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Mycotoxin testing: From Multi-toxin analysis to metabolomics

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Abstract

Mycotoxins are toxic fungal metabolites, occurring on a wide range of agricultural products. Several research projects, including the recently started European project "MyToolBox", aim for integrated approaches – combining pre- and post-harvest measures with efficient monitoring tools for control. The latter is crucial to provide food safety for the consumers and to determine the efficacy of mitigation measures to reduce mycotoxins. Analytical chemistry, in particular mass spectrometry, has evolved with a tremendous pace. While years ago, only single toxins could be measured, a clear trend is towards multi-toxin methods, providing a far more detailed picture. One example is a multi-analyte LC-MS/MS method which has recently been developed by us and which is capable of determining some 380 fungal, bacterial and plant metabolites, respectively, in cultures, cereals, food and feed products. LC-MS- based screening has also been playing a vital role in the discovery of novel mycotoxin conjugates - so called "masked" - forms of mycotoxins.

Metabolomics has emerged as the latest of the so-called –omics disciplines and shows great potential to determine hundreds to thousands of metabolites at once over a wide range of concentrations. After measurement of biological/food samples treated with a 1+1 mixture of labelled and non-labelled precursors, labelling-specific isotopic patterns can be reliably and automatically detected by means of the novel software tool ("MetExtract"). In a preliminary study, the great potential of the presented approach is further underlined by the successful and automated detection of novel plant-derived biotransformation products of the most prevalent *Fusarium* mycotoxin deoxynivalenol.

Introduction

Fungal, and subsequently, mycotoxin contamination in various crops is of major concern regarding its relevance for food and feed safety, food security and international trade. Recent global survey data indicate that up to 80% of agricultural commodities could be contaminated with mycotoxins[1),2),3),4)].

Grain and foods based on these grain (e.g. pasta, bread, bakery products) are the main source for mycotoxin exposure in all age classes of the EU population, in particular due to the mycotoxins produced by *Fusarium* spp. (deoxynivalenol (DON), T-2/HT-2 toxins, zearalenone (ZEN) and fumonisins), but also ochratoxin A (OTA). In 2015, the European Rapid Alert System for Food and Feed's (RASFF) notifications on mycotoxin contamination exceeding the EU legislative limits increased significantly as compared to 2014[5)], accounting for 28.2% of all border rejection notifications reported to RASFF[6)].

Furthermore, mycotoxins may lead to substantial economic losses in livestock production[7)]. Mycotoxicoses of agricultural animals, caused by rather short-term over-exposure to mycotoxins, are regularly reported[8)]. Besides, there is a pressing moral obligation to curb these significant food and feed losses, given that "one in eight persons worldwide is suffering from chronic undernourishment"[4)].

It is difficult to accurately estimate the market loss associated with lower productivity due to impacts on

animals' health[7], and loss of animal feed. In a recent feed survey, up to 139 different mycotoxins were detected, demonstrating the importance of multi-analyte methods in mycotoxin analysis[9]. Overall, (multi-) mycotoxin-testing plays a key role in determining the natural occurrence of mycotoxins in food and feed and the exposure of humans and animals to these toxic secondary fungal metabolites. In addition, accurate quantification of mycotoxins is crucial to evaluate the success of measures to reduce the mycotoxin levels along the food and feed chains. Furthermore, metabolomics has emerged as a novel scientific discipline in the area of mycotoxin analysis, which provides for a holistic picture of all the secondary metabolites of plants and fungi that are present in the interaction of different organisms.

Mycotoxin occurrence and detection

Various international institutions and organisations (e.g. European Commission, US Food and Drug administration, World Health Organisation, United Nations Food and Agricultural Organisation) adopted regulatory limits for major mycotoxin classes and individual mycotoxins, given their potential health risks to human and animal health[10]. To comply with such standards, validated analytical methods for determining these toxins have been developed for batches of different food and feed commodities. Similarly, sampling plans[11] were developed for such batches intended for human or animal consumption. However, sample separation and selection of the proper method of analysis have to cater to a wide range of agricultural commodities *vis a vis* a large chemical diversity of mycotoxins, thus posing a challenge for determining mycotoxins in some commodities.

Despite the enormous progress in mycotoxin analysis, there are major challenges, such as the determination of modified mycotoxins[12] including masked mycotoxins[13], the signal enhancement due to matrix effects observed when performing liquid chromatography – (tandem) mass spectrometry (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. Furthermore, climate-change effects such as extreme weather events result in increasing numbers of unexpected findings of mycotoxins in food and feed commodities. Such unexpected mycotoxins are referred to as so-called emerging toxins[14], which include enniatins, moniliformin and Alternaria metabolites, fusaproliferin or beauvericin, but also ergot alkaloids and masked mycotoxins[1]. The impact of these cocktails of secondary metabolites in food and feed, and the potential additive or synergistic effects of these substances are still mostly unknown.

