Aibo Wu *Editor*

Food Safety & Mycotoxins

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Foreword

The topic of mycotoxins concerning food safety is becoming increasingly popular in China and worldwide. In the meantime, mycotoxins are truly involved in a variety of intersecting multidisciplinary directions, such as agriculture, food, and microbiological sciences, as well as analytical chemistry and bioinformatics. Dr. Aibo Wu's "Food Safety & Mycotoxins" is an important summary of theoretical and technical advances regarding the detection, risk assessment, and control of mycotoxins contaminating the entire food chain, thus arousing food-safety problems.

This book is divided into 4 parts and 10 chapters, where it mostly covers the research aspects of mycotoxins and food safety. To be specific, the details about mycotoxins from research done in China are freshly included. The progress of various studies in 2014–2019 and prospective trends in this scope are also thoroughly discussed. Hopefully, this book will, as expected, greatly benefit the audience from research institutes, universities, or even policy makers in governmental agencies and farmers targeting practical uses.

Dr. Joseph Jen
Editor of Food Control, the former Under Secretary
of the U.S. Department of Agriculture, USA
Washington, DC, USA
June 2019

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Abbreviations

AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
AOH	Alternariol
AME	Alternariol monomethyl ether
BEA	Beauverin
CAT	Catalase
CE	Capillary electrophoresis
CHOP	CCAAT/enhancer-binding protein homologous protein
CIT	Citrinin
DAS	Diacetoxyscirpenol
DOM	Deepoxy-deoxynivalenol
DON	Deoxynivalenol
D3G	Deoxynivalenol-3-glucoside
ESI	Electrospray ionization
FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisins B3
FB4	Fumonisin B4
GJIC	Gap junctional intercellular communication
GRP78	Glucose-regulated protein 78
GR	Glutathione reductase
GPx	Glutathione peroxidase
HFB1	Hydrolyzed fumonisin B1
HT-2	HT-2 toxin
H-SRM	High-selective reaction monitoring
HPLC	High performance liquid chromatography
JNK	C-Jun N-terminal kinase

LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
LLE	Liquid-liquid extraction
ME	Matrix effect
MWCNT	Multi-walled carbon nanotube
MRM	Multiple reaction monitoring
MDA	Malondialdehyde
NMR	Nuclear magnetic resonance
NIV	Nivalenol
OTA	Ochratoxin A
PA	Purity angle
PT	Purity threshold
RSD	Relative standard deviation
RSDr	Intra-day relative standard deviation
RSDR	Inter-day relative standard deviation
ROS	Reactive oxygen species
SPE	Solid phase extraction
S/N	Signal/noise
SOD	Superoxide dismutase
Sa	Sphinganine
So	Sphingosine
TCA	Tricarballylic acid
T-2	T-2 toxin
TeA	Tenuazonic acid
T-SRM	Timed-selective reaction monitoring
TLC	Thin layer chromatography
TBARS	Thiobarbituric acid reactants
UHPLC	Ultra high performance liquid chromatography
UV	Ultraviolet detector
ZEN	Zearalenone
ZAN	Zearalanone
Z14G	Zearalenone-14-glucoside
Z16G	Zearalenone-16-glucoside
α-ZAL	α-Zearalanol
β-ZAL	β-Zearalanol
α-ZOL	α-Zearalenol
β-ZOL	β-Zearalenol
3ADON	3-Acetyl-deoxynivalenol
15ADON	15-Acetyl-deoxynivalenol
8-OH-dG	8-Hydroxydeoxyguanosine

Part I
Detection

Chapter 1
Chromogenic Platform-Based Lateral Flow Immunoassay

Na Liu and Aibo Wu

Abstract In general, several instrumental analytical methods (i.e., TLC, HPLC, CE-MS, LC-MS, MS-MS) of chromatography and rapid detection approaches of ELISA and FLA formats have been widely developed and proved to be sensitive and reliable for diverse occasions and purposes. In the recent decade, there is a still increasing interest in developing new strategic platforms which are simple, portable, disposable, and inexpensive, enabling to rapidly and simultaneously detect the target analytes in the fields of clinical diagnosis, environmental monitoring, and food safety control. Especially, those expected detection formats are extremely useful and highly welcome in remote settings, with high specificity and affinity to the target molecules showing more prospective to fulfill the above objective for these typed rapid analysis, which had been proved to be applicable for detecting clinical diseases and for monitoring environmental and food-based mycotoxins. We here propose some schematic immunoassay via a chromogenic platform-based FLA, attaining to the abovementioned expectations regarding developing ideal rapid tests of mycotoxins, especially for large-scale monitoring for industrial uses.

Keywords Mycotoxin · Rapid detection · Immunoassay · Lateral flow assay · Chromogenic quantitation

1.1 Introduction

Up to now, the most classic and long-term applicable analytical methods are commonly referring to thin layer chromatography (TLC) (Swanson et al. 1984), high-performance liquid chromatography (HPLC) (Tanaka et al. 1985), gas

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chromatography (GC) (Kinani et al. 2008), liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Cavaliere et al. 2005), enzyme-linked immunosorbent assay (ELISA) (Friguet et al. 1985), and lateral flow assay (Smits et al. 2001). As we are all known, there are some unavoidable drawbacks for the individual analysis. To be specific, TLC has poor separation efficiency, as well as low sensitivity and accuracy, which actually limits its application. With the availability of chromatographic instruments of HPLC and GC and linked with different detectors or MS/MS, more satisfactory performances in terms of accuracy, precision, sensitivity, and reproducibility are certainly achieved. However, due to the indispensable highly expensive equipment and strong expertise, as well as laborious procedure for sample preparation before loading, they are not appropriate for large-scale and on-site fast screening in short time. ELISA is one of the main rapid methods for screening analysis, but still in need of reader and relatively complicated sample pretreatment.

Since its initial development in the early 1980s, the paper-based lateral flow immunoassay (LFA) has gained wide applications in rapid clinical diagnosis, food safety, environmental monitoring, and agricultural field for its outstanding advantages of clearly visible result observation, user-friendly format, easy storage, and convenient transportation. The technology of lateral flow immunoassay based on the specific and sensitive antigen-antibody reaction generally provides a simpler and more economical alternative to instrumental approaches for the determination of chemical compounds, including mycotoxins, virus, and large analyses. However, the lateral flow immunoassay has achieved a broad penetration in various areas for its excellent advantages of more reproducible, more sensitive, easier to manufacture, and easier to operate in the last three decades.

To be simplified, LFA strips at least physically consisted of an absorbing pad, a gold conjugate pad, a sample pad, and a nitrocellulose membrane (Fig.1.1). The immunoassay formats can be distinguished into competitive and direct immunoassay according to the character of analytes. Competitive formats are typically applied when detecting for the small molecules, such as antibiotics and mycotoxins which would be exemplified in this paper. In this case, a positive result could be derived from the absence of a test line. In stark contrast, direct formats are always used for

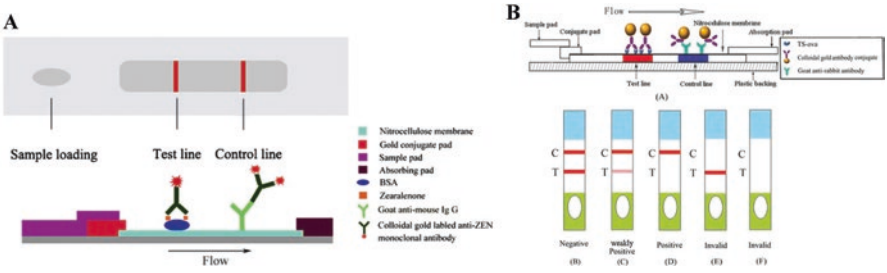

Fig. 1.1 The simplified schematic design, inner structure, and lines for reading results of the classic format of lateral flow immunoassay (LFA). (**a**) Different sectors on the horizontal plat of LFA; (**b**) the detection results read by visible lines for small molecules

testing large compounds, such as hCG. In this format for a single assay, a positive result is indicated by the presence of a test line.

In the latest decade, LFA has been widely used for rapid detection of multiple mycotoxins as small molecules, including aflatoxin B1 and B2 (Tang et al. 2009); ochratoxin A (Lai et al. 2009; Laura et al. 2011); deoxynivalenol (Kolosova et al. 2008; Xu et al. 2010); fumonisin B1, B2, and B3 (Li et al. 2012); zearalenone (Kolosova et al. 2007; Liu et al. 2012); and T-2 and HT-2 (Molinelli et al. 2008). Nevertheless, in the previously reported cases for LFA against the target mycotoxins, they are commonly for qualitative analyses and visible reading via naked eyes. It is not well known whether the feasible and applicable candidate LFA-related approach simultaneously fulfill the needs for both rapid analysis and sensitive quantitation in this aspect. Also, when we select the suitable antibody as sensing materials for the given toxin, we have to comprise the high sensitivity, assay specificity (cross-reactivity), and wide detection range, plus the complex matrices.

To overcome the above posed difficulties, the ultimate goal of chromogenic platform-based lateral flow immunoassay is to open a novel channel based on the most ubiquitously applicable LFA; to develop one combinatorial means for rapid and sensitive quantitation of ZEN contamination, which could be commonly acceptable for a wide variety of agricultural matrices, with simple sample preparation, capability for quantitation, satisfactory detection limit, sufficient accuracy, and stability; and to meet the higher requirements when applying in practical uses on some specific occasions.

1.2 The Selected Antigen and Antibody

The technical basis of LFA is the interaction between antibodies and antigens. Thus the availability of antigens and antibodies with good characteristics in terms of sensitivity, affinity, specificity, and purity will consequently ease the rest of the establishment of LFA strips. Immunogenicity and purification are key factors of antigens. Indeed, impurities will give rise to relatively low-immune response and poor quality of the antibody. Antigens of the larger molecular size usually give a good immune response, while smaller ones could not. For this reason, antigens of smaller size are generally coupled to proper carrier proteins, often bovine serum albumin (BSA), ovalbumin (OVA), or keyhole limpet hemocyanin (KLH).

The availability of a new highly sensitive monoclonal antibody against the target molecule plays a key role in LFA systems, highly determining the detection sensitivity and specificity for cross-reactivities. During the whole procedure of preparing the antibody, at the first-run screening, more than 50 positive clones against the selected coating antigen (-BSA or -OVA) were firstly screened. As followed, the clones with desirable performance to recognize the antigen are selected and characterized by SDS-PAGE. As shown in Fig. 1.2a, the anti-ZEN had good purity revealed by the apparently clear heavy and light chains at 50 and 20 KD, respectively. For further analyses, the competitive indirect ELISA system was applied to test the

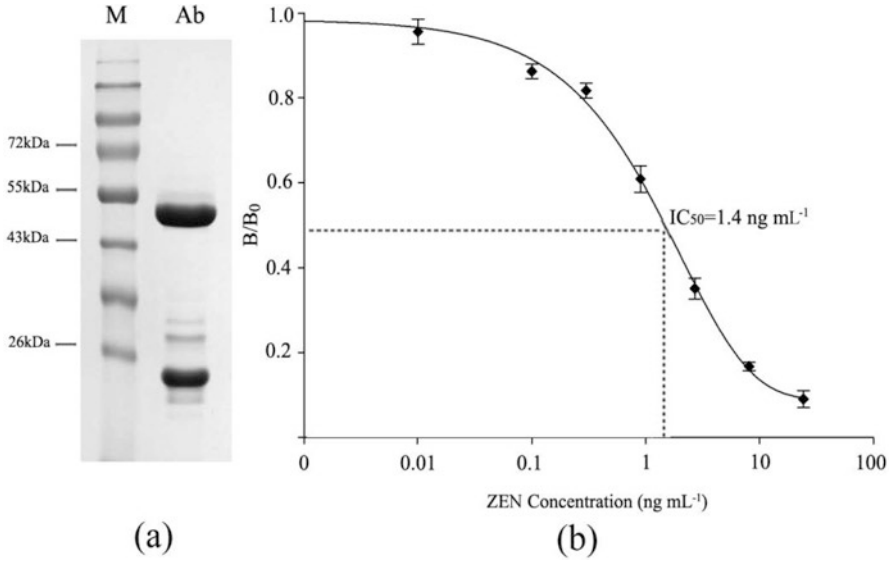

Fig. 1.2 Characterization of the selected monoclonal antibody on SDS-PAGE (**a**) and the performance of the anti-ZEN using the competitive ELISA. Each point represents the mean of three replicates (**b**). (Liu et al. 2012)

sensitivity of more anti-ZEN, in order to get the best clone which could produce the anti-ZEN with the highest sensitivity to ZEN. Finally, as indicated in Fig.1.2b, the IC50 value was 1.4 ng mL^{-1}, and the antibody titer was 1:256,000 (Liu et al. 2012).

Recently, for replacement of routine mouse monoclonal antibody, rabbit has been used to produce rabbit monoclonal antibody (RabMAbs) due to the bigger spleen and better responsiveness for broad classes of antigens (Yu et al. 2010; Spieker-Polet et al. 1995). Moreover, RabMAbs were considered to be superior to MAbs from mouse origin owing to various advantages such as a wider repertoire, simpler structure, higher binding affinity, more robust reproduction, and easier to be humanized (Feng et al. 2011; Rocha et al. 2008).

Combinatorial strategies for a single mixture immunization, screening, and rabbit hybridoma cell technology were described (Fig.1.3). Six fluoroquinolones (FQs) were conjugated with bovine serum albumin and used as immunogens for subsequent immunization, while a mixture of all was injected for co-immunization. The hybridomas against the individual and multiple FQs were obtained via elaborated screening and used for the production of diverse varieties of rabbit monoclonal antibodies (RabMAbs) against the target analytes. The approach opens a new way for simultaneously obtaining functional monoclonal antibodies which are capable of recognizing both individual and multiple analytes in a single preparation circle.

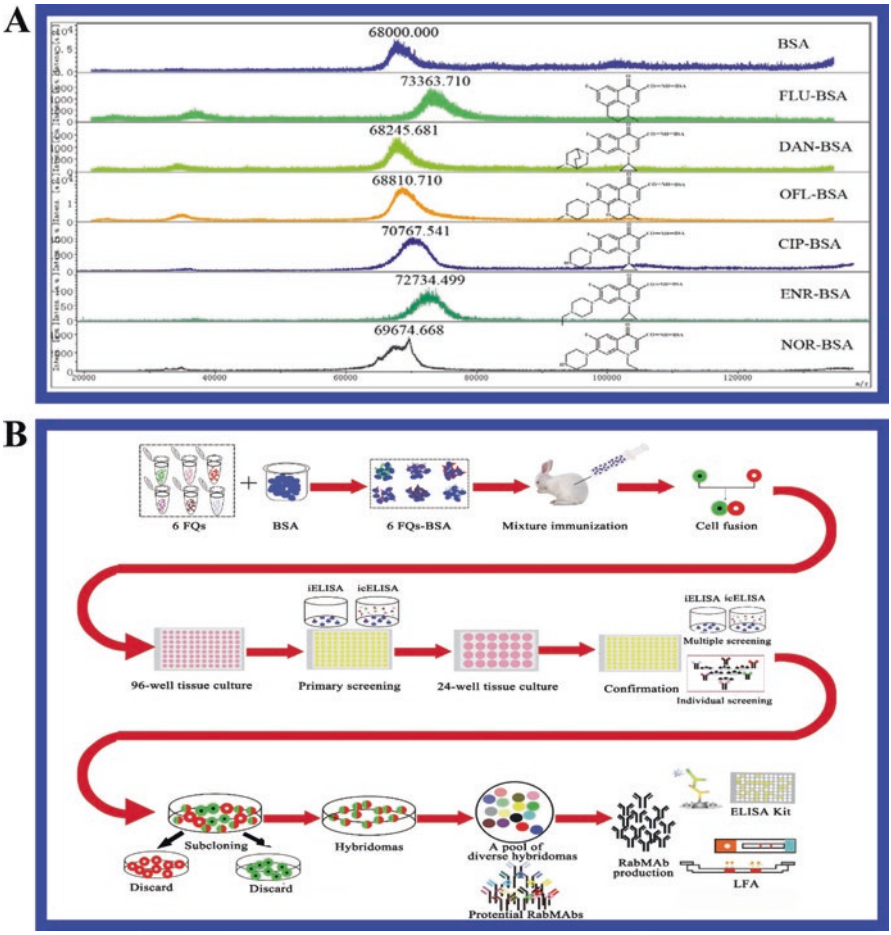

Fig.1.3 (**a**) MALDI-TOF-MS identifications of BSA, FLU-BSA, DAN-BSA, OFL-BSA, CIP-BSA, ENR-BSA, and NOR-BSA from the top down. (**b**) The scheme of simultaneous acquisition of various RabMAbs with diverse recognition functionalities against the target small molecule of fluoroquinolones (FQs). (Liu et al. 2016)

1.3 Chromogenic Reader Designed for LFA Applications

In order to overcome the limitation of misjudgments of positive samples caused by visual difference of individuals, convenient devices were invented by recording and comparing the color intensity values between the test line and control line.

1.3.1 LFA Applied for the Rapid Determination

In this case, ZEN, known as an essential mycotoxin, was screened by a colloidal gold-based competitive LFA integrated with a CHR-100 chromogenic reader (Kaiwood, Taiwan, China).

Practically, to test the accuracy of the established chromogenic platform -based FLA, we select series standard solutions of ZEN of quantitation with different CI values (Fig. 1.4). The lowest level was set at 1.0 ng/mL, which was obviously lower than the limits of ZEN in Chinese standards or even worldwide regulation concentrations. Of course, the final check using real samples contained ZEN was also consistent with the results of in-parallel LC-MS/MS analyses.

With the availability of three class-specific monoclonal antibodies, a multiplex LFA platform for simultaneous multi-class qualification or semi-quantification of AFB1, ZEN, DON, and their analogs (AFs, ZENs, DONs) was established (Fig.1.5). Moreover, the technical platform offers multiple advantages for simplicity, rapidity, sensitivity, cost-effectiveness, and time-efficiency.

Matrix effect is one of the major problems, which significantly affects the real application of the developed rapid detection platforms. For the evaluation of matrix effects, four varieties of blank samples to the blank matrices of different matrices were prepared and pretreated.

The achieved data (not shown) indicated that the samples of wheat and baby food displayed negligible matrix effects, and the sensitivity of detection also remained at

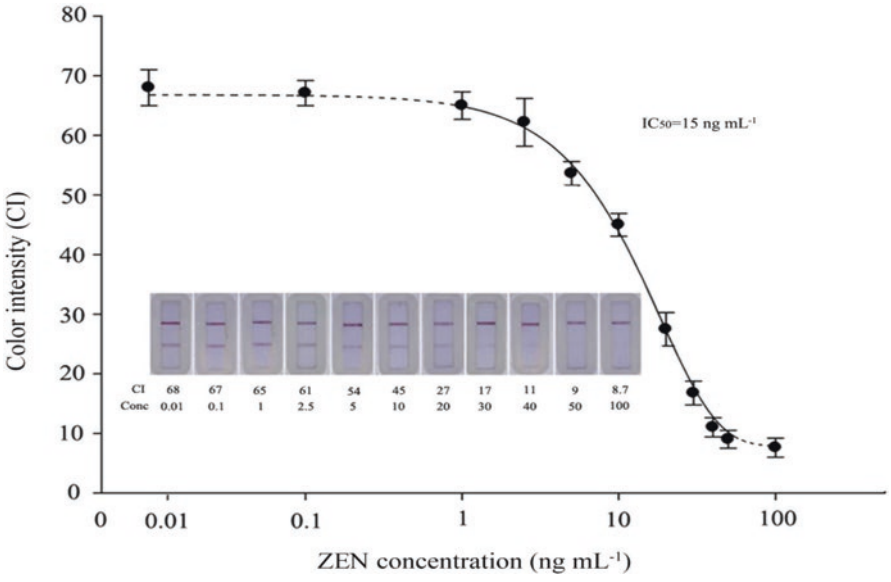

Fig. 1.4 LFA of a series of ZEN concentrations (0.01–100 ng mL^{-1}) in PBS. All points are means±s.d. of four independent measurements for each concentration. (Liu et al. 2012)

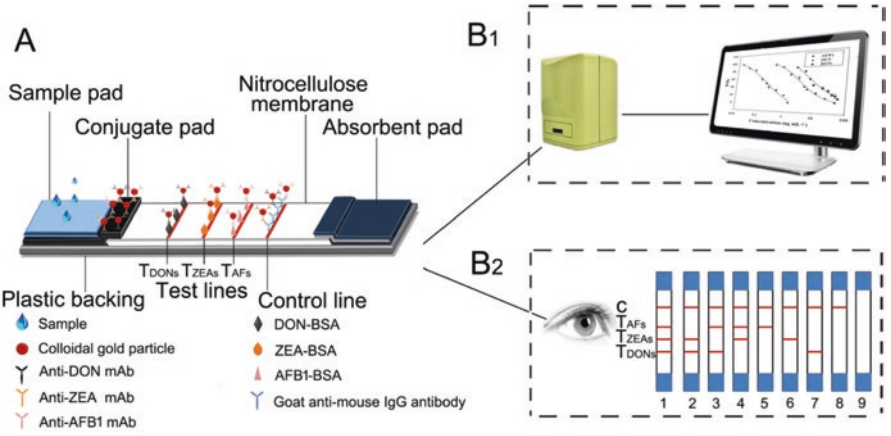

Fig.1.5 A multiplex LFA platform for qualitative and semi-quantitative determination of aflatoxin B1 (AFB1), zearalenone (ZEN), deoxynivalenol (DON), and their analogs (AFs, ZENs, DONs). (**a**) Scheme of the multiplex lateral flow immunoassay (LFA) for multiplex mycotoxins. (B_1) Semi-quantitative analysis platform for LFA. (B_2) Qualitative analysis platform for LFA. Strips 1–9 are the schematic illustrations of detection results. (1) AFs (−), ZENs (−), DONs (−); (2) AFs (+), ZENs (−), DONs (−); (3) AFs (−), ZENs (+), DONs (−); (4) AFs (−), ZENs (−), DONs (+); (5) AFs (−), ZENs (+), DONs (+); (6) AFs (+), ZENs (−), DONs (+); (7) AFs (+), ZENs (+), DONs (−); (8) AFs (+), ZENs (+), DONs (+); (9) invalid result. (Song et al. 2014)

a similar level. On this contrast, the detection of ZEN could be seriously affected by the matrices of feed and maize extracts since the curves constructed in these two matrices were different from the curve constructed in PBS. Despite the existence of the serious matrix effects, the developed test strips based on the chromogenic platform, other than the naked eyes, were still sensitive enough (1 ng mL^{-1}) to recognize the detectable ZEN in most cases, and the concentrations of ZEN could be accurately calculated in those complicated matrices.

1.3.2 Comparative Analyses of LC-MS/MS and Chromogenic LFA in Real Samples

To finally assess the accuracy of the developed chromogenic membrane-based LFA, the proper tests for its detection performance were thoroughly verified by in-parallel LC-MS/MS analyses in real samples which could contaminate the target mycotoxins.

In total, 32 food and feed samples were selected for via LC-MS/MS evaluation, where 7 positive ones were found, with the contamination levels ranging from 14.3 to 131.9 μg kg^{-1}, while the values of chromogenic LFA were 20.4–94.0 μg kg^{-1}. According to case-by-case comparison, the data and detection results are almost identical and consistent. This is the most compelling evidence to demonstrate that the developed chromogenic platform-based LFA is fast, reliable, and sensitive, supporting its application for both qualitative measurement and quantitative analyses.

1.3.3 Storage Stability

Normally, the developed LFA strips have above 12–18-month shelf-life and even could be used as far as it is calibrated before use. Moreover, the measured CI values indicate that the LFA strips remain almost 85% efficiency, supporting its effectiveness.

1.4 Conclusion

LFA strips have been proved to be the most ubiquitously applicable formats of rapid detection in practical uses, in China and other developing countries or even in developed countries for large-scale screening analyses in industrial plants. Previously available LFA strips have been used to qualitative assays against single molecule on result observation as positive or negative via naked eyes, with the commonly acceptable cutoff values. The existed insufficiencies on sensitivity and incapability for quantitation seriously limited the wide applicability of LFA, which could not fulfill the currently strictest regulation requirements in mycotoxin monitoring. This combinatorial means of LFA and chromogenic device present enables to perform quantitative analysis against three target molecules in a wide range of biomatrices, simultaneously carrying the desirable accuracy, which was thoroughly confirmed via in-parallel LC-MS/MS analyses. More importantly, this methodological platform that will benefit not only to rapid analysis of small molecules or proteins but also provide new insights to utilize the developed LFA for remote quantitative determination counting on the output images, which would be proved generally applicable to a wide range of biological systems, related to food safety management, environment monitoring, and diagnostically clinical services.

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Chapter 2
Sample Preparation and Chromatographic Analysis

Lan Wang, Zheng Yan, and Aibo Wu

Abstract Globally, mycotoxins in food and feedstuff have been considered public food safety concerns because they could seriously affect animal production and human health. Mycotoxins could also cause detrimental damages to various organs such as the spleen, liver, heart, and immune system, eventually resulting in a substantial reduction of the animals and accordingly economic losses. Furthermore, multiple mycotoxins have been found in a variety of different foods, which might lead to acute and chronic effects on humans. Thus, the authentic methods for accurate determination of multicomponent mycotoxins have gained great attention from researchers to policy-makers. We summarized the combined analytical procedure of sample preparation and mycotoxin determination in different mycotoxin-producing matrices such as rice, maize, cereals, feedstuff, milk, fruit, and vegetable, as well as biological samples. As expected, it will be valuable references to the routine analytical protocols for confirmational lab analysis.

Keywords Sample preparation · LC-MS/MS · HPLC · GC · Mycotoxin

2.1 Introduction

Mycotoxins are a group of secondary fungal metabolites and thus can contaminate a wide range of food and other commodity crops at each step in the supply chain (Bennett and Klich 2009). These natural contaminants may lead to acute and chronic effects, both in humans and animals, including immunotoxicity, hepatotoxic, nephrotoxicity, carcinogenic, and genotoxicity (Nicholson et al. 2004). The most frequently found mycotoxins in food and feed are generally produced by the genera *Aspergillus*, *Penicillium*, and *Fusarium*, including aflatoxins, deoxynivalenol, fumonisins, zearalenone, and ochratoxin A (Irena Kralj and Helena 2009; Andrade

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et al. 2017). These chemical compounds are extremely stable in nature, and thus, they can also be co-occurring in fully processed foods. Therefore, they represent a major concern for animal and human health. Many studies have shown that multi-mycotoxins had been found in different matrices such as cereals, maize, rice, fruit, and vegetable and biological samples (Al-Taher et al. 2017; da Luz et al. 2017; de Oliveira et al. 2017; Juan et al. 2017a; Fan et al. 2019). In order to determine mycotoxin residues in various food matrices to protect consumers' health, different analytical methods have been proposed with high sensitivity and accuracy. Recently, there is a growing interest in analytical methods for simplified sample preparation and techniques for the determination of single and multiple mycotoxins in foods.

2.2 Sample Preparation

In general, the most common methods applied to quantification on the levels of mycotoxins were based on first-step extraction with organic solvents of acetonitrile and methanol, followed by cleanup and dilution step (Almeida et al. 2012; Chia-Ding et al. 2013; Frenich et al. 2009). However, none of these methods could avoid matrix effects altogether. Matrix effect is a complex from samples and analyte-specific phenomenon observed in the analytical method for mycotoxins that can be compensated by matrix-matched calibration, isotope-labeled internal calibration, and sample dilution (Slobodchikova and Vuckovic 2018). The isotope-labeled internal standards could compensate for any changes in ionization caused by the presence of matrix interferences. Therefore, the best means is to cope with matrix effects and thus ensure reliable results. However, the generic application of isotopically labeled internal standards was terribly restricted by the high costs and limited availability.

In the field of mycotoxin analysis, regardless any chosen technique, cleanup after extraction is usually required, which is very crucial for reliable results finally obtained. This procedure required high precision and accuracy in order to isolate mycotoxins of interest from the matrix interferents. For matrix removal and analyte enrichment, the main sample preparation techniques that have been used to determine mycotoxin contaminations in various food matrices are as follows: solid phase extraction (SPE), immunoaffinity columns (IAC), liquid-liquid extraction (LLE), and quick, easy, cheap, effective, rugged and safe (QuEChERS) (García-Moraleja et al. 2015a, b; Liao et al. 2015; Natalia et al. 2013).

2.2.1 Solid Phase Extraction

Effective extraction of multiple mycotoxins from real complicated samples is the promise to perform either qualitative or quantitative analyses. Solid phase extraction (SPE) is by far sample preparation method used in routine analysis of

mycotoxins. This method can enrich the target analytes from different matrices. However, the drawback of SPE is time-consuming, complicated, and unsuitable for multiclass analysis (Xu et al. 2015; Arroyo-Manzanares et al. 2015). To overcome the limitations of the solid phase extraction, tremendous efforts have been made to improve this method. Recently, magnetic solid phase extraction (MSPE) has been developed to effectively separate mycotoxins (Hashemi et al. 2014; Cassandra et al. 2014). More recently, Zhao et al. have reported an analytical method based on magnetic solid phase extraction (MSPE) (Zhao et al. 2017). In their study, Fe_3O_4@$nSiO_2$@$mSiO_2$, a magnetic nanoparticle, was prepared and dispersed into the sample solution to extract mycotoxin, and the absorbent together with the mycotoxin can be conveniently separated from the solution when applying an external magnetic field. Under the optimized MSPE conditions, the limits of detection were obtained for mycotoxins at 0.210 μg/kg for FB1, 0.0800 μg/kg for OTA, and 1.03 μg/kg for ZEA, respectively. The proposed MSPE procedure serves not only for sample cleanup but also for mycotoxin enrichment that greatly enhances the assay sensitivity.

Efforts have been made to improve this method by Du et al. (2018). In their research, microwave-assisted dispersive micro-solid phase extraction has been applied for the extraction to detect multiple mycotoxins in different matrices. The optimum sample extraction conditions include 5 mL of sample solution dispersant with 2.5 μg/mL of nano zirconia. When shaking vigorously for 2 min, the obtained analytes were desorbed by 100 μL of chloroform. The optimized method for determination of multi-mycotoxins by MA-d-μ-SPE coupled with UHPLC-Q-TOF/MS showed the lower limits of detection (0.0036–0.033 μg/kg for solid samples).

2.2.2 Quick, Easy, Cheap, Effective, Rugged, and Safe Method (QuEChERS)

In order to minimize the sample pretreatment but prevent more exposure to matrix effects, a quick, easy, cheap, effective, rugged, and safe (QuEChERS) method is a suitable alternative. It is an effective simple method applied for extracting mycotoxin from a different food, both in solid samples and in liquid samples (Miró-Abella et al. 2017; Arroyo-Manzanares et al. 2015; Zhou et al. 2018). Its extraction method was based on partitioning via salting-out between aqueous and organic layer, and its cleanup was based on dispersive SPE (dSPE) by combining $MgSO_4$ and different sorbents (i.e., C18, primary, secondary amine, etc.).

Miró-Abella et al. have developed for the simultaneous determination of 11 mycotoxins in plant-based beverage matrices, using a QuEChERS extraction (Miró-Abella et al. 2017). In their method, 10 mL of sample was added to a 50 mL centrifuge tube mixed with 10 mL ACN with 1% HCOOH and shaken for 3 min. It was then 4 g of $MgSO_4$ and 1 g of NaCl were added into the solution and shaken vigorously for 3 min. Method quantification limits were between 0.05 μg/L (for aflatoxin G_1 and aflatoxin B_1) and 15 μg/L (for deoxynivalenol and fumonisin B_2).

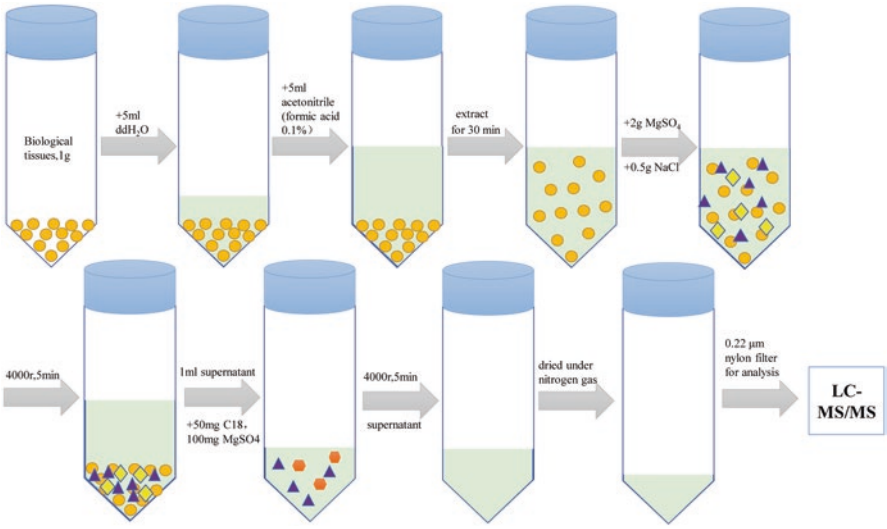

Fig. 2.1 Schematic diagrams of quick, easy, cheap, effective, rugged, and safe (QuEChERS) method for extracting mycotoxins

In 2018, Zheng Yan et al. developed and validated a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of ZEN family mycotoxins including ZEN and most relevant key metabolites in different tissues (Wang et al. 2018). In this study, the QuEChERS method was used to extract the analytes, and the procedure was shown at Fig. 2.1.

