An Overview of Testing for Mycotoxins and Plant Toxins in Food Using LC-MS/MS

Waters™

Introduction

WHAT ARE MYCOTOXINS?

Mycotoxins are secondary toxic metabolites produced by fungal species found in a wide range of agricultural commodities, that can enter the food chain due to infection of crops in the field, after harvest, and during storage, and can be present when processed into food and animal feed. Although it has been reported that 25% of world grain production was infected with mycotoxins[1], this figure greatly underestimates the true occurrence (up to 60–80%)[2]. Mycotoxins have acute and chronic health impacts both in humans and animals that are fed contaminated feed[3] and represent one of the highest risks recognized by the European Rapid Alert System for Food and Feed (RASFF)[4].

"REGULATED" MYCOTOXINS

The most common mycotoxins that pose a concern to food safety are regulated in many countries of the world after thorough risk assessment, considering toxicity, occurrence, and consumption data.

They include:

- Aflatoxins (B1, B2, G1, G2, and M1)
- Ochratoxin A
- Fumonisins (B1, B2, and B3)
- Zearalenone
- Trichothecenes (nivalenol, deoxynivalenol, T-2, and HT-2 toxin)

This eBook aims to provide a short background on the advantages of using LC-MS/MS for the determination of mycotoxins and plant toxins in foodstuffs.

"EMERGING" MYCOTOXINS

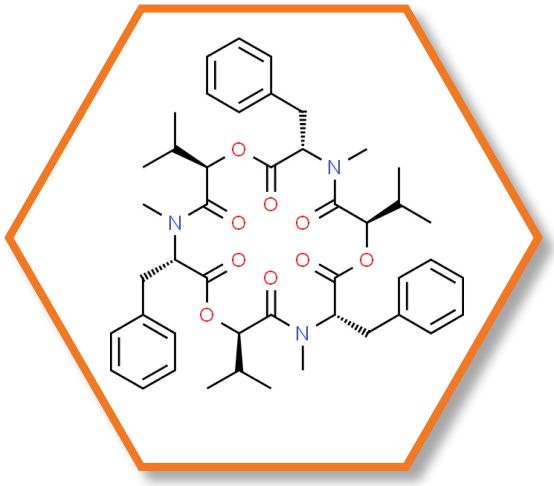
There are many other chemically diverse mycotoxins for which no current regulations exist. The co-occurrence of multiple mycotoxins in food and feed samples is a matter of concern and evidence of their incidence is increasing. Data on occurrence is needed and regulation of some of these compounds is likely to be created at some stage in the future.

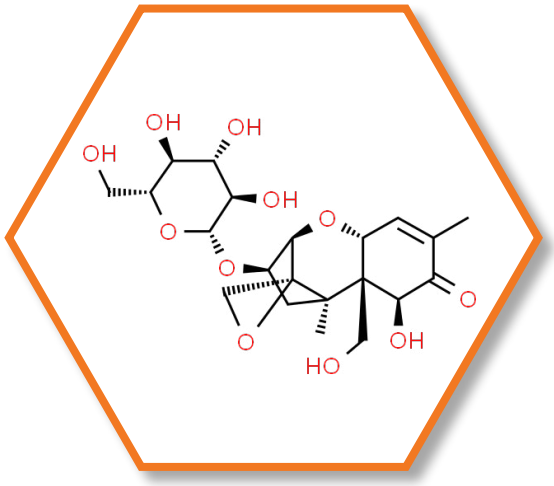
"MODIFIED" MYCOTOXINS

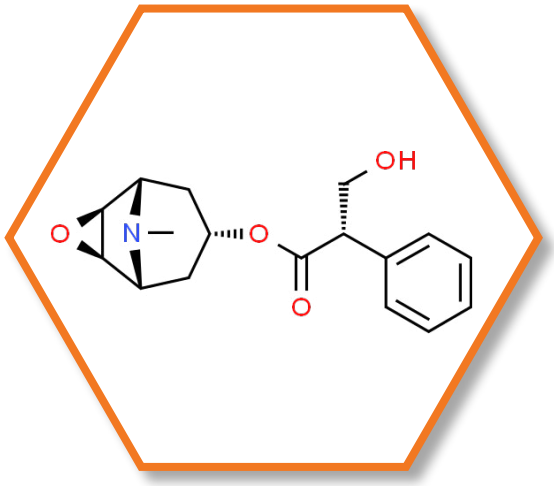
The term applies to any metabolites of the parent mycotoxins. For example, modified forms of deoxynivalenol (DON) include 3- and 15-acetylDON and DON-3-glucoside. Monitoring for modified forms is needed as they can often be reconverted to the parent toxin during the animal and human metabolism, contributing to the toxicity of the sample.

PLANT TOXINS

Plant toxins can occur in edible crops, while others enter the food chain due to contamination of edible crops with weeds. This can have an impact on human health. Some toxins occur only in specific plant genera or are even species specific, others are present in several plant families.







Drivers for Mycotoxin and Plant Toxin Testing

WHY TEST FOR TOXINS IN FOOD AND FEED?

The risk of food and feed contamination by mycotoxins is one of the most crucial global food safety concerns. Mycotoxins also generate significant economic losses for the food industry due to reduced crop yields, lost trade revenues due to failure to comply with regulatory limits, and livestock illnesses.

Since temperature and humidity are important parameters for the growth of fungi, climate change is anticipated to have an impact on the presence of mycotoxins.