In addition, global warming affects the occurrence map of common mycotoxins such as aflatoxins and DON. For example, Romania, Serbia and Croatia reported aflatoxin M1 (AFM1) contamination of milk in February and March 2013, for which maize as feed was found to be the source of contamination. In particular in Serbia, severe droughts in 2012 resulted in 70% of the maize crop being contaminated with aflatoxins[15], which was fed to dairy cattle, and thus led to high levels of AFM1 in milk, up to twice the EU legal limit. The milk scandal was fuelled when the permitted level of AFM1 in milk in Serbia was temporarily raised to 0.5 µg/kg milk, 10 times the EU legal limit. Also, countries in Northern Europe are facing increasing occurrence of DON in their wheat harvests.

Multi-mycotoxin analysis

Various methods to detect individual mycotoxins or mycotoxin classes are available[16]. For example, rapid screening methods for single mycotoxins or mycotoxin classes, which are largely based on immunochemical techniques, have been developed to deal with the increasing demand for mycotoxin analyses along the food and feed chain.

A shift from thin layer-, ELISA- and LC-methods to LC-MS-based methods[18] has been observed over the last decades in mycotoxin analysis. Until about 10 years ago, most of the available analytical methods (e.g. HPLC-UV/FLD) for the determination of these toxic metabolites only covered single mycotoxin classes (e.g. aflatoxins, type-B trichothecenes or fumonisins). In the meanwhile, mass-spectrometry-based analytical methods have been key for the determination of a variety of mycotoxins and their metabolites in plants and foods and for the investigation of the metabolism of these toxic compounds in body fluids such as serum and urine. One example is a multi-analyte LC-MS/MS method which has recently been developed by Sulyok et al.[19], and which is capable of determining more than 380 fungal, bacterial and plant metabolites, respectively, in cultures, cereals, food and feed products. LC-MS based screening is also playing a vital role in the discovery of novel conjugated (masked) mycotoxins, in the past, which is also believed to continue in the future. This unique mycotoxin LC-MS method has also been employed in BIOMIN´s Spectrum 380® screening programme[20]: For the first time, surveys can be conducted routinely which include concentration levels of more than 380 mycotoxins and fungal metabolites. Spectrum 380® employs latest state-of-the-art liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) in a single analytical step. This program revealed that a typical agricultural commodity contains on average 30 different mycotoxin metabolites. The results of a worldwide survey utilizing this fully in-house validated LC-MS/MS method have very recently been published[1].

Several decisions have to be taken, before the successful development of an LC-MS based multi-myco-

toxin method can start. First of all, the set of analytes must be selected: Which toxins/fungal metabolites are mandatory to determine and which would be nice to have included additionally? This decision is equally important as the actual analysed matrix, but of course the matrix determines the occurrence of mycotoxins to a large extent. The next decision is heavily influenced by the availability of existing mass spectrometric equipment: Is it important to quantify mycotoxins in trace levels (e.g. in baby food), or are (far) higher levels expected (e.g. raw materials or feed stuffs)? Depending on the equipment and concentration range, samples are needed to be concentrated or diluted. As matrix effects severely hamper accurate quantification in LC-MS, concentration of samples without previous sample clean-up is strongly discouraged. On the other hand, no single (effective) method can clean-up a multitude of chemically very different substances. Recent literature reviews[16),21)] on the analysis of food and feed for mycotoxins describe several strategies.

