. Trends in the determination of mycotoxins by rapid strip tests

have been summarized by Krska and Molinelli (2009a). Tests for mycotoxins, which allow for the screening of agricultural commodities with results within less than 20 minutes are gaining acceptance and are being increasingly integrated into routine quality monitoring procedures (Delmulle et al., 2005). Enzyme linked immunosorbent assays (ELISAs) have become a standard tool for the rapid monitoring of mycotoxins (Molinelli et al., 2009b). Microtitre plate ELISAs offer the advantage of speed, ease of operation, sensitivity, and high sample throughput. Faster and more easy-to-use immunoassay-based tests are preferred for applications where on-site use is necessary. Rapid disposable assay tests have been developed in multiple formats such as flow through tests (Urraca et al., 2005), dip sticks (Stephan et al., 2002) and strip tests (Molinelli et al., 2008) also called lateral flow devices (LFDs). Often these technical expressions are not used consistently. LFDs are usually based on a test format which includes sample flow along an analytical nitrocellulose membrane due to capillary forces and allow performing fast and easy-to-handle immunoassays. They can be both qualitative (with a defined cut-off level) and quantitative when used with a photometric strip reader. A major the advantage of rapid immunoassay-based tests is that sample clean-up can generally be omitted. Sample extraction must consider, however, both extraction efficiency of mycotoxins and solvent compatibility with the antibodies applied in the test.

Strip tests for mycotoxins are based on a competitive immunoassay format where a labeled antibody is used as signal reagent. In addition to the classical enzyme-linked immunoassay approach, a variety of reagents have been used for signaling such as colored latex particles, colloidal gold particles, fluorescent labels such as e.g. carbon nanoparticles (Van Dam et al., 2004), as well as magnetic beads as previously reviewed elsewhere (Chan et al., 2008). Colloidal gold is used in the majority of the available strip tests for mycotoxins due to its ready availability, ease of production and ease of conjugate formation with antibodies (Krska & Molinelli, 2009). A reproducible production method has been developed for the synthesis of well characterized colloidal gold particles to be employed in LFDs (Cvak et al., 2012).

Most of the strip tests developed constitute qualitative assays. Nevertheless, a trend can be seen towards (semi-) quantitative tests (Molinelli et al., 2008) driven by strong demand from industry, as well as multi-mycotoxin approaches such as a lateral-flow immunoassay aiming at the rapid simultaneous detection of several mycotoxins. A rising amount of commercially available test kits for mycotoxins confirms the trend towards screening tests which are easy-to-use and allow rapid on-site decision-taking based on quantitative results. Strip-test based test kits are available commercially for several mycotoxins including aflatoxins (qualitative and quantitative), deoxynivalenol (semi-quantitative and quantitative), fumonisins (qualitative and quantitative), ochratoxin A (quantitative), and zearalenone (quantitative). Very recently, a multiplex dipstick immunoassay based method for the simultaneous semi-quantitative determination of major *Fusarium* toxins, namely zearalenone, T-2 and HT-2 toxins, deoxynivalenol and fumonisins in wheat, oats and maize has been developed. The dipstick format was based on an indirect competitive approach. Four test lines and

one control line were located on the strip membrane. The optimized immunoassay was able to detect target mycotoxins at cut off levels equal to 80% of EU maximum permitted levels in maize, wheat and oats (Lattanzio et al., 2012). A membrane and a gel based flow-through enzyme immunoassay for the detection of four mycotoxins (ochratoxin A, fumonisin B1, deoxynivalenol and zearalenone) in peanut cake, maize and cassava flour samples have been developed very recently and their performance compared (Ediage et al., 2012). Both assays did not make use of any equipment and provided a yes/no response indicating whether the toxins were present or not above the maximum permitted levels of these analytes.

Problems with the reproducibility and sensitivity especially with complex food and feed matrices, however, often limit the application of strip tests (Chan et al., 2007; Krska and Molinelli, 2009). Moreover, differences observed between spiked samples and naturally contaminated samples contribute to calibration and validation problems with shifts of the cutoff level in the strip test or shifts of relative reflectance readings of the test line in a quantitative strip test. Strip test optimization and validation should, therefore, be performed using naturally contaminated material and a convenient reference method such as LC-MS/MS (Sulyok et al., 2006) should be employed. Certified reference materials for quality control measurements are available for selected commodities and mycotoxins and should be employed for testing the trueness of any analytical methods including rapid assays for the determination of mycotoxins (Krska et al., 2008; Josephs et al., 2004). There are, however, no general validation protocols available for qualitative test methods. Solely the cut-off level is defined as the concentration threshold below which positive identification becomes unreliable (Eurachem, 2002).