Plant toxins in food has been recognised as an emerging issue of concern, especially in Europe. The situation is exacerbated by the existence of complicated supply chains, which increase the chances for cross-contamination, and changes to consumption habits.

The main driver for testing is to check compliance with the stringent regulations for food safety control. Testing is carried out by governmental bodies and by the food/feed industry for due diligence - being able to prove that a business has taken reasonable steps to prevent food safety breaches.

An additional driver for testing is the response to calls for data on occurrence for risk assessment purposes.

Food Safety Controls for Mycotoxins and Plant Toxins

CONTROLS TO MINIMIZE EXPOSURE TO MYCOTOXINS FROM FOOD AND FEED

- Ensuring that good practice is undertaken during growing, harvesting, and storage of foods
- Many countries have maximum permitted levels for mycotoxins that pose the highest risk in different food stuffs
- Monitoring programs to check regulatory compliance
- Discarding contaminated batches
- Regulations

The most common mycotoxins are regulated in many countries of the world, considering toxicity, occurrence, and consumption data. Regulations typically include values that are specified in various food and feed commodities, which are either maximum allowed levels or guidance values. Although values vary in the different regions of the world, the maximum levels set for mycotoxins in food are very low due to their severe toxicity. The current regulatory limits rarely consider the exposure to multiple mycotoxins (co-occurrence), and they are either based on the risk assessment of a single compound or on their sum.

The European Commission have introduced maximum levels for some plant toxins in specific food groups: pyrrolizidine alkaloids, tropane alkaloids, glycoalkaloids, opium alkaloids, erucic acid, and hydrocyanic acid.[5]

Country/Region	Established Limits in Peanuts (µg/kg)	
	AFB1	Total AF (B1, B2, G1, and G2)
Codex Alimentarius	15	NA
Australia and New Zealand	NA	15
China	20	NA
EU	8	15
EU	2	4
India	NA	30
Japan	10	NA
USA	NA	20

Multi-Mycotoxin Methods

WHY MULTI-MYCOTOXIN ANALYSIS?

A variety of testing solutions exist for mycotoxin analysis. Easy to use, rapid tests are utilized at points of production and distribution, but samples are typically sent to a laboratory for analysis to check for regulatory compliance during monitoring for official control or by the food industry for due diligence.

The co-occurrence of many mycotoxins and plant toxins in food and feed samples is a matter of concern. Multi-mycotoxin, and even multi-toxin methods, using generic extraction conditions, optional clean up steps, followed by determination by LC-MS/MS, are now commonly used for the determination of a wide range of regulated, “modified” and “emerging” mycotoxins, as well as plant toxins, in a variety of agricultural commodities and finished products.

Multi-mycotoxin and multi-toxin methods offer superior specificity, sensitivity and analyte coverage, which results in a simplified sample preparation. In addition, it remains essential to include the mitigation of isobaric interferences and matrix effects to ensure accurate quantification.

Sampling and Sample Preparation

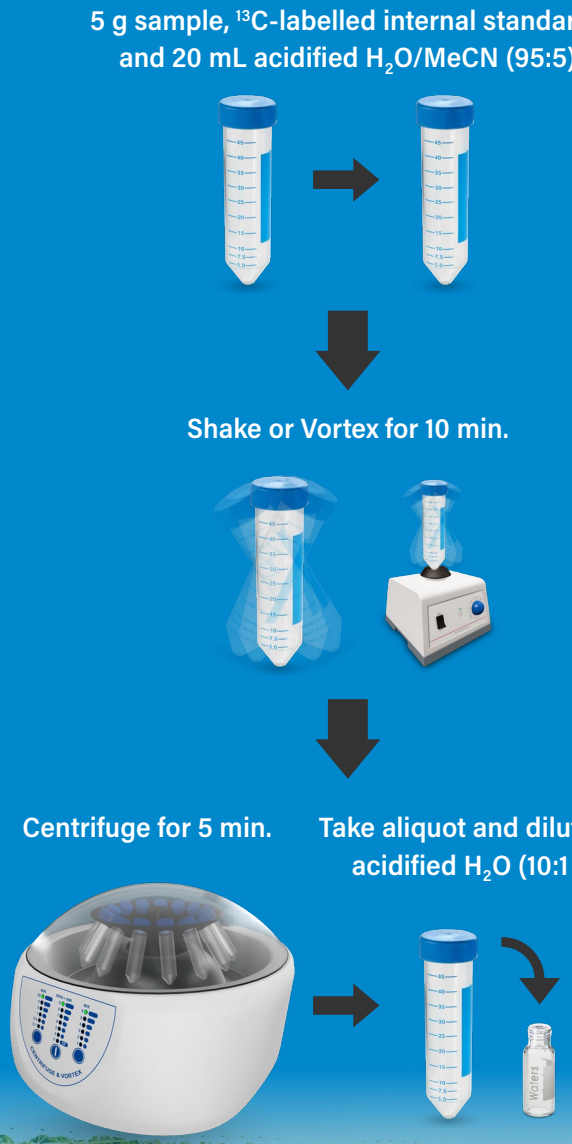
The heterogeneous distribution of mycotoxins in cereals, nuts, grains, and other commodities is often the largest error in determining concentrations of mycotoxins in food commodities. Adequate sampling, defined by the number and size of incremental sampling units, and appropriate sample preparation is needed to get a homogenous and representative sample as basis for accurate analysis.

SAMPLING

Many small incremental samples are taken at random from various places distributed throughout the consignment or lot to obtain a representative aggregate sample, which is then mixed, homogenized (see below) and divided into laboratory samples. In some cases, official procedures for sampling foodstuffs for mycotoxin analysis are laid down in regulations. An equally important facet of the whole sampling procedure is the accurate labelling and storage of samples.