2.3 Major Techniques for the Determination of Mycotoxins

Accurate quantitation of mycotoxins at trace levels in real samples is comprehensively important for subsequent studies on confirmation legal judgments and exposure assessment. Therefore, it is very extremely important to improve and guarantee the performance of the applied analytical methodologies. To date, numerous methods and quantitative techniques for mycotoxins have been well established to investigate the occurrence of multiple mycotoxins in different matrices, to evaluate their toxicokinetics and toxicological dynamics in cell models and experimental animals. Previously, enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), gas chromatography (GC), and thin-layer chromatography (TLC) are commonly used for quantitation of mycotoxins (Gutzwiller et al. 2014; Tamura et al. 2015; Rodríguez-Carrasco et al. 2014). These analytical methods are sensitive and selective but unsuitable for on-site monitoring because of bulky instrumentation in lab. In addition, these methods are relatively expensive and require well-trained, skilled persons for the operation and laborious sample preparation and cleanup procedures. At the same time, due to the case that several groups

of mycotoxins are frequently co-occurring even in the same sample, effective and reliable analytical methods are strictly required to detect multicomponent mycotoxins. Recently, with the fast developments in mass spectrometry (MS) technology, it enables to have the higher selectivity and sensitivity and provided the capacity to distinguish multiple contaminates from co-extractives in one individual sample. As an tendency, instrumental analysis on quantification of different mycotoxins requires the minimal sample loading volume via the utmost simplified green procedure, without much chemical solutions involved. Universally, the triple-quadrupole tandem MS (MS/MS) is considered the gold standard for quantitative analysis of multiple compounds (Koesukwiwat et al. 2014). Therefore, LC-MS/MS has been widely developed and employed for the analysis of single mycotoxin or group of mycotoxins. Previous studies demonstrated that LC-MS/MS is capable of simultaneously detecting multiple mycotoxins in different feedstuffs and foods, such as cereals (wheat, maize and rice), wheat-derived products, animal-borne feed, milk, and plant-based beverage (Oliveira et al. 2017; Suzane et al. 2017; Zhao et al. 2015; Jiang et al. 2018).

2.3.1 Cereals and Grains

Cereals are main staple foods in diets worldwide, which are highly valued as a source of nutrients, vitamins, minerals, and complex carbohydrates. One of the major challenges of the quality and safety of cereals is the natural contamination by mycotoxin-producing fungi post invasion into the host plants. This contamination can feasibly occur in the crops and worsen during harvesting, transportation, drying, processing, and/or further developing during storage of cereals and its derived products (Irena Kralj and Helena 2009). *Fusarium* head blight (FHB) mainly caused by *Fusarium graminearum* spp. was the common fungal disease in wheat and cereals (Prandini et al. 2009). The most common mycotoxins causing FHB were considered to be fumonisins, zearalenone, and deoxynivalenol (Krska et al. 2003; Ramirez et al. 2004). In addition to the parental mycotoxin forms, some food could also contaminate derivative/transformed compounds during food processing or as results of plant/animal metabolisms (Rychlik et al. 2014). Thus from now, it should be possible to suggest that accurate and reliable methods for the determination of mycotoxins and their modified forms in cereals are totally required in order to ensure the quality of these products and at most preserve consumer health.

Different analytical techniques have been proposed to monitor the large range of mycotoxins in cereals. Table 2.1 has summarized recent studies of method for determination of multi-mycotoxins in cereals and maize by LC-MS/MS. A multicomponent method to determine aflatoxins, deoxynivalenol, fumonisins, zearalenone, ochratoxin A, and some metabolites in cereals was developed by Andrade et al. (2017). In this study, acidified ACN used as the extraction solvent followed by LC-MS/MS analysis proved to be suitable, rapid, and cost-effective for the multi-mycotoxin determination in wheat, maize, and rice products. This method

Table 2.1 Representative studies on analytical methods and limit of quantification (LOQ) in cereals and grains

Matrix	Limit of quantification (μg/kg)													Sample preparation	Detection method	References
	AFB_1	AFB_2	AFG_1	AFG_2	DON	15-AcDON	3-AcDON	D3G	FB_1	FB_2	FB3	OTA	ZEN			
Maize	1.2	0.7	0.7	0.5	39.0	121.0	77.0	84.0	19.0	8.0	32.0	4	24	SLE-LTP	LC-MS/MS	Andrade et al. (2017)
Grain	0.3	4.0	1.0	3.8	18.4				14.7	16.0		1.1	0.9	IAC	LC-MS/MS	Rodríguez-Carrasco et al. (2016)
Maize									12.5	12.5	12.5			SPE	LC-MS/MS	Wang and Li (2015)
Wheat					125	125	125		63	63	63		125	SPE	LC-MS/MS	Blesa et al. (2014)
Sorghum	5	5	10	10	25	62.5	31		25	50	50	2.5	6	SPE	LC-MS/MS	Ediage et al. (2015)
Wheat					3.9		11.1	6.6					5.7		LC-MS/MS	Berthiller et al. (2005)
Rice	0.5	1.2	1.0	1.6	40	72	48	84	21	12	24	3	16	SLE-LTP	LC-MS/MS	Andrade et al. (2017)
Cereal					75.2				2.89	2.36		0.65	38.6	QuEChERS	LC-MS/MS	Arroyo-Manzanares et al. (2015)

Corn					7.8	7.3	5.3	8.4						SPE	LC-MS/MS	Zhiyong et al. (2014)
Rice	1.7	1.7	1.7	3.3	16.7				3.3	1.7		1.7	1.7	QuEChERS	LC-MS/MS	Koesukwiwat et al. (2014)
Feedstuff	2	4	4	4	250	100	100		375	125		25	50	SPE	UPLC-MS/MS	Romera et al. (2018)
Poultry feed					5.0	5.3	6.4	7.4						SPE	LC-MS/MS	Zhao et al. (2014)
Swine feed	0.2	0.2	0.2	0.2	25	20	25		20	20		0.2	10	MSPE	LC-MS/MS	Zhao et al. (2015)
Swine feed					5.9	10.0	8.8	5.5						SPE	LC-MS/MS	Zhao et al. (2014)

was satisfactorily validated for the simultaneous quantitation of multi-mycotoxins, with LOQs ranging from 0.5 to 121 μg/kg listed in Table 2.1. Natalia Arroyo-Manzanares et al. proposed an UHPLC-MS/MS method combined with a sample treatment based on QuEChERS (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate and 0.5 g disodium hydrogen citrate sesquihydrate) for the determination of multi-mycotoxins in cereals (Arroyo-Manzanares et al. 2015). The analytical method for detecting fusarenon-X, T-2 and HT-2 toxin, and citrinin was also developed in this study with low LODs and LOQs. A UPLC-ESI-MS/MS method for the simultaneous determination of multi-mycotoxins including zearalenone, aflatoxins, and deoxynivalenol in feedstuffs was developed by David Romera et al. (2018). In their study, acetonitrile-water-formic acid (80:19:1, v/v/v) was carried out to extract target and nontarget mycotoxins in feedstuff and then detected by combining QTOF-MS and MS/MS detectors with UPLC. In 2019, E.J. Llorent-Martínez et al. developed an alternative flow-through luminescence optosensor for the quantification of ZEA (Llorent-Martínez et al. 2019). With this purpose, a QuEChERS procedure was carried out for the extraction of ZEA from different cereal samples and feedstuff materials. Solid-phase spectroscopy (SPS) in a multi-commutated system was implemented by making use of three-way solenoid values, which provides an enhancement in the sensitivity and selectivity of the method. An ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI+/−-MS/MS) method for the simultaneous analysis of citrinin (CIT) and ochratoxin A (OTA) in feed was first developed by Celine Meerpoel et al. (2018). In their study, the involved mycotoxins were extracted from these matrices using a QuEChERS-based extraction method without any further cleanup step. Furthermore, they detected citrinin (CIT) and ochratoxin A (OTA) in the selected matrices including pig feed and wheat flour with low LOQs. A study proposed a liquid chromatography-tandem mass spectrometry method using the selected reaction monitoring mode for determining fumonisins B1 and B2 (Oliveira et al. 2017). They modified the extraction procedure based on a matrix solid-phase dispersion approach, using 1 g of silica gel for dispersion and elution with 70% of ammonium formate aqueous buffer (50 mmol/L, pH 9), demonstrating a simple, cheap, and chemically friendly sample preparation procedure.

2.3.2 Milk and Beverage

Milk is an important source that provides nutrition for the growth, development, and maintenance of human health, particularly to younger children and infants. For an instance, breast milk is considered to be the ideal food for infants since it provides the best original health nutrition for their development and growth. However, milk can also be a carrier of mycotoxins and their metabolites, causing various physiological problems in individuals. Additionally, mycotoxins present in milk could transform into the blood and tissues of breeding mammals and thus brought to the forefront for milk safety (Degen et al. 2017). The most studied mycotoxins in milk

are aflatoxins and ochratoxin A and to a lesser extent, trichothecenes and zearalenone (Flores-Flores and González-Peñas 2015; Huang et al. 2014). Previous studies have shown considerable amount of AFM1 in milk and dairy products. As confirmed, aflatoxin M1 is produced by lactating animals and mothers when the cows consumed the feeds or foods contaminated with aflatoxin B1 (AFB1) through cytochrome P450-associated enzymes in the liver. Then, AFM1 was secreted through urine and milk (Dashti et al. 2009). In the last decades, several validated analytical methods have been developed for multi-mycotoxin determination in milk, which allow studying the presence of mycotoxins in milk. Table 2.2 has listed recent studies of method for determination of multi-mycotoxins in milk and milk product by LC-MS/MS. A reliable and universal QuEChERS-based UPLC-MS/MS method was newly developed for the determination of multi-mycotoxins in milk (Zhou et al. 2018). As for the choice of the mobile elution phase, MeOH and ACN were considered as candidates. The results showed that compared with MeOH, ACN provided sharper peak profiles and facilitated the elution of analytes.

Since milk samples always present complex matrix with multiple components, complex interaction should exist among the detected mycotoxins and matrix, as well as other functional nutrients. Herein, several methods have been proposed to alleviate matrix effects. For example, an m-μdSPE method using Fe_3O_4@pDA NPs has been applied for the extraction of six mycotoxins before their LC-MS analysis from different milk and yogurt samples in the study of Javier González-Sálamo et al. (2017). Moreover, a highly sensitive and specific multiclass method by combining multi-mycotoxin IAC enrichment procedure and UHPLC-Q-Orbitrap was developed and validated to be capable of quantifying 14 mycotoxins in raw milk (Mao et al. 2017). Interestingly, multi-mycotoxin IAC cleanup utilized there could produce the extract-alleviating matrix effects, while the addition of acetonitrile in milk was found to be beneficial for the extraction. As proved, the developed method has been successfully applied to routine analyses for quantitation of mycotoxins in raw milk.

2.3.3 Fruits and Vegetables

Fruits and vegetables are highly susceptible to be infested by pathogenic fungi due to their high water content and abundance of nutrients. Among all fruits, berry fruit (i.e., small berries such as strawberries, blueberries, and red currants) is especially susceptible to be contaminated by fungi considering that their soft and fragile skin is susceptible to small lesions, which allow the growth of spoilage fungi. They may decompose during any processing stage of growth, pre- and post harvests, as well as storage and transportation. Therefore, several classes of mycotoxins can simultaneously contaminate fruits and vegetable (Sanzani et al. 2016). Mycotoxins associated with fruits and vegetables mainly include *Alternaria* toxins, ochratoxin A (OTA), patulin (PAT), and trichothecenes. Although consumers are easily tend to cut out the visible rotten areas of the infected fruits and vegetables before consumption, several

Table 2.2 Representative studies on analytical methods and limit of quantification (LOQ) in milk product

Matrix	Limit of quantification (μg/kg)										Sample preparation	Detection method	References
	AFM_1	AFM_2	AFG_1	AFG_2	AFB_1	AFB_2	ZEN	ZAN	OTA	OTB			
Cow milk							2.15	1.57			m-μdSPE	LC-MS	González-Sálamo et al. (2017)
Liquid milk	1				0.1		5		2		SPE	LC-MS/MS	Wang and Li (2015)
Milk powder	5				0.5		25		10		SPE	LC-MS/MS	Wang and Li (2015)
Milk	0.01	0.02	0.01	0.02	0.01	0.01	0.2	0.1	0.01	0.01	QuEChERS	UPLC-MS/MS	Zhou et al. (2018)
Milk	0.001	0.001	0.003	0.003	0.003	0.003	0.025	0.01	0.001	0.001	IAC	LC/Q-Orbitrap	Mao et al. (2017)
Milk	0.02				0.03		0.04	0.15	0.02		SPE	LC-MS/MS	Jiang et al. (2018)
Milk		0.1	0.02	0.02	0.02	0.02	1		0.1	0.05	MSPE	LC-MS/MS	Zhao et al. (2015)

mycotoxins, especially those mentioned above, could still be transferred into the remaining parts indicating that mycotoxins could be present in fresh fruit and healthy processed products with low concentrations (Monbaliu et al. 2010). The potential adverse effects on human health could be caused by the presence of mycotoxins, considering the concurrent intake of different contaminated food and drinks by the population. Thus, sensitive, robust, and accurate analytical methods for specific analysis of multiple mycotoxins in fruits and vegetables are indeed necessary. Recently, high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) has been increasingly accepted for mycotoxin analyses owing to its high selection, sensitivity, and anti-interference ability. Table 2.3 has summarized several studies of method for determination of multi-mycotoxins in fruits and vegetables by LC-MS/MS. A rapid and accurate analytical method for the determination of three *Alternaria* mycotoxins in fruit and juice was developed and validated by Charalampos K. Myresiotis et al.(Myresiotis et al. 2015). The QuEChERS extraction technique coupled with HPLC-DAD was used for the simultaneous determination of three *Alternaria* mycotoxins. Hao Dong et al. have developed a rapid and robust UHPLC-MS/MS method for the determination of AOH, AME, TEA, TEN, DON, PAT, and OTA in fresh fruit and vegetable (Dong et al. 2019). QuEChERS technique was finally applied for effective purification, and extraction was performed with acetonitrile under acidic conditions. In this method, a C18 column by gradient elution was applied to separate mycotoxins. Mass spectrometry analysis was scanned by ESI+ and ESI− dynamic switching using multiple reaction monitoring (MRM). In 2016, Meng Wang et al. have developed a highly sensitive, rapid, and reliable method for the simultaneous determination of eight mycotoxins in fruits by ultra-high-performance liquid chromatography-tandem mass spectrometry based on homemade “MCX + NH2” SPE cleanup (Wang et al. 2016). Before SPE cleanup, acidified aqueous acetonitrile and an additional salt-out step using NaCl were employed. In their study, ESI positive mode was selected to analyze four mycotoxins including TEA, ALT, OTA, and CIT, and ESI negative mode was selected to analyze the other four mycotoxins including AOH, AME, TEN, and PAT. In addition, the CORTECS C18 column was carried out in this method. In 2018, Sara De Berardisa et al. have developed a liquid chromatography-tandem mass spectrometry method for the determination of four *Alternaria* toxins, i.e., AOH, AME, TEN, and TEA in tomato-based and fruit-based products (De et al. 2018). The use of an organic solvent (acetonitrile) as an extraction solvent was particularly appropriate for TeA extraction in this method. QuEChERS extraction method was performed and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was carried out in the study of Juan et al. (2017b). The simultaneous quantification of OTA and *Alternaria* toxins in berry by-products, jam and juice, was successfully proposed and validated, with good sensitivity to detect these mycotoxins.

Table 2.3 Representative studies on analytical methods and limit of quantification (LOQ) in fruit and vegetable

Matrix	Limit of quantification (μg/kg)									Sample preparation	Detection method	References
	AOH	AME	TEA	TEN	DON	PAT	OTA	ALT	CIT			
Tomato	3.5	3.5		1.75						LLE	LC-MS/MS	Rodríguez-Carrasco et al. (2016)
Sweet cherries	1.0	1.0	1.0	1.0		5.0	1.0	1.0	1.0	SPE	UPLC-MS/MS	Wang et al. (2016)
Apples	1.0	1.0	1.0	1.0		5.0	1.0	1.0	1.0	SPE	UPLC-MS/MS	Wang et al. (2016)
Tomato	1.0	1.0	1.0	1.0		5.0	1.0	2.0	1.0	SPE	UPLC-MS/MS	Wang et al. (2016)
Orange	1.0	1.0	1.0	1.0		5.0	1.0	1.0	2.0	SPE	UPLC-MS/MS	Wang et al. (2016)
Tomato	16	20	4	160						QuEChERS	LC-MS/MS	De et al. (2018)
Jam berries	7	15		5			3.5			QuEChERS	LC-MS/MS	Juan et al. (2017b)
Juice berries	5	9		2			5			QuEChERS	LC-MS/MS	Juan et al. (2017b)
Vegetable	1.0	0.2	1.0	0.4	1.5	10.0	5.0			QuEChERS	LC-MS/MS	Dong et al. (2019)

2.3.4 Biological Samples

In general, external exposure assessment with the scope of risk assessment is used to food contamination and consumption data to estimate human exposure to different mycotoxins for different human populations such as infants and children. However, food and feedstuffs cannot accurately estimate the real disease risk to the human beings. Therefore, determination of mycotoxins and its derivatives in biological samples such as plasma and urine can monitor the mycotoxin levels directly and promote effective exposure assessment, which is crucial for the establishment of regulatory limits. Several findings of direct biomonitoring showed that a higher prevalence of some mycotoxins was observed (Gerding et al. 2015; Huybrechts et al. 2015). These studies clearly emphasized the importance of direct biomonitoring of mycotoxins in biological samples.

For example, a liquid chromatography-tandem mass spectrometry method for multi-mycotoxins in fish plasma was developed (Tolosa et al. 2016). However, LOQs of this method ranged from 1 to 17 ng/ml and are not suitable for the determination of mycotoxins in human plasma. Irina et al. developed a sensitive liquid chromatography-mass spectrometry method for 17 mycotoxins in human plasma (Slobodchikova and Vuckovic 2018). In their study, they studied the effect of a different method of sample preparation (solvent precipitation, SPE, and LLE) and LC separation on simultaneous analysis of mycotoxins. Finally, in order to eliminate the need for immunoaffinity extraction and minimize matrix effects, they developed the method based on three-step liquid-liquid extraction with ethyl acetate. In this method, 15 of these mycotoxins met the accuracy and precision of 80–120% and ≤20% RSD at different concentrations tested. The obtained LOQs of all mycotoxins were in the range of 0.1–0.5 ng/ml except NIV (3 ng/ml).

2.4 Conclusion

Till now, some effective and reliable analytical methods have been well established to determine mycotoxins contaminated in food and feedstuff at legislated levels. For more emphasis, proficiency testing (PT) is an effective procedure for quality assurance and performance verification in different laboratories from different countries at the global level, ensuring that laboratory validation and inter- or intra-laboratory procedures are working satisfactorily (Girolamo et al. 2016). To be specific, the maize samples contaminated with multiple mycotoxins, including DON, FB1, FB2, ZEA, T-2, HT-2, OTA, AFB1, AFG1, AFB2, and AFG2, and the wheat samples contaminated with DON, ZEA, T-2, HT-2, and OTA were selected as real materials. As resulted, the majority of laboratory participants had the ability to provide acceptable results for the simultaneous analysis of DON, fFB1 and B2, OTA, ZEN, T-2 and HT-2, and AFB1 and G1 in maize and DON, OTA, ZEN, and T-2 and HT-2 in wheat, respectively. In addition, acidified ACN-water as extraction dilution and

injection without cleanup extract were of the best performance. However, only a few laboratories were capable to analyze AFB2 and AFG2 and FB1 together with other mycotoxins in maize and T-2 in wheat. More importantly, some modified or derived forms of different mycotoxins were reported and proved to have identical toxicities to the parental molecule, such as D3G and 3- and 15-ADON, respectively. To be specific, via the currently used LC-MS/MS method, it is too tough for absolute separation and identification of 3- and 15-ADON. Also, it is very difficult to obtain the standards of the masked forms of the key mycotoxins (D3G, Z14G, and Z14 S), which have aroused as great challenges regarding the typed chromatographic analyses.

In one word, further improvements on sample preparations and optimization of equipment parameters of LC-MS/MS methodologies to gain higher sensitivity, fewer matrix effects, and more targets involved are still required for ongoing multi-mycotoxin analysis.

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Part II
Risk Assessment

Chapter 3
Toxicity Evaluation Using Animal and Cell Models

Yunxia Yang, Wenda Wu, and Aibo Wu

Abstract Toxicology is a science that studies various physical and chemical and biological harmful factors, which especially on human beings. The main task is to evaluate the possible health hazards of exogenous chemicals to the contactors and ultimately to provide the basis for controlling the hazards of chemical substances and strengthening the management of chemical substances. Toxicology plays an important role in safeguarding human health, maintaining ecological balance, and improving the environment through hazard assessment of exogenous chemicals. In this chapter, we discussed the current and future plans for the toxicity evaluation using classic and new strategies in vivo and in vitro model for toxicological evaluation of specific mycotoxin contaminants arousing food safety issues.

Keywords Toxicology · Toxicity assessment · Methods · In vivo models · In vitro models

3.1 Models and Methods for In Vivo Toxicity

3.1.1 Introduction

In this part, we describe the methods of toxicity studies. To investigate the mechanism and degree of toxic substances which can induce different toxicities for humans, animals, or the environment, a variety of studies are conducted in different species of laboratory animals. Most testing commonly uses rats and mice, but in some cases, the studies are carried out on rabbits, guinea pigs, dogs or primates, and

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so on. The toxicity testing we introduce is of the following types: acute toxicity studies, repeat-dose toxicity studies, carcinogenicity studies, reproductive and developmental toxicity studies, neurotoxicity studies, and genotoxicity studies.

3.1.2 Acute Toxicity Studies

Acute toxicity is defined by Globally Harmonized System (GHS) as those adverse effects occurring following oral or dermal administration of a single dose of a substance, or multiple doses given within 24 h, or an inhalation exposure of 4 h. It is a short-term assessment that evaluates of these test substances (Monosson 2013). Tests are adopted to detect the hazardous effects (corrosion, skin irritancy, eye irritancy, and sensitization; topical or local toxicity) induced by a substance which gets in contact with the skin and eyes through oral, dermal, and inhalation exposures (Table 3.1).

In the past few decades, acute systemic toxicity was indicated by data from lethal-dose tests, in which the oral dose causing the death of 50 percent of the treated animals (the LD_{50} value) was determined. At least 30 animals are employed in such tests, which adopt the death of the experimental animals as an endpoint. Therefore, the LD_{50} tests for acute oral toxicity were deleted from the Organization for Economic Cooperation and Development (OECD) manual of internationally accepted test guidelines. Instead, other alternative methods have been improved, which use fewer animals and in some cases replace death as the endpoint with signs of significant toxicity.

Table 3.1 Basic parameters of acute toxicity studies (OECD 2002a, b, 2008a, 2009, 2017)

Species—oral and inhalation tests: rats
Skin and eye irritancy tests: rabbits
Skin sensitization tests: guinea pigs, mice
Age: young adults
Number of animals: at least 10 animals (5 female and 5 male) for each test group
Dosage: three dose levels generally
Exposure time: oral and dermal tests: single doses or fractionated doses up to 24 h
Inhalation tests: 4 h
Observation period: 14 days

3.1.3 Repeat-Dose Toxicity Studies

These tests are conducted to predict the occurrence and level of the tumorogenesis in humans at much lower levels by using nonhuman species exposed to high dose or exposure level (Gad 2007; Early et al. 2013) no-observed-adverse-effect level (NOAEL) and point of departure for establishment of a Benchmark Dose (BMD), and to determine the doses induce harmful effects (Table 3.2).

3.1.4 Carcinogenicity Studies

These tests are conducted to predict the occurrence and level of the genesis of tumor in humans at much lower levels using nonhuman species exposed to high dose or exposure level (Gad 2007). It is similar to chronic toxicity tests. Larger groups of experimental animals are needed in longer period. However, the assessment of carcinogenicity is always combined with repeat-dose studies so as to reduce the use of experimental animals (Table 3.3).

Table 3.2 Basic parameters of subacute and subchronic toxicity studies

Subacute toxicity studies (OECD 1981a, 2018a, b)
Species: rodents (rats preferably) are used in oral and inhalation tests; dermal studies are intended for use with the adult rat, rabbit, or guinea pig
Age: young adults
Number of animals: at least 10 animals (5 female and 5 male) for each test group
Dosage: at least three tests groups should be used
Observation period: 28 days
Subchronic toxicity studies (OECD 1981b, 2018b, c)
Species: oral and inhalation tests, rodents (usually rats); non-rodents (usually dogs) are recommended as a second choice
Dermal studies tests: rats, rabbits, and guinea pigs
Age: young adults
Number of animals: at least 20 animals (10 female and 10 male) for each test group
For non-rodents, four of each sex should be used per dose level
Dosage: three dose levels and a control group generally; a toxic dose level and NOAEL
Group included
Exposures period: 90 days
Observation period: 90 days (same as exposure period)

Table 3.3 Basic parameters of carcinogenicity studies (OECD 2018d, e)

Species: rat and mice are preferred due to relatively short life spans
Age: young adults
Number of animals: at least 100 animals (50 female and 50 male) for each test group
Dosage: three dose levels generally; the minimal toxic dose should be set as the highest dose group
Exposure period: At least 10 months and 24 month for mice and rats, respectively
Observation period: 18–24 months and 24–30 months for mice and rats, respectively

Table 3.4 Basic parameters of reproductive and developmental toxicity studies

Reproductive toxicity studies (OECD 2018f)
Species: rat is preferred
Age: young adults
Number of animals: at least 40 animals (20 female and 20 male) for each test group
Dosage: three dose levels generally; the highest dose should be toxic but not lethal for parents; the lowest dose should not be toxic
Assay process: test substance should be given to sexually mature males and females (P1) 2 weeks preceding mating and continuously through mating, gestation, and weaning of the pups (F1); test substance should then be further administered to selected F1 offspring from weaning to adulthood. Clinical observations and pathology examinations are performed on all animals as signs of toxicity. This test can be extended to include an F2 generation, in which the procedures for F1 animals are similar to those for the P1 animals
Developmental toxicity studies (OECD 2018g)
Species: rodent (rat and mice preferably) and non-rodent (rabbit and hamster preferably)
Age: young adult females
Number of animals: this test should contain a sufficient number of females to result in approximately 20 female animals with implantation sites at necropsy
Dosage: at least three concentrations should be used
Assay process: the test compound should be given to pregnant animals at least from implantation to 1 day prior to the day of scheduled kill. Some measurements (weighing) and clinical daily observations are viewed as the results. The females are killed 1 day prior to the expected day of delivery, after which the uterine contents are examined, and the fetuses are evaluated for soft tissue and skeletal changes

3.1.5 Reproductive and Developmental Toxicity Studies

Animal models can be used to study a variety of reproductive phenomena, which are critical for studying the effects of various agents on general reproduction (including developmental toxicology) and subsequent assessments of the potential effects of poison on many species. The reproductive and developmental toxicity studies are designed to offer an evaluation of reproductive and developmental effects which may occur as a result of pre- or postnatal toxic compound exposure as well as an evaluation of systemic toxicity for females in gestation and lactation period and young and adult offspring (Table 3.4).

The animal models used in the evaluation of developmental and teratogenic effects are usually rats and mice; however, in recent years, the zebrafish embryo is becoming an important model in developmental toxicology. In the study of zebrafish, in different developmental stages of zebrafish embryos, the researchers evaluated the presence and morphological development of somites, tail detachment, and otoliths, eyes, heartbeat, and blood circulation. After hatching, larvae were evaluated for skeletal deformities, body position, and their ability to swim (Yuan et al. 2014; Truong and Harper 2011).

3.1.6 Neurotoxicity Studies

The nervous system is the most complex system of structure and function in the body. Due to the heterogeneity of the nervous system, different neurotoxic substances can affect neural and behavioral functions in different and specific ways. Therefore, the neurotoxicity of the neurotoxic substances can be evaluated from different aspects, that is, neuropathology evaluation, which is a classical method to confirm neural injury, peripheral nerve conduction testing motor activity measurement, and neuroethology method (Table 3.5).

Table 3.5 Testing for neurotoxicity studies

Neuropathology evaluation: testing for nerve damage by microscopic examination. The basic processes are as follows: fixation of nerve tissue → material extraction → dehydration → embedding → sectioning → HE staining → histopathological evaluation	
Peripheral nerve conduction: this test is conducted to evaluate the electrical conduction in motor and sensory nerves, in which the test animals (usually rodents) are treated with test substances for 90 days	
Motor activity: testing for decreased motor activity, such as cage movement	
Rat and mice are recommended. Zebrafish model can also be used	
Neuroethology method: evaluation for behavior of offspring rats via battery system (Kihara et al. 2000)	
Preweaning	**Postweaning**
Cliff avoidance	Rotarod
Negative geotaxis	Open field
Swimming development	Conditioned avoidance learning
	Underwater T-maze
	Reproduction

3.1.7 Genotoxicity Studies

Generalized genotoxicity refers to the detrimental effect induced by genetically toxic substances that cause, that is, specific changes in the molecule structure of a biological cell genome or variation in genetic information. It can also be briefly summarized as the ability to damage DNA and alter DNA. Therefore, genotoxicity manifests as mutations caused by DNA damage and increased error rates in the genome replication process and changes in gene expression patterns. According to this, genotoxicity can be divided into four categories, DNA damage, gene mutation, chromosome structure change, and chromosome number change, which can be used as an endpoint for genotoxicity tests. There are three methods for detecting genotoxicity: gene mutation detection, chromosomal aberration detection, and DNA damage detection. A large variety of tests have been developed to measure genotoxicity.

3.1.7.1 Transgenic Mutation Assays (TG Assays)

Transgenic (TG) mouse mutation assays have originated with two λ phage-based TG mice named Muta Mouse (Gossen et al. 1989) and Big Blue Mouse (Kohler et al. 1990). The systems using Muta Mouse and Big Blue Mouse employ bacterial lacZ or lacI gene, respectively, as a reporter gene for mutations. The cII gene, a novel reporter gene, is advantageous over the lacI or lacZ genes, which is more compact enough to routinely identify mutations by DNA sequencing (Jakubczak et al. 1996). Moreover, to efficiently detect deletion mutations, Spi^- selection has been introduced in the TG assays. To accomplish the Spi^- selection, a novel TG mouse named gpt delta has been established (Nohmi et al. 1996). Among the transgenic mouse system, the Spi^- selection is for deletions and 6-thioguanine (6-TG) selection is for point mutations such as base substitutions and frameshifts (Fig. 3.1).