## 3.5. LC-MS(/MS) based methods

### 3.5.1. INTRODUCTION

Until the mid 1990s, most instrumental analytical methods for the determination of mycotoxins used HPLC in combination with FLD (Schuhmacher et al.,1998) or UV detection and GC-ECD (e.g. trichothecenes) (Weingärtner et al.,1997) or GC-FID. The confirmation of the presence of mycotoxins in real-world samples was carried out almost exclusively by a combination of GC with MS (Schwadorf et al., 1991; Plasencia et al., 1990; Plattner et al., 1990). During the last 10-15 years, the online coupling of HPLC and MS (LC-MS) has developed to one of the most powerful techniques for the analysis of mycotoxins. There has been a clear trend towards the use of LC-MS-based multi-analyte methods in the field of mycotoxin analysis which often involved no or minimal clean-up procedures and the use of matrix matched calibration or (C13-) labeled mycotoxin standards in the last decade.

Developments in the application of LC-MS/MS for the trace determination of organic residues and contaminants including mycotoxins were summarised by

Schuhmacher et al. (2008). In addition to a short presentation of the most frequently applied MS instruments, advances in HPLC instrumentation, as well as three major current topics in LC-MS(/MS) -- namely matrix effects, multi-target methods and the confirmation of positive results -- were discussed in Schuhmacher et al., 2008.

The main technical problem which had to be solved with respect to the direct coupling (interfacing) of HPLC with MS consisted in the incompatibility of liquid mobile phase flow rates (ml/min) with the vacuum required in the mass spectrometer. Different types of interfaces such as the moving belt (Millington et al., 1980) or particle beam interface (Willoughby et al., 1984; Winkler et al., 1988) were developed in which the liquid mobile phase was evaporated before the analyte reached the ion source of the mass spectrometer. Unfortunately, these instruments lacked robustness, as well as sensitivity, and were, therefore, not well suited for the routine measurement of trace contaminants like mycotoxins. The real breakthrough was achieved by the development of robust interfaces in which the analyte ionization was achieved under atmospheric pressure conditions (API). Today, electrospray ionization (ESI) (Yamashita et al., 1984; Fenn et al., 1989) and atmospheric pressure chemical ionization (APCI) (Bruins, 1991) are the most common API interfaces for the determination of mycotoxins and are used routinely in many analytical laboratories. In API, ions are formed by a mixture of solvent evaporation and low energy collision processes, both of which afford ions having little excess of internal energy (Johnstone et al., 1996), resulting in API mass spectra which are dominated by (quasi)molecular ions such as $[M+H]^+$, $[M+Na]^+$, $[M-H]^-$ without much fragmentation. Therefore, tandem mass analysers (MS/MS) are used nowadays for trace analysis of mycotoxins to enhance both method selectivity and sensitivity (Spanjer et al., 2005; Sørensen et al., 2005; Biselli et al., 2005; Berthiller et al., 2005; Kokkonen et al., 2005; Tanaka et al., 2006; Rundberget et al., 2002; Sulyok et al., 2007).

Regarding the latest developments in HPLC, the introduction of columns filled with sub-2-µm particles and ultra-performance liquid chromatography (UPLC) instruments capable of handling the resulting high back pressures of up to 1200 bar are the major technical achievement (Schuhmacher et al., 2008). Ventura and colleagues (Ventura et al., 2006), for example, used the advantage of the UPLC and developed a QqQ based method for aflatoxins $B_1$, $B_2$, $G_1$, $G_2$ and ochratoxin A in beer, affording the separation and quantification of five toxins in only 3.2 minutes. Ren and co-workers (Ren et al., 2007) also used UPLC-QqQ for the analysis of 17 *Aspergillus*, *Fusarium* and *Penicillium* toxins. Ten toxins were chromatographed in 6.5 minutes in the positive mode. The separation of the other seven toxins in the negative mode was achieved in only 4 minutes. MS analysers which have short cycle times such as QqQ in SRM mode and Qq-TOF instruments are best suited for hyphenation as the increased separation power in UPLC leads to typical chromatographic peak widths of only a few seconds.

