Health and Food Safety

Fast and reliable answers regarding aflatoxins in foods

If you are going to determine the concentration of mycotoxins in foods, in pharmaceutical products, or in raw materials used in their production, you will probably rely on Solid Phase Extraction (SPE) combined with LC/MS analysis. This approach ensures that detection limits will be lower than the maximum concentrations allowed by law. While long-established manual SPE procedures may leave little room for further optimization, automation of the process can provide laboratories with more reliable results in less than half the time.

The well-stocked cheese counter may seem to tell a different story, but whoever consumes moldy foods – other than mold cheese – is putting his or her health at risk. This is due to mycotoxins: Toxins that are created as metabolites by certain molds. Mycotoxins can lead to acute illness as well as chronic ailments, caused by carcinogenic, mutagenic and hormone active properties that are especially harmful to infants and toddlers.

To date, more than 300 mycotoxins, formed by approximately 250 mold types, have been found. For food safety purposes, however, only a few mycotoxins are of importance, such as those of the genus Aspergillus flavus and Aspergillus parasiticus. These molds thrive, especially under humid-warm conditions, on oily and starchy seeds such as peanuts, walnuts, hazelnuts, pistachios, almonds, figs, coco, grains, rice, corn and soy, as well as on dried fruits and spices.

High concentrations of a group of mycotoxins called aflatoxins have been found, for example, in pistachios, figs and cereals. Aflatoxins are among the most potent human carcinogens found in plants. The aflatoxins B1, B2, G1, G2 and M1, produced by Aspergillus flavus and Aspergillus parasiticus, belong to the most potent mycotoxins that exist. Aflatoxin B1 poses the greatest hazard of all due to its carcinogenic properties. Because of the extreme toxicity of aflatoxins, EU legislation specifies very low acceptable daily intakes and low maximum residue limits.

The risk of acute poisoning through high mycotoxin concentrations is relatively low in most of the developed world thanks to the overall good food quality. In Africa and parts of Asia, things can be quite different: Conditions for growing, storing and transporting agricultural products are frequently bad, resulting in moldy peanut or corn products. Consumption of moldy products regularly results in acute and even fatal aflatoxin poisoning. According to literature references, the lethal dose for an adult is 1 to 10 mg per kilogram body weight.

Pervasive food contamination resulted in government regulation

Mycotoxin contamination of food and feed is a global problem. The UN Food and Agricultural Organisation (FAO) estimates that up to 25% of the world's food production is contaminated with mycotoxins. Approximately 20% of the EU's cereal harvest contains detectable amounts of mycotoxins.

Due to the health risk posed by molds and due to their universal presence in cer-

Further Information about Aflatoxins

USFDA
http://vm.cfsan.fda.gov/~mow/chap41.html
Cornell University
http://www.ansci.cornell.edu/plants/toxicagents/aflatoxin/aflatoxin.html
Colorado State
http://www.ext.colostate.edu/pubs/CROPS/00306.pdf

tain food products, maximum concentration values for mycotoxins have been established in the range of a few micrograms per kilogram (µg/kg): For peanuts, indehiscent fruits (mainly nuts), dried fruits and grain intended for direct consumption or for use in food products, maximum allowable concentrations of 2 µg/kg aflatoxin B1 or 4 µg/kg total of B1, B2, G1 and G2 apply. The concentration of aflatoxin M1 in milk is not allowed to exceed 0.05 µg/kg. Regulations limit the acceptable quantity in foods for infants and toddlers to 0.05 µg/kg aflatoxin B1, and 0.025 µg/kg M1.

„Fully automated Solid Phase Extraction using the GERSTEL SPE system provides reliable results in less than half the time it takes to perform the extraction manually." Norbert Helle, Ph.D.

Faster results and lower detection limits

The method of choice for reliable and sensitive detection of aflatoxins is Solid Phase Extraction (SPE) or affinity chromatography, combined with LC/MS analysis. This approach ensures that detection limits will be lower than the maximum concentrations allowed by law.