3.1.7.2 In Vivo Comet

Single-cell gel electrophoresis (SCGE), also called comet assay, is a sensitive microgel electrophoresis technique to detect DNA damage at the level of a single cell. In the technique, cells are embedded in agarose placed on a microscope slide. These cells are lysed by detergents and high salt so as to remove membranes and soluble cell constituents, most histones included, leave the DNA supercoiled and attached to a nuclear matrix as a nucleoid, the electrophoresis is conducted later. In the electrophoresis tank, cells with an increased frequency of DNA double-strand breaks displayed increased migration of DNA toward the anode. Nuclei and removed DNA fragments can be observed with fluorescent staining, forming “comets.” Both of the relative content of DNA in the comet tail (% tail DNA), the tail length and the

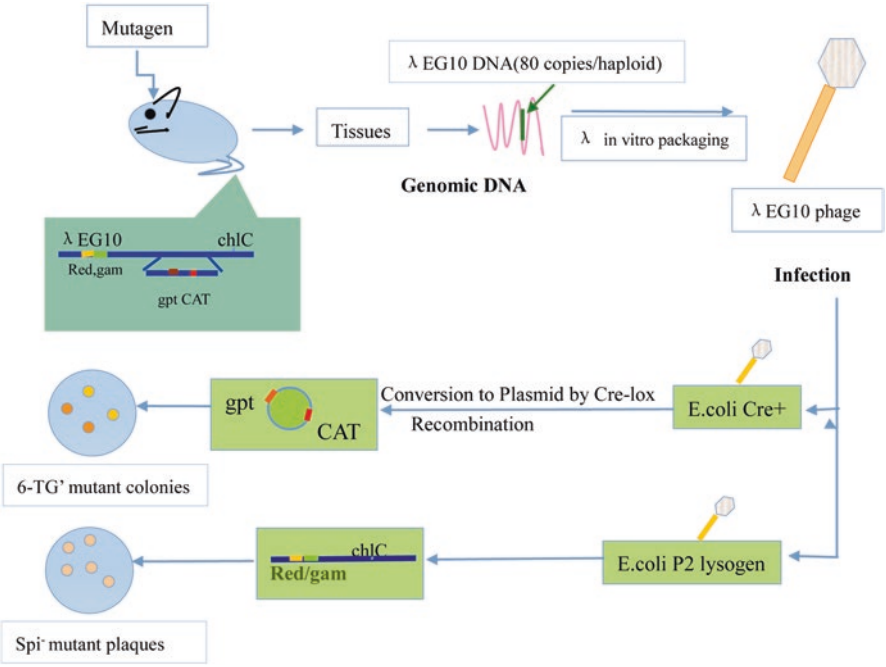

Fig. 3.1 Protocol of gpt delta transgenic mouse mutagenicity assay

tail moment, indicate the frequency of breaks (Fairbairn et al. 1995; Kumaravel and Jha 2006; Dusinska and Collins 2008).

3.1.7.3 Micronucleus Assay in Bone Marrow Polychromatic Erythrocytes

Micronuclei (MN), a biomarker of chromosome breakage and whole chromosome loss, is an acentric fragment of a chromosome or free agglomerates formed in the cytoplasm of intermitotic daughter cells. It results from hysteresis chromosomes and cannot enter the nucleus of daughter cells in the late stage of cell division. The main nucleus of erythroblasts is eliminated when it develops into red blood cells, then becomes polychromatic erythrocytes (PCEs), which are basophilic for 24 h, after that these cells become normal chromatic erythrocytes (NCE), and enter into circulating peripheral blood subsequently. MN can be detected in the cytoplasm in the course of main nucleus excretion, and maintained for a while during the excretion. Therefore, the MN in PCE can be the indicator of chromosome breakage spindle damage.

3.1.7.4 Cytokinesis-Block Micronucleus (CBMN) Assay

The cytokinesis-block micronucleus (CBMN) assay, another micronucleus assay, is a comprehensive system for measuring DNA damage, and the MN can be measured as the endpoints. In the CBMN assay, cytochalasin-B is used as an inhibitor of cytokinesis, and it will not have an impact on nucleus division, which renders the once-divided cells are recognized by their binucleated appearance. The number of binuclear cells and MN-contained cells is counted, and the changes of MN frequencies can be measured as indicator for genotoxicity.

3.2 Models and Methods for In Vitro Toxicity

3.2.1 Introduction

With the development of science and technology and the increasing number of new chemicals, traditional toxicological evaluation methods are facing new challenges. With advocacy implementation of the 3Rs (replacement, reduction, and refinement) (Knight 2008) for experimental animals and the transformation of biomedical model, whole animal experiments are facing severe challenges. The in vitro model study, instead of animal experiments, has become an important direction of toxicological development. In vitro model research has replaced animal experiments as an important direction of toxicology research. Whether from a scientific or economic point of view, in vitro substitution method is of great significance to the evaluation and management of the hazards of exogenous chemicals. It can reduce the effective factors of in vivo experiments, reduce the use of animals, shorten the experimental cycle, and reduce costs and other advantages.

In recent years, in vitro experiments have covered many toxicity endpoints, such as general toxicity, genotoxicity, organ toxicity, etc. Means of research have also extended from general cell and tissue culture to genomics, proteomics, metabolomics, and computer-assisted simulation evaluation system. Various detection tools have been developed, which may replace animals in toxicity testing. The Environmental Protection Agency has undergone a series of in vitro tests to assess large quantities of chemicals, and many in vitro tests have been widely recognized (Walum et al. 1994). At present, some alternative toxicological methods have been verified by relevant authoritative bodies and have been popularized and applied by the European Union, the United States, and the Organisation for Economic Co-operation and Development (OECD).

With the development of science and technology, there is an urgent need to improve existing in vitro methods or new alternatives to non-animal models. All in

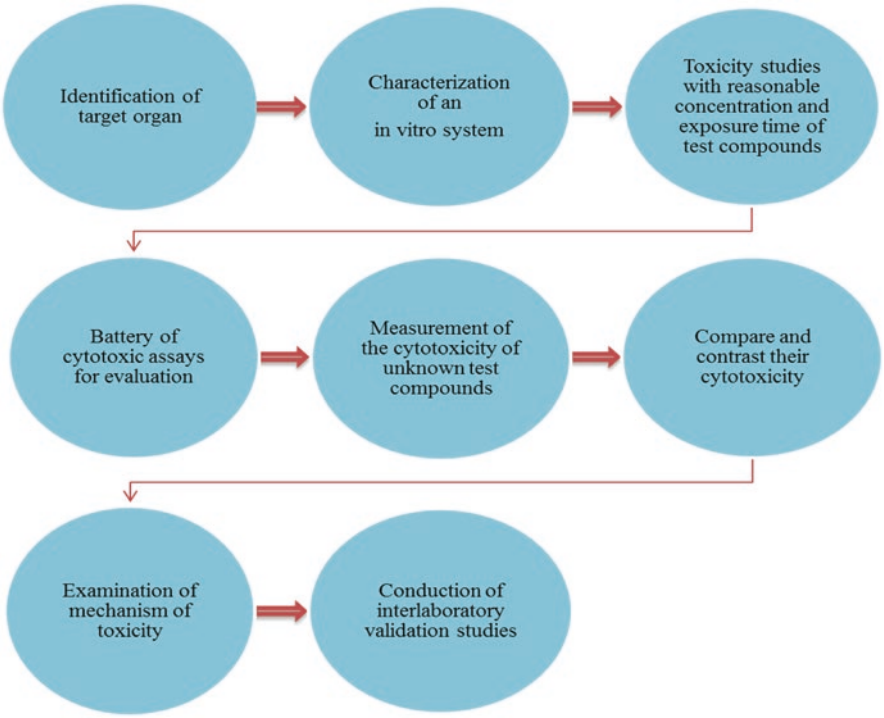

Fig. 3.2 Systematic representation of phases involved in elucidating the in vitro toxicity of a test compound. (Alok Dhawan and Seok Kwon 2018)

all, in vitro test models established worldwide are essential to elucidate the mechanisms and causes of various diseases (Fig. 3.2).

3.2.2 Basic Methods of In Vitro Substitution

3.2.2.1 In Vitro Technology

In vitro methods are considered to be the most common and important alternative ways for animal experiments. Rather than depending on the use of intact animals, they use lower levels of tissue, such as primary cultured cells, tissues, and organs. Many in vitro experimental techniques have been developed rapidly, such as coculture technology to reproduce the cell population of tissues or organs, including the reproduction of intestinal barrier, skin cells, normal mature keratinocytes, and so on. Pluripotent stem cells from embryos or various mature tissues can continue to differentiate into different types of cells under appropriate culture conditions and cell signal regulation due to their omnipotence. Researchers used iPSC-derived hepatocytes, cardiomyocytes, and nerve cells for toxicity tests and drug screening

(Csöbönyeiová et al. 2016). Cell engineering provides a new tool for toxicological research, expanding immortal cell lines to special functional cell lines. Through these efforts, we can reconstruct in vitro the complexity in vivo and reproduce the physiology and structure-activity relationship of cells in vivo, so as to better study the mechanism of action and risk identification. In vitro toxicity information can be used for toxicity studies at cellular and molecular levels, as well as for the study of biomarkers of toxicity, but its biodynamic processes need to be considered.

3.2.2.2 Physicochemical Methods and the Use of Computer

There is a certain relationship between the biological activity of chemical substances and their physical and chemical properties. Researchers mostly focus on the toxic mechanisms of lipid-water partition coefficient, bio-heat, molecular size, and electrophilicity, which are diverse and complex. In recent years, quantitative structure-activity relationship (QSAR) (Gerner et al. 2005) model has been applied in toxicological prediction and evaluation, especially in acute toxicity prediction. QSAR method is highly automated; can quickly classify and label substances, toxicity rating, and risk assessment; effectively reduce the use of experimental animals; and has broad prospects for development. Therefore, new compounds with certain structures and properties designed by computer, mathematical models of physiological, biochemical, pathological, and toxicological research can be established, many life processes in vivo can be simulated, and data obtained from other in vitro experiments can be supplemented and strengthened.

3.2.2.3 Toxicity Evaluation Using Caco-2 Cells Model

The intestinal and gastric epithelia, the most important organs of digestion, play an important role in the absorption and transport of various endogenous and exogenous materials. Intestinal epithelial cells are a single layer of cells arranged in the intestinal cavity, whose function mainly includes two aspects: barrier function, preventing harmful substances (including foreign antigens, microorganisms, and toxins) through the barrier in the intestinal cavity, and transport function, selectively allowing nutrients, electrolytes, and water from food material through the intestinal cavity into the circulatory system (Alam and Neish 2018; Groschwitz and Hogan 2009). The material transport of intestinal epithelial cells is mediated by two main pathways: transepithelial and cellular bypass transport. The structure, function, and material transport pathway of intestinal epithelial cells are shown in Fig. 3.3 (Akbari et al. 2017).

Human colon cancer cell line caco-2 is an in vitro model system used to study the small intestine for material transport and barrier function. In the process of culture, they undergo self-differentiation and develop into a structure that is functionally similar to the small intestine. Caco-2 cells grew in transwell, through 21 d culture cycle, to develop a monolayer model. This monolayer model is used to evaluate the

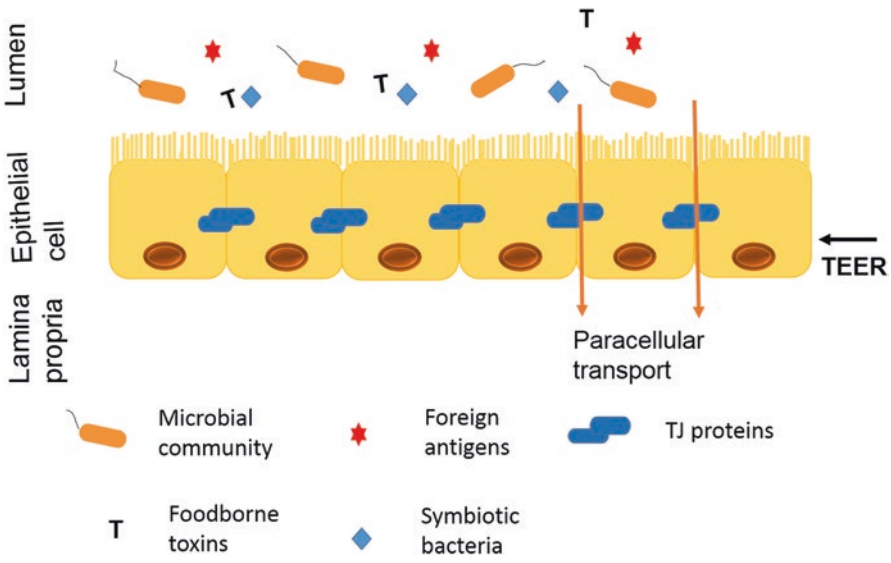

Fig. 3.3 Intestinal epithelial cell structure and paracellular transport schematic. Various mycotoxins induced the intestinal barrier breakdown and increased paracellular transport. *TEER* transepithelial electrical resistance, *TJ* tight junction

absorption and toxicity of drugs or toxins in vitro toxicity of validation. Cell differentiation was tracked by measuring changes in cell resistance. Transepithelial electrical resistance (TEER) is a fine parameter of barrier integrity in caco-2 cell model. Because the measurement of cell resistance is simple and fast, the change of cell resistance is an important evaluation index in various Caco-2 cell experiments (Akbari et al. 2017; Srinivasan et al. 2015).

The effects of mycotoxins on the integrity and barrier function of monolayer intestinal epithelial cells were mainly achieved by the evaluation index of TEER value, tracer flux analysis, and tight junction protein expression. The measurement of cytotoxicity of toxins was evaluated by cell viability.

Cell tracer flux analysis was a common method used to verify the integrity and permeability of caco-2 monolayer cell model. The marked substance meets the following requirements: no cytotoxicity, no charge, water soluble, not absorbed or metabolized by cells. Therefore, the integrity of a single barrier can be verified by measuring the mass of substances passing through the cell (transmembrane transport or cell bypass). Common markers include fluorescent compounds (fluorescein), compounds that bind to fluorescent markers (Fluorescein isothiocyanate glucan) (González-González et al. 2019).

The main functional element of epithelial barrier function is TJ protein, which forms a network of junctions near the surface of the lumen, sealing the cells between the epithelial cells. In this way, the transport of hydrophilic molecules and ions with small molecular weights is restricted. TJ proteins of Caco-2 cells in layered single stroma mainly include occludin protein, tight junction protein

(zonula occludens-1,-2, ZO-1,-2), and claudin family protein (claudin-1, claudin-2, claudin-3, claudin-4). The structure distribution of the tight junction protein between cells was shown in Fig. 3.4. The barrier that may be destroyed by drugs or toxins was found through the expression level of TJ protein that is detected by qRT-PCR and Western blot. Moreover, fluorescent dyeing observation of the subcellular localization of TJ protein is another method to identify the integrity of the intestinal epithelial barrier (McLaughlin et al. 2004; Kawauchiya et al. 2011). TJ proteins, which constitute the epithelial monolayer, are one of the most significant functional parts of the intestinal barrier, and the abnormal expression of different TJ proteins is verified in all major chronic intestinal inflammatory diseases as shown in Fig. 3.5.

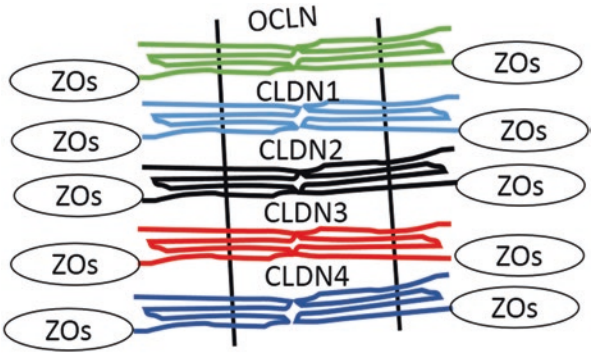

Fig. 3.4 Structure distribution of the tight junction protein between cells. TJ proteins: OCLN, CLDN1, CLDN2, CLDN3, CLDN4. ZOs zonula occludens

Inflammatory disease		Expression of TJ proteins
Crohn's disease	→	↓ OCLN, ↓CLDN3, ↓CLDN5, ↓CLDN8, ↓JAM ↑CLDN2
Ulcerative colitis	→	↓OCLN, ↓CLDN1, ↓CLDN4, ↓JAM, ↓Tricellulin ↑CLDN2
Celiac disease	→	↓OCLN, ↓ZO-1 ↑CLDN2, ↑CLDN3
Irritable bowel syndrome	→	↓OCLN, ↓CLDN1, ↓ZO-1

Fig. 3.5 Aspect of TJ proteins expression in chronic intestinal inflammatory diseases

Cell viability is an important indicator in evaluating the cytotoxicity of toxins. The cells are incubated with toxins for a period of time (generally 12, 24, 48, 72 h). Different methods are used to determine the amount of enzymes that can reflect the cell viability (e.g., succinic acid dehydrogenase, lactate dehydrogenase) (Romero et al. 2016).

The toxicity of AFB1 and AFM1 in Caco-2 cell model mainly includes the decrease of TEER value, the accumulation of reactive oxygen species (ROS), and DNA damage (Zhang et al. 2015). AFB1 could cause a significant decrease of the expression levels of TJ protein claudin-3 and occludin expression. Current studies suggested that the main toxic target organs of AFs are in the liver (Zheng et al. 2018), but the results in caco-2 cell model also indicated AFB1 And AFM1 damage the intestinal epithelial cell barrier causing ROS imbalance and DNA damage.

OTA can impair the barrier function of intestinal epithelial cells. The researches using caco-2 cell model found that OTA can cause a significant decrease of TEER value, leading to increased permeability of cell monolayers and the decreased protein expression levels of claudin family, causing apoptosis (McLaughlin et al. 2004; Sergent et al. 2005). The decreased TEER value depends on the concentration of OTA in caco-2 cells (Romero et al. 2016). OTA inhibited the absorbing ability of glucose and fructose by caco-2. After treatment with 100 mol/L OTA for 48 h, the inhibition rate of saccharide absorption was more than 30% (Maresca et al. 2001). OTA also result in the decreased protein expression levels of tight junction protein claudin-3, claudin-4, and occludin. Caco-2 cells are treated with 30 mol/L OTA. The expression levels of claudin-3, claudin-4, and occludin in Caco-2 cells decreased by more than 80%, seriously destroying the single-layer cell screening barrier (McLaughlin et al. 2004). OTA also induced the increase of ROS levels and oxidative damage in cells, leading to cell DNA damage and resulting in cell apoptosis. The results were verified in human kidney epithelial cells (hk-2), primary rat proximal renal tubular cells, proximal endonephric tubule cells (LLC-PK1 cells), human hepatocellular carcinoma (HepG2), and caco-2 cells (Kamp et al. 2005).

The interaction between DON (deoxynivalenol) and caco-2 cells was mainly manifested in the increase of cell permeability and decreased protein levels, including the production of TJ protein, leading to inflammation that destroys the intestinal tract. DON damages the intestinal barrier function; mainly the TEER values of caco-2 cell showed a significant decrease after DON treatment for 24 h, and the resistance decreased by 70%. 5 g/ml DON-treated cells decreased resistance by more than 80% (De Walle et al. 2010; Sergent et al. 2006). Treatment of DON results in the reduction of the total protein amount of the cell: At 5 g/ml DON, the total protein was reduced by about 20%. The protein expression level of claudin-4 declined with the increasing concentration of DON. DON can also drive the expression of pro-inflammatory genes, so that caco-2 cells produced inflammation reaction. DON activates the MAPK pathway through phosphorylation of various cytokines (Erk1/2, p38MAPK, and JNK/SAPK), which selectively transcribe by binding to the promoter of pro-inflammatory gene and changing the expression of mRNA, driving translation, inducing expression of apoptotic factor and cell apoptosis (Pestka et al. 2005). In the same way, DON can damage the intestinal barrier

function and tightness junction protein of porcine small intestinal epithelial cells (ipec-j2) and induces the activation of MAPK p44/42 and the activation of this pathway also resulting in the decrease of claudin-3 (Springler et al. 2016).

In the caco-2 cells, ZEN (zearalenone) induces apoptosis and cell cycle arrest. ZEN significantly reduces cell activity, but does not cause obvious cell membrane damage. Caco-2 cells treated with ZEN induced the high expression levels of caspase-3/7 that indicated that ZEN induced apoptosis (Wentzel et al. 2017). ZEN in a manner of concentration dependence induce cell DNA fragmentation, forming apoptotic bodies and inducing apoptosis. In Vero cell, caco-2 cell, and DOK cell, OTA-induced cell cycle arrest mainly manifested as the increase of cell number in G2/M phase (Abid-Essefi et al. 2003). In IPEC-1 cells, the toxicity of Zen is lower than that of its metabolite (alpha-beta-zen), which shows that NO significantly reduces transmembrane resistance. However, under low concentration of toxin, the difference of gene expression is significantly induced, including the up-regulation of cytokines involved in inflammation and the down-regulation of tumor suppressor gene expression, indicating potential inflammation and carcinogenicity (Taranu et al. 2015; Marin et al. 2015).

PAT first interacts with gastrointestinal epithelial cells after ingestion. Because of its qualifying for a variety of enzyme inhibition effect, PAT has strong toxic effect to the intestinal epithelial cells and can be quickly absorbed, so that the epithelial barrier function is destroyed, leading to mucosal ulceration and inflammation (McLaughlin et al. 2009). PAT reduces the TEER value of caco-2 monolayer cells in a concentration- and time-dependent manner. With 50 μmol/L of PAT treatment for 6 h, the cell TEER value of caco-2 was reduced by more than 30%. The TEER value was reduced by 90% after 5 h treatment at 100 μmol/L PAT; when PAT was added to both sides of layer cells, different resistance values were changed. PAT can induce the phosphorylation of tight junction protein zo-1 and significantly reduce the content of zo-1 (Kawauchiya et al. 2011; McLaughlin et al. 2009). Treatment of caco-2 cells with 50 μmol/L PAT resulted in the upregulation of MLC2, causing dysfunctional epithelial barrier. Simultaneously, the upregulated expression of procaspase-3 induced apoptosis (Assunção et al. 2016). PAT-treated human colon cancer cells (HCT116) and embryonic kidney cells (HEK293) generate ROS and induced endoplasmic reticulum stress reaction, mitochondrial apoptosis, and cell apoptosis (Boussabbeh et al. 2015).

3.2.2.4 Toxicity Evaluation Using Cell-Based Biosensors

With the development of science and technology, traditional cell assays and sensor technology are integrated. A variety of new technologies and new methods of cell sensors have emerged. Cell sensors are shown in Fig. 3.6, which are mainly composed of sensor, transducer, and signal analysis and processing system. Cells as the receptor are fixed to the interface when stimulated by external drugs and other changes in cell physiological activity; these changes are transformed into the photoelectric signal by the transducer, and the results are obtained through identification

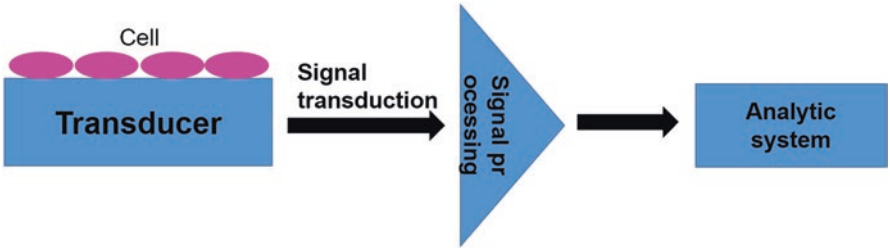

Fig. 3.6 Illustration of cell-based biosensors

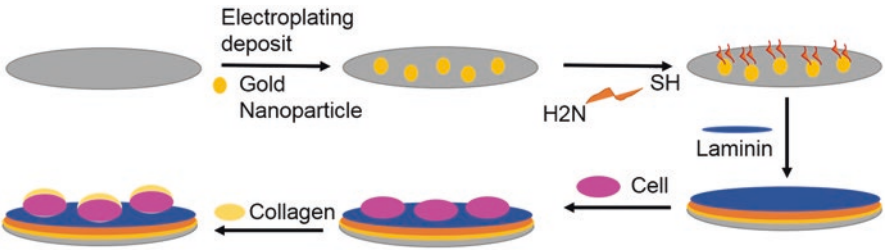

Fig. 3.7 Schematic illustration of the preparation of cell-based biosensor

and analysis of analysis system (Asphahani et al. 2008). Hence, depending on the signal variation to conducted qualitative analysis on the toxic stimulation of cells is a commonly used toxicity assessment method (Curtis et al. 2009). Combining the cell sensing and electrochemical impedance technology (EIS), when the surface properties of the electrode were changing, it will be sensitively captured by the electrical impedance and finally reflected in the changes of the impedance value. So monitoring the change of cell impedance at the electrode interface can present the physiological state of the biological cell in a timely and continuous manner and reflect the action of cells to poison (Asphahani and Zhang 2007). The EIS technique is unlabeled, noninvasive, and highly sensitive to cells during monitoring; it is widely used in the study of cell physiology, morphology, and functional changes. The construction method and steps of the cell sensor are shown in Fig. 3.7.

Cheng et al. constructed an impedance cell sensor and successfully monitored the effect of dengue fever virus on mouse kidney cells BHK 21 in real time. The effect of the virus on the cell is directly reflected in the impedance signal. Cell sensors to the virus are more sensitive compared with the traditional method (Cheng et al. 2015). Daza et al. constructed a kind of microelectrode cell sensor to detect cell size, morphology, number, proliferation, migration, and inhibition effect of ubiquitin proteasome inhibitors through impedance signal. Indicators were analyzed to achieve the expected test results. Morefield et al. immobilized rat embryonic auricular cortical neurons on an electrode array; different concentrations of cannabinoid-induced neurons changes were quantitatively measured by recording the potential changes of neurons stimulated by cannabinoid (Liu et al. 2014). To

evaluate the toxicity of the drug, BEL7402 cells are fixed to gold electrode surface to construct the impedance sensor. The toxicity of DON and ZEN was monitored by MTT and flow cytometry in real time, and the results are consistent. Wang et al. synthesized multiwalled carbon nanotubes/gold nanoparticles/chitosan composite solution. It can be used to modify the magnetoelectric pole and rapidly evaluate the lipopolysaccharides of foodborne pathogens and toxic effect of LPS on the cells by detecting the changes of electrochemical signals.

Screen printing electrode, SPE, due to its simple production process, low cost, portability, few samples, batch production, and other excellent performances, are now widely used (Kafi et al. 2011). However, some products have already been commercialized and are attracting much attention in the field of sensors (Hernández-Ibáñez et al. 2016). Meanwhile, another focus in the field of cellular sensing is the use of biocompatible materials. Biocompatible materials have a large surface area, can absorb more proteins and other biological molecules to maintain biological activity, more truly reflect the human ecological environment, and have been widely used in cell modification and immobilization in cell sensing (Robertus et al. 2009). Hui et al. used EIS to study the effect of taste enhancer in bitter taste receptor cells that inoculated in SPE as a sensing element using the EIS to study the effect of stimulating by taste enhancer on taste (Hui et al. 2013; Banerjee and Bhunia 2009). When electrochemical impedance cytosensor is applied to evaluate the function and toxicity of the substance to be tested, it had a rapid and dynamic advantage and showed a broad application potential in drug development, toxicology testing, environmental monitoring, and other fields (Xing et al. 2005; Robertus et al. 2009).

Xia et al. used Fe (CN)6 $^{3-/4-}$ as a the REDOX probe, the SPE as a working electrode, and plated the human liver cancer cell on the electrode surface to perform the electrochemical impedance test to evaluate the cell toxicity of mycotoxin (Xia et al. 2017). Cytotoxicity was assessed using DON, ZEN, and AFB1 (the three most common mycotoxins) alone and in combination. EIS electrochemical impedance technique was used to determine the impedance values of different doses of three mycotoxins alone, and the IC 50 values of toxin stimulation after 24 h. AFB1 3.1 μg/ml, DON 48.5 μg/ml, ZEN is 59.0 μg/ml, respectively. For Hep G2 cell, AFB1 is the most toxic. DON is the second, ZEN is the weakest. Combining with the joint index method to calculate the CI Index to evaluate the combined action of the three mycotoxins: DON and ZEN coexistence in Hep G2 Cells are synergy. DON and AFB1, when coexisting, mostly show additive effects. ZEN and AFB1 combination and triple combination actions were antagonistic at the tested dose. The sensor evaluation method is compared with the traditional cell evaluation method (CCK 8 method), and it was found that the sensor evaluation method was more sensitive to low dose of toxin. Biological experiments indicated that EIS electrochemical impedance technique correlated with $[Ca^{2+}]i$ concentrations and the ratios of apoptosis and necrotic cells, therefore affecting the electrochemical signals. Hence, this technology is a simpler, faster, and more convenient alternative method with the advantage of sensitive response.

Gu et al. firstly utilized EIS measurements, the cell-based electrochemical biosensor to assess the individual and combined toxicity of DON and ZEN on BEL-7402 cells (Gu et al. 2015). Their results showed that the values of EIS decreased with the dose of DON and ZEN in the range of 0–20 and 0–50 μg/ml with the limit of detection of 0.03 and 0.05 μg/ml, respectively. Moreover, the combined two mycotoxins mean an additive effect. The results of the electrochemical method suggested that this EIS electrochemical impedance technique is more sensitive, real-time, accurate, and simple and it could be developed as a convenient method to evaluate the toxicity of mycotoxins.

3.2.2.5 Toxicity Evaluation Using Model *Caenorhabditis elegans*

In vitro toxicity studies can provide a lot of powerful information for scientific research, but due to the complexity of organisms, it is necessary to speculate the results of an animal experiment to humans. The in vivo toxicity evaluation of mycotoxins can be summarized as acute toxicity test, chronic toxicity test, subacute toxicity test, subchronic toxicity test, and dynamics of toxicity test (Wang et al. 2017). Subacute and subchronic toxicity trials are a large part of all studies, 40% literature reports belong to subacute researches, other toxicity tests ratio as shown in Fig. 3.8 (data from the PubMed database). So far, the most commonly used method is to obtain preliminary data from animal experiments in vitro and further to establish a model to infer the toxic effect of the tested substance in the human body. Despite the current development of many cell lines such as in vitro models which can replace model animals to conduct partial in vivo toxicity test, there are no in vitro test models available to completely replace the traditional in vivo tests.

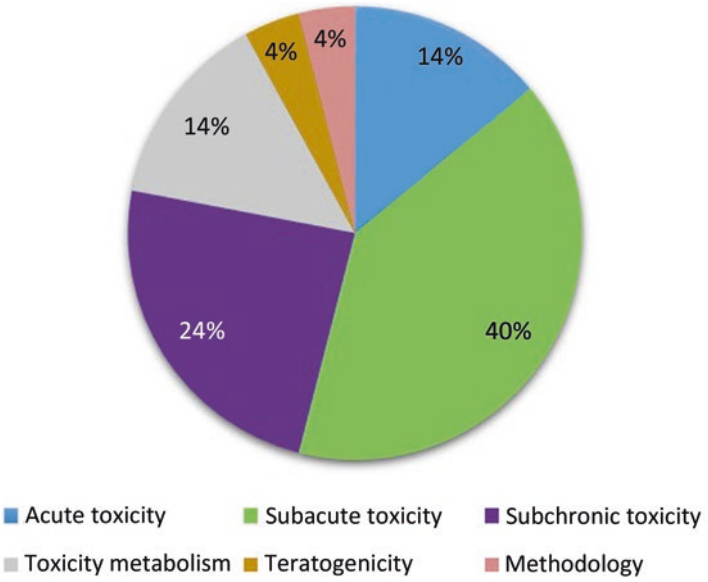

Fig. 3.8 Percentage of the different studies according to the design condition

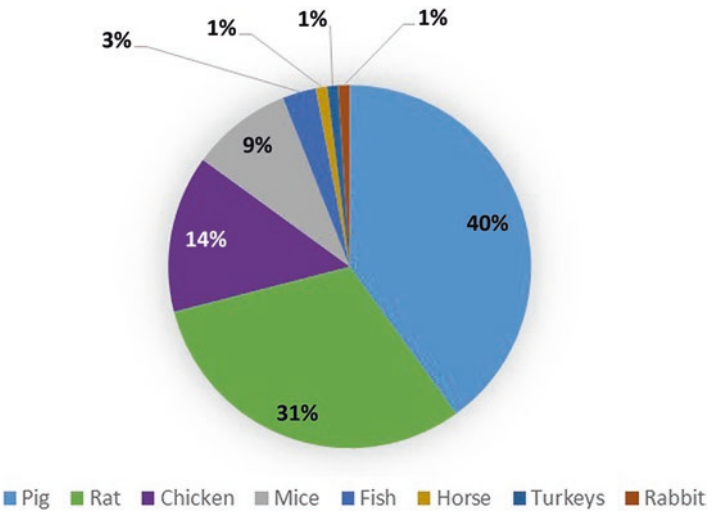

Fig. 3.9 Percentage of mycotoxins studies according to the animal models used

Hence, as the carriers of in vivo experiments, the status of various biological models is particularly important. As shown in Fig. 3.9, pigs (piglets, long-white pigs, weaning young pigs) and rats (Sprague-Dawley rats, Wistar rats, Fisher rats) were the most commonly used animal models in laboratory studies of mycotoxins, followed by broilers and mice, as well as fish (zebrafish, trout), horses, turkeys, and rabbits (New Zealand rabbits). In recent years, a few studies were reported on fruit flies, *elegans*, and invertebrates (Tiemann et al. 2006).