Moreover, the use of hydrophilic interaction chromatography (HILIC) columns or mixed mode reversed phase-weak anion exchange (RP-WAX) columns for the separation of very polar analytes has been shown to be a good alternative to conventional RP stationary phases (Hemström et al., 2006). The retention behaviour

of 79 mycotoxins and fungal secondary metabolites showing a wide range of polarities on different commercially available stationary phases such as C18-RP, amino type WAX and RP-WAX was compared by Apfelthaler et al. (2008) for the first time. It was demonstrated that RP-WAX columns offer the potential to separate mycotoxins by exploiting a combination of various chromatographic interaction modes, which is not accessible with conventional RP or WAX columns. Ionic (attractive/repulsive), RP-like hydrophobic, as well as hydrophilic retention mechanisms can be used for separation of the analytes. The retention behaviour on the RP-WAX phase proved to be largely controlled by composition (nature and amount of organic modifier), pH and ionic strength of the eluent.

In the last couple of years, there is a clear trend towards the development of universal multi-mycotoxin methods to enable reliable and fast estimation of mycotoxin contamination of food and feed.

### 3.5.2. MULTI-ANALYTE APPROACHES

Until recently, most of the available analytical methods only covered single mycotoxin classes (e.g. aflatoxins, type-B trichothecenes or fumonisins) (reviewed by Zöllner et al., 2006 and Sforza et al., 2006). However, in contrast to LC-MS/MS based pesticide and drug residue analysis, where comprehensive multi-target methods have been developed earlier (Alder et al., 2006; Müller et al., 2005), papers on multi-mycotoxin methods for food and feed were not available until a couple of years ago. The first attempts towards the development of multi-mycotoxin methods were made by Danish researchers as early as 1987 (Frisvad et al., 1987). Frisvad and Thrane developed a standardised HPLC method for the detection of 182 mycotoxins and other fungal metabolites. Their approach was based on alkylphenone retention indices and diode array detection (DAD) for the identification of the analytes in fungal cultures. This method is appropriate for qualitative screening of mycotoxins produced in fungal cultures, but not for the quantitative determination of trace levels of toxins in food and feed extracts (Pittet, 2005). Later on, Nielsen and Smedsgaard extended this method to 474 metabolites using LC-UV and LC-ESI-TOF simultaneously (Nielsen et al., 2003).

More recently, various methods for the determination of different toxin classes produced by the same fungal genus (e.g. *Fusarium*, *Penicillium* or *Aspergillus*) and multi-toxin approaches, which focus on the simultaneous determination of compounds with established legal maximum permissible limits (i.e. trichothecenes, aflatoxins, ochratoxin A (OTA), ZEN, fumonisins (FUMs) and patulin) (European Commission, 2006b; van Egmond et al., 2007), have been developed. Royer and colleagues, for example, developed a method for the quantitative determination of deoxynivalenol (DON), ZEN and fumonisin $B_1$ in maize using a two-stage SPE clean-up step (Royer et al., 2004). An LC-MS/MS method for the quantification of several type-A and type-B trichothecenes and ZEN in wheat and maize was developed in 2005 (Berthiller et al., 2005; Schuhmacher et al., 2005b). Cavaliere and co-workers extended the method

of Berthiller et al. by neosolaniol, α-zearalenol and the fumonisins $B_1$, $B_2$ and $B_3$ (Cavaliere et al., 2005). Multi-immunoaffinity columns were used in combination with LC-MS/MS for the simultaneous determination of all regulated mycotoxins plus T-2 and HT-2 toxin in maize, in addition to conventional SPE (Lattanzio et al., 2007).

Sulyok et al., (2007) described the development and application of a comprehensive LC-MS/MS method for the determination of 87 mycotoxins and fungal metabolites. It is based on their initial multi-mycotoxin method for the determination of 39 mycotoxins in maize and wheat (Sulyok et al., 2006). This method includes the most relevant members of the following toxin classes: type-B- and type-A trichothecenes, zearalenone (ZEN) and derivatives, fumonisins, beauvericin, enniatins, moniliformin, ochratoxins, aflatoxins, ergot alkaloids and patulin. Additionally, further bioactive fungal metabolites produced by *Penicillium*, *Claviceps* and *Alternaria spp.* are covered by this method. In-house validation was carried out for the model matrix breadcrumbs and the method was applied to a semi-quantitative screening of mouldy food samples (Sulyok et al., 2007). This method has recently been extended to the determination of 187 fungal and bacterial metabolites (Vishwanath et al., 2011). A typical LC-MS/MS chromatogram obtained from such a multi-mycotoxin method is displayed in figure 3.1.