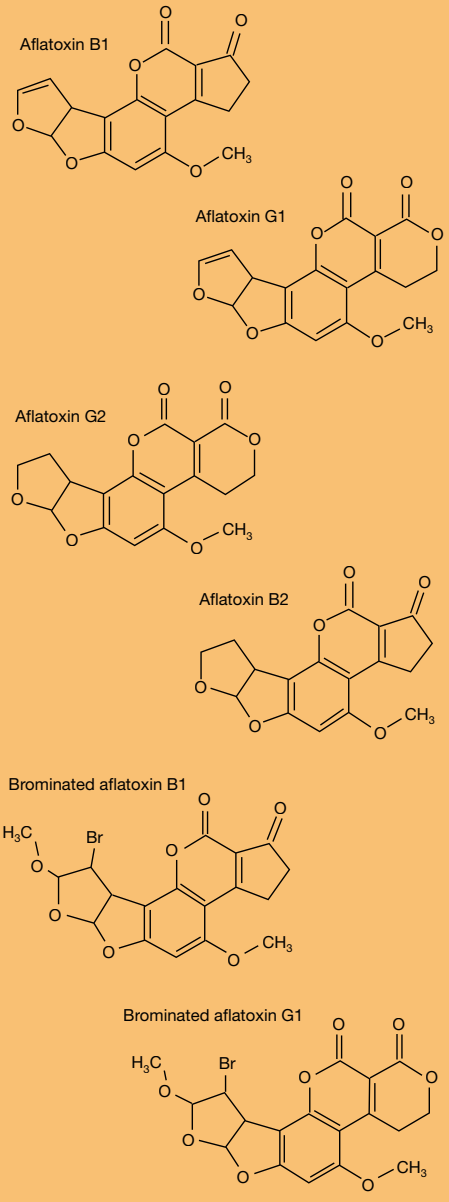
Norbert Helle, Ph.D., food safety analysis expert and owner of TeLA GmbH, a German contract laboratory based in Bremerhaven, explains the background behind some of his recent work on the determination of Aflatoxins in foods: „Established sample preparation methods used in LC/MS determination of aflatoxin levels provide only limited scope for optimization, but reliable and useful analysis results can be obtained in less than half the time if the SPE process is automated. In the case of the aflatoxin determination, manual processing requires on the order of 4 hours for eight samples. The GERSTEL SPE requires only 80 to 95 minutes to prepare the same number of samples, according to Dr. Helle.

„All steps from standard addition and derivatization through Solid Phase Extraction to LC/MS analysis are fully automated", says the applications expert, while adding: "Software-controlled parallel processing of sample preparation and analysis ensures that there is only negligible analyte decomposition. The preparation steps for each and every sample are performed at exactly the same point in time prior to analysis. The GERSTEL SPE system provides on-time sample prep for best possible results".

Dr. Helle has developed an LC/MS method for the determination of B1, B2, G1 and G2 aflatoxins in foods such as pistachios, bell pepper seasoning and various fruits. Following clean-up on an SPE affinity column, the two aflatoxin compounds

Chemical structure of aflatoxins

Aflatoxins are a group of more than 20 different fluorescent heterocyclic compounds, consisting of a dihydro or tetrahydrofuran unit connected with a substituted coumarin ring. The toxicologically relevant compounds are Aflatoxins B1, B2, G1 and G2, with B1 occurring most frequently. Aflatoxin M1, found for example in milk and milk products, is a metabolite of Aflatoxin B1. M1 is formed in humans or in animals when they have consumed food or feed contaminated with B1. The toxicity of Aflatoxin M1 is comparable to Aflatoxin B1; however, M1 is significantly less carcinogenic than B1.