Caenorhabditis elegans is an ideal model animal to study the toxicology of exogenous toxicants (Honnen 2017; Hunt 2017). It has many advantages such as simple construction, transparent body, and short life cycle. From embryonic development to life expectancy and aging research and in 2002 years, the research of Brenner, Horvitz, and Sulsto on organ development and gene regulation mechanism of programmed cell death in *C. elegans* has obtained Nobel Prize in physiology or medicine (Gowrinathan et al. 2011). *Caenorhabditis elegans* is free to live in soil or water, and its adult body length is approximately 1.0–1.5 mm, which feeds on bacteria. In the natural state, *Caenorhabditis elegans* has two genders (Fig. 3.10), hermaphrodite and males, of which males were less than 0.2% (Corsi 2015).

Although *C. elegans* is a very simple invertebrate, it at least has three independent and conserved signal transduction regulation pathways of immune regulation that are similar to that of humans and higher animals: insulin pathway/IGF-1, c-jun N-terminal kinase pathway, and PMK-1 protein kinase pathway. These signaling pathways are involved in immunity response regulation, reproductive development, aging, and other physiological functions (Klass et al. 1982). And these pathways play an important role in the corresponding regulation of nematode extrapolating to higher animals and humans. *Caenorhabditis elegan* owns the simple body structure, and its individual cell possesses the development and death at a fixed time and place. It is a good

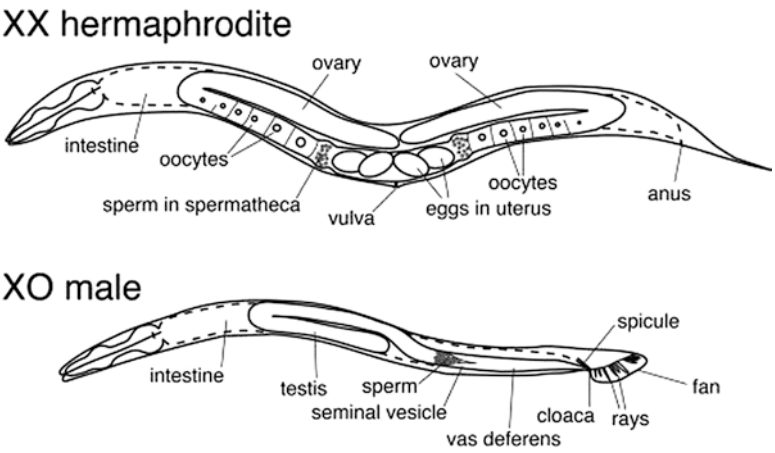

Fig. 3.10 The two sexes of *C. elegans*. Quotation from http://www.ncbi.nlm.nih.gov/books/NBK19759/

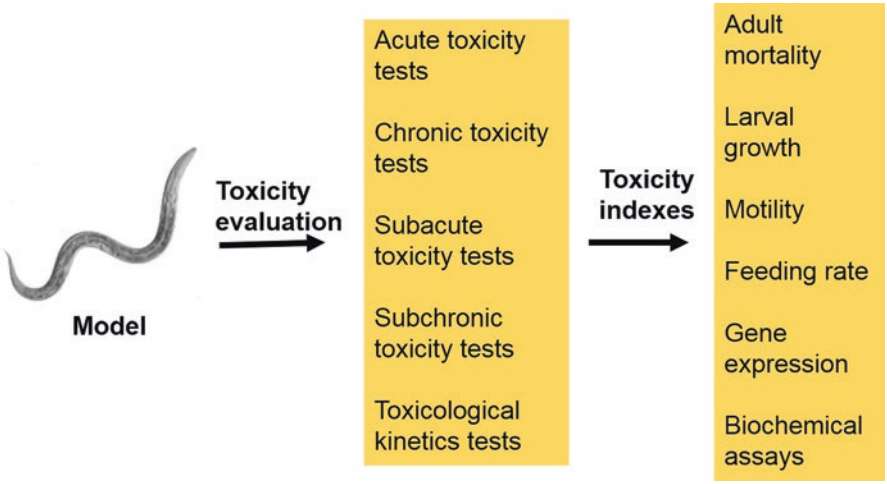

Fig. 3.11 The evaluation index of toxicity testing in *C. elegans* can provide a bridge to mammalian in vivo testing

model for studying the development of body organs and reproductive embryos (Muhammed et al. 2012). *C. elegans* strains are readily available for some target genes; this model has great advantage for the assessment of human relevant pathways of toxicity (Fig. 3.11).

Leung et al. studied the aflatoxin B1 toxicity effects on cytochrome P450 (CYP) series in *Caenorhabditis elegans* (Leung et al. 2010). Research results showed that the significant effects of AFB1-induced *Caenorhabditis elegans* CYP1, CYP2, and

CYP3 family enzymes were consistent with those in mammalian models. Gowrinathan et al. have shown that DON can inhibit the number of *Caenorhabditis elegans* wild-type N2 and mutant strains AU1 offspring, and the rate at which they develop from embryo to larva has also decreased (Gowrinathan et al. 2011). There was a significant dose-dependent relationship between the exposure concentration and the number of offspring. Moreover, this kind of reproductive inhibition does not only exist in DON exposure but may also exist in the effects of the crude extract of *Fusarium* mycotoxin. Therefore, it is speculated that the effects of *Caenorhabditis elegans* exposed to *Fusarium* mycotoxins may have reproductive toxicity.

Yang et al. reported that the common mycotoxins of aflatoxin B1 (AFB1), deoxynivalenol (DON), fumonisin B1 (FB1), T-2 toxin (T-2), and zearalenone (ZEN) were assessed in *Caenorhabditis elegans* model for their toxic effects on lethality, growth, and reproduction, as well as life span (Yang et al. 2015). They showed that the lethality effects of *Caenorhabditis elegans* to T-2 were more sensitive, and with a EC50 at 1.38 μg/ml, growth inhibition was more sensitive for AFB1 toxic effects, and reproductive inhibition of elegans to mycotoxin AFB1, FB1, and ZEN was relatively sensitive. In addition, the life span inhibitions of *C. elegans* to all five tested mycotoxins were all sensitive. These results provided an important viewpoint on the evaluation of mycotoxin toxic effects, especially for evaluating developmental and reproductive toxic effects, utilizing the *C. elegans* model.

Feng et al. observed the AFB1 toxic effect on DNA damage, germline apoptosis, growth, and reproductive ability in *Caenorhabditis elegans* model (Feng et al. 2016). Results suggested that AFB1 induced DNA damage, germline apoptosis, and significant inhibition of growth and reproduction on the nematodes in a dose-dependent manner. And in the DNA repair-deficient xpa-1 nematodes, AFB1 inhibited growth or reproduction more obviously compared to the wild-type N2 strain.

In conclusion, tissue culture, computer technology, immunology technology, and physical and chemical methods have developed rapidly as alternatives to animal experiments. In addition, animal experiments are still needed to verify the data obtained in vitro in the final stage of biomedical research.

3.2.3 In Vitro Models and Assessment Methods for Different Toxicities

3.2.3.1 In Vitro Models and Assessment Methods for Cytotoxicity

Cytotoxicity is considered primarily as the potential of a compound to induce cell death. There are a number of in vitro models specifically designed for cytotoxicity analysis of various chemicals, including monolayer coculture, multilayer coculture, xenograft models, and tissue excision. Cytotoxic model systems can be used to control intercellular contact, cell adhesion, cell interaction, and cell shape and provide significant benefits over conventional in vitro systems. Most in vitro cytotoxicity tests measure necrosis. However, apoptosis is an equally important cell death

Table 3.6 Applications of common in vitro cytotoxicity models

In vitro models	Applications	References
Two-dimensional (2D) cell culture model	Testing the basic functions of cells	Bourdeau et al. (1990)
	Testing number of parameter such as vital solution, cytosolic enzyme release, efficiency of cell growth and cloning, and integrity of cell membranes	
	Determine the maximum permissible dosage of new drugs	
	Determine the minimum lethal dosage of toxin	
2D coculture model	Investigate heterotypic cell-cell interaction in bone regeneration in vitro coculture system	Burguera et al. (2010) and Lu and Wang (2007)
Three-dimensional (3D) culture model	Investigate the multiple feedback mechanism	Kim et al. (2004)
	Investigate the function of adhesion molecule in invasion/metastasis	
Tissue slice/grafting models	Study 3D representation of tissue slice, cell-to-cell, and cell-to-matrix relationship	Lipman et al. (1992)
	Study synergistic, additive, or antagonistic effect of chemicals	
Microscale cell culture model	Study in vivo situation very closely	Sung and Shuler (2010)
	Helps to study multiorgan interaction with blood circulation	
	Study the whole body response to drug along with 3D cell dimension	

mechanism that requires different assessment methods. Several in vitro cytotoxic models and their applications are listed in Table 3.6.

3.2.3.2 In Vitro Models and Assessment Methods for Neurotoxicity

The nervous system is the most complex system in the internal structure and function of the organism. It is more sensitive to the toxicity of environmental chemicals and is easily attacked by exogenous toxic substances. Due to the heterogeneity of the nervous system, different neurotoxic substances can affect neural and behavioral functions in different and specific ways, such as direct damage to neurons, interference with synaptic transmission, disruption of ion channels, axonal degeneration, myelin damage, edema, and so on. Therefore, the neurotoxicity of the environmental chemicals can be evaluated from different aspects.

In recent years, in addition to using traditional animal models in neurotoxicology research, researchers continue to create alternative schemes, such as tissue culture in vitro, mathematical models, and so on. In vitro tissue culture plays an important role in neurotoxicity assessment (Harry et al. 1998). Various culture systems are known for neurological evaluation, including cell lines and primary cultures derived from nervous system, explants of organ type cultured by regrouping, and synaptosomes cultured (Fedoroff and Richardson 2001) Various nervous system cultures

Table 3.7 Neurological system culture model in vitro

Type	Common materials	Advantages and disadvantages
Dispersed cell culture	Primary dispersion: neurons, neural stem cells, astrocytes second-generation dispersion: processes from the cells mentioned above or cell lines, explants	Maintaining the original characteristics of cells, having electrophysiological activity of single cells, can be cocultured
		Limited in vitro survival time, usually no organ type
Whole embryo culture	Rodents, chicken embryos, usually preferred rats	Screening for developmental neurotoxins, existing complete embryonic metabolism, and normal brain development
		High technical difficulty
Organ culture	Hippocampal slice, phrenic nerve	Longer survival time; the metabolism of brain tissue can be studied directly through the blood-brain barrier
		Possible damage to brain cells, High technical difficulty
Aggregation test	Suspension culture of organ types	Providing a large number of cells for morphological evaluation, longer survival time
		The growing environment is not easy to determine. A lot of embryos are needed
Whole organ explants	Autonomic ganglion of peripheral nervous system	Culturing more mature cells; limited number
Acellular system	Brain homogenate, synapse	Easy to make, maintaining a certain metabolic capacity
		Limited time to maintain vitality, uncertain cell type

Adopted From Peng et al. (2008)

in vitro have their advantages and disadvantages and should be selected or used in combination (Table 3.7).

At present, there are many methods to evaluate neurotoxic effects, including traditional morphology, electrophysiology, pathology, biochemistry, and neurobehavioral studies. Technology, biochemistry, and molecular biology have become useful tools for toxicologists to study neurotoxicity. For example, neural electrophysiological measurement is one of the sensitive indicators for detecting neurotoxicity. Patch clamp technique is often used to study and evaluate the effects of toxicants on ionic channels of nerve cell membranes and signal transduction processes (Liu 2006). For example, in vitro marker gene expression can be used to analyze the specification of neuronal and glial cell types as well as neuronal subtype (e.g., γ-aminobutyric acidergic neurons, glutaminergic neurons) (Brosamle and Halpern 2002). Certain molecules can be used as early indicators to predict and estimate nerve damage, for example, the increased activities of β-glucuronidase and β-galactosidase have been

reported as early signs of peripheral nervous system (PNS) and central nervous system (CNS) degeneration (Dewar and Moffett 1979).

3.2.3.3 In Vitro Models and Assessment Methods for Immunotoxicity

The immune system differentiates the self from the nonself through a series of precise, balanced, and complex multicellular mechanisms in vivo, generates corresponding immune responses, maintains the stable environment of the body, and protects the body from infectious diseases. Immunotoxicity means that exogenous chemicals and physical factors directly damage the structure and function of immune cells; affect the synthesis, release, and biological activity of immune molecules; or disrupt the nerve-endocrine-immune network by interfering with the nervous system and endocrine system, so that the immune system reacts too high or too low to antigens. The immune system can target the immune toxicity of various exogenous chemicals including environmental pollutants such as polychlorinated biphenyls, pesticides, heavy metals, drugs, and so on (Krzystyniak et al. 1995). Therefore, exposure of such chemicals can induce immunosuppression that leads to decreased resistance to viral, bacteria, fungal, and other infectious agents. Because of the highly complex composition and function of the immune system, no single experiment can fully demonstrate the effects of exogenous chemicals on it.

Most quantitative and functional assays have been developed and validated to quantify interferons, cytokines, and other factors secreted by immune cells (Luster et al. 1993). Monocyte line, lymphocyte, and other immune cells are usually used for immunotoxicity research. There are many methods to detect immunosuppression, such as determining the content of lactate dehydrogenase to understand the destructive ability of NK cells to target cells and judging the proliferation of T lymphocytes and B lymphocytes by MTT method. Enzyme-linked immunosorbent assay (ELISA) and flow cytometry quantitatively activated $CD4^+$ and $CD8^+$ T cell subsets can clearly demonstrate that chemicals induce immune imbalance in the body (Krzystyniak et al. 1992; Gleichmann et al. 1989).

3.2.3.4 In Vitro Models and Assessment Methods for Liver Toxicity

The liver is one of the important organs in vertebrates for maintenance of homeostasis and metabolic activity. It is the main site for the body's anabolic metabolism of sugars, proteins, fats, and vitamins, as well as the main detoxification organ in the body. Catabolism, inactivation, and excretion of many endogenous and exogenous chemicals are carried out in the liver.

The liver has two blood supply channels, including the hepatic artery and the portal vein, and two output channels, including the hepatic vein and the biliary tract. Venous blood from the gastrointestinal tract flows into the liver through the portal vein and enters the systemic circulation. Therefore, many nutrients and toxic sub-

stances absorbed by the gastrointestinal tract can enter the liver through the portal vein. The liver is also the main organ for biotransformation of exogenous chemicals. The liver metabolizes certain chemicals to produce nontoxic metabolites and excretes them. Other chemicals produce more toxic or more toxic metabolites that cause liver damage. This unique vascular layout and metabolic transformation of the liver makes it the primary target organ for toxins.

Liver toxicity has been a toxicological problem for more than 100 years. The evaluation of the damage effect is continuously developed with the development of life science and technology and has undergone a transformation from the whole animal to the organ cell molecular level. Traditional in vivo experiments are indispensable for the evaluation of hepatotoxicity. The whole animal can observe the effects of chronic toxicity on the body in a long-term dynamic period. However, the shortcoming is that it is difficult to distinguish primary or secondary liver injury by factors such as endogenous and exogenous compounds, body fluids, and neuroendocrine; the existing in vivo test period is longer. In vitro testing of hepatotoxicity can rule out the effects of interacting systems such as neuroendocrine systems by controlling experimental conditions. Common in vitro test models include isolated liver perfusion models, precision liver section models, primary hepatocyte models, and hepatic cell line models.

Since isolated perfused liver has the advantage of organ level and the advantages of whole animal experiments and in vitro experiments, it has been widely used in metabolic and toxicity study of drugs and other exogenous chemicals. Deters (Deters et al. 1998) used the isolated rat liver perfusion model to study the liver toxicity of n-butanol, $CdCl_2$, t-Butyl hydroperoxide, and cyclosporine. These exogenous chemicals were found to cause significant hepatotoxicity, manifested by increased leakage of LDH and transaminase in perfusate, decreased oxygen consumption, hepatic bile suppression, ATP and GSH depletion, and a significant increase in lipid peroxidation products. Yokoyama (Yokoyama et al. 2006) used this model to study the hepatotoxicity of naproxen. It was found that naproxen can cause oxidative stress in the liver and affect the physiological function of the liver by damaging bile excretion.

The precision-cut liver slices model utilizes both the tissue microtome and the dynamic organ culture system. The thickness of the liver slice obtained by the former is uniform, the damage to the liver tissue is very small, and the establishment of a dynamic incubation system solves the shortcomings of insufficient oxygen and nutrient supply during liver slice culture, enabling liver slices to survive for several days. The emergence of precision-cut liver slices model establishes a sub-organ level bridge between organ perfusion and cell culture. The precision-cut liver slices model plays an important role in elucidating the metabolic conversion of drugs and poison, predicting the liver toxicity of exogenous chemical substances, and comparing the differences in metabolism and toxicity between different species. Stoff-Khalili et al. (2006) studied the hepatotoxicity of replicable adenovirus preparations using precision-cut liver slices model of isolated human and mouse. The results showed that this model could cause significant liver damage, which is reflected by increased transaminase leakage and hepatocyte apoptosis. Booth et al. (2004) com-

pared the metabolic differences between ethylene glycol and glycolic acid in female SD rats, rabbits, and human liver slices. It was found that the amount of glycolic acid produced by the metabolism of ethylene glycol in rabbit liver was much lower than that of rats, but in human liver, no formation of glycolic acid was detected. Hepatic acid can be metabolized to glyoxylic acid by liver slices of three species, but human liver slices have the highest metabolic efficiency.

The primary hepatocyte model preserves and maintains the intact morphology of hepatocytes and the in vitro metabolic activity of hepatocytes, which can truly reflect the metabolism of the body. The metabolism and toxicity of chemical substances can be studied under the condition of close physiological state; the influence of other organs and tissues is excluded. Monostory et al. (2005) studied the in vitro metabolism of deramciclane in mouse, rat, rabbit, canine, and human primary hepatocytes. The results showed hydroxylation of the first side chain of deramciclane in rat and human primary hepatocytes and detection of a large amount of deramciclane N-oxide in mouse and human primary hepatocytes. The product of binding of the hydroxylated deramciclane derivative to glucuronide was not detected but was detected in rats, rabbits, and dogs. Fahring (Fahrig et al. 1998) was cultured in rat and human hepatocytes with S9 as a metabolic activation system, and hepatocytes were cocultured with K562 and V79 cells, respectively. The genotoxicity of benzopyrene and dimethylbenzindole (DMBA) was tested using the comet assay and the hypoxanthine! phosphoribosyl transferase assay (HRPT). The results showed that with S9 and hepatocytes as the activation system, DMBA could induce gene mutation in V79 cells and both benzopyrene and DMBA could induce DNA fragmentation in K562 cells.

The hepatocyte cell line is characterized by relatively infinite growth ability and high survival rate. It can display genotype and phenotypic characteristics similar to normal hepatocytes and is mostly used for toxicity assessment. Its functional enzyme regulates xenobiotic I (Scheers et al. 2001; Brandon et al. 2006). The hepatic cell lines HepG2, Hep3B, HBG, and HeparG are the most widely used, and they are commonly used as hepatic immortalized cell lines for in vitro evaluation of hepatotoxicity studies (Guguen-Guillouzo et al. 2010; Guguen-Guillouzo and Guillouzo 2010). The hepatocyte lineage model is the first consideration for toxicological and pharmacological studies to detect toxic chemicals and assess their cytotoxic mechanisms. Hepatotoxicity means that chemically derived damage results in several acute and chronic liver diseases. Some liver in vitro models are used to understand liver toxicity.

3.2.3.5 In Vitro Models and Assessment Methods for Kidney Toxicity

Exogenous compounds need to be excreted from the kidneys after entering the body through various routes. The kidneys also can transform foreign compounds. Most of the metabolic enzymes present in the liver can also be found in the kidneys. Therefore, the kidney is one of the main target organs for the toxic effects of compounds. Due to the complexity and heterogeneity of kidney tissue structure, it is

difficult to study the toxicity of kidneys with strong compensatory ability. Therefore, the evaluation of renal toxicity is a relatively complex research content in the evaluation of compound toxicity.

Due to the complex neuroendocrine regulation and cell-cell interactions in vivo, traditional in vivo animal experiments are difficult to determine the exact mechanism of nephrotoxic damage of exogenous compounds such as mycotoxins. Finding alternative methods with short cycle, high sensitivity, and specificity has become an important part of studying the nephrotoxicity of mycotoxins. In recent years, various in vitro culture models, such as tissue culture, primary culture of kidney cells, and slice culture, have been gradually used for the evaluation of renal toxicity of compounds. For example, immortalized renal tubular epithelial cells have abundant metabolic transformation and biotransformation enzymes, maintain the differentiation state of precursor cells in vivo, and have long-term exposure to mycotoxins under controlled conditions, which has become a reliable cell model for studying nephrotoxicity. With the development of molecular biology technology, more and more in vitro alternative methods will gradually be applied to the study of nephrotoxicity.

The primary renal proximal convoluted tubule cell culture model was developed on the basis of glomerular and renal tubular culture. Renal proximal convoluted tubule epithelial cells have rich metabolic transformation and biotransformation functions, maintain the differentiation state of precursor cells in vivo, and are very sensitive to the damage of exogenous toxicants, which is the most common site of nephrotoxic damage (Bakker et al. 2002). Different cells can be isolated according to different specificities of renal tubular epithelial cell surface antigens. This method is mainly applied to the evaluation and research of toxicity of exogenous compounds but it is affected by the age, sex, and strain of kidney donors. There are certain differences between cell lines, so the application is limited and gradually replaced by immortalized renal tubular cells.

The immortalized renal cell model establishes a renal proximal tubular epithelial cell line to optimize screening of lead compounds early in drug development and compares the relative nephrotoxicity of structurally similar compounds. The commonly used immortalized renal cell lines include MDCK from the epithelial cells of dog kidney collection tube, the proximal convoluted tubule epithelium cells of possum from USA, pig renal epithelium cells near convoluted tubule lc-pk1, human renal proximal convoluted tubule epithelium cells HK-2. These cell lines are characterized by the unique structure and function of renal epithelial cells, which can partially express the tubular specific marker enzyme, and have a long survival time and are easy to subculture (Yokoo et al. 2007). The OK cell line was originally established to study the inactivation of the X chromosome. It has been widely used in the study of nephrotoxicity mechanisms of aminoglycoside antibiotics, cisplatin, aristolochic acid, etc., especially when it comes to the study of transport systems. Cells are often used as models for studying sodium-hydrogen ion exchange systems and para-aminopurine transport systems (Park et al. 2002). The LLC-PK1 cell line is derived from a single layer of polar cells of the porcine kidney epithelium. It has a non-sodium-dependent hexose transport system and a polyamine transport system

in addition to the sodium-dependent transport system and is now the main cell for nephrotoxicity studies. One of the models is used for the study of drug transmembrane transport and renal cytotoxicity of drugs such as β-lactam antibiotics.

3.3 Challenges and Considerations

In recent years, with the advocacy and implementation of the 3R principle of experimental animals, biomedical research model has changed, and the whole animal experiment is facing severe challenges. The in vitro model research of replacing animal experiments has become an important direction of toxicology development. With the development of toxicological alternatives, in vitro surrogate models have covered multiple toxicity endpoints such as general toxicity, genotoxicity, and organ toxicity, including research on tissue, cell culture, genomics, proteomics and metabolomics, and computer-aided simulation evaluation system. However, the in vitro replacement model is a simplified model of a very complex in vivo system, and there are always limitations in toxicological situations in which in vivo data is inferred by in vitro model studies. A typical problem with in vitro model systems is the deficiency of translational studies. In addition, the authenticity of in vitro cell lines remains problematic because in vitro models are always at risk of contamination and the type of cell line may be erroneous. The culture environment employed in the in vitro model is not steady state compared to the animal as a whole, such as nutrient consumption, accumulation of metabolic waste, and limitations of dissolved oxygen supply, which sometimes may cause anaerobic culture. At the same time, compared to the actual tissue, the in vitro model culture cell density is less than 1%, which breaks the nonphysiological conditions of intracellular signal transduction and simulation, such as differences in animal body temperature and blood electrolyte concentration. In vitro characterization of cell-based assays should be specifically considered so that a possible understanding of the reaction can be developed in each cell of a particular assay.

3.4 Conclusion

In vivo toxicology studies and evaluations generally use a variety of in vivo tests as the final reference standard. Therefore, various in vitro screening, testing, and evaluation methods must be accepted as a standard in vivo test alternative method through a series of steps such as establishing methodology, standardization of test procedures, multi-laboratory pre-assessment, international collaborative pre-certification, and international collaborative certification. Therefore, the establishment of a new in vitro replacement model still requires many efforts. Subsequent studies of these models are expected to facilitate more understanding of the toxic mechanisms and increase their analytical capabilities and value in risk assessment.

These studies may help elucidate the corresponding mechanisms by which cytotoxicity acts on the human body. By combining these studies with many existing toxicological principles and methods, it is definitely clear to increase the credibility of extrapolation from experimental animals to human beings.

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Chapter 4
Risk Assessment and Profiling of Co-occurring Contaminations with Mycotoxins

Lan Wang and Aibo Wu

Abstract Food safety has generally remained a basic but compulsory needs to human beings, while ensuring the safety of foods has been a major focus of international and national actions. Humans can be externally and internally exposed to mycotoxin contaminations through a variety of routes, such as contaminations from the circumstantial environment, water, and food consumption. Among those mentioned exposure, food consumption is highly recognized as the main resource of human exposure to mycotoxin contaminations. More obviously, the direct exposure is almost through the consumption of contaminated cereal-based foods and animal-borne foods. Mycotoxin's exposure evaluation through total diet studies (TDS) has been conducted in several countries worldwide.This chapter features an overview of occurrence and dietary exposure assessment through TDS studies of co-occurring mycotoxins in a variety of foods. Also, little progress via biomarker analysis in human urine had been made regarding to more accurate risk assessment via the so-called internal exposure assessment, likely on DON and ZEN for different populations in Europe and China.

Keywords Risk assessment · Risk profiling · Consumption data · Exposure assessment · Mycotoxins

4.1 Introduction

In recent years, food safety policies based on the updated scientific data have been implemented in the whole food production to utmostly protect the human health and rights of consumers. The presence of mycotoxins has been highly reported in many

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studies (Juan et al. 2017; Rodríguez-Carrasco et al. 2016). Mycotoxins, an environmentally persistent compounds produced by pathogenic fungi, have a potential adverse health effects on human beings and animals (Khan et al. 2008). Humans can easily be exposed to a multitude of mycotoxins, and dietary intake is generally considered to be a major pathway. However, exposure data tend to focus on individual levels of various mycotoxins in food commodities and often fail to directly assess the population's exposure to them in total. In this sense, international committees of EFSA, FAO, and WHO regularly conduct harmonized guidance document including Provisional Maximum Tolerable Daily Intake (PMTDI) for different mycotoxins to improve dietary exposure assessment's efficiency and accuracy.

On the commonsense, risk assessment includes a four-step conceptual framework for assessing the specific hazards due to the exposure of fungal toxins, monitoring exposure, and estimating associated health risks. Based on the acquired scientific evidence, risk assessment is providing risk managements with sufficient informational strategies for effective interventions, which are critical to help authorities and decision-makers to take appropriate actions to reduce health risks in different populations. Therefore, De Nijs et al. suggested that food consumption surveys, food surveillance studies, repetitive diet studies, and total diet studies (TDS) should be all considered as key factors to exposure assessment on mycotoxin contaminations in foods (De Nijs et al. 2016).

4.2 Risk Assessment

The combined toxicity of mycotoxins is a complex problem, but in general, co-exposure to several kinds of mycotoxins can cause unavoidable harm to the human body through an additive or synergistic effect, especially when the co-occurred mycotoxins exert their toxicities by a common mode of action. Till now, several methods for the hazard and risk assessment of individual mycotoxin have been proposed. However, risk assessments on human health performed on individual mycotoxin might underestimate the total risks aroused by the co-occurring mycotoxins contaminated in the actually consumed foods. Even so, the most direct way to assess the co-toxicity of the multi-mycotoxins is to follow single mycotoxin assessments. No special methodology is required in this method. However, several bottle-necked problems were observed, the most one of which is to obtain the mixture of mycotoxins. Recently, the combinatorial concepts of independent action (IA) and concentration addition (CA) were applied to assess the risk of various contaminants (Borg et al. 2013; Sarigiannis and Hansen, 2012). Namely, IA supposes that the individual component acts independently of each other, whereas CA assumes that the mixture components act equally only differing in the concentrations for eliciting their toxic effects. The latter approach seems much more suitable for the cumulative risk assessment of the concomitant mycotoxins of one family mycotoxins such as DON, 3-ADON, and 15-ADON with the similarities of actions and toxicities. Zhu et al. and Wang et al. first developed the liquid chromatography-tandem mass spectrometry

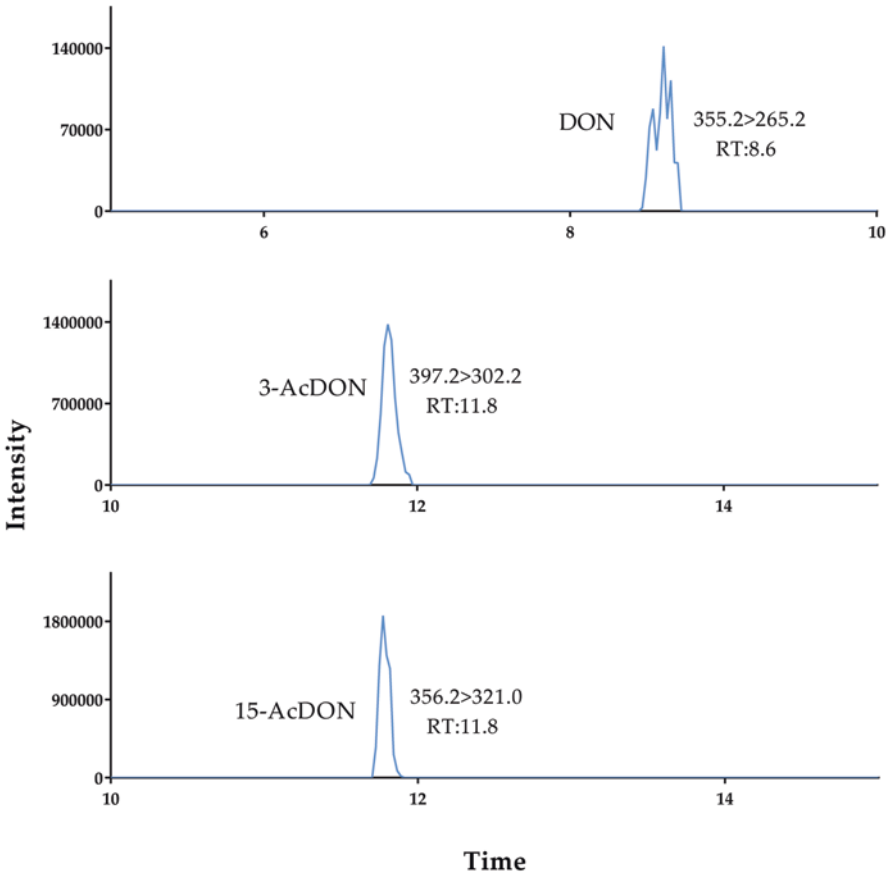

Fig. 4.1 The extracted ion chromatograms of DON (62.5 ng/mL) at the retention time of 8.6 min and 3-AcDON (125 ng/mL) and 15-AcDON (75 ng/mL) at the same retention time at 11.8 without no difference

(LC-MS/MS) method based on QuEChERS for determination of DON, 3-ADON, and 15-ADON in eggs and tissues (Zhu et al. 2015; Wang et al. 2018). Furthermore, the subsequent analysis of the estimated intake of DONs (DON, 15-ADON, and 3-ADON) was demonstrated in their study. Figure 4.1 showed the chromatograms of the DONs (DON, 15-ADON, and 3-ADON) in the chicken tissues.

4.2.1 Total Diet Studies (TDS)

Risk assessment, with the basis of the achieved data combined with mycotoxin levels in foods and consumption levels of foods, is of great importance for identifying mycotoxins as detrimental hazards at both an individual and a total level. Therefore,

it is the premise for risk assessment that international and regional specific data on mycotoxins was representatively collected in order to identify the representative groups of foods mainly contributing with mycotoxins exposure in different human populations. Exposure to fungal toxins in the diet of a given population can be assessed by different models and methods. The most accurate way to obtain data on the mycotoxin concentration of foods used for dietary exposure assessment is to purchase and analyze the foods that people eat.