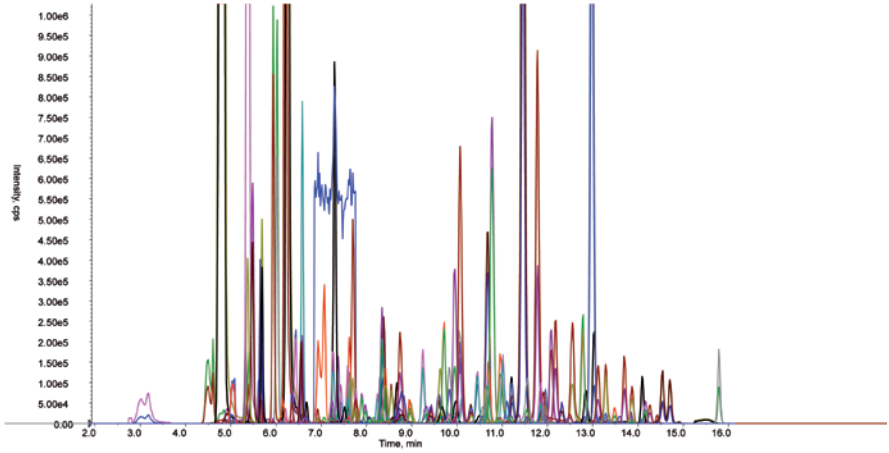


**Figure 3.1** - LC-ESI(+)-MS/MS chromatogram of 187 analytes in the positive mode

In contrast to single class methods which usually apply some kind of clean-up (Krska et al., 2008), a clean-up isfor multi-analyte approaches do not allow for extract purification not possible as a consequence of the wide range of toxin properties in a comprehensive approach as developed by Sulyok et al. (2007). Moreover, the conditions for extraction, HPLC separation and detection in such an approach cannot be optimal for each target analyte. For some of the acidic toxins, e.g. moniliformin and citrinin, extraction efficiencies were only 55% and 30% respectively. The method detection limits of the procedure described by Sulyok et al. (2007) ranged from below

1 µg/kg (e.g. for the enniatins and ergot alkaloids) to about 200 µg/kg for neosolaniol. In most cases, the limits of detection were below the regulated values of mycotoxins in food and the apparent recoveries complied with official guidelines with few exceptions (Sulyok et al., 2007). Spanjer also described a method without sample clean-up for the quantification of 22 toxins in different foodstuffs. After extraction with a mixture of acetonitrile/water, the samples were diluted with water prior to LC-MS/MS measurement (Spanjer et al., 2005).

One of the major problems in the quantification by LC-MS and LC-MS/MS is that compound and matrix-dependent signal response suppression or enhancement may occur (Niessen et al., 2006; Antignac et al., 2005; Gosetti et al., 2010). This so-called matrix effect is caused by the presence of endogenous or exogenous co-eluting components in the ion source of the mass spectrometer. Competition for "charges" between analytes and interfering compounds, co-precipitation of analytes with non-volatile material, formation of strong ion pairs and change of viscosity/surface tension of the droplets in the ion source are generally accepted as the main mechanisms of matrix effects (Niessen et al., 2006; Antignac et al., 2005). Ion suppression and its negative effects on method accuracy and precision can be reduced or eliminated by improvement of sample clean-up (and, therefore, removal of interfering matrix components) or the change of HPLC conditions (e.g. gradient programme, column length, mobile phase) (Niessen et al., 2006). However, matrix calibration is time consuming and labour intensive and, therefore, not frequently applied in routine analysis. As long as the number of target compounds is limited, the addition of appropriate internal standards (ISTDs) can be applied (Niessen et al., 2006). Sulyok et al. (Sulyok et al., 2007; Sulyok et al., 2006; Sulyok et al., 2007) have extensively investigated the matrix effects in LC-MS/MS. It has become feasible to differentiate between analyte loss during extraction and ion suppression/enhancement in the MS-ESI source (Häubl et al., 2007; Sulyok et al., 2007), following a procedure, suggested by Matuszewski and co-workers (Matuszewski et al., 2003). Raw extracts were diluted 1+1 with a mixture of acetonitrile/water to reduce matrix effects. In conclusion, the so-called dilute and shoot approach often requires matrix calibration for accurate quantification. Moreover, the choice of a representative matrix for a certain set of samples is also very important in order to obtain reliable quantitative results (Sulyok et al., 2007; Sulyok et al., 2006; Sulyok et al., 2007).