SAMPLE PREPARATION

Solid products, such as kernels and nuts, must be ground to a powder form or aqueous slurry to provide a homogenous sample for analysis but also to enlarge the surface area to make them accessible for extraction solvents.



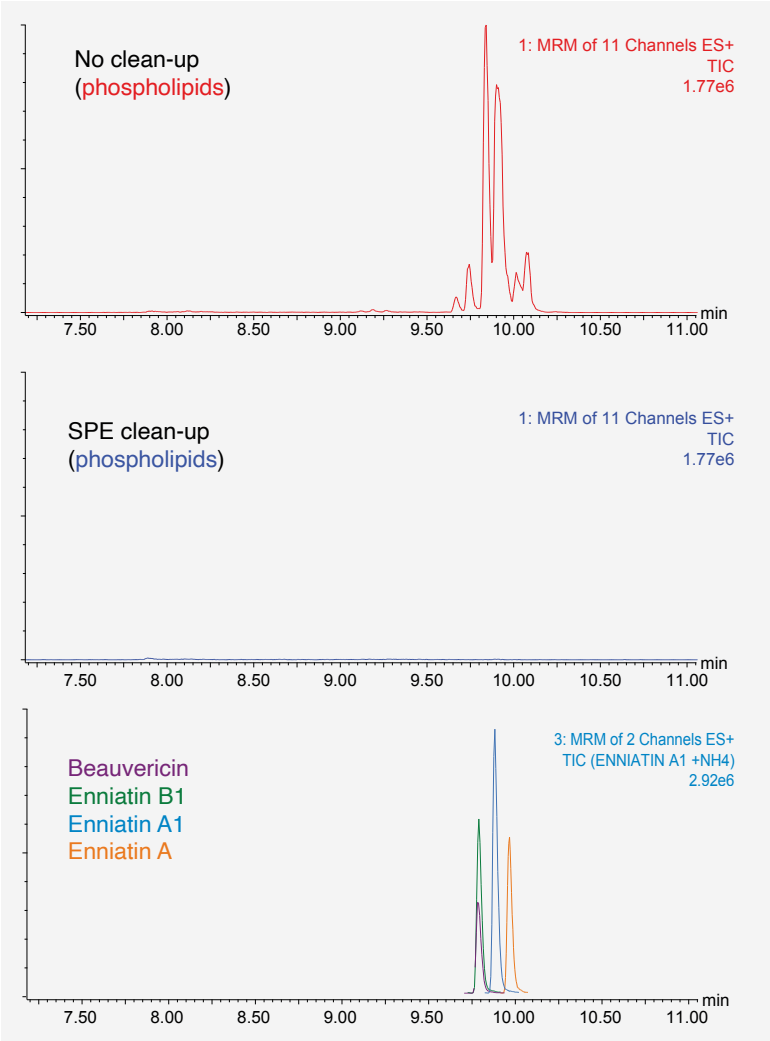
Sample Extraction

Due to extremely low concentrations of mycotoxins in samples, the determination must be very sensitive to meet regulatory limits. Therefore, mycotoxins need to be extracted from the sample into a solvent that is suitable for subsequent clean-up or direct injection. The complexity and varying composition of food matrices constitute a challenge to seeking a suitable protocol.

Methods for analysis of multiple mycotoxins in a range of agricultural commodities require generic extraction conditions, typically using acetonitrile (MeCN) or methanol (MeOH) as the organic solvent, with small amounts of water (H_2O) and/or acids such as formic acid (HAc), to enhance extraction efficiency and to ensure adequate recovery of the different types of mycotoxins.

There are many different generic extraction protocols in common use for multi-mycotoxin methods, but procedures using acidified MeCN/H_2O are common such as:

- MeCN/H_2O:HAc (79:20:1, *v/v/v*) with a 1:4 sample/solvent ratio[6]
- Acidified versions of QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe)[7]. The use of salts increases the ionic strength of the aqueous mixture and induces phase separation with MeCN. Acidic conditions are used to favor the partition of the more acidic analytes (such as fumonisins and ochratoxins) into the MeCN.



Sample Clean-up

Mycotoxins are sought in complex commodities so method performance can be negatively impacted by matrix effects (ion suppression/ enhancement) and isobaric interference (poor selectivity). Continuous analysis of crude extracts may necessitate timely interventions for planned system maintenance or be faced with unexpected loss of performance requiring immediate attention and often repeat analysis. Reducing the level of co-extractives by using clean-up helps to obtain reliable results and reduces system contamination and subsequent instrument maintenance.

Selecting appropriate clean-up is one of the most critical aspects of the analysis.

- It should remove the co-extracted components, mitigating the impacts of both isobaric interferences and matrix effects, without significantly affecting the recovery of the target analytes.

A number of approaches have been applied to the determination of mycotoxins:

- Immunoaffinity chromatography (IAC) columns
- Solid phase extraction (SPE), in "trap and elute", "dispersive" or "pass through" forms
- "Dilute and shoot"

READ THIS WHITEPAPER:

Learn how sample clean-up reduce the amount of unwanted co-extractives introduced into the UPLC™-MS/MS system

Immunoaffinity Chromatography Columns

Immunoaffinity Chromatography (IAC) Columns are packed with activated solid phase bound to a specific antibody for a given mycotoxin(s). When the extract passes through the column, the mycotoxin binds to the antibodies, while other co-extractives are removed by a washing step. Mycotoxins are then eluted with a miscible solvent such as methanol or by antibody denaturation.