Up to now, the exposure data could be collectively achieved by a total dietary study (TDS) approach, in which the occurrence and concentration of mycotoxins in the targeted foods are used for a refined dietary exposure assessment. The resulting exposure data obtained is representative and accurate for a specific food item or a specific population because TDS data are based on actual food consumed in the whole diet. In the last decade, the TDS approach has been widely applied worldwide to assess the risk of multi-mycotoxins in foods (Egan et al. 2002; Sirot et al. 2013; Raad et al. 2014). TDS are designed to measure the average amount of a given food consumed by the studied population. This public-health-oriented approach differs from traditional chemical-monitoring programs in that it focuses on chemicals in the overall diet rather than specific target foods. Also, taking into account, in part, the effects of home cooking on the breakdown or formation of chemicals are prepared for consumption before analysis. TDS possesses two main characteristics, (1) the representativeness of the samples and (2) preparation of "consumed" samples, making it a representative public health risk assessment tool in terms of food safety and nutrition. TDS is implemented in certain people; the key four steps include (1) the analysis of the target food; (2) the average and high food consumption data; (3) the sampling, preparation; and (4) the exposure assessment data obtained from consumption data multiplied by food chemicals concentration data. For example, total aflatoxins exposure levels through consumption of rice were 21.7 ng/kg bw/day, which highlighted that it is necessary to assess the total dietary exposure to major mycotoxins using TDS data (Huong et al. 2016a).

4.2.2 Simulation via Point Evaluation and Monte Carlo

Ubiquitously at the global level, two mathematical approaches of point evaluation (deterministic approach) and Monte Carlo simulation (probabilistic analysis) are carried out for computation of the risk assessments for mycotoxin contaminations. The point evaluation model and the Monte Carlo assessment model are both based on the following equation:

$$y = x \times c / w \tag{4.1}$$

As abovementioned, y is the dietary intake (DI) values of the mycotoxins (μg/kg BW/day), while x is the mean consumption of the different foods (g/day). Besides, c refers to the concentration levels of the mycotoxins in different food (mg/kg), and

w is the body weight (kg). In the present study, non-detected referred to the levels of mycotoxins that were less than LOD values. The values of non-detected were defined as half of LOD values (LOD/2) for the mycotoxin dietary exposure assessment based on the criteria (Scoop et al. 2004). The Monte Carlo assessment model combined the mean food consumption data with the distributions of mycotoxins contaminated in the foods, which involves a scenario in which the levels of mycotoxins are modeled as distributions. Best fit distributions were formed for mycotoxins individually and in combination in food and also to the respective consumption data. The @RISK software package, version 7.0 (Microsoft, USA), was usually used to run a simulation of 10,000 iterations to perform the Monte Carlo assessment.

4.3 Occurrence and Risk Assessment of Aflatoxins in Different Foods

Among mycotoxins, aflatoxins (AFs) produced by *Aspergillus flavus* and *Aspergillus parasiticus* molds are contaminants of a wide range of crops, such as cereals, maize, and milk. The evaluation of epidemiological and confirmatory laboratory results demonstrated that there is sufficient evidence in humans for the carcinogenicity of naturally occurring mixtures of AFs, which have also been classified as a group I carcinogen by the International Agency (Pittet 1998). One of the metabolites of AFB_1 that can be detected in foodstuffs is aflatoxin M_1 (AFM_1). AFM_1 is a toxic metabolite of AFB_1. As we all know, milk is a kind of food of great nutritional values, which provides macro- and micronutrients for human growth, development, and health maintenance. The occurrence of AFM_1 in milk and dairy products has addressed the prevalence and consequent global concern. From the view of management on food safety, the potential contamination of milk by AFs is of great concern to the regulatory agencies, considering the exposure to humans through the daily intake (DI) of various forms and types of milk or derived products, as well as the products in which it is incorporated together. On this purpose, the European Commission (EC) has set up the maximum level (ML) for AFM_1 in raw bovine milk and heat-treated milk at 50 ng kg^{-1}.

Since AFM_1 is stable at high temperature and cannot be removed from milk by heating process, this toxin can be transferred into the milk products such as liquid milk, powdered milk, cheese, yogurt, and other milk-based products (Nadira et al. 2016; Shuib et al. 2017). Several studies have been published to estimate the dietary exposure of aflatoxins in different foods such as cereals and milk, and these results were summarized in Table 4.1. In 2016, Huong et al. collected a total of 1134 food samples and took a total dietary study approach in order to estimate the dietary exposure of AFB_1 and OTA for adults living in Lao Cai province, Vietnam (Huong et al. 2016b). The obtained results demonstrated that the dietary exposure to AFB1 (39.4 ng/kg bw/day) was much higher. Subsequent studies are needed to perform on evaluation of the efficiency of different storage and other techniques to reduce

Table 4.1 Occurrence and dietary exposure assessment by point evaluation of aflatoxins (AFs) in different foods

Country	Analyzed food (years)	Mycotoxins	Total samples	Positive samples	Mean value (μg/kg)	Range (μg/kg)	References
Lao Cai province, Vietnam	Rice (2016)	Aflatoxin B_1	81	55	2.69	2.4–3.02	Huong et al. (2016b)
	Wheat (2016)		27	0	1	0–2	
	Cereals (2016)		27	27	3.2	3.2	
	Beans (2016)		108	81	2.86	2.61–3.11	
	Meat (2016)		297	267	4.07	3.99–4.17	
	Eggs (2016)		108	108	5.33	5.33	
	Vegetables (2016)		27	27	2.47	2.47	
Rio Grande, Brazil	Whole milk (2016–2017)	Aflatoxin M1	26	19	/	0.06–3.67	Gonçalves et al. (2018)
	Skimmed milk (2016–2017)		11	7	/	0.04–1.05	
	Semi-skimmed milk (2016–2017)		12	9	/	0.09–1.4	
	Infant milk (2016–2017)		10	6	/	0.88–2.8	
Assomada City, Cape Verde)	Whole milk (2016–2017)	Aflatoxin M1	3	0	/	/	Gonçalves et al. (2018)
	Skimmed milk (2016–2017)		17	10	/	0.32–0.72	
	Semi-skimmed milk (2016–2017)		16	11	/	0.21–2.21	
	Infant milk (2016–2017)		17	17	/	1.28–2.89	

(continued)

Table 4.1 (continued)

Country	Analyzed food (years)	Mycotoxins	Total samples	Positive samples	Mean value (μg/kg)	Range (μg/kg)	References
Terengganu, Malaysia	Powder milk (2013)	Aflatoxin M1	11	3	/	0.003–0.086	Nadira et al. (2016)
	Liquid milk (2013)		12	4	/	0.007–0.012	
	Cultured milk (2013)		7	4	/	0.003–0.1	
	Yogurt (2013)		5	2	/	0.007–0.025	
	Cheese (2013)		2	2	/	0.005–0.022	
Punjab, Pakistan	Chicken meat (2011–2012)	Total AFs	115	40	2.40	0.05–8.01	Iqbal et al. (2014)
	Eggs (2011–2012)		80	22	1.97	0.05–4.46	

mycotoxin contamination. In 2018, 112 milk samples (whole, skimmed, semi-skimmed, liquids, and powders) were collected from a local commercial in southern Brazil and Assomada City, Cape Verde, by David, M. G. K et al. (Gonçalves et al. 2018). Sixty-eight percent of milk samples were contaminated by AFM_1 at concentrations of 40–3670 ng/L, and 16% of them were detected with concentrations ranging from 40 to 600 ng/L.

4.4 Occurrence and Risk Assessment of Ochratoxin A

Ochratoxin (OTA) was mainly produced by *Penicillium verrucosum*, *Aspergillus carbonarius*, and *A. ochraceus* (Wu et al. 2014). Several research have been previously reported that it is carcinogenic in the kidney of different experimental animals, which lead to numerous specific toxic effects on humans and animals including hepatotoxicity, teratogenicity, and immunosuppression (Mally 2012; Peter et al. 2015). OTA has been found in a wide variety of foods, such as grapes, cereals, beans, dried fruits, as well as their derived products (Amézqueta et al. 2009). Considering the toxicity of ochratoxin A, the European Food Safety Authority (EFSA) have set a provisional maximum tolerable daily intake (PMTDI) for it at 17 ng/kg body weight/day. OTA constitutes a potential risk for human and animal health due to their heat stability, diverse chemical and biological properties, and toxic effects, which are extremely variable. Therefore, the contamination levels should be assessed in further estimation of the consumers' exposure. A set of studies have been reported to estimate the dietary exposure of OTA in a variety of foods,

Table 4.2 Occurrence and dietary exposure assessment by point evaluation of ochratoxin A in different foods

Country	Analyzed food (years)	Mycotoxins	Total samples	Positive samples	Mean value (μg/kg)	Range (μg/kg)	DI/PMTDI (ng/kg body weight/day)	References
Lao Cai province, Vietnam	Rice (2016)	OchratoxinA	81	0	0.95	0–1.9	7.9/17	Huong et al. (2016b)
	Wheat (2016)		27	0	0.95	0–1.9	0.3/17	
	Cereals (2016)		27	0	0.95	0–1.9	0.3/17	
	Beans (2016)		108	54	0.97	9.21–10.16	0.2/17	
	Meat (2016)		297	190	2.69	2.34–3.03	5.0/17	
	Eggs (2016)		108	81	3.16	0.29–3.4	1.4/17	
	Vegetables (2016)		27	0	0.95	0–1.9	0.6/17	
Valencia	Fish (2016)	OchratoxinA	53	2	0.27	/	0.34/17	Carballo et al. (2018)
	Meat (2016)		22	4	8.09	/	9.34/17	
Shanghai, China	Cereals (2012)	OchratoxinA	100	21	0.94	0–7.22	1.093/17	Zheng et al. (2013)
	Beans (2012)		100	14	0.59	0–1.59	0.029/17	
Punjab, Pakistan	Chicken meat (2011–2012)	OchratoxinA	115	47	1.41	0.06–4.7	/	Iqbal et al. (2014)
	Eggs (2011–2012)		80	28	1.17	0.06–2.98	/	

and these results were summarized in Table 4.2. For example, the mean value of daily intake (DI) of OTA was 1.147 ng/kg body weight/day, which was lower than all the reference standards (Zheng et al. 2013). However, DI value (8.566 ng/kg body weight/day) in the high percentile (97.5th) was obviously higher than the PTDI (5 ng/kg body weight/day) proposed by Scientific Committee on Food. To conclude, the results of their study suggested that strict control of food should be of considerable importance in reducing the risks to human health.

4.5 Occurrence and Risk Assessment of Deoxynivalenol and Its Acetyl Derivatives

To date, the most frequently found mycotoxins are deoxynivalenol (DON) and its acetylated derivative of 3-acetyldeoxynivalenol (3-ADON) and 15-acetydeoxynivalenol (15-ADON) in maize and wheat (Jin et al. 2010; Wang et al. 2012). Deoxynivalenol could lead to chronic and acute human diseases such as nausea, vomiting, gastrointestinal upset, and diarrhea (Marin et al. 2013). As reported, acetylated DONs showed a stronger toxicity because these toxins were more rapidly absorbed into the intestine (Pinton et al. 2012). In addition, the acetylated products of DON could be excreted directly, and thus consumer health risks could be originated by hydrolysis of these conjugates during mammalian digestion (Boevre et al. 2013). Consequently, the acetylated forms (3-ADON and 15A-DON) have received increasing attention and are considered as a critical contribution to total dietary exposure to the DON family mycotoxins. In 2010, the previous Provisional Maximum Tolerable Daily Intake (PMTDI) of 1 μg/kg BW/day for DON has been extended to a group PMTDI for the three compounds by JECFA in 2010 (Ng 2010).

Several studies have been performed to evaluate the total health risks of DON, 3-ADON, and 15-ADON, as single family compounds (Sirot et al. 2013; Soubra et al. 2009). However, these compounds in a mixture can work synergistically and certainly cause synergistical effects greater than the individual component. Hence, assessing mycotoxins singly should underestimate the total risks of DON family mycotoxins. In a word, the cumulative health risks of concomitant exposure via dietary intake (DI) to multiple mycotoxins occurred, namely, DON and its acetyl derivatives of 3-ADON and 15-ADON, based on the concentration addition (CA) concept.

Table 4.3 summarizes the occurrence and dietary exposure assessment of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), and 15-acetyldeoxynivalenol (15-ADON) in different foods. Zheng Han et al. assessed the health risk of DON and its acetyl derivatives 3-ADON and 15-ADON using samples collected from 2009 to 2012 (Han et al. 2014). A total of 330 samples involving heat (n = 198) and maize (n = 132) were purchased from located supermarkets in Shanghai, China (Han et al. 2014). In their study, mycotoxins (DON, 3-ADON, 15-ADON) were individually assessed for the health risks, and the combinations of these mycotoxins were divided into four groups: DON +3-ADON, DON +15-ADON, 3-ADON +15-ADON, and DON +3-ADON +15-ADON. They recommended that the derivatives of DON should be included in routine monitoring and control programs of food safety administration. Raw barley (n = 31) and soup of barley (n = 21) were purchased from different local markets in Tunisia between November 2015 and January 2016 in the study of Juan C. et al. (Juan et al. 2017). They investigate the presence of DON and its derivates, and the result reported that 62% of barley samples and 71% of soup of barley samples presented contents of mycotoxins. Even if no toxicological concern is arising for Tunisians through barley

Table 4.3 Occurrence and dietary exposure assessment by point evaluation of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), and 15-acetyldeoxynivalenol (15-ADON) in different foods

Origin	Analyzed food (years)	Mycotoxins	Total samples	Positive samples	Mean value	Range (μg/kg)	DI/PMTDI (ng/kg body weight/day)	References
Shanghaii, China	Maize (2011–2012)	DON	50	50	116.0	0.5–584	1.89/1000	Han et al. (2014)
		3-ADON	50	50	2.0	0.7–8.4	0.03/1000	
		15-ADON	50	50	23.9	0.5–242	0.39/1000	
		Total	50	50	141.9	3.3–834.4	2.32/1000	
	Wheat (2011–2012)	DON	38	38	106.5	0.5–604.0	152.6/1000	
		3-ADON	38	38	10.3	0.7–35.2	14.8/1000	
		15-ADON	38	38	1.4	0.5–6.2	2.01/1000	
		Total	38	38	118.2	3.0–628.8	169.4/1000	
Tunisia	Barely (2015–2016)	DON	31	7	2.9	1.7–6.1	0.14/1000	Juan et al. (2017)
		3-ADON	31	0	0	0	0	
		15-ADON	31	2	7.5	7.3–7.7	0.37/1000	
	Soup of barely (2015–2016)	DON	21	6	3.3	1.6–5.6	0.06/1000	
		3-ADON	21	0	0	0	0	
		15-ADON	21	4	7.5	7.3–7.7	0.22/1000	
Valencia	Cereals (2016)	DON	88	53	3.39	/	2.9/1000	Carballo et al. (2018)
		3-ADON	88	3	0.08	/	0.006/1000	
		15-ADON	88	13	0.21	/	0.01/1000	
	Legumes (2016)	DON	24	5	1.75	/	0.2/1000	
		3-ADON	24	0	0	/	0.002/1000	
		15-ADON	24	2	1.15	/	0.13/1000	
	Vegetables (2016)	DON	141	19	1.53	/	3.06/1000	
		3-ADON	141	0	0	/	0.04/1000	
		15-ADON	141	3	0.27	/	0.54/1000	
	Fish (2016)	DON	53	10	1.19	/	1.53/1000	
		3-ADON	53	5	0.15	/	0.19/1000	
		15-ADON	53	5	0.35	/	0.45/1000	
	Meat (2016)	DON	22	4	1.64	/	1.87/1000	
		3-ADON	22	0	0	/	0.02/1000	
		15-ADON	22	0	0	/	0.02/1000	

and derived products consumption, mycotoxins could represent potential threat for infants. A study on ready-to-eat meals including cereals, legumes, vegetables, fish, and meat was carried out to determine the natural presence of multi-mycotoxins via the developed LC-MS/MS method coupled with QuEChERS extraction (Carballo et al. 2018). In their study, a total of 328 samples, representative of the Mediterranean diet, were randomly collected from the university restaurant mainly from September to December 2016. DON was the most frequently found mycotoxin in the vegetables, meat, fish, and cereals, with an incidence of 13%, 18%, 19%, and 60%, respectively, and the highest mean levels were found in fish (1.19 μg/kg) and vegetable (1.53 μg/kg), respectively. Palacios, Sofía A. et al. collected durum wheat (n = 84) samples from three different areas in South Argentina (Palacios et al. 2017). Also, DON was found in all collected samples at the concentrations varying between 50 μg/kg and 9480 μg/kg. Furthermore, the acetylated derivatives of DON were also detected but at a lower frequency of 49%. Consequently, further studies are required for more strict food safety policies worldwide.

4.6 Conclusion

Humans could be naturally and frequently exposed to a multitude of mycotoxins, but health risk assessments are usually performed on individual mycotoxin, which might underestimate the total risks. Therefore, studies of exposure assessment and toxicological evaluations are needed to determine the health effects of co-occurrence and exposure to different classes of mycotoxins. Assessing human exposure to mycotoxins is a key step in risk assessments on public health. A better understanding of these risks could lead to the evidence-based decision-making process, which provides improved risk management at national and international levels. Recently, quantitative cumulative health risk assessments have been conducted using dietary intake (DI) of multiple mycotoxins, considering the high toxicity and co-occurrence of the multi-mycotoxins in target foods to different human populations.

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Chapter 5
Metabolism of Mycotoxins and the Potential Biomarkers for Risk Assessment

Bingxuan Jia, Song Yu, Qinghua Wu, and Aibo Wu

Abstract Currently, most metabolic research on mycotoxins focuses on four classes of toxins, aflatoxins, trichothecenes, zearalenone, and fumonisin. These types of mycotoxins are widely contaminated by food crops. Ingestion by human and animal intake can cause different types and degrees of harm. For example, deoxynivalenol from members of the trichothecene family can cause nausea, vomiting, and refusal to eat; zearalenone can cause animal infertility, miscarriage, stillbirth, and liver dysfunction; and fumonisin can cause kidney damage, etc., which have caused great losses to the animal husbandry and may even threaten human health. Till now several metabolites from a few of mycotoxins have been roughly identified. Simultaneously, the cytotoxicities of mycotoxins and related metabolites were evaluated by experimental animal and various cell tests. The molecular mechanisms of the toxicities were further explored to achieve more data of toxicities from one single or mixed mycotoxins on some occasion. This would provide some new venues or menace reminding from possible thresholds legislation of mycotoxins and related metabolites to guarantee safer foods in the future.

Keywords Metabolism · Mycotoxins · Biomarker · Risk assessment

5.1 Introduction

Mycotoxin contamination is a serious problem for the world. Trichothecene is a group of mycotoxins produced by *Fusarium* genus, which have four types (A–D) in nature (Table 5.1) (Wu et al. 2017). T-2 toxin and deoxynivalenol (DON) are the

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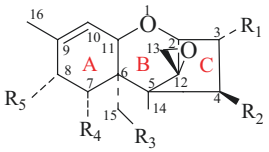
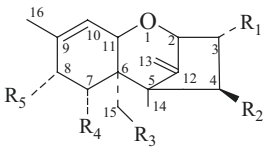
Table 5.1 Chemical structure type A and type B trichothecenes



Trichothenene



Deepoxy-trichothecene

Compound	R_1	R_2	R_3	R_4	R_5
Type A					
T-2 toxin	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
Acetyl-T-2 toxin	OAc	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
Acetyl-HT-2 toxin	OAc	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
Neosolaniol (NEO)	OH	OAc	OAc	H	OH
15-Deacetyl-NEO	OH	OAc	OH	H	OH
T-2 triol	OH	OH	OH	H	$OCOCH_2CH(CH_3)_2$
T-2 tetraol	OH	OH	OH	H	OH
3′-OH-T-2 toxin	OH	OAc	OAc	H	$OCOCH_2C(OH)(CH_3)_2$
4′-OH-T-2 toxin	OH	OAc	OAc	H	$OCOCH_2CH(CH_3CH_2OH)$
3′-OH-HT-2 toxin	OH	OH	OAc	H	$OCOCH_2C(OH)(CH_3)_2$
3′-OH-T-2 triol	OH	OH	OH	H	$OCOCH_2C(OH)(CH_3)_2$
8-Acetyl-T-2 tetraol	OH	OH	OH	H	OAc
15-Acetyl-T-2 tetraol	OH	OH	OAc	H	OH
Deepoxy-HT-2 toxin	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
Deepoxy-T-2 toxin	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
Type B					
Diacetoxyscirpenol (DAS)	OH	OAc	OAc	H	H
3,15-Diacetoxyscirpenol (acetyl-DAS)	OAc	OAc	OAc	H	H
Scirpentriol (SCP)	OH	OH	OH	H	H
Deepoxy-SCP	OH	OH	OH	H	H
Calonectrin	OAc	H	OAc	H	H
Fusarenon-X (FX)	OH	OAc	OH	OH	=O
Deoxynivalenol (DON)	OH	H	OH	OH	=O
3-Acetyl-DON	OAc	H	OH	OH	=O
15-Acetyl-DON	OH	H	OAc	OH	=O
Deepoxy-DON (DOM-1)	OH	H	OH	OH	=O
Nivalenol (NIV)	OH	OH	OH	OH	=O
Trichodermin	H	OAc	H	H	H
Trichodermol	H	OH	H	H	H
Trichothecin	H	$OCOCHCHCH_3$	H	H	H

most important ones in the trichothecenes. T-2 toxin has the highest toxicity, whereas DON has a profile of wide contamination in nature and are harmful for humans and animals (Juan-García et al. 2019). Zearalenone is widely distributed and is contaminated to varying degrees in most parts of the world. It is commonly found in several foods and feeds in temperate regions of Europe, Africa, Asia, America, and Oceania. It has been detected in corn, wheat, sorghum, rice, etc. (Zinedine et al. 2007). ZEN and its derivatives are estrogen analogs that compete with estrogen for binding to estrogen receptors with strong estrogenic and reproductive toxicity (Gratz 2017).

The conjugated mycotoxins, also called masked mycotoxin, are quite crucial now in the field of mycotoxins due to their hard detection (Khaneghah et al. 2018). There are many forms of fumonisins, of which fumonisin B1 is one of the most contaminated and most harmful toxins and has been rated as a Class 2B carcinogen by the International Agency for Research on Cancer (IARC). Aflatoxins are mainly produced by *Aspergillus*, and most metabolites are identified, for example, AFB1, B2, G1, etc. (Ghadiri et al. 2019; Zarba et al. 1992). The most commonly occurring ones in fungi cultures are aflatoxins B_1, B_2, G_1, and G_2 and then aflatoxins M_1 and M_2 in milk.

In this chapter, we will summarize the metabolic pathway of the major mycotoxins including T-2 toxin, DON, AFB1, ZEN, and FB. The related metabolites would be selected as biomarkers after mining and further confirmation. All these information will provide abundant information for toxicology and food safety assessment.

5.2 Deoxynivalenol

Deoxynivalenol (DON) is a type B trichothecene and one of the most common mycotoxins found in cereals (Table 5.1). The metabolic studies of trichothecenes including DON are mainly performed in the 1980s. DON can be transformed to deepoxy metabolite, DOM-1. It is a very common metabolite in most species; however, the researchers have not found this metabolite in livers, and most reports are in the gut (Wu et al. 2014).

DOM-1 was reported in the urine and feces of rats. For example, in the earlier study of Lake et al. (1987), DOM-1 (Fig. 5.1) was identified in urine and feces after administrated of DON with dose 10 mg/kg. As mentioned, this metabolite was not identified in liver homogenate, suggesting that the deepoxy product can only produce by the microorganism in the gut (Worrell et al. 1989).

Pigs are more vulnerable to DON toxicity (Wu et al. 2009). The research group of Eriksen et al. (2003) has not found the deepoxides in urine and plasma of pig. Moreover, this metabolite is considered to be less toxic to DON. DON can be metabolized to DOM-1 in chicken gut (Yunus et al. 2012). Ross broilers at 7 day of age were fed either a basal diet (0.265 ± 0.048 mg of DON/kg), a low-DON diet

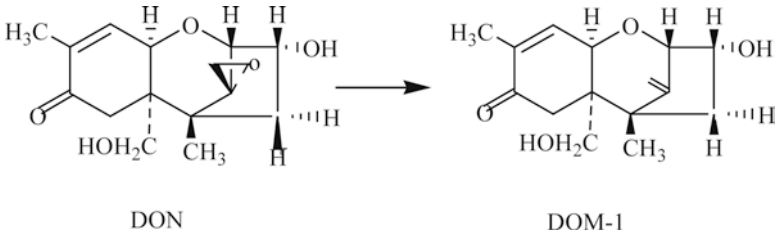


Fig. 5.1 Metabolic pathway of deoxynivalenol (DON) in rats

(1.68 mg of DON/kg), or a high-DON diet (12.209 ± 1.149 mg of DON/kg). Upon challenging the birds with a fixed amount of DON after week 5 of exposure, higher amounts of DON and the detoxification metabolite (DOM-1) were found at 5 h post-challenge in the guts of birds raised on the DON diets (Yunus et al. 2012).

Currently, there are some reports of DON metabolism in humans. For example, in the study of Warth et al. 2013, DON-15-glucuronide is the major metabolite besides DON-3-glucuronide. Eriksen et al. (2003) also noted that DON can be found when ingesting the 3-acetyl-DON-contaminated foods. However, the deepoxy metabolite DOM-1 has not found in these reports, indicating that human lacks the microflora for DOM-1. A recent study, showed that DON in chickens can be widely distributed in most tissues, and some metabolites including DON-3 alpha-sulfate were identified.

The biotransformation of masked DON is also highly concerned in humans. In the study of Nagl et al. (2012), DON-3-glucoside (D3G) can be biotransformed into DON and DOM-1 in feces. The authors also suggested that D3G has lower toxicity than that of DON. Also D3G can be metabolized into free DON during the process of digestion and increase the level of DON with higher toxicity (Nagl et al. 2014). In the study of Nagl et al. (2014), DON, DOM-1, and DON-15-G are the metabolites of D3G.

D3G cannot be degraded by acidic process in vitro, suggesting that this product cannot be degraded in the stomach. Warth et al. (2013) have not identified the free DON in rat urine, whereas in human, D3G to DON process is a very common metabolism process (Gratz et al. 2013), which increases the human toxicity burden. The proposed phase II biotransformation of DON in animals and humans is summarized in Fig. 5.2.

Currently, more studies have investigated the potential of DON or its metabolites as biomarkers in humans and animals for the risk assessment (Turner et al. 2008). Meky et al. (2003) found that 37% of DON is excreted in urine, and DON-glucuronide seems to be the major metabolite. From Linxian, China, DON were detected in human. Deng et al. (2018) found that in 151 healthy Chinese, DON and DOM-1 were detected in the urine samples. Dietary exposure to DON from contaminated cereal crops is also frequent in Europe. In the study by Turner et al. (2010), in French farmers, DON are detected in most samples, with less extent of DOM-1 as the metabolite. In another study, Turner et al. (2011) also reported that in UK adults, free DON were detected with mean 2.4 ng/ml, whereas urinary

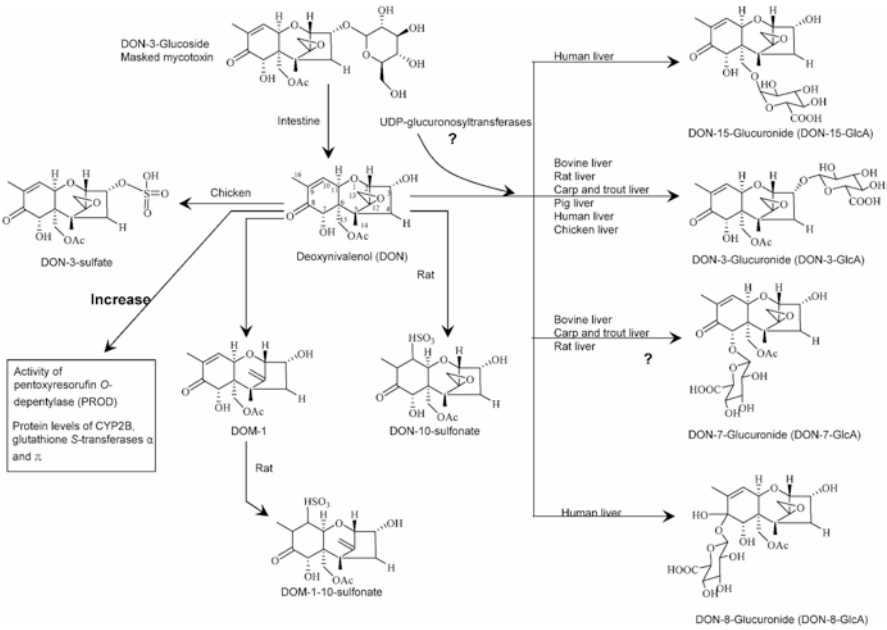


Fig. 5.2 Phase II metabolism of DON in animals and humans

DOM-1 was detected in 1/34 of individuals. A recent study also found that contamination level with above of 0.25 ng/ml DON is detected in 90% of tested elderly UK men and women. A recent human intervention study was conducted to unravel the urinary excretion profile and metabolism of DON and D3G (Vidal et al. 2018). In this study, DON-15-G was the most prominent urinary biomarker followed by free DON and D3G. DOM-3-sulfate was also detected in human as new biomarker of DON (Schwartz-Zimmermann et al. 2015; Warth et al. 2016).

5.3 T-2 Toxin

T-2 toxin is the most toxic one in trichothecenes. The metabolism of T-2 toxin is mainly studied in the 1980s. The liver and intestine are the major places that T-2 toxin can be biotransformed. Basically the major metabolic pathways of T-2 toxin are hydroxylation, hydrolysis, deepoxidation, and conjugation. Glucuronide-conjugated products of T-2 toxin are also reported. In the intestine, T-2 toxin can be biotransformed into HT-2 toxin, 3-OH-HT-2 toxin, 3-OH-T-2 toxin, and T-2 tetraol. T-2 toxin can be metabolized in the liver; however, there are very rare studies that investigated the metabolism of T-2 toxin in chicken liver (Young et al. 2007). In chickens, T-2 toxin can be absorbed rapidly, and most of T-2 toxin can be biotransformed into metabolites including HT-2 toxin (Sun et al. 2015).

The metabolism of T-2 toxin in humans is also reported. For example, Kemppainen et al. (1986) showed that in human skin, T-2 toxin can be metabolized into HT-2 toxin, T-2 tetral, and other metabolites that were not identified. In human fibroblasts, HT-2 toxin was the only metabolite (Trusal 1986). Besides HT-2 toxin, other metabolites including 3′-OH-T-2 and HT-2-glucuronide were detected in human colon carcinoma cells (HT-29) (Weidner et al. 2012). T-2 and HT-2 toxin both can enter the blood-brain barrier (Weidner et al. 2012). The proposed metabolic pathways of T-2 toxin in humans are summarized in Fig. 5.3.

Wu et al. (2012) had performed a study to investigate the global metabolic profile of T-2 toxin in different animal species including pigs, chicken, and carp. Metabolites including HT-2 toxin, NEO, 3′-OH-T-2, 3′-OH-HT-2, and T-2 triol were detected in these animals. Most importantly, the authors found that hydrolysis for the formation of HT-2 toxin is the major metabolic process in the land animals, whereas3′-OH-T-2 is the major metabolites in the fish. In pigs' intestine,

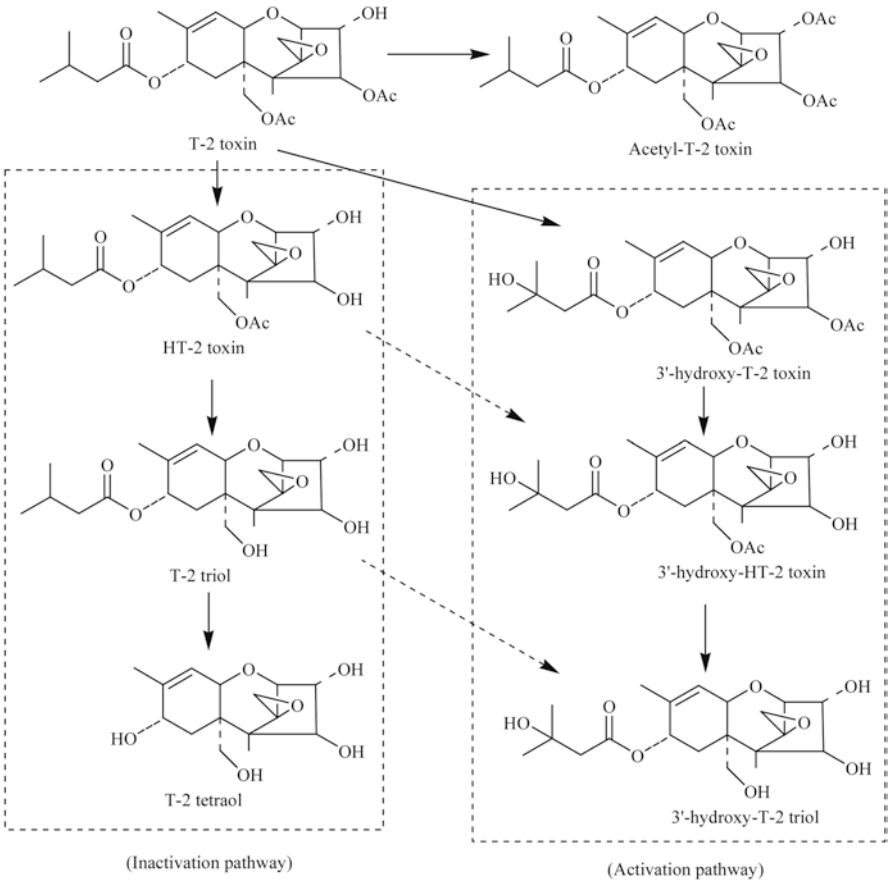


Fig. 5.3 Proposed metabolic pathways of T-2 toxin in humans

HT-2 is the major one (Wu et al. 2012), since HT-2 toxin still has high toxicity; therefore, the major toxic profile of T-2 toxin on the pigs are from HT-2 toxin and T-2 toxin together. A new metabolite HT-2-4-glucuronide was identified in pig's urine (Welsch and Humpf 2012). In addition, other new metabolites were tentatively identified by Yang et al. (2013) in rat liver microsomes, including 15-deacetyl-T-2 (Fig. 5.4). In vivo, hydroxylation at C-9 was found as the metabolic pathway of T-2 toxin for the first time.