The multi-analyte methods which cover a wide range of different mycotoxins were applied successfully to spontaneously molded food samples (including bread, fruits, vegetables, jam, cheese, chestnuts and red wine) from private households (Sulyok et al., 2007; Sulyok et al., 2008). These studies revealed the great value of the LC-MS/MS based methods. Thirty-seven different fungal metabolites were identified at concentrations of up to 33 mg/kg. Some of the analytes have never been reported in the context of moldy food products before. The results of that pilot monitoring study underline the great potential of LC-MS/MS multi-analyte methods to establish a comprehensive picture of the range of mycotoxins potentially occurring in mouldy food products. Validated multianalyte LC-MS/MS methods for the quantification

of numerous mycotoxins have in the meanwhile also been successfully applied to a variety of different food and feed matrices such as cassava flour or peanut cake.

Recently, novel approaches in the analysis of mycotoxins in cereals employing ultra-performance liquid chromatography coupled with high resolution mass spectrometry have also been introduced (Zachariasova et al., 2010). In this context an LC-high-resolution FT-Orbitrap mass spectrometric technique was evaluated for the quantification of selected mycotoxins and the simultaneous screening of fungal metabolites in food (Lehner et al., 2012).

### 3.5.3. ISOPTOPE DILUTION ASSAYS

The need for sensitive, selective and accurate methods has led to increased efforts to produce stable isotope labeled mycotoxins, which can be used as internal standards (ISTDs) in MS based methods. In the isotope dilution assay, different isotopologues of the same mycotoxins are mixed and the quantification is based on the evaluation of the signal ratio of the isotopologues, stable labelling is a prerequisite for accurate quantification (see figure 3.2). In addition, the retention times of the native and the labeled toxins should be as close as possible as signal suppressing matrix compounds may elute from the HPLC column in form of a chromatographic peak. Both pre-requisites are ideally fulfilled for ($^{13}C_{24}$) T-2 toxin, which was synthesized according to Patent WO 2006105563. C-C bonds are very stable and unlikely to be cleaved during the analytical procedure. Recently, Asam et al. (Asam et al., 2006) reported the development of a SIDA for the most relevant type-A trichothecenes in food and feed. They synthesized and used ($^{13}C_2$) MAS, ($^{13}C_4$) DAS, ($^{13}C_2$) HT-2 and ($^{13}C_4$) T-2 toxin as ISTDs for the determination of the respective native toxins and achieved – depending on the toxin -- recoveries between 90% and 127%. In contrast to the use of $^{13}C$ labels, $^{2}H$ or $^{18}O$ labels can be hydrolyzed if these labels are at labile positions. Deuterium, for example, is susceptible to H/D exchange if it is activated e.g. by adjacent carbonyl groups or aromatic rings (Rychlik et al., 2008c). Moreover, isotope effects, leading to deviating retention times due to small differences of chemical and physical properties of the isotopologues, can be considered small for $^{12}C/^{13}C$- compared to $^{1}H/^{2}H$-isotopologues.

Finally, it is necessary that the analyte and its standard can be distinguished during MS/MS detection. Where there is a spectral overlap between the spectra of the labeled and native compounds, calibration is not straight forward and hyperbolic models have to be applied, for example (Jonckheere et al., 1983). Bretz and colleagues reported an isotope dilution assay for DON and 3-Ac-DON by LC-ESI-MS/MS in the negative ionization mode using ($^{2}H_1$) DON and ($^{2}H_3$) 3-Ac-DON as ISTDs (Bretz et al., 2006). They demonstrated that despite substantial spectral overlap, SRM transitions could be chosen in a way that minimal interference was observed and quantification was feasible. No spectral overlap of mass signals was observed with labeled ISTDs which were fully substituted with isotopic enrichment above 95%. As a consequence, it was