Due to their high specificity, IAC Columns produce cleaner extracts when compared to less selective SPE sorbent materials and so can be used to generate data that is less susceptible to interference and ion suppression from co-extractives.

IAC Columns can be used with LC–MS/MS to deliver highly sensitive methods for analysis of key mycotoxins in the more complex commodities, and to simplify quantification by avoiding matrix-matched calibration or internal standards.

The main disadvantage is that each IAC Column is limited to analysis of a small group of "regulated" mycotoxins in specific commodities and requires a variety of different time-consuming protocols.

READ THIS APPLICATION NOTE:

Using VICAM™ immunoaffinity chromatography cleanup for the determination of OTA and AFs by UPLC-MS/MS

Solid-Phase Extraction (SPE)

Solid-Phase Extraction (SPE) can be applied for mycotoxins extraction from liquid samples but can also be used as clean-up and/or concentration steps after a previous extraction procedure. SPE is a technique based on the specific partitioning of the analyte between being dissolved in the extract and associated with the stationary phase on particles in the cartridge.

Using a typical "trap and elute" approach, the mycotoxins are absorbed to the stationary phase and then eluted from the cartridge with an organic solvent. The wide range of sorbents offer a variety of retention mechanisms, which may be used to provide optimum recovery of different groups of mycotoxins. Oasis™ MCX is a novel, mixed-mode polymeric sorbent that has been optimized to achieve higher selectivity and sensitivity for basic compounds, such as pyrrolizidine alkaloids (PAs). The protocol makes use of the cation-exchange mechanism for protonation of the basic PAs under acidic conditions. After the washing steps, the PAs are eluted from the cartridge using an organic solvent at high pH.

READ THIS APPLICATION NOTE:

A range of plant based foodstuff extracts are cleaned-up using mixed cation exchange SPE

Dispersive Solid-Phase Extraction (dSPE)

dSPE is a rapid, simple, and straightforward technique suitable for the clean-up of extracts from a wide variety of food and agricultural commodities. An aliquot of sample extract is added to a disposable tube containing sorbent(s). The tube is shaken, centrifuged, and the supernatant is collected for further analysis. Unlike in conventional SPE, it is the co-extractives that are partitioned into the sorbent whilst the analytes remain in the solution.

The most popular commercially available kits were developed for methods using QuEChERS and typically include magnesium sulphate to remove any remaining water and one or more of three sorbents:

- Graphitized carbon black (GCB) for removing pigments
- Primary-secondary amine (PSA) for removing sugars, fatty acids, organic acids
- Octadodecyl-bonded silica (C_{18}) for removing long chain hydrocarbons, lipids, waxes)

Varying amounts of different sorbents are added depending on the commodity and mycotoxins of interest.

READ THIS APPLICATION NOTE:

dSPE is used to clean up botanical samples prior to LC-MS/MS analysis

Solid-Phase Extraction (SPE)

When using dSPE, one must be careful with the choice of sorbents to avoid loss of some mycotoxins. For example, the PSA sorbent retains acidic compounds such as fumonisins and ochratoxins. SPE using a "pass through" protocol has been shown to be a valid alternative to dSPE and is more effective at removal of phospholipids and fats. This protocol inverts the classical approach to SPE by allowing the analytes of interest to pass through the cartridge and retention of co-extracted fats, phospholipids and pigments by the sorbent. A clean eluate is achieved in only a few minutes.

The Oasis HLB PRiME Cartridge behaves as a short chromatography column. Clean-up of the extract can be optimised by evaluating the stage at which the analytes pass through the cartridge and discarding the first aliquot eluting from the cartridge.

The use of SPE in this way is an effective and efficient approach to clean up, as it involves a simple and quick pass through protocol, thus eliminating the SPE conditioning, equilibration, and washing steps.

READ THIS APPLICATION NOTE:

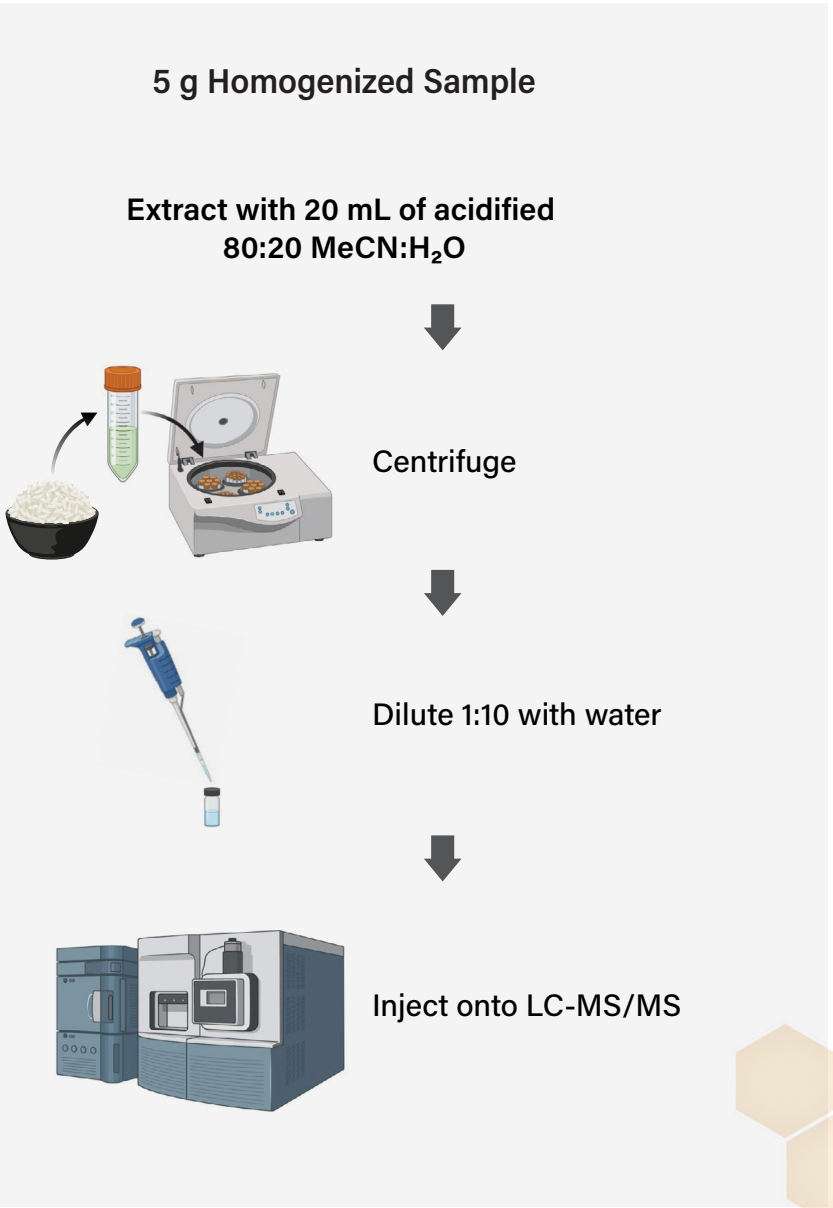
Using Pass-Through SPE and UPLC-MS/MS for the determination of multi-toxins in relevant foods and feed

"Dilute and Shoot"

"Dilute and shoot" refers to the dilution of a sample extract with a dilution solvent (diluent) suitable for analysis by LC-MS/MS. Compared with other traditional sample clean-up techniques which remove unwanted co-extracted matrix components, dilute and shoot simply decreases matrix effects while eliminating time-consuming and costly extraction procedures. Therefore, it can be considered the most straightforward and fastest clean-up method available.

The dilute and shoot approach has been used for the determination of a wide range of mycotoxins, using either matrix-matched standards and/or ^{13}C-labelled analogues as internal standards (where available), often added just before LC-MS/MS to mitigate matrix effects and improve repeatability. The scope of this approach can be extended to include some plant toxins of interest.

This strategy also allows for a reduction in cost and time of analysis, while covering a wide range of mycotoxins with high chemical diversity. Highly sensitivity instruments are required to be able to benefit from maximizing the dilution factor.



READ THIS APPLICATION NOTE:

Learn how regulated mycotoxins are quantified in cereal grains using the dilute and shoot approach

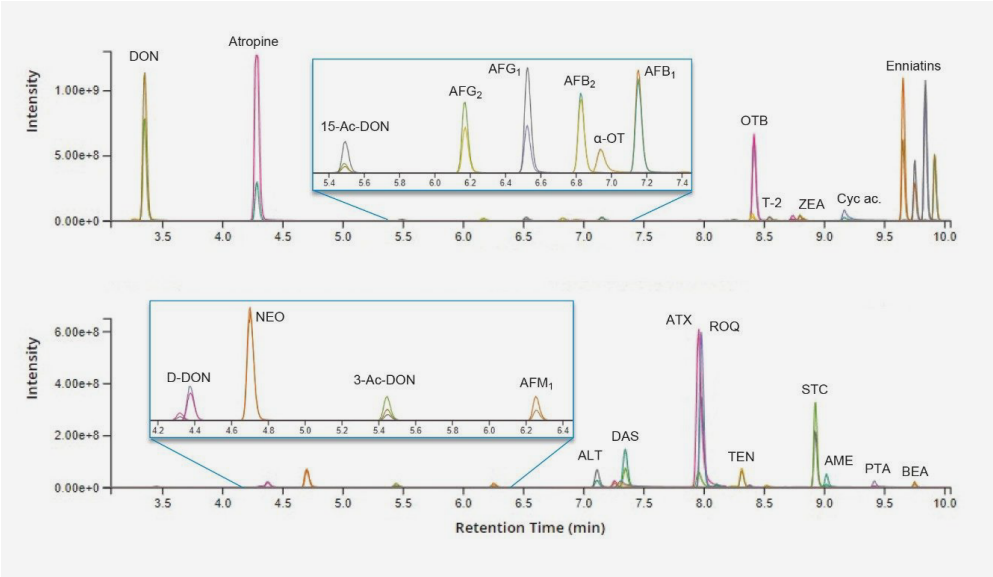
Liquid Chromatography-Tandem Quadrupole Mass Spectrometry (LC-MS/MS)

LC-MS/MS has become the most intensively used instrumental technique for the quantitative determination of mycotoxins in a single method. Its high selectivity in multiple reaction monitoring (MRM) mode, sensitivity, robustness, and multi-analyte capability, including polarity switching, allows for the simultaneous determination of many analytes from different classes of mycotoxins in a reliable manner.

Sample preparation needs to be tailored to the sensitivity achievable on the instrument to be used.