Very recently, the metabolic pathway of T-2 toxin in chicken was studied (Yang et al. 2017). In the liver microsomes, HT-2 toxin was the major product, and the authors also reported 13 new metabolites, including 3′-hydroxy-T-2 3-sulfate and 3′,4′-dihydroxy-HT-2. For more detailed information, it can be found in the reference of Yang et al. (2017). Moreover, some metabolites including NEO, HT-2 toxin, and 3′-OH-T-2 toxin were found as the major ones in chickens.

Recently, the metabolic profile of T-2 toxin-3-glucoside (T2-Glc) in humans and rats was investigated by Yang et al. (2017). In their study, T2-Glc was biotransformed into T-2 toxin; moreover, this product was mainly metabolized in the gastrointestinal tract, and hydroxylation, hydrolysis, and deconguation are the major pathways of T2-Glc in this study. In humans, HT-2 and NEO are the major metabolic products, and some other products, for example, 4-deacetylneosolaniol were also detected in low levels. The metabolic pathways of T-2 toxin in animals are shown in Fig. 5.5.

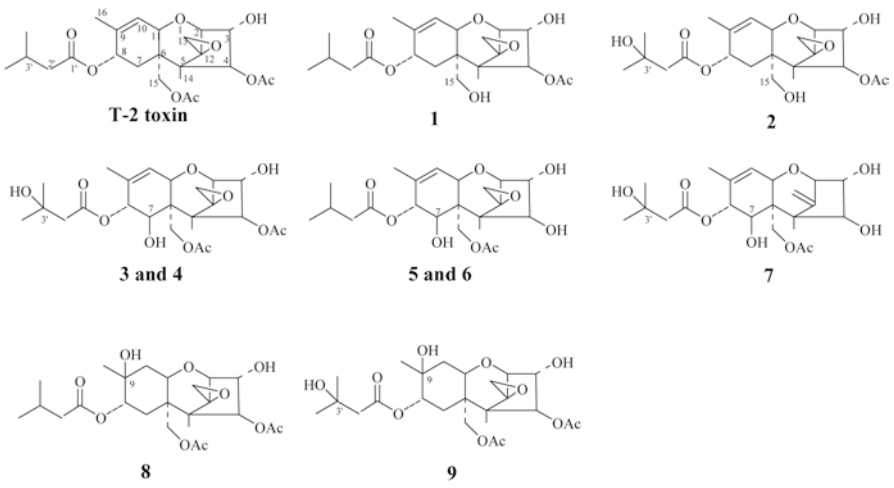


Fig. 5.4 Some novel metabolites of T-2 toxin in rats

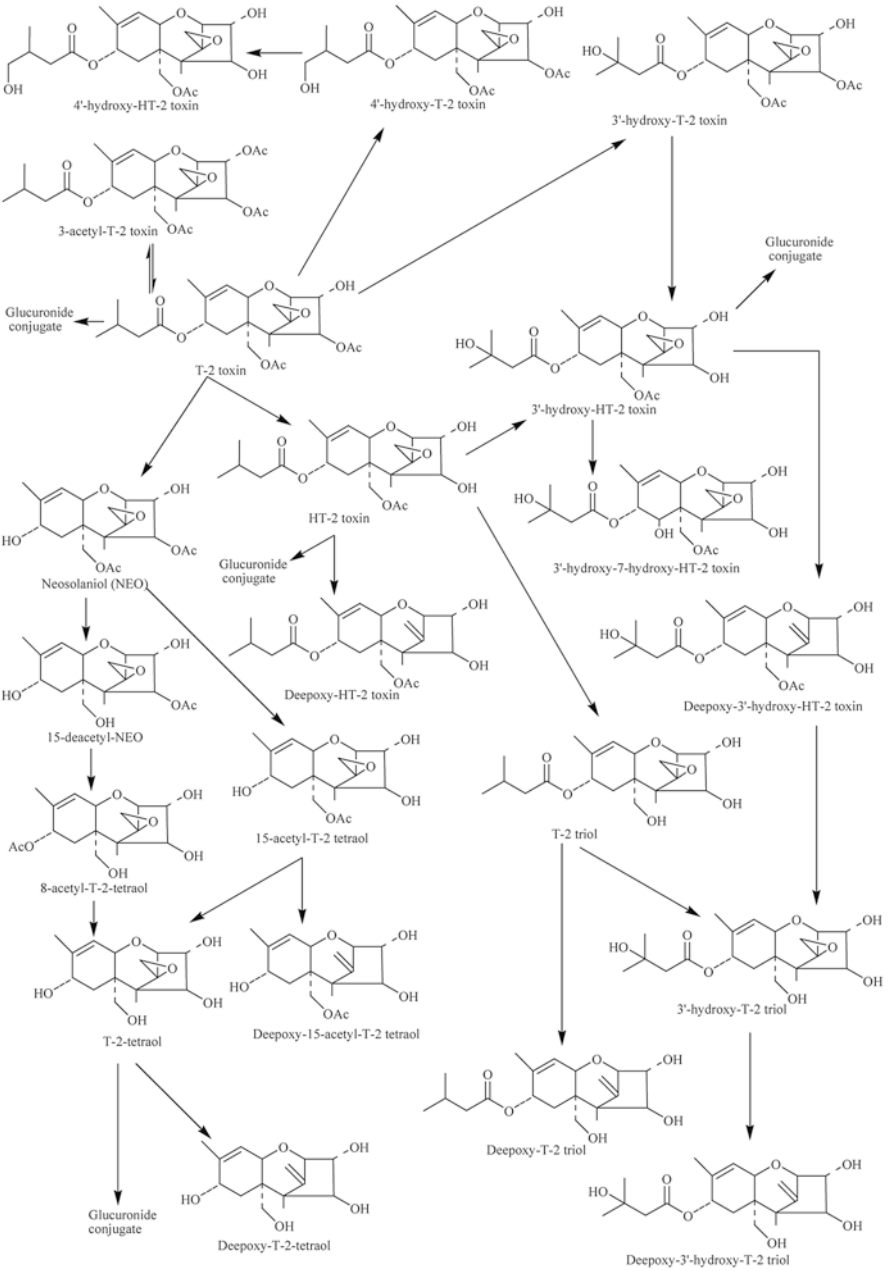


Fig. 5.5 Proposed metabolic pathways of T-2 toxin in animals

5.4 Aflatoxin B1

Aflatoxins are a group of chemically similar compounds that have been isolated, and more than 12 kinds of aflatoxins have been identified, including B1, B2, G1, G2, M1, M2, P1, Q, H1, GM, B2a, and toxic alcohols. The basic structure of aflatoxin is difuran ring and coumarin, and B1 is a derivative of dihydrofuran oxaphthalene. It contains a difuran ring and an oxaphthalene (coumarin). AFM1 is a metabolite derived from AFB1 by hydroxylation in the body. The main molecular formula of aflatoxin contains B1, B2, G1, G2, M1, M2, etc. M1 and M2 are mainly found in milk. B1 is the most toxic and carcinogenic substance (Wu et al. 2009). AFB1 can be biotransformed to other products by animals. AFQ1 is tranformed to AFH1 in the liver (Yiannikouris and Jouany 2002).

The metabolic enzymes in the metabolism of aflatoxins are studied, and most of the function enzymes are CYP450 enzymes. For example, in rat liver AFB1 can be metabolized to AFM1, AFQ1, CYP3A4, and CYP1A2 that plays an important role in the biotransformation of the aflatoxins in the human liver, whereas in the human lung, some other enzymes, including lipoxygenase and prostaglandin H synthase, seem to be more imporant in this context (Donnelly et al. 1996). CYP3A4 mainly metabolizes AFB1 to AFBO, and the activity of CYP3A5 and CYP3A7 shows much less low activity in this context. Notably, CYP450 has a role in the carcinogenic activation of AFB1 and should be highly concerned especially in the treatment of cancer (Kamdem et al. 2006). In turkey livers, CYP1A5 is the major metabolizing enzyme that responsible for AFB1 metabolism (Rawal and Coulombe 2011).

In addition, Lozano and Diaz (2006) further found that male chicken can produce more AFBO than females, and turkey and duck liver produce more AFL than chickens. The sex difference in rats is also reported (Dohnal et al. 2014), in which more AFB1-DNA bound compound are detected in males. Age is another factor that affects the metabolism, as documented by Klein et al. (2002), and younger birds seem to have more efficient metabolic activity of AFB1.

AFB1 metabolism in humans shows some region-dependent profile. In south China (Guangzhou), AFQ1 and AFM1 are the major metabolites in the human urine, whereas in Guangxi, AFP1 is the major one (Mykkänen et al. 2005; Groopman et al. 1992). In other countries, for example, less AFB1 is detected in Egyptian than that of Guinean. AFM1 is the major metabolite in these regions.

Currently, there is much evidence that an individual's susceptibility to cancer is determined by a different genetic background that detoxifies carcinogens. In fact, the carcinogenic potential of AFB1 varies with the ability of the exposed individual to detoxify its active intermediate, AFBO (Massey et al. 2000). The genetic polymorphism of the AFB1 metabolic enzyme is a major factor in the susceptibility of AFB1-associated HCC individuals. The GSTM1 or GSTT1 deletion genotype is closely related to HCC in AFB1 severely exposed areas. Molecular biomarker in urine and liver AFB1-DNA and serum AFB1-albumin adducts reflect the interaction of AFB1 terminal carcinogens with cellular target molecules (DNA and protein), which are direct products and substitutions of target molecules after damage. In addition to reflecting the intake of AFB1, the body's ability to absorb, distribute, and metabolize

should also be incorporated into evaluation criteria. Therefore, AFM1, AFQ1, and AFB1-N7-Gua adducts in urine and AFB1-albumin adducts in serum are commonly used as markers for human and animal exposure to AFB1 studies (Rawal and Coulombe 2011).

Due to the kinetic factors of metabolism of these compounds in the body, the urine AFB-DNA adduct only reflects the recent exposure level of AFB1, and the half-life of albumin in vivo is 2–3 weeks. The serum AFB1-albumin adduct is more reflective. A study of the negative results of a Taiwan survey is mentioned above followed by biomarker detection of AFB1 exposure to HCC. The results showed a dose-dependent relationship between the risk of AFM1 and HCC in urine. The high and low levels of AFB1 metabolites were five times higher in patients with chronic HBsAg (+); the risk of HCC was higher in patients with positive serum AFB1-albumin adducts than in negative ones (Dohnal et al. 2014). The major metabolic pathways of AFB1 are summarized in Fig. 5.6.

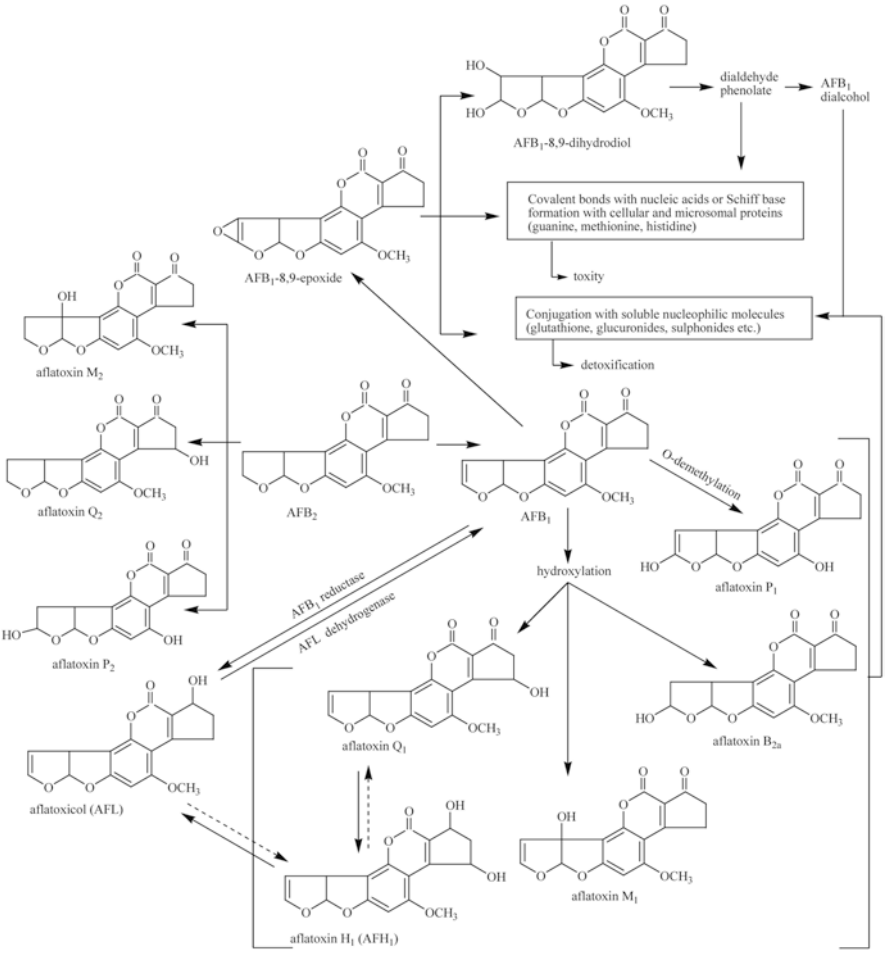


Fig. 5.6 Metabolic pathways of aflatoxin B_1 in animals

5.5 Zearalenone

Zearalenone (ZEN) is a secondary metabolite produced by the polyketone reaction by *Fusarium graminearum* (*Fusarium culmorum*, *Fusarium equiseti*, *Fusarium crookwellense*, etc.), also known as F-2 toxin (Bennett and Klich 2003). It was originally isolated from moldy corn contaminated with *Fusarium graminearum* (Stob et al. 1962). ZEN is a white crystal with a structure of 6-(10-hydroxy-6-oxo-trans-1-undecenyl) β-dihydroxybenzoic acid lactone. The molecular formula is $C_{18}H_{22}O_5$, and the melting point is 161~163 °C, insoluble in water but soluble in alkaline aqueous solutions, acetonitrile, chloroform, and other organic solutions (W.H.Urry et al. 1966). ZEN has various forms of phase I metabolism, such as α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), and zealanone (ZAN), of which the former two are the most common.

Its action on the reproductive system can lead to genital dysfunction and damage to germ cells, resulting in reduced animal offspring, stillbirth, and even infertility. In addition, ZEN has hepatotoxicity, hematological toxicity, immunotoxicity, and genotoxicity and is also associated with the development of estrogen-dependent cancers such as breast cancer (Ahamed et al. 2001).

ZEN is rapidly absorbed after oral administration, mainly in the liver and intestines. Its bioavailability is high, and it is widely absorbed in rats, rabbits, and humans. However, due to the presence of enterohepatic circulation, it is difficult to determine its absorption level (Mirocha et al. 1981). ZEN has two metabolic forms in mammals (Fig. 5.7), and enterohepatic circulation and bile excretion are important processes of ZEN metabolism, in which the enterohepatic circulation leads to prolonged retention of ZEN in the body, increasing its damage to the body:

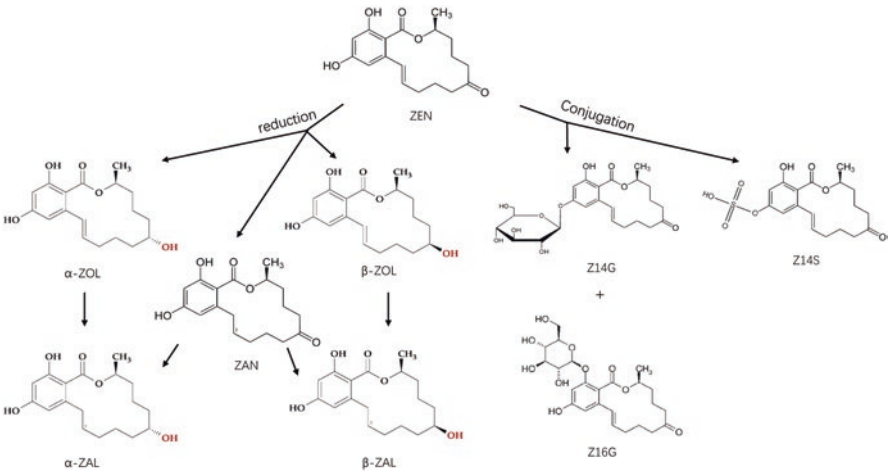


Fig. 5.7 Chemical structure of zearalenone and its metabolites

(a) Hydroxylation: reduction reaction, mainly occurring in the liver, which is reduced to α-zearalenol and β-zearalenol by the action of 3-hydroxysteroid dehydrogenase (Olsen et al. 1981). These two zearalenols can be further reduced to α-zearalanol and β-zearalanol (Rogowska et al. 2019), also known as phase I metabolites.
(b) Conjugation reaction: phase II metabolite, such as zearalenone-14-glucoside (Z14G) and zearalenone-16-glucoside (Z16G), which is catalyzed by uridine diphosphate glucuronyltransferase. In addition to the form conjugated with glucuronic acid, there are compounds which are conjugated to a sulfuric acid group, such as zearalenone-14-sulfate (Z14S) (Rogowska et al. 2019). In addition, the abovementioned conjugation reaction can also occur in the phase I metabolite of ZEN.

ZEN exerts different toxic mechanisms at different doses and in different cell types. Low concentrations of ZEN stimulate cell growth, mainly in estrogen-dependent tumor cells, as a result of estrogen effects (Gratz 2017). ZEN and its derivatives have different estrogenic activities and can be roughly arranged according to α-ZOL = α-ZAL> ZAN> ZEN = β-ZAL> β-ZOL (Parveen et al. 2009). The reason why α-ZOL and α-ZAL has stronger estrogenic activity than ZEN and other derivatives may be that α-ZOL does not bind to carrier proteins, thus increasing its affinity for estrogen receptors. In addition, due to the high proportion of α-hydroxylation in pigs, the metabolites in pigs are mainly α-ZOL, and the toxicity of α-ZOL is relatively large, so pigs are the most sensitive animals to the toxin. High concentrations of ZEN inhibit cell growth and cause cell death, including apoptosis and necrosis, primarily through damage to DNA and lead to oxidative stress in cells (Gratz 2017).

The most concerned about zearalenone is its reproductive toxicity as an estrogen analog, which seriously affects the development of animal husbandry. In addition to reproductive toxicity, ZEN also has a variety of toxicities, such as carcinogenicity, genotoxicity, immunotoxicity, etc.; the specific mechanisms are as follows:

(a) Reproductive toxicity and endocrine toxicity: As an estrogen analog, ZEN combines with estrogen receptors (ERs) to exert an estrogen-like effect. After dimerization with ERs, it enters the nucleus and binds to estrogen response elements, triggers target gene transcription and related protein synthesis, disrupts normal endocrine, and leads to endocrine toxicity. Specifically, ovarian development is promoted by activation of the ERs/GSK-3β-mediated Wnt-1/beta-Catenin pathway, and follicular growth is accelerated (Yang et al. 2018a). Estrogen-like effects in estrogen-sensitive hypothalamic AVPV and ARC nuclei further affect estrogen and luteinizing hormone levels and affect neonatal reproductive function (Parandin et al. 2017). What's more, ZEN can affect the production of testosterone by affecting the signal of estrogen receptor and the expression of Nur77, an orphan nuclear receptor, and also cause the apoptosis of sperm, testicular supporting cells, and other related cells through its cytotoxic effect, leading to reproductive disorders in male animals (Jee et al. 2010). Although some researchers have studied the mechanism of action of ZEN on

reproductive organs such as ovaries, there is still a lack of more detailed and systematic studies to explain the reproductive toxicity mechanisms of ZEN.

(b) Immunotoxicity: Damage to lymphoid organs, leading to atrophy of the thymus, leading to a decrease in multiple immune cells by inhibiting cell growth and promoting apoptosis (Hueza et al. 2014). In addition, it promotes an increase in the synthesis of the inflammatory cytokines IL-8 and IL-10 at low concentrations (Marin et al. 2015). Specifically, high concentrations of ZEN reduced serum IgG and IgM levels and affected lymphocyte proliferation and the proportion of T lymphocyte subsets (CD3 +, CD4 +, CD8 +, CD56 +) in peripheral blood (Yang et al. 2016). ZEN inhibits the expression of various cellular activation signals such as CD69, CD25, and CD71 by inhibiting the cell activity of Con a-mediated T cells and inhibits the expression of initiation regulatory proteins LAT, Lck, Zap-70, and p-Zap-70 during the activation of T cells. Meanwhile, ZEN interferes with nuclear transcription factors NFAT and nf-kb signaling pathways, and inhibits the secretion of cytokines IL-2, IL-3, IL-5, IL-6, and GM-CSF. In addition, ZEN exposure reduced the expression of the costimulatory molecule CD28 in T cells (Cai et al. 2018).

(c) Carcinogenicity: The excessive proliferation of cells by promoting the progression of the cell cycle. ZEN causes DNA damage, which further leads to mutations in DNA, which may promote oncogene expression and promote cell cycle progression, and leads to excessive cell proliferation (Zhang et al. 2018). At the same time, inhibition of gap junctional intercellular communication (GJIC) promotes oncogene expression (Ouanes-Ben Othmen et al. 2008), GJIC dysfunction is generally considered to be a precursor or prerequisite for tumors, and the invasion phase is primarily associated with loss of gap junction function (Mesnil et al. 2005). In estrogen-dependent tumor cell proliferation, mitogen-activated protein kinase signaling cascade (MAPK) is required for MCF-7 cell cycle progression (Ahamed et al. 2001).

(d) Genotoxicity: Induction of sister chromatid exchange and chromosomal aberrations and induction of DNA adduct formation in mouse liver (Ghedira-Chekir et al. 1998).

(e) Liver and kidney toxicity: ZEN is metabolized in the liver, which can induce liver damage, resulting in increased aspartate aminotransferase and alkaline phosphatase activity ($P < 0.05$) and decreased serum total protein and albumin concentrations ($P < 0.05$) (Gao et al. 2018) and causing ceramide synthesis disorders and unsaturation in hepatocyte membrane lipid fatty acids (FAs) at a small extent (Szabo et al. 2017). ZEN also induces oxidative stress in the kidney, resulting in increased malondialdehyde concentration and nitric oxide (NO) levels and decreased superoxide dismutase activity and hydroxyl radical inhibition, and also affects renal function indicators such as urea and uric acid levels, which may cause liver and kidney degenerative disease (Liang et al. 2015).

(f) Cytotoxicity: ZEN can induce oxidative stress in cells, lead to changes in mitochondrial membrane potential, promote the production of reactive oxygen species (ROS) in cells, and inhibit the expression of antioxidant system in cells (Yang et al. 2018b). At the same time, ZEN reduced Bcl2/Bax and promoted

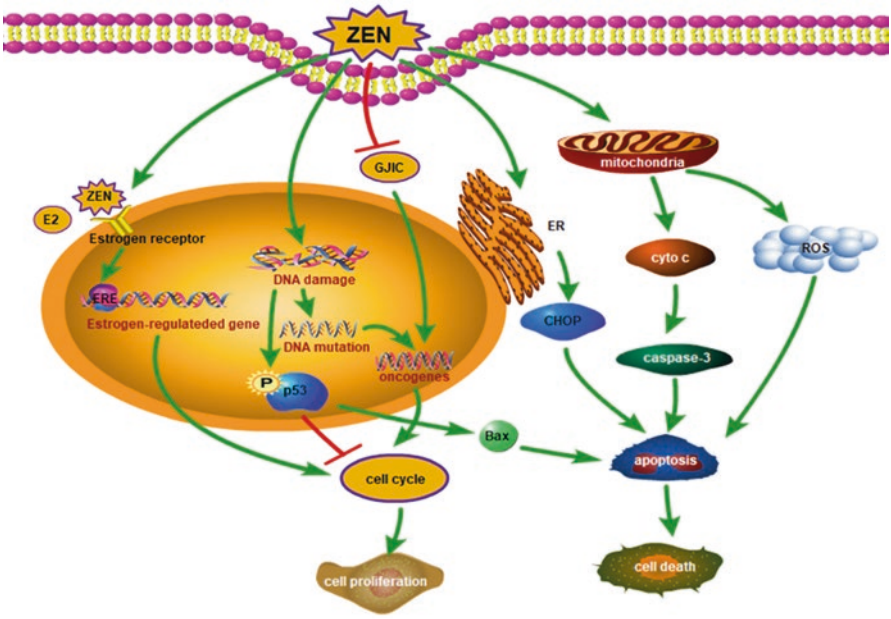


Fig. 5.8 Metabolic pathways of zearalenone in cell

caspase-3 activation, leading to apoptosis by triggering an endogenous apoptotic pathway (Yang et al. 2018b). It also causes endoplasmic reticulum stress and activates glucose-regulated protein 78 (GRP78) and CCAAT/enhancer-binding protein homologous protein (CHOP) to cause apoptosis (Chen et al. 2015). Therefore, ZEN may induce apoptosis by damaging mitochondria to promote ROS production (Li et al. 2014). At the same time, DNA is damaged, and cell cycle progression is repressed by the p53 pathway (Fig. 5.8).

5.6 Fumonisins

Fumonisin B (FUM B) is mainly a mycotoxin produced by *Fusarium moniliforme* and *Fusarium proliferatum*, which widely contaminates corn and its products (Marschik et al. 2013). There are many forms of fumonisins, of which fumonisin B1 is one of the most contaminated and most harmful toxins and has been rated as a Class 2B carcinogen by the International Agency for Research on Cancer (IARC). It has been reported that long-term consumption of fumonisin B1 in mice can cause liver and kidney tumors (Creppy 2002). Fumonisin B1 also causes defects in rat embryonic neural tube development by inhibiting the synthesis of embryonic sphingolipids (Sadler et al. 2002). In addition, fumonisin B1 can also cause reactive

oxygen species (ROS) in a variety of cell and animal models and triggers oxidative stress. High concentrations of reactive oxygen species (ROS) will cause redox homeostasis, leading to lipid peroxidation, membrane damage, DNA damage, and protein oxidation (Mishra et al. 2014).

There are four types of fumonisins, namely, FB1–FB4, which differ in the number and position of hydroxyl substitutions on the carbon chain (Table 5.2 and Fig. 5.9). Among them, the contamination rate and toxicity of FB1 are relatively high, so there are many studies of it.

The metabolism of fumonisins are mainly divided into the following types (Guerre 2015):

(a) Hydroxylation and dehydroxylation: The main difference between the four major fumonisins is the number and location of hydroxyl groups, which can be converted into each other by hydroxylation and dehydroxylation.
(b) Hydrolysis reaction: FB1 undergoes hydrolysis under the action of carboxylesterase and hydrolyzes to form its masked toxin HFB1. HFB1 is further converted to a keto group by the action of an aminotransferase to produce 2-keto-HFB1.
(c) N-acylation reaction: N-acylation reaction occurs in FB1 and HFB1 to form N-acyl-FB1 and N-acyl-HFB1, respectively (Fig. 5.10).

The kidney is the most sensitive organ to FB1. Oxidative stress can lead to lipid peroxidation on the cell membrane, leading to an increase in the levels of thiobarbituric acid reactants (TBARS) and malondialdehyde (MDA) (Klaric et al. 2007), the rise of TBARS occurs mainly in the liver (Gelderblom et al. 1992), and elevated MDA is detected in both blood and kidney, but not in the liver. Malondialdehyde

Table 5.2 Classification and structure of fumonisins

Classification	Structure
FB1	R1=OH, R2=OH
FB2	R1=OH, R2=H
FB3	R1=H, R2=OH
FB4	R1=H, R2=H

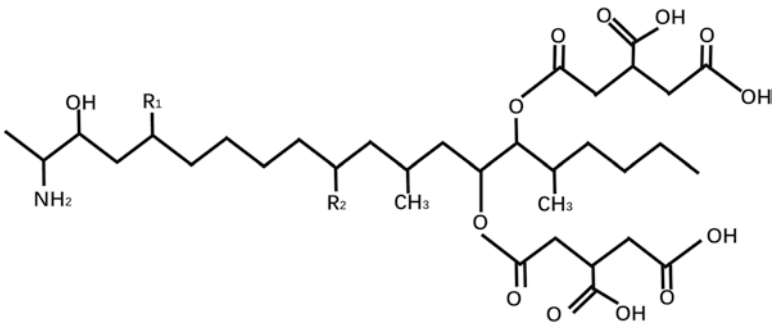


Fig. 5.9 Chemical structure of fumonisins

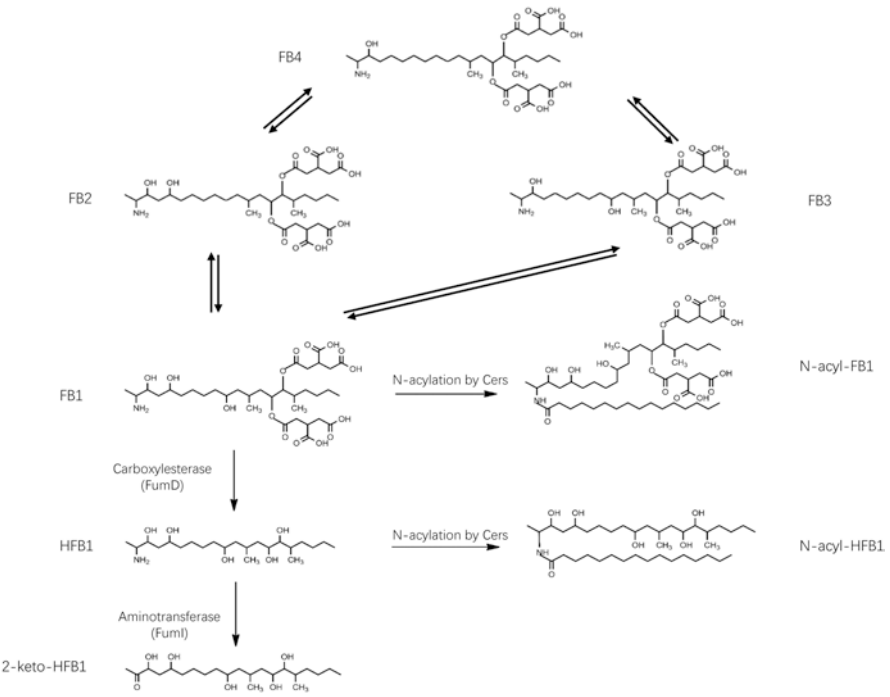


Fig. 5.10 Chemical structure of fumonisins and its metabolites

(MDA) level, as the main marker of lipid peroxidation, indicates that the kidney is more sensitive to oxidative stress caused by fumonisin B1 (Domijan et al. 2008; Domijan et al. 2007). In addition, FB1 (200 ng/kg bw and 50 μg/kg bw) was fed to male Wistar mice, which caused elevated levels of carbonyl compounds in the kidneys, but the level of carbonyl compounds in the liver remained almost unchanged, further indicating that the kidney is more sensitive to oxidative damage caused by FB1 (Domijan et al. 2007).