While the high selectivity of antibody-based clean-ups renders their use in LC-MS methods scarce, multi-functional SPE columns (e.g. MycoSep®, Bond Elut®, etc.) offer efficient matrix removal for the most important mycotoxin classes (typically fewer than 20 toxins). Equally interesting is the use of QuEChERS based extraction and purification, which was widely used before in pesticide analysis. The main idea is that while water and acetonitrile are miscible, they separate into two phases at high salt concentrations. After extraction of mycotoxins with an acetonitrile/water mixture, addition of salts leads to an acetonitrile rich fraction containing most of the mycotoxins and a water rich fraction containing lots of very polar matrix constituents. For pesticide determination often a subsequent matrix removal step using primary, secondary amines (PSA) and/or C18 material is employed. This is rarely possible for mycotoxins as PSA would also remove acidic toxins, such as fumonisins, and C18 would bind to apolar toxins such as ZEN. Yet, both strategies, multi-functional SPE columns or QuEChERS, offer sufficient matrix removals to allow enrichment of mycotoxins prior to LC-MS/MS determination. If more (than usually 20) mycotoxins/fungal metabolites are to be analysed, each clean-up would only discriminate certain compounds. Therefore, dilute-and-shoot techniques[17)] have been developed, which allow measuring hundreds of compounds, although high-end mass spectrometers with excellent sensitivity are required. Another challenge to be overcome in methods measuring hundreds of compounds is the limited cycle time. Let us assume that a chromatographic peak is about 20 s broad. In order to have 10 data points which define the peak shape and are needed for quantification, a maximum cycle time of about 2 s must be kept. This time would even be lower when UHPLC conditions are used. With two selected monitoring reaction (SRM) transitions for each analyte (quantifier and qualifier) and even a ultra-fast dwell time of 5 ms, a maximum number of 200 analytes might be squeezed into one LC-MS/MS method on a triple quadrupole system. To overcome this limitation, so called “dynamic” or “scheduled” SRM methods have been introduced. Hereby, no all SRM transitions are measured throughout the whole chromatographic run, but only at the expected retention time, greatly reducing the number of analytes which can be considered. Still, at around 1000 analytes not even dynamic/scheduled SRM methods would suffice and multiple injections for one sample would be necessary to be analysed with different methods. An interesting trend is the use of LC high resolution (HR) MS methods, with time-of-flight or Orbitrap mass analysers. While those types of mass spectrometers are not as sensitive, they offer a virtually unlimited number of compounds to be analysed. For instance, a method for the analysis of mycotoxins (but also other analytes such as pesticides) in bakery products by LC-Orbitrap-MS was developed[22)]. While good quantitative findings were obtained for the analysed mycotoxins, the limits of quantification were not suitable to verify the levels of OTA and aflatoxin B1 with respect to maximum permitted levels in the European Union. An additional advantage of LC-HR-MS method is the acquisition of full scans, which allows the detection of substances which were not considered at the time of method development and even retrospective data analysis.

Finally, all developed methods should be validated in a manner that they are proven to be fit for purpose. Typically linear range, limit of detection, limit of quantification, repeatability and recovery are considered. Sometimes also reproducibility, robustness or trueness of methods are determined during validation for each analyte. Obviously, semi-quantitative methods have lesser requirements in that aspect compared to quantitative or even very accurate quantitative methods. The latter are for instance required in official control laboratories and are strongly suggested for accredited laboratories. Besides clean-up strategies, which are barely available for a multitude of substances (see above), also other options exist to compensate for matrix effects. Very commonly, matrix-matched calibration is used instead of external calibration with standards in neat solvents. For this a representative blank matrix has to be found first, which is not always easy. Besides, individual differences of matrix effects within one commodity are not considered. An excellent alternative is internal calibration with e.g. ^{13}C-labelled mycotoxins in a so called stable isotope dilution assay (SIDA). Chemically, native toxins and their isotope labelled analogues are identical and therefore also the ionization behaviour and retention times are identical. Yet, mass spectrometry can differentiate between the two forms due to the difference in the mass. Ideally, the same SRM fragments with the same collision energies should be chosen for analyte and internal standard (of course

with a shift in mass). Uniformly ^{13}C labelled mycotoxins are commercially available since about 10 years now[23). Interestingly, it is still a common belief that internal standards are very expensive and not affordable for the average testing laboratory. The amazing speed of development in the sensitivity of mass spectrometry equipment over the last decade tells another story. With more sensitive instruments, also the concentration of the internal standard in a given sample can be reasonably low. For example, an LC-MS/MS based SIDA method for the determination of all mycotoxins which are currently regulated in solid foodstuffs within Europe was developed[24)]. A double extraction ensured high extraction recoveries and the internal standards were only required to be added into the HPLC vial just before injection in the MS/MS system to efficiently compensate for matrix effects. Authors calculated that the additional costs of the internal standards for analysis are below 2 EUR per sample.

Metabolomics of plant-fungi interactions

Metabolites are the products of cellular regulatory processes, whose levels can be regarded as the ultimate response of biological systems to genetic or environmental changes[25)]. Metabolome analysis or metabolomics has been introduced to identify the entire set of low-molecular-mass compounds, i.e. metabolites, synthesized by an organism[26)]. To determine hundreds to thousands of these metabolites at once over a wide range of concentrations, metabolomics-based approaches have become increasingly popular.