- Employing systems with highest sensitivity, allows for simple dilute and shoot of crude extracts, avoiding timely and labor-intensive solvent reconstitution and/or extract clean-up.
- Other system levels require rapid but effective approaches to clean-up to provide sufficient sensitivity.
- The use of ultraperformance liquid chromatography (UPLC), using high efficiency columns based upon sub 2 µm particles, has reduced analytical runtimes, allowing twice the samples to be analyzed by UPLC-MS/MS vs traditional LC-MS/MS.
- There has been increased focus on sustainability, footprint (physical and environmental), and reduced operational costs.

Waters ACQUITY™ UPLC Premier and Xevo™ TQ-S micro.



Chromatographic Performance

WHAT IS RETENTION TIME?

Retention time (RT) is the time between the start of an injection to the emergence of the peak maximum of the analyte(s).

WHY IS RT IMPORTANT?

If this interaction is too short, then little to no chromatography has taken place, separations will be less stable, and there will be a high chance of co-elution with matrix components. Guidelines such as SANTE/12089/2016[8] state "the minimum acceptable retention time for the analyte(s) should be at least twice the retention time corresponding to the void volume of the column."

The chromatography also needs to be very stable as the RT of the peak in the sample extract should correspond to that of the analyte reference, within a specified tolerance, for identification purposes. With UPLC, deviations are typically within ±0.1 minutes within each batch sequence.

WATCH THIS VIDEO:

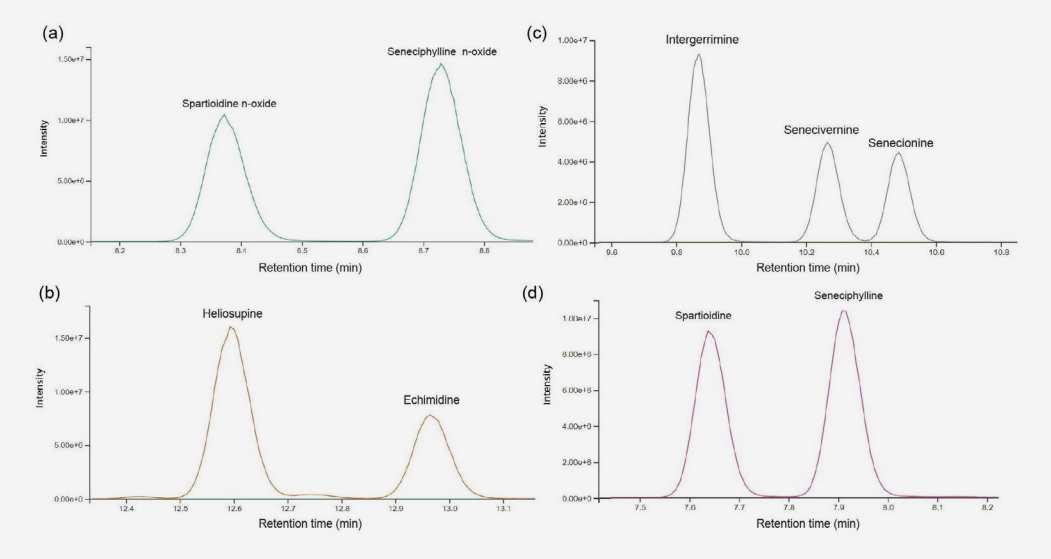
The importance of retention, and how to calculate it efficiently in order to get reliable results, are discussed.

Chromatographic Performance

Good chromatographic separation of each mycotoxin or plant toxin peak is important as it helps optimize the MRM acquisition windows to minimize function overlap, thus maximizing dwell times and performance of polarity switching, to provide precise measurements.

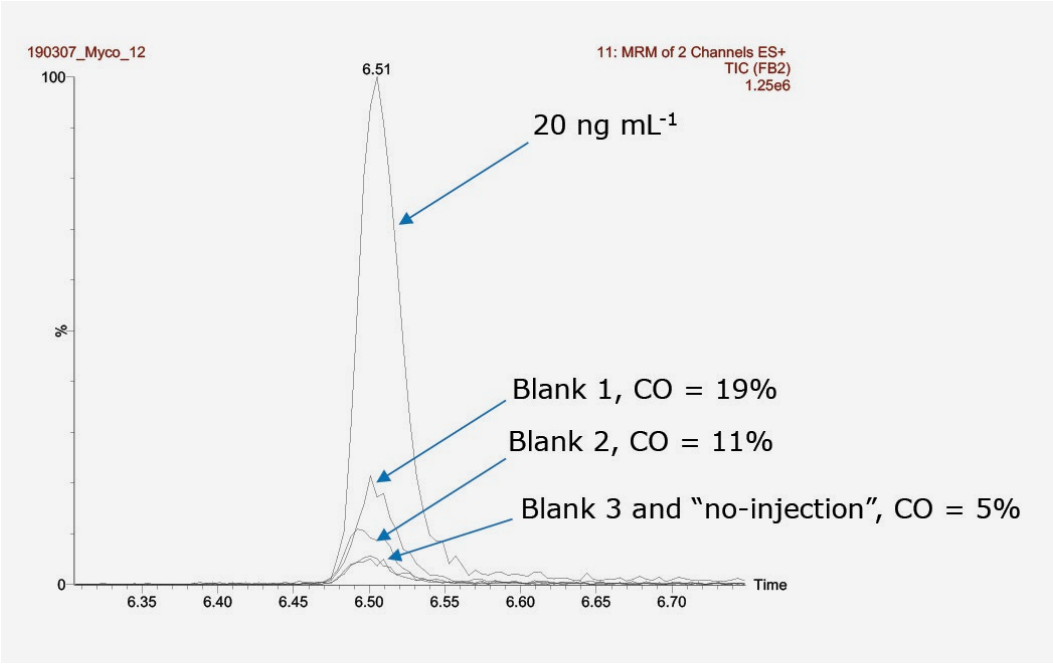
One of the main challenges in determining pyrrolizidine alkaloids and their N-oxides is the co-occurrence of isomers, which causes coelution, making it difficult to separate these compounds chromatographically. Many isomers are also isobaric, presenting the same MRM transitions, which exasperates the situation.