The specific mechanisms are as follows:

(a) Cytotoxicity: FB1 causes mitochondrial stress, endoplasmic reticulum stress, and DNA damage, resulting in increased levels of intracellular ROS while inhibiting various antioxidant enzymes such as superoxide dismutase (SOD), hydrogen peroxide, catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx), which in turn induces apoptosis or necrosis leading to cell death (Shi et al. 2015). Upon injury to DON, activation of p53 inhibits the cell cycle while promoting apoptosis by activating Bax (Mobio et al. 2003). At the same time, the disorder of intracellular redox state or the formation of ROS stimulates redox-sensitive signaling molecules such as mitogen-activated protein kinase (MAPK) and heat shock protein (HSP). The MAPK superfamily is a signal transduction cascade that is activated by many extracellular stimuli and

is involved in important regulatory roles such as in apoptosis. The three major MAPK family members in mammals are extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase, respectively. The three major MAPK family members perform different functions, such as the ERK signaling cascade can inhibit apoptosis, while the p38 signaling pathway promotes apoptosis (Wang et al. 2012; Wu et al. 2014). In addition, these proteins can also reduce ROS production, affect cellular GSH levels, and neutralize the toxic effects of oxidized proteins.

(b) Hepatorenal toxicity: The causes of hepatorenal toxicity are largely dependent on cytotoxicity, but the liver and kidney are sensitive to FB1 due to lipid peroxidation. Oxidative stress can lead to the peroxidation of lipids in cell membrane, which leads to the increase of thiobarbituric acid reactant (TBARS) and malondialdehyde (MDA) (Klaric et al. 2007).

(c) Genotoxicity: Oxidative stress causes DNA damage that produces 8-hydroxydeoxyguanosine (8-OH-dG) (Ihsan et al. 2011).

(d) Neurotoxicity: FB1 is similar in structure to sphingosine (So) and sphinganine (Sa) and can block the synthesis of sphingosine by inhibiting N-acyl sphingosine synthase and destroy the metabolism of sphingolipids or affect their functions. At the same time, it causes an increase in sphinganine, resulting in an increase in the ratio of SA/SO in tissues, blood, and urine, causing a toxic reaction (Fig. 5.11).

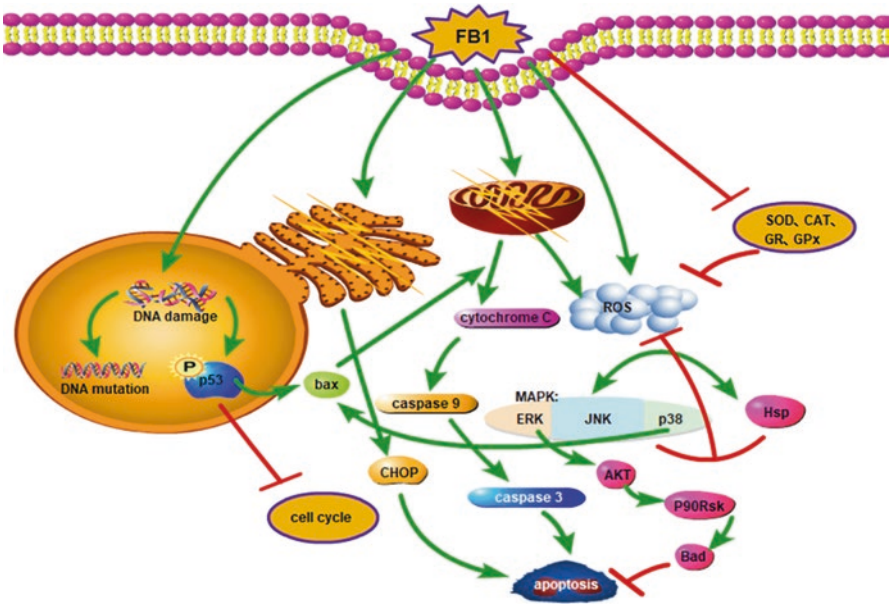


Fig. 5.11 Metabolic pathways of fumonisins B1 in cell

5.7 Conclusion

Metabolism of mycotoxins including the major trichothecenes and the most toxic one AFB1 has been deeply studied in the latest years. T-2 toxin is the most toxic one, and as dicussed in the above sections, T-2 toxin can be metabolized to less toxic compounds, however, as HT-2 toxin only reduce very less toxicity and usually show toxicity with T-2 toxin in animals and humans. Besides the free DON, more reseachers focus on the masked DON, and in the future study, the metabolism of the masked DON and other mycotoxins should be investigated. Although ZEN and FB1 are less toxic than DON, they widely contaminate corn and its products and cause serious harm to people and livestock after long-term exposure. Strict limits have been set in the United States and Europe. It should be noted that all these metabolic profiles are quite crucial for the identification of the biomarker of the mycotoxins in humans and animals for the risk assessments. More reports of human exposure of myctoxins including trichothecenes and aflatoxins are available from European countries; thus, further information is required from the United States and Asian countries.

At present, it is difficult to completely avoid the intake of mycotoixns due to the lack of economical and effective anti-fouling measures. Especially in some places including southern China, food such as corn, flowers, and rice and their products are still contaminated by T-2 toxin, DON, and AFB1 to varying degrees. The carcinogenicity of AFB1 is undoubted, and the metabolic process of mycotoxins in the human body is affected by the functional status of various metabolic enzymes. Further study of the metabolic enzyme gene polymorphism is important for the prevention of early detection and early treatment of HCC in susceptible individuals with mycotoxins.

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Part III
Control

Chapter 6
Origin of Mycotoxin-Producing Fungal Species

Dianzhen Yu, Jianhua Wang, Yan Tang, Dongqiang Hu, and Aibo Wu

Abstract Mycotoxins are certainly recognized as secondary metabolites by the fungal pathogens after invasion into the host plants, which are toxic or harmful to plants, animals and human beings. To trace back to the origin of mycotoxin contamination, the fungal pathogens are surely the targets and also the source of mycotoxin synthesis there, except for the hosts. Actually the types of produced mycotoxin are basically and genetically determined by the pathogenic microbes. Due to the expertise or more familiar characteristics, this chapter will cover more about *Fusarium* and *Alternaria* species, trying to elucidate their contribution to the network of mycotoxin biosynthesis. This would be very helpful to unveil molecular regulation mechanisms from the origins and control of mycotoxin contaminants from the basic components.

Keywords Mycotoxin-producing fungi · *Fusarium* · *Alternaria* · Mycotoxins

6.1 Introduction

To the best of our current knowledge, *Fusarium* and *Alternaria* species are the most agro-economically important phytopathogens for cereal grains and fruits, except for *Aspergillus* and *Penicellium* spp. In most cases, the fungal strains are simultaneously found on the host plants. For more food safety concerns originating from *Fusarium graminearum* and fewer reports on Alternaria toxins in fruits, we will focus more on those two types of fungal pathogens, especially for their capabilities on mycotoxin production.

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6.1.1 Fusarium graminearum

The most representative fungal phytopathogen in *Fusarium graminearum* species complex (FGSC) can cause *Fusarium* head blight (FHB) of wheat, barley, oats and other small cereal grain crops worldwide. Also, the maize ear rot caused by *Fusarium graminearum* is one of the most devastating diseases on maize. Warm and humid weather conditions at the flowering stage are conducive to disease development. Due to the changes in climatic conditions and in agricultural practices, outbreaks of FHB have occurred more and more frequently and seriously in China. From 2008 to 2015, serious yield loss of wheat caused by FHB occurred in more than five million ha each year. Over the past 20 years, economic loss due to FHB in Canada is estimated $50–$300 million annually (Dahl and Wilson 2018).

Previously, due to the failed recognition of morphological species to accurately assess the species, actual limits for the *Fusarium graminearum* species complex, FGSC, were considered as a single cosmopolitan species in nature. Applying the genealogical concordance phylogenetic species recognition (GCPSR), FGSC was first divided into seven phylogenetic lineages in 2000(O'Donnell 2000). Phylogenetic analyses of multilocus genotyping (MLGT) of DNA sequences from the portions of 13 housing keeping genes, combined with GCPSR and molecular marker technologies, reveal that this morphospecies comprises at least 16 biogeographically structured, phylogenetically distinct species (Sarver et al. 2011). After that, the species designation *Fusarium graminearum* has been sensu stricto in some conditions. Up to now, 15 of the 16 species have been formally described, including *F. acaciae-mearnsii*, *F. aethiopicum*, *F. asiaticum*, *F. austroamericanum*, *F. boothii*, *F. brasilicum*, *F. cortaderiae*, *F. gerlachii*, *F. graminearum* sensu stricto, *F. louisianense*, *F. meridionale*, *F. mesoamericanum*, *F. nepalense*, *F. ussurianum* and *F. vorosii*, and one additional species was informally recognized based on genealogical exclusivity and conidial morphology on SNA (Aoki et al. 2012). Some specific databases were created for *Fusarium* DNA sequence alignment analysis. For example, similarity searches of the obtained sequences can be performed with the Pairse DNA alignments network service of the *Fusarium* MLST database (http://www.westerdijkinstitute.nl/fusarium/), basic local alignment search tool (BLAST) network service of the *Fusarium* ID database (http://www.fusariumdb.org/index.php) and NCBI nucleotide database.

These phytopathogenic fungi are also of big concern because they can mainly produce trichothecenes, a group of mycotoxins that are very harmful to human and animal health. Up to now, more than 200 trichothecenes have been previously identified. Due to the diverse chemical structures, trichothecenes are divided into four types, namely, type A (having a single bond at carbon atom 8, C-8), B (having a keto at C-8), C (having an epoxide at C-7, 8) and D (having a macrocyclic ring between C-4 and C-15). All trichothecenes share a common tricyclic 12,13-epoxytrichothec-9-ene, and they are derived from the isoprenoid intermediate farnesyl pyrophosphate via a series of biochemical reactions in *Fusarium*. Among these mycotoxins, type B trichothecenes (Fig. 6.1) are the most common detected in cereal grains and

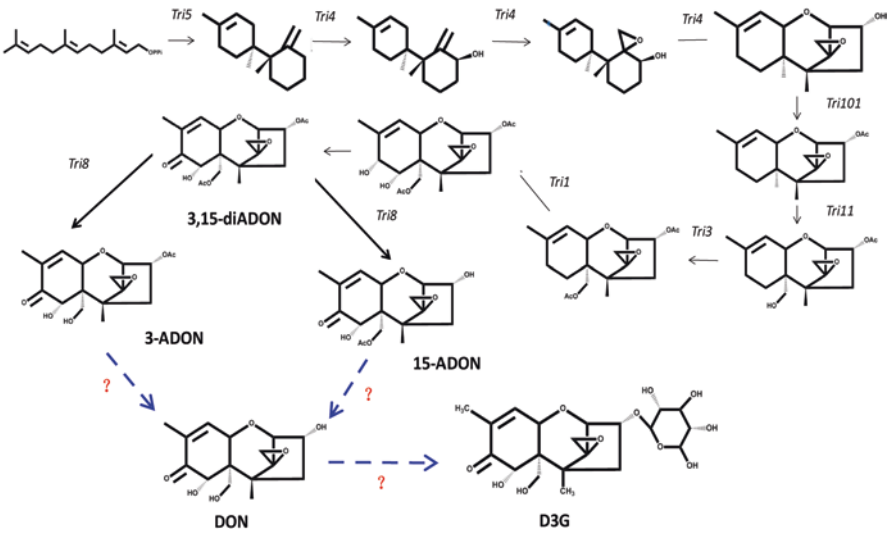

Fig. 6.1 The key genes required for the biosynthesis for the prototype deoxynivalenol (DON), its derivatives (3- and 15- ADON) and the masked form (D3G)

their related products. They are distinguished from type A by the presence of a keto function at C-8 and include deoxynivalenol (DON) and its acetylated forms 3-acetyldeoxynivalenol (3ADON) and 15-acetyldeoxynivalenol (15ADON), as well as nivalenol (NIV) and its acetylated form 4-acetylnivalenol (4ANIV). DON is characterized by the absence of a hydroxyl function at C-4, whereas NIV is characterized by the presence of a hydroxyl function at C-4. 3ADON and 15ADON are the acetylated forms of DON at C-3 and C-15, respectively. Meanwhile, NIV and 4ANIV can be differed by the absence (NIV) and presence (4ANIV) of an acetyl function at C-4 (Fig. 6.1).

Due to the ability of *Fusarium* strains to produce different kinds of trichothecenes, as majority, three strain-specific trichothecene chemotypes (genotypes) were identified in FGSC: the 3ADON chemotype produces DON and 3ADON, the 15ADON chemotype produces DON and 15ADON and the NIV chemotype produces NIV and its acetylated derivatives (Eriksen et al. 2004). The species composition and chemotype prevalence of FGSC vary widely in different regions, which reflects the level of risk factors in feed/food safety. Investigations on FGSC isolated from wheat, barley and maize crops have been reported in the last two decades (Reynoso et al. 2011; de Kuppler et al. 2011; Duan et al. 2016; Zhang et al. 2012). Dynamic changes in species composition and chemotype proportion have been found in different agricultural ecosystems worldwide. Prior to 2000, strains from the United States and Canada were almost exclusively 15ADON producers, while they have been increasingly replaced by the 3ADON producers in some major wheat-growing regions (Varga et al. 2015). Typically, the frequency of the 3ADON chemotype in western Canada increased more than 14-fold between 1998 and 2004

(Ward et al. 2008). The study by Barker (2009) indicated that the *F. graminearum* 15ADON chemotype is being replaced by the 3ADON chemotype. Also Kelly et al. (2015) analysed the trichothecene genotypes of FGSC collected from winter wheat fields in the eastern parts of the United States. They revealed an increasing gradient in 3ADON distribution from south to north and closer to Canada. The epidemiology data indicated that 3ADON chemotype dominates in Northern Europe, while 15ADON chemotype dominates in North America, Central Europe, southern Russia and some parts of Asia (Kelly et al. 2015). Type B trichothecenes are of the greatest concern in wheat- and barley-growing regions worldwide, because they can represent a major threat to food and feed safety. These toxins are potent inhibitors of protein synthesis and are responsible for neurologic, gastrointestinal, immune function and other disorders (Kuhn and Ghannoum 2003). For example, DON is associated with feed refusal, vomiting and suppressed immune functions, and NIV is more toxic to humans and domestic animals than DON (Pestka and Smolinski 2005). It was revealed that NIV was approximately four times more toxic than DON to human cells (Minervini et al. 2004). Conversely, DON is 10–24 times more toxic to plant cells than NIV (Shimada and Otani 1990). Determination of these chemotype variations is extremely important because the toxicity of DON and NIV might vary much according to the eukaryotic organism affected.

In a recent study, oral administration (PO) of infected wheat grains to mice revealed significantly higher toxicity of 3ADON compared to 15ADON (Broekaert et al. 2015). They are therefore also potent phytotoxins and act as virulence factors of pathogenic fungi and thus facilitate tissue colonization on sensitive host plants, e.g. of DON produced by *Fusarium graminearum* in wheat (Broekaert et al. 2015). Although only slight difference exists between the type B trichothecenes in the pattern of hydroxylation or acetylation, their toxicity and biological activity can be different greatly (Wu et al. 2010), indicating that chemotype differences may have important health consequences. Chemotype characterization has been extensively used to characterize FGSC for their toxigenic potential (Pasquali et al. 2010). Due to the toxicological differences between DON and NIV, it is important to monitor the population and determine the chemotypes of strains present in any given geographic region. Mycotoxin-producing capability of a certain strain could be established both through biochemical and molecular techniques. The biochemical approach involves the incubation and extraction of mycotoxins, the methods being complicated and time-consuming. The molecular techniques are based on detection of specific gene by using specific primers.

The genome sequences of several *F. graminearum* strains have been published. The trichothecene core gene cluster nucleotide sequences of many strain representatives of 3ADON, 15ADON and NIV genotypes have also been deposited in the GenBank. The availability of the information makes it possible to reveal the structural features and allowed the selection of several primer sets used successfully in PCR assays for the molecular characterization of various chemotypes. The ability to distinguish 3ADON, 15ADON and NIV chemotypes by a single PCR assays was reported by us previously (Nielsen et al. 2012).

The strains with different chemotypes showed different fitness to the ecological environment, such as the hosts, temperature, rotation and so on. 3ADON producer was more aggressive than 15ADON population in susceptible wheat, and also the 3ADON isolates exhibit a higher DON production than the 15ADON isolates. Similar conclusions revealed that *F. asiaticum* strains with 3ADON chemotype revealed significant advantages over the strains that produce NIV in pathogenicity, fungal growth rate and trichothecene accumulation. The achieved data also indicated that the growth of rice might be a crucial factor for the presence of *F. asiaticum* (Zhang et al. 2012). The fitness of three chemotype *Fusarium* strains were compared, and it was found that 15ADON producers had the advantage in perithecia formation and ascospore release, whereas more DON were produced by the 3ADON chemotypes (Liu et al. 2017). Duan et al. (2018) estimated the effects on rice or maize as former crops for mycotoxin accumulation in wheat grains, where they concluded that rice-wheat rotation favoured DON accumulation (Duan et al. 2018). Changes in DON chemotype distribution were reported for FGSC from Canada, the United States and Northern Europe. Recently, Nicolli et al. (2018) assessed a range of fitness-related traits (perithecia formation, mycelial growth, sporulation and germination, pathogenicity and sensitivity to tebuconazole) with 30 strains representatives of 3ADON, 15ADON and NIV producers. The pathogenicity assay results indicated that strains with the DON chemotypes were generally more aggressive than the NIV ones (Nicolli et al. 2018).

Modified form of trichothecenemycotoxins should be concerned with regard to food and feed safety. Now it is very clear that metabolization of mycotoxins in plants can lead to the generation of modified mycotoxins, likely with the conversion of DON to DON-3-glucoside or to DON-sulphates (Nobis et al. 2019; Warth et al. 2015; Poppenberger et al. 2003). These conjugates may be metabolized in vivo to DON and thus present a possible health hazard to consumers. Natural occurrence of a DON glucoside, DON-3-glucoside, in wheat has been reported (Nobis et al. 2019). More recently, EFSA considered it appropriate to assess human exposure to the modified forms of various toxins in addition to the parent compounds, because many modified forms are hydrolysed into the parent compounds or released from the matrix during digestion after consumption (Broekaert et al. 2017). Hence, modified mycotoxins could still pose a potential risk to food and feed safety. Also, a new series of type A trichothecene, NX-toxins, produced by FGSC were characterized. The distribution of NX-toxin producer in different wheat-growing areas and toxicity of these toxins should be further studied and accurately evaluated.

The knowledge about the mycotoxins chemotypes could contribute to a better management of fungal infections and breeding of resistance, in order to obtain grains of better quality. The results will also contribute to improve our understanding of the ecology and epidemiology of FGSC members, which may be of value for improving models for assessing the risk or epidemics and mycotoxin production.

To reveal the regulation mechanism, comparative proteome analyses were conducted to determine deoxynivalenol mycotoxin production difference in various FGC isolates. The mycotoxin phenotypes of three FGC isolates with different genetic backgrounds were determined in various media, including PDA, PDB, rice

and wheat in room and inoculated in the field. Analysis of characteristic proteins separated by two-dimensional gel electrophoresis revealed 80 proteins with significant differences in abundance ($P < 0.05$ and greater than threefold) in three selected FGC isolates. The 80 proteins were then identified through MALDI-TOF/TOF MS/MS analysis. As resulted, they were mainly involved in primary metabolism, protein metabolism and oxidoreductase activity. The identified proteins associated mainly with ten biological processes including biosynthesis, small molecular metabolism, cell nitrogen component metabolism, etc. Pathway analysis showed that 12 DEP were closely associated with the pathways linking with *Tri* genes of FG, which may be potentially new regulators of *Fusarium* mycotoxin production. The proteins involved in mycotoxin biosynthesis were proposed, which would provide scientific solid base for determining the molecular regulatory mechanisms on the production of deoxynivalenol (DON) family mycotoxins including the derivatives and masked forms in FG (data not shown).

6.2 *Alternaria* Toxin-Producing Species

As previously reported, *Alternaria alternate* and *Alternaria tenuissima* are the main pathogenic *Alternaria* species. They enable the production of different *Alternaria* toxins which are normally present in fruits and vegetables. Generally speaking, among varied *Alternaria* toxins, tenuazonic acid (TeA), alternariol (AOH) and alternariol monomethyl ether (AME) are the most considerable toxic metabolites according to the laboratory experiments and field investigations. Although no clarified standards for any of the *Alternaria* toxins, the US Food and Drug Administration identified the AOH as a new photosensitizing and DNA cross-linking agent, and TeA was also applied as a useful herbicide, suggesting its deep damage for organisms.

TeA was first identified by STICKINGS CE (Stickings 1959) in 1959; actually, from then on, little reports about its synthesis and regularity were reported. Until in 2015, Yun et al. (2015) elucidated the whole synthesized pathway of TeA via two TeA-inducing conditions of a low-producing strain in *Magnaporthe oryzae*. Non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) are responsible for the production of TeA; condensation, adenylation and peptidyl carrier protrein domain of NRPS are the enzymes, that catalyse the isoleucine and CoA to cooperate a linear structure of amino acid derivative. The ketosynthase domain of PKS is responsible for the final reacted step; double bond forms after the Dieckmann cyclization, and then we get the mature TeA. Actually we also identified similar enzymes in *Alternaria* spp.; the four domains were clustered in the same genome scaffold in sequence. Different from other typical fungal PKS-NRPS hybrid enzymes, TeA synthetic enzymes include more than one PKS domain and a reverse order of two enzyme clusters (Fig. 6.2).

AOH series is another important mycotoxin in *Alternaria* spp., which includes two main benzopyrones, AOH and AME. While the latter compounds were all

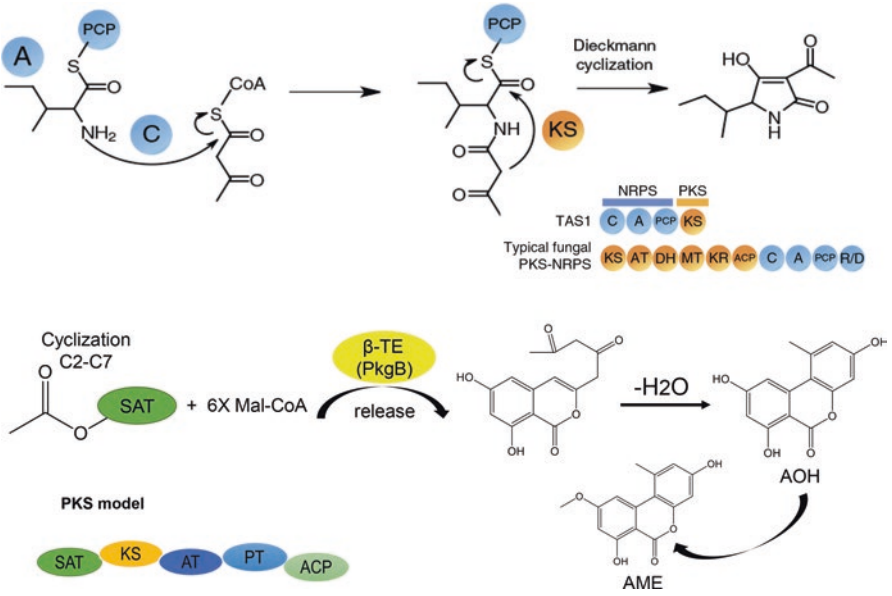

Fig. 6.2 Synthetic schemes of TeA (upper) and AOH (lower) and their representative catalysed modules

modified from AOH, omt1, a FAD-dependent monooxygenase was responsible for the catalysation of AOH to AME (Wenderoth et al. 2019). Just like TeA, little reports about AOH have been uncovered since the first report of its discovery by Raistrick et al. (1953). In 2012, Manmeet Ahuja et al. (2012) elucidated the synthesized steps of AOH incidentally via replacing the promoters of series PKS genes in *Aspergillus nidulans*, uncovering the mystery of its synthesis process. Different from TeA, PKS module is enough for the production of AOH. Starter unit-ACP transacylase, acyl transferase and acyl carrier protein consisted the catalysation of its original unit, Mal-CoA; after six times of such cyclization of C2-C7, basic structure of AOH forms. The final step of dehydration release is catalysed by another collateral gene, β-lactamase-type thioesterase, and then we get the mature product AOH (Fig. 6.2).

6.3 Conclusion

In order to understand or predict the production patterns and contamination profiles of mycotoxins in food, it is better to trace from the origin of mycotoxin-producing fungal strains, which normally co-existed with the host plants. As priorities, the genetic background elucidates the regulatory mechanism on biosynthesis of the target mycotoxins before and after invasion into the host plants and storage under different circustantial conditions. However, it is not so easy to completely know the network for mycotoxins produced in various stages, even from the same origin

isolates, due to the changing population, and so many forms of mycotoxins are produced. Especially for F. graminearum, the specific genes, proteins and small metabolites are needed to unveil in subsequent studies, to correlate with the source of mycotoxins which could around the food safety problems. Also, for *Alternaria* species and some host fruits, there are much things that are unknown, including the biosynthesis pathway of *Alternaria* toxins and the related genetic background responsible for each toxin. The obtained knowledge would be very helpful to predict and evaluate the risk potential and to propose the effective approaches for control of these target mycotoxins from the beginning of mycotoxin production.

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Chapter 7
Enzymes for Degradation of *Fusarium* Mycotoxins

Md Shofiul Azam, Dianzhen Yu, and Aibo Wu

Abstract *The Fusarium* mycotoxins are one of the agro-economically important toxins that seriously and frequently contaminate cereal-derived food and feedstuffs. The currently biological enzymes are widely applied in practice with ubiquitous acceptance for the desirably specific degradation of target mycotoxin contamination. Simultaneously, biotransformation of target mycotoxins by application with the specific enzyme is a suitable and practically favorable prevention measure. Major *Fusarium* mycotoxins studied in this section are the following: trichothecene, zearalenone, and fumonisins groups. The possible bi- or trifunctional enzymes for degrading various target mycotoxins are also first mentioned as the trend. This section also discussed various resources of these degradation enzymes. In the future perspective, as predicted, this enzymatic control measure needs to target the actual co-occurring mycotoxins to strengthen its industrial and economic importance. From this control strategy, especially through the large-scale application, cereal-derived food and feedstuffs will be obviously protected and will minimize the food loss to ensure food security. Ultimately, food safety problems will be somewhat resolved worldwide.

Keywords Enzyme · Control · Contamination · Degradation · Transformation · Mycotoxins

7.1 Introduction

Fusarium mycotoxins are the most agro-economically important fungal toxins. Trichothecenes (DON, NIV, T-2, HT-2, etc.), zearalenone, and fumonisins are the major representatives and most studied *Fusarium* mycotoxins (Mankeviciene et al.

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2006). *Fusarium* mycotoxins mostly contaminate cereal grains, a great variety of food products and feed, which seriously cause huge economic losses and pose a threat to animal production and human health worldwide. Depending on the type, toxicities and related mechanisms of *Fusarium* mycotoxins have been well investigated. More specifically, epigenetic modifications (DNA methylation, histone modifications, and regulation of noncoding RNA) have been implicated in various human diseases and the toxicities in animals caused by *Fusarium* mycotoxins, such ascarcinogenesis, genotoxicity, and reproductive disorders. Based on very recently documented data, this section discussed the relationship between epigenetic modifications and *Fusarium* mycotoxin-induced toxicities (Huang et al. 2019). The European Commission (EC) has already set legislative limits for DON and ZEN in cereal grains and cereal-based products intended for human consumption early in the year of 2006.

To the best knowledge we have right now, biodegradation of these mycotoxins has been considered as one of the best strategies to decontaminate food and feedstuffs. Biodegradation employs the application of microbes or functional enzymes to the contaminated food and feedstuffs. Several microbes from different niches have been previously reported to have a biotransformation capability. Biotransformation or cleaving and detoxifying mycotoxin molecules by microbes or enzyme is an effective and safer method for mycotoxin control (Upadhaya et al. 2010).

Mycotoxin biotransformation is defined as "the degradation of mycotoxins into nontoxic metabolites by using bacteria/fungi or enzymes." On some occassions, biotransformation referred to metabolism is the structural modification of a chemical by enzymes in the body. This represents a valid strategy, especially if multi-step reactions are required or if the microorganism is already implemented within industrial processes. On the other hand, in case of high levels of mycotoxin contamination, the increase and physiology of such microorganisms might be altered or inhibited, as a consequence requiringlonger time for adaptation before achieving satisfactory decontamination levels (Coward-Kelly and Chen 2007; Loi et al. 2017).

Enzymes are specific proteins that catalyze chemical reactions and extensively employed in biotechnological sectors. A protein is simply a polypeptide composed of amino acids linked by a peptide bond, while the term generally, but not always, refers to the folded conformation. To understand how an enzyme functions, including its binding and functional properties, it is necessary to know the properties of the amino acids and how the amino acids are linked together, including the torsion angles of the bonds and the space occupied. The interactions of the atoms lead to the final conformations of the folded protein. Only in the folded state can a protein function effectively as an enzyme to bind substrates and act as a catalyst (Lee and AJ 2003). This evidence proves the effective application of microbial enzymes for biodegradation of mycotoxins.

$$A \xrightarrow{E1} B \xrightarrow{E2} C \xrightarrow{E3} \ldots \xrightarrow{\text{En}} \text{metabolite}$$

This section focuses on the biotransformation of mycotoxins performed with purified enzymes isolated from bacteria, fungi, and plants, while the enzymatic activity was validated via in vitro and in vivo assays (Loi et al. 2017).

7.2 Enzymes that Degrade *Fusarium* Mycotoxins

7.2.1 Zearalenone(ZEN)

Zearalenone (ZEN), mainly produced by *Fusarium* molds, has been associated with hyperestrogenism and other reproductive disorders in pigs, sheep, and other farm animals. Figure 7.1 illustrated the chemical structure of ZEN.

Enzymes from the *Acinetobacter* sp. SM04 extracellular extracts of liquid cultures were isolated by Sephadex G-100 column with oxidation process (Yu et al. 2011). Lactonase catalyzed the hydrolysis. For instances, a ZEN-degrading enzyme-encoding gene zhd101was isolated from *Clonostachys rosea*, and its encoding products could specifically cleave the lactone ring of ZEN (Wang et al. 2018; Takahashi-Ando et al. 2002). After incubation of the enzyme against ZEN, we detected the earlier elusive major reaction product of hydrolyzed ZEN (HZEN) by LC-MS/MS, after purification by pre-HPLC, and confirmed its postulated structure ((E)-2,4-dihydroxy-6-(10-hydroxy-6-oxo-1-undecen-1-yl)benzoic acid) by nuclear magnetic resonance (NMR) techniques. Spontaneous decarboxylation to DHZEN ((E)-1-(3,5-dihydroxyphenyl)-10-hydroxy-1-undecen-6-one) was observed (Vekiru et al. 2016). The maximal activity of ZHD101 toward ZEN was measured at approximately 37–45 °C and pH 10.5 (k cat at 30 °C, 0.51 s^{-1}). The enzyme was irreversibly inactivated at pH values below 4.5 or by treatment with serine protease inhibitors (Takahashi-Ando et al. 2004). The pathways for ZEN biodegradation were proposed as follows: ZEN underwent a cleavage of the lactone ring, followed by a decarboxylation, indicating that ZEN degradation may be partially contributed to esterase activities (Fig. 7.2).

In the previous study, a novel detoxifying agent which used rice husk (RH) to immobilize ZEN-degrading enzyme (ZDE) was produced to reduce ZEN from *Aspergillus niger* FS10 (He et al. 2016). Table 7.1 shows ZEN degradation enzyme and resource organism.