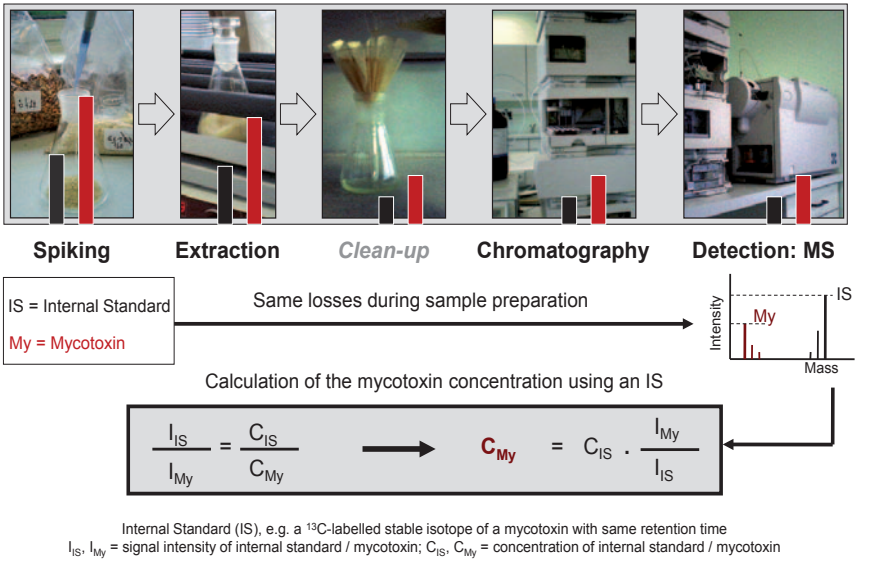


**Figure 3.2** – Schematic overview of an isotope dilution assay method
[Copyright by: Krska and Häubl, 2008]

possible to use simple linear regression for calibration of DON and T-2 toxin for any potential SRM transition (Häubl et al., 2007).

Häubl et al. (2007) present the characterization and application of fully $^{13}$C isotope labeled ($^{13}C_{24}$) T-2 toxin for the determination of T-2 toxin in maize and oats. This compound represents an ideal ISTD for the quantitative determination of T2-toxin, but has not been commercially available until recently. In their paper, the first stable isotope dilution assay (SIDA) for the determination of T-2 toxin by use of ($^{13}C_{24}$) T-2 toxin as ISTD is presented. The method was evaluated with and without conventional clean-up and validated in-house for maize and oats. Both cereal types showed significant matrix enhancement effects, which could be compensated by application of the isotope-substituted ISTD.

The ideal ISTD consists of a stable isotope labeled isotopologue of the native analyte and can be used to compensate for random variations, as well as systematic errors, during each step of an analytical method. However, the correction for analyte loss during extraction of natural solid samples (e.g. wheat) is not straightforward. It can be assumed that a spiked standard can be extracted more efficiently than the naturally occurring analyte which might be bound to matrix components of the same sample (e.g. Liu et al., 2005). The use of SIDAs in mycotoxin analysis has been reviewed (Rychlik et al., 2008). So far, stable $^{2}$H and/or $^{13}$C labeled derivatives of the following mycotoxins have been used for the development and application of SIDAs:

type-B trichothecenes (DON, 3-acetyl-deoxynivalenol (3-Ac-DON), 15-acetyl-deoxynivalenol (15-Ac-DON), nivalenol, fusarenon-X), type-A trichothecenes (T-2 toxin, HT-2 toxin, diacetoxyscirpenol, 15-monoacetoxyscirpenol), patulin, fumonisin $B_1$, $B_2$, $B_3$ and ochratoxin A. For ZEN a so-called alternate isotope-coded derivatisation assay (AIDA) has been published, which is based on sample splitting and derivatisation of ZEN with labeled and non-labeled acetic anhydride (Sforza et al., 2006).

The successful use of fully $^{13}C$ labeled ISTD for the quantification of DON in wheat and maize (Häubl et al., 2006a; Häubl et al., 2006b). In this study the apparent recovery of DON in wheat was 29±6% (n=7), whereas for maize 37.5±5 % (n=7) were obtained when no clean-up was used and the ISTD was not considered as ISTD. However, the quantification of DON in certified wheat reference material and a matrix reference maize material yielded 95±3% (wheat) and 99±3% (maize) when the labeled toxin was used for quantification.