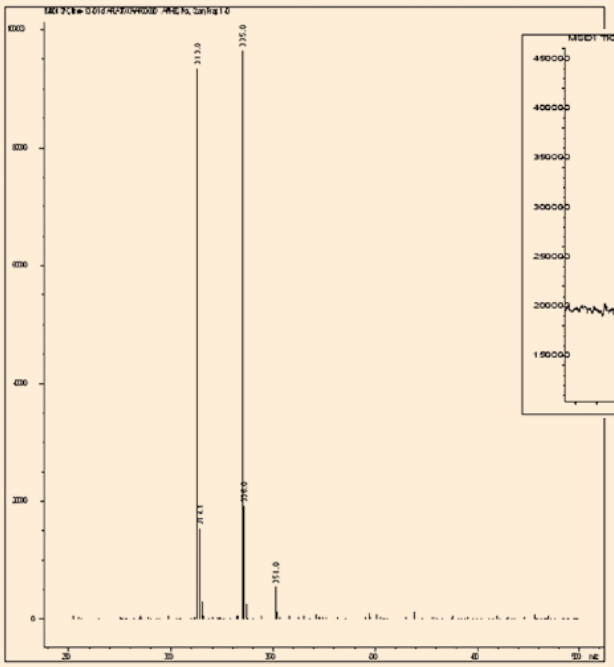
PrepBuilder method for automated SPE. The MPS is used for sample preparation to determine the concentrations of individual aflatoxins in various food products such as pistachio, ground chilli and fruits.
All steps are selected by mouse-click from a menu and added to the list.

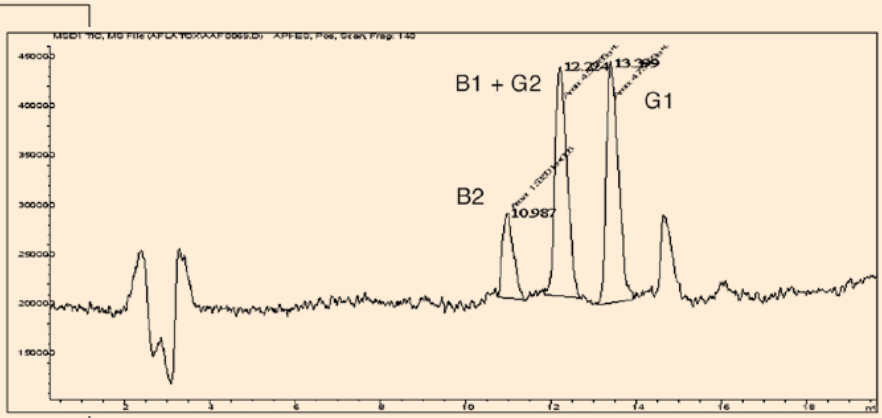
MOVE	Transport sample vial into SPE Vial position Transport cartridge to the SPE Waste position
ADD 1	Add 4 mL sample to the cartridge; flow 50 µL/s
ADD 2	Rinse cartridge with 20 mL H2O; flow 50 µL/s
SPE-SHIFT	Slide SPE carriage with cartridge from SPE Waste to SPE Vial position
ADD 3	Elution of aflatoxins with 0.5 mL MeOH; flow: 30 µL/s
ADD 3	Elution of aflatoxins with 0.5 mL MeOH; flow: 30 µL/s
ADD 3	Elution of aflatoxins with 0.5 mL MeOH; flow: 30 µL/s
WAIT	Wait 30 seconds for the eluent to transfer completely
MOVE	Discard SPE cartridge into the cartridge disposal container
SPE-SHIFT	Slide SPE carriage from SPE Vial to SPE Waste position
MOVE	Transport sample vial from SPE Vial position back to tray

GERSTEL SPE

Dr. Norbert Helle developed his method on an Agilent Technologies Series 1100 LC/MSD and a GERSTEL MultiPurpose Sampler (MPS) with SPE option. The MPS was also used for derivatization and sample introduction. The LC/MS system was operated with electrospray ionisation in positive ion mode. The column used was a Phenomenex Synergi Max-RP (250*2.1 millimetre, 4 µm particle size) operated at a flow of 0.3 mL/min. Separation is performed using a solvent gradient (eluent A: 0.1 % formic acid, eluent B: acetonitrile). Control of the complete system, from sample preparation and sample introduction to LC/MS analysis, is handled from the Agilent Technologies ChemStation software using the integrated GERSTEL MAESTRO software.