Modern mass spectrometric techniques (LC-HR-MS/MS) are applied for both metabolomics and metabolite profiling. In contrast to mere metabolite profiling, metabolomics ideally shows fitness for a functional genomics context. To yield new insights into the behaviour of biological systems, all studies in functional genomics aim to narrow the gap between gene sequence and gene function. Metabolomics can be an extremely useful tool that finds applications in many aspects of drug discovery, food safety issues and disorders of cells and organisms. Ideally, a direct link between resistance genes and culture's ability to detoxify the relevant mycotoxin into its non-toxic glucosidic form can be discovered as demonstrated for DON-3-glucoside[27)]. Advanced research in the area of metabolomics shall lead to better understanding and predictability of the behaviour of complex biological systems such as plants and microbes.

The untargeted detection of yet unknown secondary metabolites of mould and metabolic products of mould-resistant plants becomes feasible by applying non-directional metabolomics-methods. Within a tracer-fate study, plants are treated with native and ^{13}C-marked mycotoxins, whose metabolic products are then traced by HR-MS[28)]. Following the measurement of biological/food samples treated with a 1+1 mixture of labelled and non-labelled precursors (Fig. 1), a novel software tool ("MetExtract")[29)], can detect the labelling-specific isotopic patterns reliably and automatically. The successful and automated detection of eight novel plant-derived biotransformation products of the most prevalent Fusarium mycotoxin DON proved the great potential of that approach: Besides DON-3-glucoside, DON-S-glutathione was identified, the latter representing the discovery of a new form of conjugated (masked) mycotoxins as a result of an alternative plant defense mechanism derived from the glutathion pathway[30)]. However, the relevance of these novel metabolites for food and feed safety is still to be investigated[31)].

Conclusion and outlook

In order to reduce and control mycotoxins and their metabolites along the entire food and feed chain, integrated approaches will be increasingly sought. Such approaches must combine effective pre- and

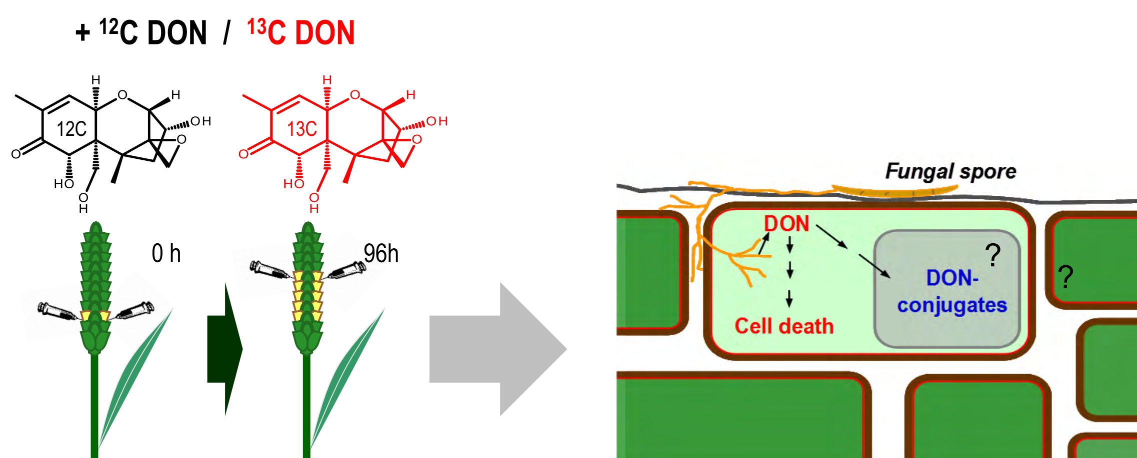

Fig. 1 Studying the metabolization of DON in wheat plants using stable isotopic-labeling assisted metabolomics.

post-harvest measures and monitoring tools for the determination of mycotoxins. The MyToolBox project (www.mytoolbox.eu), a four-years project funded by the EU's Horizon 2020 programme, is dedicated to develop such technologies and measures for stakeholders along the food and feed chain which will feed into a web-based e-toolbox. In terms of mycotoxin determination it is expected that the need for both rapid screening methods as well as tailored confirmatory methods will remain high. For the latter, LC-MS/MS and lately also LC-HR-MS methods have become popular, allowing screening for and quantification of hundreds of fungal metabolites in food and feed samples. Metabolomics studies will complement such integrated approaches to reveal the fundamental biological processes of mycotoxin production and its reduction.

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