Häubl et al. (2007) also described the unambiguous identification of the prepared ($^{13}C_{24}$) T-2 toxin by product ion MS/MS and NMR, as well as the assessment of its purity by LC-UV (99%). A direct comparison of the MS/MS and MS3 spectra (obtained for the labeled and non-labeled T-2 toxin) enabled the elucidation of the fragmentation behaviour of the two isotopologues in the collision cell of the MS instrument. MS3 spectra were recorded using selected MS/MS fragments as precursors. The number of C-atoms was deduced by direct comparison between corresponding signals of labeled and non-labeled fragments in the $MS^3$ spectra for each observed neutral loss fragment (Häubl et al., 2007).

Most recently, a stable isotope dilution assay for the accurate determination of 11 mycotoxins currently regulated in maize and other cereal-based food products in Europe by UHPLC-MS/MS was developed and validated by Varga et al. (2012).

### 3.5.4. OUTLOOK

It can be expected that further improvements of LC-MS/MS instrumentation (e.g. new combinations of mass analysers and new software features) and its availability at lower price will further contribute to LC-MS/MS becoming the major tool for the analysis of multi-contaminants, such as mycotoxins in grains, food and biological samples.

LC-MS-based screening has also been playing a vital role in the discovery of novel mycotoxin conjugates so-called “masked” forms of mycotoxins in the past and it is believed that this will also continue in the future (Berthiller et al., 2009). Awareness of such altered forms of mycotoxins is increasing, but still reliable analytical methodology, (certified) standards, occurrence and toxicity data are lacking. Modern analytical methods employ LC-MS either directly or after conversion to the precursor of masked mycotoxins.

Moreover, the use of LC-MS methods can lead to completely new insights in resistance breeding against fungi and the use of biocontrol through monitoring changes

in the overall metabolite spectrum of plants especially treated with mycotoxins and fungal strains. Employing LC-MS/MS for the determination of mycotoxin biomarkers can lead to improved exposure assessment of humans and animals to mycotoxins. Moreover, new detoxification routes can be revealed through the study of microbial interactions of free and bound toxins by utilizing novel analytical tools including multitoxin-approaches based on LC-MS/MS and high-resolution mass spectrometry.

The established state-of-the-art chromatography-based methods for the determination of mycotoxins are increasingly being complemented by a rising number of methods for fast and cost efficient analysis, including rapid strip tests. The optimization and validation of rapid test systems incl. strip tests will contribute to meeting contract or legislative specifications for maximum acceptable levels of mycotoxins in foods and feeds through effective screening.

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## THE AUTHORS

### 1. John L. Richard

John received his PhD from Iowa State University in 1968. His research career began at the National Animal Disease Center, USDA, ARS in Ames, Iowa in 1963 investigating toxic fungi from stored corn. He was part of a team that pioneered investigating the effects of mycotoxins on immune phenomena in animals. He authored over 250 research papers and chapters in or was editor of various books on mycotoxins.

After 27 years at Ames, Iowa, he became research leader of the Mycotoxin Unit, National Center for Agricultural Utilization Research, Peoria, IL for seven years before retiring from government service. He became Executive Vice President, President and CEO of Romer Labs. He is a consultant to Romer Labs and an independent consultant. He is an Honorary Diplomate of the American College of Veterinary Microbiologists and a member of the honor societies of Phi Kappa Phi, Phi Zeta and Sigma Xi. He was U.S. Chair of the UJNR Panel on Toxic Microorganisms from 1985 to 1995. He authored or edited the first Romer Guides to Mycotoxins.

### 2. Thomas B. Whitaker and Andrew B. Slate

#### Thomas B. Whitaker

Tom is a native of North Carolina. He received his BS and MS from North Carolina State University in the Agricultural Engineering Department and his PhD from Ohio State University. Tom was a research engineer with the Agricultural Research Service of the US Department of Agriculture for 42 years until his retirement in Feb 2009. Currently, Tom holds the position of Professor Emeritus, Biological and Agricultural Engineering Department, North Carolina State University.

Tom's research has led to the development of industry wide mycotoxin sampling programs for commodities such as peanuts, almonds, corn, and pistachios. Internationally, the techniques he developed have been used by Codex to develop international sampling programs to detect mycotoxins in peanuts, almonds, pistachios, hazelnuts, and Brazil nuts.

Tom has received the Secretary of Agricultures Group Honors Award for his efforts that resulted in China lifting its embargo of US wheat because of TCK contamination and the second for efforts resulting in the EU lifting the "Special Measures" imposed on the import of California almonds due to aflatoxin contamination.