Using an ACQUITY™ UPLC BEH™ C_8 Column, under acidic conditions, we achieved baseline separation for 27 of the 35 analytes of interest, whilst four pairs of coeluting isomers, lycopsamine/indicine, rinderine/echinatine, intermedine N-oxide/indicine N-oxide, and integerrimine N-oxide/senecivernine N-oxide, were quantified as sum of the pairs.



READ THIS APPLICATION NOTE:

The chromatographic separation of a number of pyrrolizidine alkaloids is described



Carryover

WHAT IS CARRYOVER?

Carryover is the result of analyte(s) left over from previous injections leading to ghost peaks in the following injections, which can lead to inaccurate quantification and possible false positives. Troubleshooting carryover can be frustrating, especially if compounds interact and are adsorbed on the metal surface within the flow path. Enniatins, fumonisins, sterigmatocystin, and beauvericin are all susceptible.

STRATEGIES TO MITIGATE THE PROBLEM

These include the careful selection of washing solutions, metal free needles, more regular changing of the injector seal or adding chelating agents to the mobile phase, needle wash solutions, and sample diluent.

Technology, such as MaxPeak™ HPS, provides a highly effective barrier that mitigates undesired interactions with the metal surfaces in the flow path, allowing for the removal of metal chelators, and also improved analytical throughput, as fewer washing cycles are needed within the same sequence.

READ THIS APPLICATION NOTE:

The benefits of ACQUITY Premier UPLC for multi-mycotoxin methods

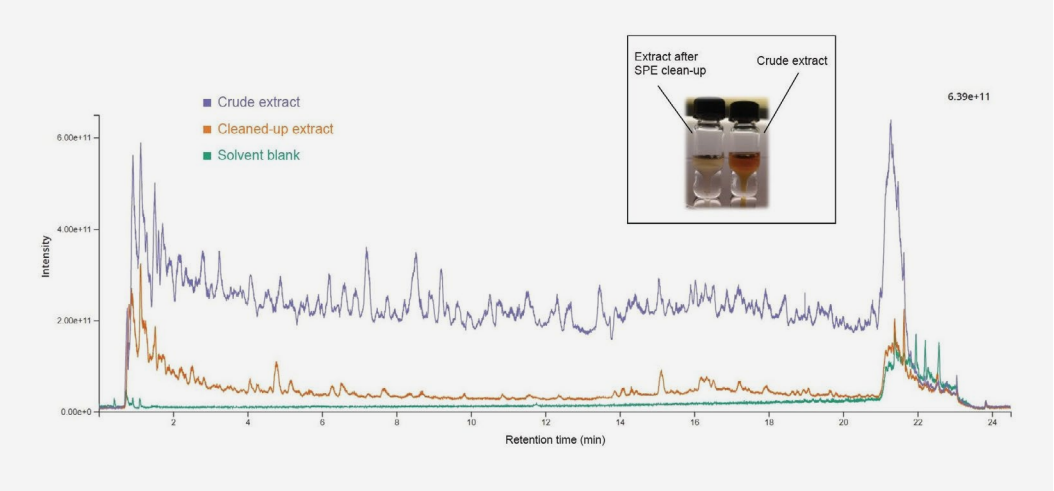
Understanding Sample Complexity

INVESTIGATING THE PRESENCE OF MATRIX CO-EXTRACTIVES

Laboratories dealing with mycotoxins and plant toxins in complex agricultural commodities and foodstuffs have to make tricky decisions on whether to spend time and money on clean-up. The “dilute and shoot” approach is simple, quick, and allows multiple toxins to be determined simultaneously. The presence of matrix co-extractives should be evaluated as they can cause isobaric interference, matrix effects, and impact method reliability.