with an isolated, non-conjugated, double bond, B1 and G1, are brominated by stirring the extract with a 3 % solution of bromine in chloroform. The mass spectra indicate that bromination results only in the formation of 1-methoxy-2-bromo-substituted compounds. Under the chosen experimental conditions, dibromo-substituted aflatoxins are not detected. The 1-methoxy-2-bromo-substituted compounds show longer retention times in reversed phase chromatography than the non-brominated species, resulting in baseline separation for the four aflatoxins with minimal interference from residual matrix. Additionally, the derivatized compounds yield significantly better MS responses and the characteristic bromine pattern in the mass spectra provides improved differentiation from background signals and thus a better signal to noise ratio. These combined advantages enable the system to reach detection limits below 0.01 µg/kg for the aflatoxins.





Monobrominated aflatoxins exhibit longer retention times than the non-brominated compounds, resulting in better separation of the four aflatoxins and reduced interference from matrix components (fig. above). Derivatization of aflatoxins B1 and G1 results in significantly improved MS responses combined with characteristic bromine patterns in the mass spectra (fig. left):
The detection limits for the examined aflatoxins are below 0.01 µg/kg.

When mycotoxin poisoning is suspected

The Centre for Information To Counter Poisoning, in the state of North-Rhine Westphalia, Germany's most populous state (population 18 million), is located at the University of Bonn. Last year, the centre received around 20 calls concerning cases of food poisoning caused by moldy foods. Cases of acute and severe poisoning caused by mycotoxin ingestion are rare. When symptoms call for it, patients are properly diagnosed by performing chemical laboratory tests on their blood serum. When moldy foods are ingested, the mycotoxins present are absorbed via the digestive tract and then released into the blood stream from which they reach the rest of the body. Aflatoxins are among the most potent human carcinogens found in plants. Aflatoxin B1, the most carcinogenic among the mycotoxins of Aspergillus flavus, unleashes its effect when metabolized to an epoxide in the human body. The epoxide attaches itself to the human genetic material causing potentially carcinogenic mutations. The accepted lethal dose of aflatoxin B1 is 1 to 10 mg/kg bodyweight for adults. Some types of molds can produce toxins that directly damage the liver, i.e. these are hepatotoxic.

Typical acute symptoms of aflatoxin poisoning are digestive problems such as nausea, diarrhoea and vomiting. Animal experiments have shown that long-term exposure to aflatoxins can trigger liver carcinomas; it is widely assumed that aflatoxins have a similar effect on humans. The responsible *Aspergillus* species appear mainly in nuts and bread.

Exposure to molds can also trigger allergic reactions, especially in predisposed persons. The manner in which the mold reaches the organism would not make a difference, it could be absorbed via the lungs or digestive tract - or through contact with skin and mucous membranes. Health professionals would not refer to this as a case of poisoning, however.

When mycotoxin poisoning is suspected, for example when moldy foods have been ingested, it is recommended to consult a physician or an organization specialized in helping patients in cases of poisoning. When mycotoxin poisoning is diagnosed, frequent tests of the blood and liver values are performed, symptomatic therapy of the liver function is performed, and in some cases medication is administered to limit uptake of mycotoxins in the liver. ■

Scrape off the mold or just throw the food away?