### Andrew B. Slate

Andrew Slate is an Agricultural Engineer with the Biological and Agricultural Engineering Department at NC State University. After receiving his BS degree from NC State in 1981, he joined the ARS, Market Quality and Handling Research Unit in Raleigh, NC where he served 23 years as support engineer for Dr. Tom Whitaker. His responsibilities included: research project planning and protocol design; development of computer programs for statistical data analysis, data management, and statistical modeling of testing procedures for mycotoxins; assisting the Agricultural Marketing Service with sampling/grading equipment design, electronic control, standardization and validation; and development of presentation, report and scientific documents.

## 3. Rudolf Krska and Rainer Schuhmacher

### Rudolf Krska

In 2010 Rudolf Krska has become director of the Department for Agrobiotechnology IFA-Tulln (140 co-workers) at BOKU-University of Natural Resources and Applied Life Sciences, Vienna. He is also head of the Center for Analytical Chemistry of the IFA-Tulln since 1996 and has been appointed full professor for (Bio-)Analytics and Organic Trace Analysis at the BOKU in 2008. Rudolf Krska is an internationally recognized expert in food and feed analysis by chromatographic, mass spectrometric and immunoanalytical techniques.

Rudolf has established intensive interdisciplinary cooperation with universities and companies and was coordinator of three European Commission funded projects dealing with the determination of mycotoxins and allergens in food. From 2002 to 2009 he also supervised the Christian Doppler Laboratory for Mycotoxin Research in Tulln. As member of JECFA (Joint Expert Committee for Food Additives) of the FAO/WHO he has evaluated the impact of trichothecenes on humans. From 07/2009 to 07/2010 Rudolf Krska worked as Acting Chief of the Food Research Division at Health Canada in Ottawa, where he was responsible for the research on chemical contaminants in foods carried out within Health Canada´s National Food Chemical Safety Laboratory Network in Ottawa, Montreal, Toronto, Winnipeg and Vancouver. Rudolf received 6 scientific awards and is (co-)author of more than 250 scientific publications and has given more than 240 presentations at international conferences.

## Rainer Schuhmacher

Rainer Schuhmacher, PhD, Assoc. Prof. is head of the working group Metabolomics and Bioactive Compounds at the Department for Agrobiotechnology (IFA-Tulln) of University of Natural Resources and Life Sciences, Vienna (BOKU). Rainer graduated in Chemistry from University of Konstanz, Germany and worked at a pharmaceutical company in Konstanz before doing his PhD at Vienna University of Technology on the development of analytical methods for the determination of mycotoxins. He dealt with the development and validation of analytical methods for determination of organic trace residues and contaminants as well as quality assurance in analytical chemistry. In 2009 he earned his habilitation in Analytical Chemistry from BOKU University, where he is currently Associate Professor. Rainer´s research interests are: liquid- and gas chromatography – mass spectrometry based metabolomics of microbes and plants, study of the interaction between living organisms as well as qualitative and quantitative analytics of bioactive compounds.

*Levels of chemical contaminants in foods and feeds can originate from natural sources such as mycotoxins, which are toxic secondary metabolites produced by fungi on agricultural commodities in the field and during storage. More than 300 mycotoxins have been identified so far with widely different chemical structures and differing modes of action. The potential health risks to animals and humans posed by food- and feed-borne mycotoxin intoxication have been recognized by national and international organisations, which have addressed this problem by adopting regulatory limits for major mycotoxin classes and selected individual mycotoxins. The Romer Labs Guide to Mycotoxins is a timely reflection of the research progress related to the significance, sampling and determination of mycotoxins. In this respect, the Romer Labs Guide will not only provide a thorough introduction into the history of mycotoxins and mycotoxicoses, but will also discuss the chemistry and toxic effects of the most common mycotoxins. The book will also provide guidance for the development of proper sampling and sample preparation techniques to obtain representative samples for the subsequent analysis of the food and feed commodities to be checked for mycotoxins. The analytical part of the Romer Labs Guide to Mycotoxins covers proper extraction and clean-up procedures, separation and detection techniques including latest state-of-the-art rapid strip tests. Special emphasis is put on multi-toxin methods based on liquid chromatography coupled with mass spectrometry for the simultaneous determination of a wide variety of secondary fungal metabolites which allow for a comprehensive assessment of the wide range of mycotoxins humans and animals are exposed to.*

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