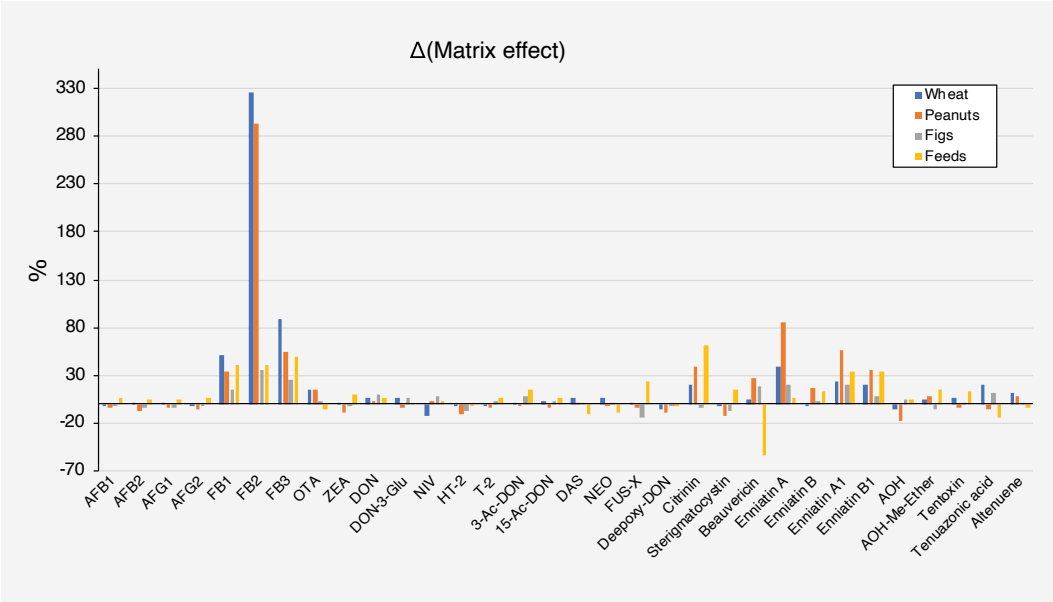
RADAR

RADAR is an acquisition mode whereby MRM transitions and full scan data are simultaneously acquired without significant degradation of MRM signals. This tool can be used during method development to investigate the presence of co-extractives in the chromatogram to better inform mitigation strategies to cope with coelutions without the need for multiple injections.



READ THIS APPLICATION NOTE:

Method Development and Validation for the Determination of Pyrrolizidine Alkaloids



Matrix Effects

WHAT ARE MATRIX EFFECTS?

Matrix effects that alter response of analytes are well known when using LC-MS/MS, especially with electrospray ionization. Matrix effects typically manifest as suppression or enhancement of the signals for target compounds by co-eluting, co-extracted, unwanted components from the matrix. Co-eluting matrix compounds can change the physical properties of the electrospray droplets slowing down desolvation and compete for the limited number of charged sites on the surface of the electrospray droplet. Matrix effects should not be confused with matrix isobaric interference, which is a response at the expected retention time on an MRM channel from another, (partially) co-eluting, compound that contributes to the analyte response.

MITIGATION

The presence and magnitude of matrix effects should be assessed and the following steps should be taken to mitigate their impact:

- Use of matrix-matched calibration
- Stable isotopically labelled analogues as internal standards
- Suitable clean-up
- Standard addition

WATCH THIS VIDEO:

How to determine matrix effects in complex food samples.

Data Processing and Review

Multi-mycotoxin methods, each with two MRM transitions, create a significant amount of data for review. Along with peak integration, retention time, ion ratios, signal to noise, calibration curve data, and quality control standard performance must be assessed. Data review can create significant bottlenecks for routine testing laboratories without some form of automation.

Data processing software with Exception Focused Review (XFR) functionality allows customized rule-sets based upon required acceptance criteria to be automatically applied to the data. Benefits include:

- Review of data more efficiently and consistently
- Enable multiple chromatograms to be viewed and assessed at the same time without having to navigate lots of windows
- Automatic flagging of outliers for further inspection
- Visually and quickly identify samples with suspected residues
- Submission of the sample results for approval with confidence

READ THIS WHITE PAPER:

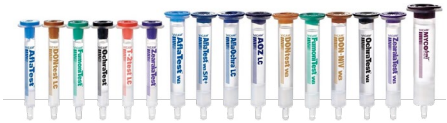
The benefits of data review using the MS Quan application

Enabling Technologies and Services Across The Analytical Workflow

Because of climate change, studies reveal that many parts of the world can expect unpredictable periods of potentially harmful levels of mycotoxin contamination in crop harvests in the future. In addition, plant toxins, can be detected in certain food and feed materials. With an extensive regulatory framework in place across the globe, government laboratories, food manufacturers and contract testing services need flexible, fast and reliable testing solutions for various natural toxins in a diverse range of different agricultural commodities and food ingredients classes.

Waters offers fully supported, complete analytical solutions, suitable for determination of multiple natural toxins in different commodities, using generic extraction, various approaches to clean-up, coupled with UPLC-MS/MS, maximizing operational efficiency with customizable informatics workflows to best meet requirements.

Waters offers flexible finance and service options and Analytical Professional Services for application support.

Screening	Confirmation	Multi-toxin Analysis	Data Analysis
 Qualitative Screening	 HPLC with FLD	 LC-MS/MS	 waters_connect for Quantitation
 Quantitative, Eco-Friendly Rapid Detection	 Immunoaffinity Columns		

References

1. Boutrif, E and Canet, C (1998). Mycotoxin prevention and control: FAO programmes. *Rev. Med. Vet.* **149**:681–694.

2. Eskola, M *et al.* (2019). Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate' of 25%, *Crit. Rev. Food Sci. Nutr.* **60(16):**2773–2789

3. Eskola, M *et al.* (2020). Towards a dietary-exposome assessment of chemicals in food: An update on the chronic health risks for the European consumer. *Crit. Rev. Food Sci. Nutr.* **60**:1890–1911

4. Pigłowski, M (2019). Comparative analysis of notifications regarding mycotoxins in the Rapid Alert System for Food and Feed (RASFF). *Qual. Assur. Saf:* **11(8):**725–735

5. EC. (2023). Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. European Commission. *OJ* L **119**:103–157

6. Malachova, A.*et al.* (2014). Optimization and validation of a quantitative liquid chromatography-tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices. *J. Chromatogr. A* **1362**:145–156.

7. Sun J *et al.* (2016). QuEChERS Purification Combined with Ultrahigh-Performance Liquid Chromatography Tandem Mass Spectrometry for Simultaneous Quantification of 25 Mycotoxins in Cereals. *Toxins* **8(12)**:375

8. SANTE/12089/2016. Guidance document on identification of mycotoxins in food and feed

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