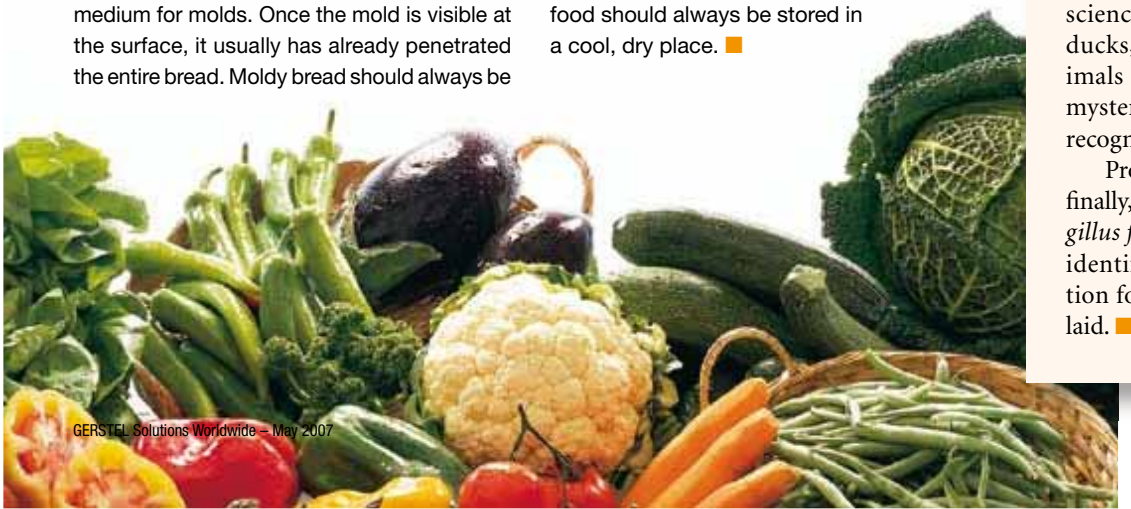
Moldy foods are in fact spoiled and should be discarded, not consumed. Exceptions are special mold cultures used for cheeses, for example, Camembert, Roquefort, Gorgonzola or Stilton. Even some specialty Italian salamis can have been processed using special mold cultures to provide them with their unique flavor.

White or colored spots are usually a sign of unhealthy mold growth. The roots of the mold, also called the mycelium, spread inside the food, invisible to the naked eye. Both the visible molds and the mycelium can contain mycotoxins, such as aflatoxins from Aspergillus flavus, which has a severe carcinogenic effect.

Fungi spread through the release of spores. When these reach a nutrient-rich medium, they can multiply on the surface and/or inside this medium. Bread, for instance, provides a good medium for molds. Once the mold is visible at the surface, it usually has already penetrated the entire bread. Moldy bread should always be discarded immediately. Due to the high moisture content, molds also spread quickly in fruits and vegetables once they have gained access. At this stage, mold spores have easy access to the nutrition they need in order to proliferate. When in doubt, the entire fruit should be cautiously discarded, without breaking, squeezing or damaging it. Such actions would only lead to further spreading of spores.

Jams or preserves with a sugar content of more than 50% do not provide a good medium for molds, since sugar acts as a preservative at high concentrations. For these products, it is often sufficient to generously remove mold spots and the surrounding food. The same approach applies for hard cheeses that just have mold on the surface.

To minimize the risk of mold growth, food should always be stored in a cool, dry place. ■



Molds and mycotoxin research

Since the beginning of time, mankind has been struggling with molds and their impact on human life. The earliest known reference to mold-contaminated rooms and objects, along with corresponding recommendations for hygiene, are found in the Old Testament, Third Book of Moses (Leviticus). In medieval times there were regular occurrences of poisonings, many of them fatal, following consumption of rye bread made with flour that contained ergot. Even in the middle of the 20th century, thousands of people died after consuming bread contaminated with Fusarium mold.

Prof. Manfred Gareis

„Mycotoxin Poisoning has been known for ages - even over the course of the last centuries it occurred quite frequently", explains Prof. Manfred Gareis of the German Federal Agency for Nutrition and Food, Department of Microbiology and Toxicology. However, the triggers for the diseases and for the symptoms remained in the dark.

It took massive fatalities among English turkeys in the 1960's, and the associated huge economic loss, to get science involved in the matter. When ducks, pheasants and other farm animals also started perishing from the mysterious Turkey-X disease, experts recognized the connection.

Prof. Manfred Gareis: „At that stage, finally, highly toxic metabolites of *Aspergillus flavus fungi* - the aflatoxins were identified as the cause." The foundation for mycotoxin research had been laid. ■