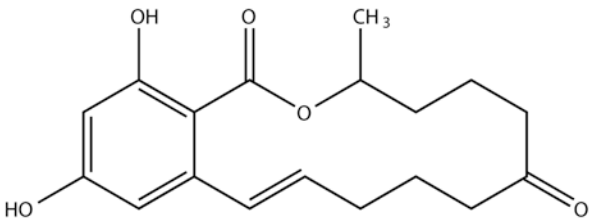


Fig. 7.1 The chemical structure of zearalenone (ZEN)

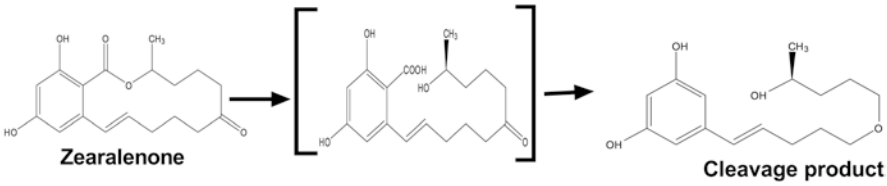


Fig. 7.2 Detoxification of ZEN. A hypothetical pathway for the detoxification of ZEN. Structures of ZEN (compound 1) and 1-(3,5-dihydroxyphenyl)-10h-hydroxy-1h-undecen-6h-one (compound 2) are indicated. A putative unstable intermediate is shown in square brackets

Table 7.1 ZEN controlling degradation enzymes

Enzyme	Producing organism	References
Laccase	*Trametes versicolor*	Novozymes (2009a)
Laccase	*Streptomyces coelicolor*	Novozymes (2009b)
Lactono hydrolase	*Clonostachys rosea*	Takahashi-Ando et al. (2002, 2005)
2cys-peroxiredoxin	*Acinetobacter* sp. SM04	Yu et al. (2012)
Lactonase	*Neurospora crassa*	Bi et al. (2018)

7.2.2 Fumonisins

Fumonisins are mainly produced by the phytopathogenic filamentous fungi of *Fusarium verticillioides* and *Fusarium proliferatum*. They frequently contaminate corn and corn-based products and cause, ingested with food or feed, several severe diseases in humans and animals. Fumonisins are associated with several mycotoxicoses, including equine leukoencephalomalacia, porcine pulmonary edema, and experimental kidney and liver cancer in rats. Chemically, fumonisins are diesters of propane-1,2,3-tricarboxylic acid and similar long-chain aminopolyol backbones. Structurally they are similar to the sphingoid bases sphinganine (Sa) and sphingosine (So), with tricarboxylic acid groups added at the C14 and C15 positions. This structural similarity is responsible for the action mechanism. It was once described to act using demanding the sphingolipids metabolism, with the aid of inhibiting the enzyme ceramide synthase and leading to accumulation of sphinganine in cells and tissues (Loi et al. 2017).

7.2.2.1 Fumonisin B1

Fumonisin B1 (FB1) is the most prevalent fumonisin and holds the highest risk for human and animal nutrition (Fig. 7.3). FB1 was shown to be carcinogenic and teratogenic and be linked with the etiology of esophageal cancer and neural tube defects in humans (Heinl et al. 2010).

Upadhaya et al. (2010) reported two genes, frame, encoding a carboxylesterase, and FumI encoding an aminotransferase which is responsible forFB1 degradation

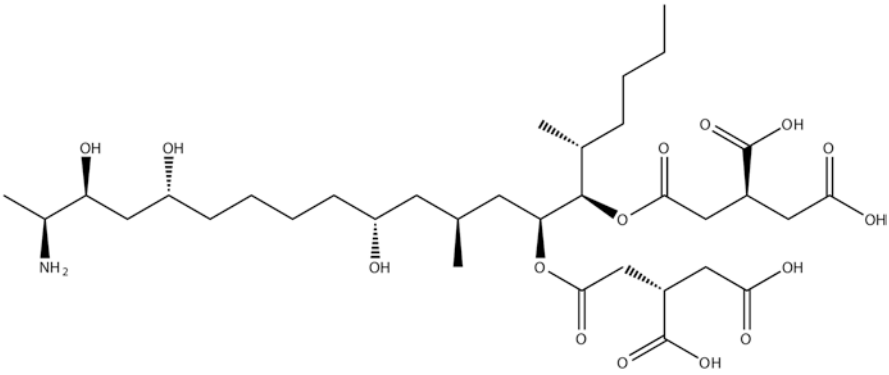


Fig. 7.3 The chemical structure of fumonisin B1 (FB1)

Table 7.2 The reported degrading enzymes of fumonisin B1 (FB1)

Enzyme	Producing organism	References
Carboxylesterase and aminotransferase	*Sphingomonas* sp. ATCC55552	Pioneer (1985)
Carboxylesterase B and aminotransferase	*Sphingopyxis* sp. MTA144	Heinl et al. (2009)
Carboxylesterase FumD	Gastrointestinal tract of turkeys and pigs	Masching et al. (2016)
Fumonisin esterase	*Sphingopyxis* sp. MTA144	EFSA (2014)

by *Sphingopyxis* sp. MTA144 (Upadhaya et al. 2010). New fumonisin-metabolizing bacterial strains have been isolated and characterized. Recombinant enzymes from *Sphingopyxis* sp. caused hydrolysis of FB1 to HFB1 by carboxylesterase with loss of the two tricarballylic side chains, followed by deamination of HFB1 by aminotransferase in the presence of pyruvate and pyridoxal phosphate (Heinl et al. 2010).

Enzymatic characteristics of aminotransferase FumI of *Sphingopyxis* sp. MTA144 were assayed for deamination of hydrolyzed FB1. With this basis, a technological application of FumI, in combination with the fumonisin carboxylesterase FumD for hydrolysis of fumonisins, for decontamination and detoxification of hydrolyzed fumonisins seems possible, if the enzyme properties are considered (Hartinger et al. 2011). Table 7.2 shows FB1 biotransformation enzyme and its origin organism.

The interactive effects of combined DON, ZEN, and FB1 on the fungal growth of brewing yeasts were examined. Yeast growth was assessed by measurement of dry weight or relative growth, cell number, viability, and conductance change of the growth medium using direct and oblique techniques. The interactive effect of a combination of these mycotoxins was subject to the ratio of toxins as the mixture and the toxicity of individual toxin on yeast growth. When a combination of mycotoxins at low concentration was added into the growth media, no significant inhibitory effect on growth was observed when compared to controls. However, when a

combination of high concentrations of DON and ZEN which individually inhibited yeast growth was examined, the interactive effect was shown to pass from antagonism to synergism depending on the ratio of the mixed toxins. The ability to reduce *Fusarium* toxins by fermentative bacteria was evaluated in vitro (Niderkorn et al. 2006).

Initial steps of FB1 degradation pathway of *Sphingopyxis* sp. MTA144: FB1 (2-amino-12,16-dimethyl-3,5,10-trihydroxy-14,15-propan-1,2,3 tricarboxyicosane) are the substrate of the fumonisin carboxylesterase FumD, which catalyzes hydrolytic cleavage of both tricarballylic acid (TCA) chains off the core chain to produce HFB1 (2-amino-12,16-dimethylicosane-3,5,10,14,15-pentol) and tricarballylic acid (1,2,3-propanetricarboxylic acid). Aminotransferase FumI transfers the 2-amino group from HFB 1 to pyruvate, producing 2-keto-HFB 1(3,5,10,14,15-pen tahydroxy-12,16-dimethylicosane-2-one) and alanine (Hartinger et al. 2011). The equation listed below shows the FB1 degradation process.

$$\text{FB1} \xrightarrow{\text{Carboxylesterase FumD}} \text{HFB1} \xrightarrow{\text{aminotransferase FumI}} \text{2 Keto HFB1}$$

7.2.2.2 FB2

The empirical formula of Fumonisin B2 is C34H59NO14 and its molecular weight is 705.83. FB2 belongs to the family of toxins known as fumonisins. FB2 is a structural analog of FB1, but it is more cytotoxic than the latter, and it inhibits sphinganine-N-acetyltransferase (ceramide synthase). FB2 is a carcinogenic mycotoxin generally present on corn-based food and feedstuff, which is produced by *Fusarium verticillioides* and *Fusarium moniliforme*. FB2 could also be detected in *Aspergillus niger* (Frisvad et al. 2007). Presence of FB1 and FB2 was reported in cattle milk also due to the consumption of contaminated feed or fodder (Scott 2012). Figure 7.4 shows the chemical structure of fumonisin B2.

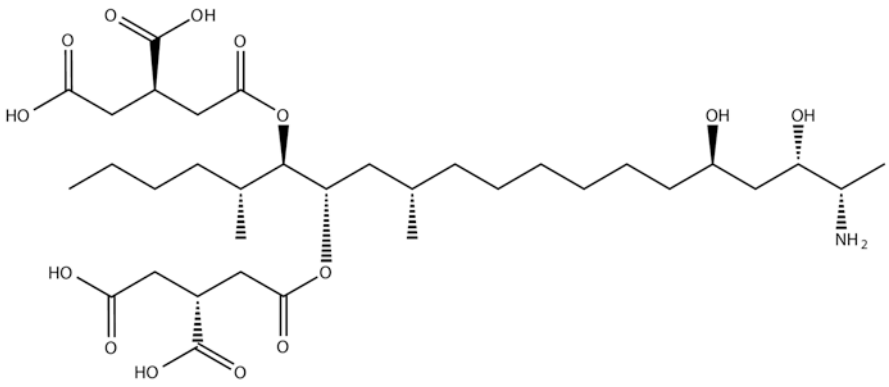


Fig. 7.4 The chemical structure of fumonisin B2 (FB2)

Cholestyramine, a bile acid sequestrant and preventative of diarrhea, was shown to be an effective binder for fumonisins – both in vivo, using the increase in the sphinganine/sphingosine (Sa/So) ratio in rat urine and tissues as a biomarker, and in vitro, using a dynamic gastrointestinal model. These results provide a basis for an enzymatic detoxification process. Lactic acid bacteria (*Bacillus subtilis* and *Micrococcus luteus*) bind FB1 and FB2. Peptoglycan is the likely binding site, and more FB2 is bound than FB1, with at least one tricarballylic acid moiety involved in the binding (Scott 2012; Niderkorn et al. 2006). FB2 was rapidly eliminated from the plasma of velvet monkeys dosed iv with 2 mg FB2/kg body mass. The concentration of FB2 in plasma after the iv dose was characterized by an initial distributional phase and a subsequent elimination phase with a mean half-life of 18 min (Shephard and Snijman 1999).

7.2.3 Trichothecenes

Trichothecenes are sesquiterpenoids mainly produced by the genera *Fusarium*, *Trichothecium*, *Myrothecium*, *Trichoderma*, and *Stachybotrys* fungi. Trichothecenes are produced by *Fusarium* species (*F. sporotrichioides*, *F. graminearum*, *F. poae*, and *F. culmorum*). It can also be generated by members of other genera via *Trichothecium* together with Tri101, which inactivates trichothecenes (Takahashi-Ando et al. 2002).

7.2.3.1 T-2 Toxin (T-2)

Poisoning of fungus toxin is based on the finding of T-2 toxin accumulation in grains, which may cause the disease. T-2 toxin is the major toxin produced by *Fusarium poae* and *F. sporotrichioides* in suitable environments and can endanger human health. T-2 toxin is present widely in nature and pollutes maize, wheat, barley, oat, and winter rye grain crops. When individuals eat the mildew food contaminated by T-2 toxin, the latter can reach the particular cartilage, where T-2 toxin can interfere with the DNA metabolism of chondrocytes and inhibit the synthesis of collagen and glycosaminoglycans, eventually leading to multiple articular cartilage lesions and chondrocyte necrosis. Moreover, T-2 toxin can provoke degenerative events in chicken embryo chondrocytes and alter the collagens and proteoglycans that are components of the cartilage matrix (Yu et al. 2017; Mankevičienė et al. 2006). Figure 7.5 shows the chemical structure of T-2 toxin.

T-2 causes cytotoxicity, which reduces cell viability and induces intracellular lactate dehydrogenase (LDH) release. It also leads to oxidative stress in cells via enhancing the generation of reactive oxygen species (ROS) (Yang et al. 2019). Table 7.3 shows T-2 toxin-controlling enzyme and its organism source.

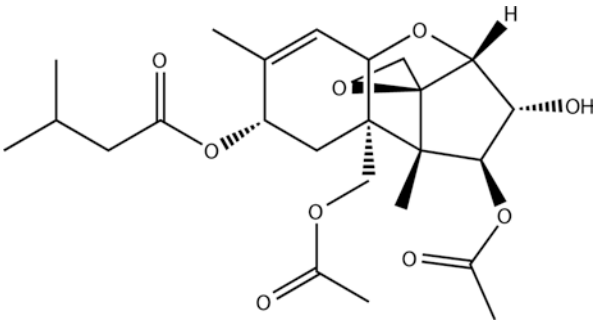


Fig. 7.5 The chemical structure of T-2 toxin (T-2)

Table 7.3 Detoxification enzymes of controlling T-2 toxin contamination

Enzyme	Producing organism	Final products	References
Acetyltransferases	*Blastobotrys proliferans*	3-acetyl T-2 toxin	McCormick et al. (2012)
Glucosyltransferase	*Blastobotrys muscicola*	T-2 toxin 3-glucoside	McCormick et al. (2012)
Cytochrome P450 (CYP 3A4)	*Homo sapiens*	3′-OH T-2	Lin et al. (2015)
Carboxylesterase	Tissue-infiltrating plasma cells	HT-2	Lin et al. (2015)
Hydrolyzing	*Curtobacterium* sp. strain 114-2	HT-2	Ueno et al. (1983)

7.2.3.2 HT-2 Toxin (HT-2)

HT-2 toxin is the main metabolite in vivo of T-2 toxin, where hepatic carboxylesterases are responsible for the specific deacetylation of T-2, resulting in HT-2 as the major metabolite (Medina and Magan 2011). Figure 7.6 shows the chemical structure ofHT-2 toxin.

7.2.3.3 Deoxynivalenol (DON)

Deoxynivalenol (DON), also known as vomitoxin, belongs to the large family of trichothecenes as potent inhibitors of protein synthesis. DON is mainly produced by *F. graminearum* and *F. culmorum*, which is a common contaminant of barley, wheat, oats, and corn all throughout the world (Mankevičienė et al. 2006). Deepoxidase was stated to be responsible for detoxifying DON (Upadhaya et al. 2010). Figure 7.7 shows the chemical structure of DON.

3-O-acetylation of the trichothecene ring in DON leads to its inactivation. Gene Tri101 encoding trichothecene-3-O-acetyl-transferase from *F. graminearum*was characterized. The previous study cloned trichothecene 3-O-acetyl-transferases

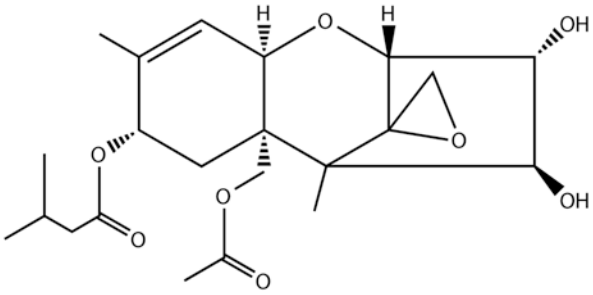
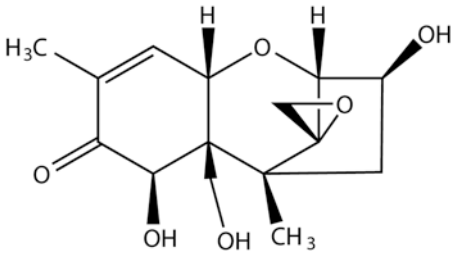


Fig. 7.6 The chemical structure of HT-2 toxin (HT-2)

Fig. 7.7 The molecular structure of deoxynivalenol (DON)



genes from *Fusarium* species and compared the properties of them to identify an optimal source of the enzyme for biotechnological applications. A UDP-glucosyltransferase from *Arabidopsis thaliana* catalyzed the transfer of glucose from UDP-glucose to the hydroxyl group at C3 of DON (Poppenberger et al. 2003). However, whether acetylation of C3-OH or conjugation by glycosylation can be considered as detoxification is controversial, because acetylated and conjugated mycotoxins may be hydrolyzed and regenerated the toxins in the digestive system of animals and human beings (Poppenberger et al. 2003).

DON degradation by *Aspergillus oryzae* and *Rhizopus oryzae* in a submerged fermentation system was found to correlate with the activity of oxydo-reductase enzymes (Garda-Buffon et al. 2011) and the catabolizingbacterial Cytochrome P450 system (Ito et al. 2013), as well as the peroxidase enzyme, which was extracted from rice bran (Feltrin et al. 2017).

As reported, the *Devosia* mutans 17-2-E-8 (*Devosia* spp. 17-2-E-8) was capable of transforming DON to the nontoxic stereoisomer 3-epi-deoxynivalenol, along with the earlier reported bacterial species capable of oxidizing DON to 3-keto-DON, generating great interests on the possible mechanism of recognition and enzyme(s) involved. An understanding of these details could pave the way for novel strategies to manage this widely present toxin. It was previously shown that DON epimerization proceeds through a two-step biocatalysis. Significantly, this report describes the identification of the first enzymatic step in this pathway. The enzyme, a dehydrogenase responsible for the selective oxidation of DON at the C3 position, was shown to readily convert DON to 3-keto-DON, a less toxic intermediate in the

DON epimerization pathway. DON detoxification enzymes have the following classification: de-epoxidation, oxidation, epimerization, and glycosylation (Tian et al. 2016). Table 7.4 shows the enzymes of DON detoxification and their origin. In addition, DON mitigation by the PQQ dependence of the enzyme could be a new feasible strategy (Carere et al. 2018).

7.2.3.4 Diacetoxyscirpenol (DAS)

Diacetoxyscirpenol (DAS), also called anguidine, is a mycotoxin from the group of type A trichothecene. It is a secondary metabolite of the genus *Fusarium* and may cause toxicosis in farm animals. DAS has been detected in agricultural products worldwide and persists in products after processing (Fig. 7.8). In humans as well as in animals, DAS consumption has been shown to induce hematological disorders (neutropenia, aplastic anemia). DAS is metabolized in animals to

Table 7.4 Detoxification enzymes of controlling DON contamination

Type of enzyme	Origin	References
Deepoxidase	*Bacillus* sp. LS100 derived from chicken digesta	Li et al. (2011)
Deepoxidase	Bacteria derived from intestines of chicken	Young et al. (2007)
Deepoxidase	A strain of *Bacillus* derived from the intestinaltract of fish	Guan et al. (2009)
Deepoxidase	A mixed microbial culture including six bacterial genera found from soil	Islam et al. (2011)
Deepoxidase	Fecal microbiota derived from intestines of human	Gratz et al. (2013)
Oxidase and epimerase	*Nocardioides* sp. strain WSN05-2 derived from a wheat field	Ikunaga et al. (2011)
Oxidase and epimerase	Genus of *Nocardioides* and *Devosia* derived from field soils and wheat leaves	Sato et al. (2012)
Oxidase and epimerase	*Devosia* mutans 17-2-E-8 isolated from an agricultural soil	He et al. (2015)
UDP-glucosyltransferase	*Arabidopsis thaliana*	Poppenberger et al. (2003)
UDP-glucosyltransferase	*Triticum aestivum* L. cv. Wangshuibai	Lulin et al. (2010)
UDP-glucosyltransferase	Barley	Schisler et al. (2011)
UDP-glucosyltransferase	*Arabidopsis thaliana*	Shin et al. (2012)
UDP-glucosyltransferase	Barley	Li et al. (2015)
Deoxynivalenol hydroxylase	*Sphingomonas* sp. strain KSM1	Ito et al. (2013)
Peroxidase	Rice bran	Feltrin et al. (2017)

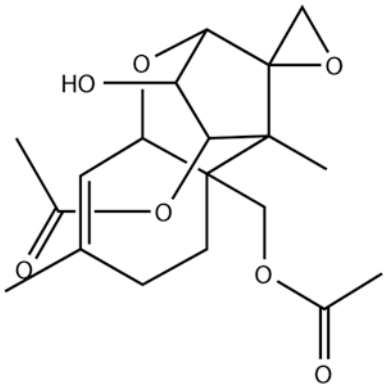


Fig. 7.8 The chemical structure of diacetoxyscirpenol (DAS)

Table 7.5 Detoxification enzymes of controlling diacetoxyscirpenol (DAS) contamination

Enzyme	Producing organism	Final products	References
Deepoxidase	Intestinal microflora in rats, cattle, and swine	Deepoxy MAS, deepoxy scirpentriol	Matsushima et al. (1996)
Deepoxidase	*Mucor racemosus*	Deepoxy scirpentriol	Bocarov-Stancic et al. (2011)
Carboxylesterase	Mouse liver microsomes	15-monoacetoxyscirpendiol, scirpentriol (Triol)	Wu and Marletta (1988)

15-monoacetoxyscirpenol (15-MAS) via C-4 deacetylation and then transformed to scirpentriol (SCP) via C-15 deacetylation (Wu et al. 2010; Pronk et al. 2002; Lautraite et al. 1997). Table 7.5 shows DAS controlling enzyme and enzyme source organism.

7.2.3.5 Nivalenol (NIV)

Nivalenol (NIV) is one of the trichothecene mycotoxins commonly contaminating cereals. This toxin has been associated with the poisoning of animals and humans, and many toxicological studies have been performed on *Fusarium* sp. Fn-2B, a well-known nivalenol producer (Hedman and Pettersson 1996) (Fig. 7.9). The impact of DON and NIV on the increase of *Saccharomyces cerevisiae* strains has been studied. The toxins were added to the growth medium in low and high concentrations. Yeast growth was assessed by the size of dry weight or relative growth, cell number, viability, and conductance change of the growth medium using direct and indirect methods. The inhibitory effect of both DON and NIV on yeast growth was dependent on toxin concentration. Additionally, when the extent of inhibition of yeast growth caused by high concentrations of both toxins was observed, it was subject to a yeast strain, length of incubation, and approach used to investigate yeast growth. The lowest concentrations of mycotoxin causing significant inhibition on

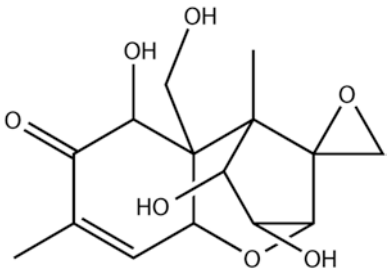


Fig. 7.9 Structure of nivalenol (NIV)

Table 7.6 Detoxification enzymes of controlling NIV contamination

Enzyme	Source of organism	References
UDP-glucosyltransferase, HvUGT13248	Barley	Li et al. (2017)
Cytochrome P450 system (Ddna + Kdx + KdR	*Sphingomonas* sp. strain KSM1	Ito et al. (2013)
Glutathione transferase, microsomal cytochrome P450	Animal liver	Gouze et al. (2007)

the growth of brewing yeasts were 100 μg/mL of DON for the lager strain, 50 μg/ml for the ale strain, and 50 μg/mL of NIV for the ale strain (Pronk ME. et al. 2002). Table 7.6 shows NIV degrading enzymes and its origin organism.

7.2.3.6 Beauvericin (BEA)

Beauvericin (BEA) and enniatins (ENNs) are prominent cyclic hexadepsipeptide mycotoxins primarily manufactured by the fungi of *Fusarium* species such as *F. oxysporum*, *F. avenaceum*, *F. poae*, and *Beauveria bassiana*. They are fundamentally interconnected and comprising of three alternating hydroxyisovaleryl and N-methylamino acid residues. Its molecular formula is C45H57N3O9. According to the scientific opinion on the risks to human and animal health related to the presence of BEA and ENNs in food and feed, 29 naturally take place ENN analogs have been identified but only four ENNs including enniatin A (ENA), A1 (ENA1), B (ENB), and B1 (ENB1) (Han et al. 2019; Maranghi et al. 2018; Wu et al. 2018, 2019). These compounds have antibiotic, insecticidal, and ionophoric properties and different bioactivities (Liuzzi et al. 2017). This study investigated the degradation of BEA by intracellular raw enzymes of four strains of *Saccharomyces cerevisiae*, namely, LO9, YE5, A34, and A17 (Meca et al. 2013). In the Figure 7.10 shows the chemical structure of BEA.

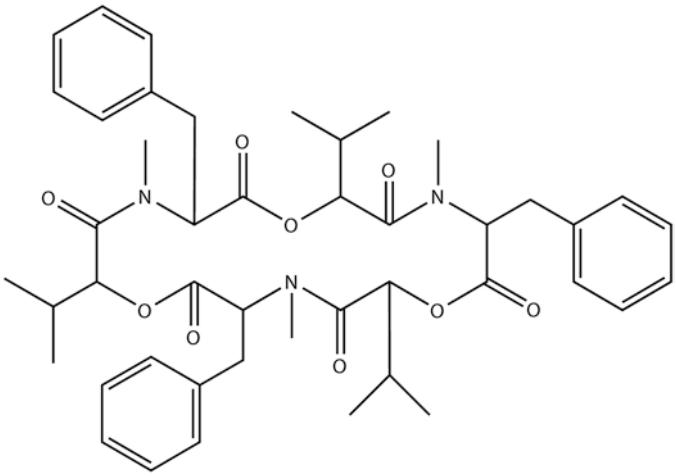


Fig. 7.10 The chemical structure of beauvericin (BEA)

7.3 Source of Degradation Enzymes Against Mycotoxins

7.3.1 Natural Source of Enzyme

Except for intact microbes or cell-free enzymatic preparations as feed additives, the expression of the respective genes in genetically manipulated organisms has opened new avenues for the protection of the health of farm animals. Examples of such procedures consist of the genetic engineering of ruminal microorganisms and feeding transgenic corn with reduced mycotoxin production to pigs (Upadhaya et al. 2010).

7.3.2 Recombinant Fusion Enzyme (Expression, Purification)

Recombinant fusion proteins are created artificially by recombinant DNA technology for use in biological research or therapeutics. A fusion protein is a recombinant protein created through genetic engineering of a fusion gene consisting of at least two genes. This naturally involves removing the stop codon from a cDNA sequence coding for the first protein, then appending the cDNA sequence of the second protein in the frame through ligation or overlap extension PCR. That DNA sequence will then be expressed by a cell system as a single protein. The protein can be engineered to include the full sequence of both original proteins or only a portion of either fusion enzyme technology, based on fusion protein design, which is frequently used in multifunctional enzyme construction and enzyme proximity control (Huang et al. 2012). The detoxification genes may be cloned and expressed in

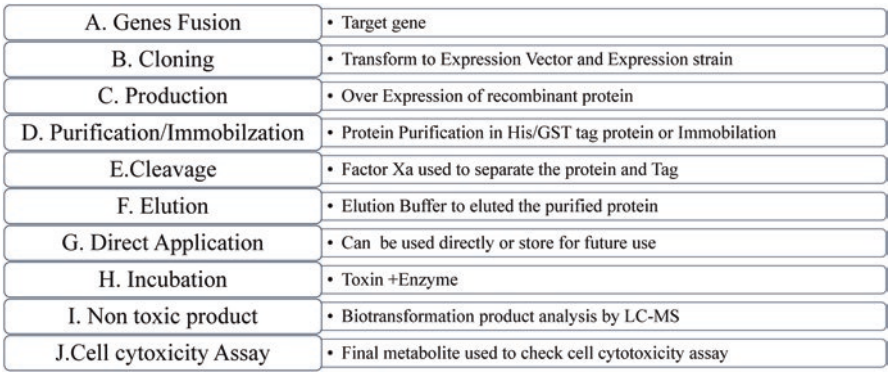


Fig. 7.11 A schematic outline of enzyme processing and application in the food industry

microorganisms to produce recombinant microorganisms that are suitable in an industrial scale enzyme production and purification (Altalhi 2007). Figure 7.11 shows the schematic outline of enzyme processing and application in the food industry.

As the whole procedure, the industrial production of enzymes from microorganisms generally involves culturing the microorganisms in huge tanks where enzymes are secreted into the fermentation medium as metabolites of microbial activity. And then, the enzymes are extracted, purified, and used as processing aids in the food industry. Purified enzymes are cell-free entities and should not contain any other macromolecules such as DNA residues. There are two common kinds of cells that are used for protein expression, namely, *E. coli* and yeast (Upadhaya et al. 2010).

7.3.3 Chemical Synthesis

The chemical synthesis of enzymatic proteins is feasibly completed by chemical ligation, where the key is the chemoselective reaction of unprotected synthetic peptides (so-called chemical ligation).

Notably, native chemical ligation enables the reaction of two unprotected peptides in aqueous solution at neutral pH to form a single product. Full-length synthetic polypeptides are folded to form the defined tertiary structure of the target enzyme, which could be further characterized by analysis of mass spectrometry, NMR, and X-ray crystallography, in addition of assays on biochemical activities (Kent 2003, 2009).

7.4 Basic Enzymatic Characteristics

7.4.1 Enzyme Activity

The most important property of one enzyme is the ability to increase the rates of reactions occurring in living organisms, known as "catalytic activity." The enzymatic activity is tediously affected by the factors that disrupt protein structure including temperature and pH value and affect catalysts consisting of reactant or substrate concentration and catalyst or enzyme concentration. The enzymatic activity can be measured by monitoring either the rate at which a substrate disappears or the rate at which a final product forms (Dubey 2018; Butko et al. 2012).

7.4.2 Enzyme Kinetics

The kinetics of one enzyme is the branch of enzymology that deals with the factors affecting the rates of enzyme-catalyzed reactions. An enzyme catalyzes the rate of a reaction without changing the equilibrium concentration of the reactants and products. The kinetics are included at two stages: (1) at pre-steady state, also known as the transient state, which monitors the microscopic events along the reaction pathway during the first round of the reaction (prior to enzyme turnover) and (2) at steady state, which monitors multiple rounds or turnovers of an enzymatic reaction (Lee and AJ 2003).

7.4.3 Enzyme Inhibitor

As would be anticipated with an enzyme system of such broad-based specificity, competition between ligands for a particular isoform is rife within the P450 family, with competitive inhibition between mutual substrates being a common pharmacological phenomenon (Meighen 2005). Compounds that influence the rates of enzyme-catalyzed reactions are called modulators, moderators, or modifiers. Usually, the effect is to reduce the rate, and this is called inhibition. Sometimes the enzyme reaction is increased, and this is called activation. Accordingly, the compounds are termed inhibitors or activators (Stojan 2005).

7.4.4 Enzyme Immobilization

One enzyme is seriously limited within its phase allowing its re-usability. The lack of purification and efficient recovery is the most critical and challenging aspect, which renders them enormously expensive for industrial costs. Aiming to tackle these problems, magnetic nanoparticles (MNPs) have gained a special place as versatile carriers and supporting matrices for immobilization purposes, owing to the exceptional properties of MNPs, such as larger surface area, larger surface-to-volume ratio, and more mobility, as well as higher mass transference (Bilal et al. 2018).

7.5 What Conditions Affect the Success of the Enzyme for the Degradation of Mycotoxins?

7.5.1 Enzyme Safety

Each enzyme has its own specific mechanism while it could work as usual with its biochemical properties. The enzymes have far fewer side effects and possible unknown reactions than other compounds, supplements, or medications. With the exception for the potential skin and irritating eye effects of some proteases and the well-documented potential for respiratory sensitization in the case of workplace exposure, enzymes, in general, do not produce acute toxicity, dermal sensitization, genotoxicity, or repeated dose oral toxicity. Acute inhalation, reproduction, chronic toxicity, and carcinogenicity are not relevant for enzymes. Several hundred mutagenicity studies have been conducted on bacterial and mammalian cells using a variety of enzymes. No positive findings were observed (Ladics and Sewalt 2018; Spök 2006). In the past, the safety of some food enzymes was assessed by the Scientific Committee on Food (SCF). Since 2003, EFSA has replaced the SCF and is currently undertaking the evaluation of all food enzymes. Only FE for which the proposed uses are considered safe will be on the EU list.

7.5.2 Enzyme Safety Dose Level

Enzymes are approved as safe biological ingredients in the manufacturing of many everyday products. Enzymes are regulated worldwide and are approved as safe biological ingredients in the manufacturing of numerous products such as detergents, textiles, and food. As with other proteins such as pollen or flour, enzymes may cause allergic reactions for people working with them in industrial processes, if not handled correctly. No known toxicity has been demonstrated at any level of enzyme dosing in animal or human beings. As reported, the rats were fed enzymes

equivalent to a human dose of 2500 tablets daily for a short period, and the rats only seemed a little fatigued (Palfey 2005).

7.5.3 Safe Handling of Enzymes

Safe handling of enzymes during the manufacturing process is vital because, if inhaled in their raw state, in the form of dust or aerosols, they may cause respiratory allergy similar to other well-known allergens like pollen, house dust mites, and animal dander. When added to detergent products, enzymes are treated, for example, by encapsulation, making them safe for use.

7.5.4 Difficulties During Mycotoxin Biotransformation

Biodegradation of mycotoxins with microorganisms or enzymes is considered as the best strategy for detoxification of food and feedstuffs. This approach is also pointed as an environmentally friendly approach in contrast to physicochemical techniques of detoxification. Since ruminants are a potential source of microbes or enzymes for mycotoxin biotransformation, isolation of pure culture using enriched media or screening of candidate genes from the metagenomic library seems to be a good strategy. Furthermore, genetic engineering techniques will not only improve the efficiency with which enzymes can be manufactured from these organisms or producing the engineered organism having the target genes, but they also increase their availability, bioavailability. Thus, the use of enzymes or engineered microorganisms as processing aids in the food industry would be proved to have an overall beneficial impact (Upadhaya et al. 2010).

7.5.5 Challenges and Limitations of and During Degradation by Enzymes

The use of enzymes in the food and feed industries is not the new thing, while the enzymes as biocatalysts have been increasingly used in recent years. They are usually applied to reduce the employment of hazardous chemicals, to use mild working conditions, to increase specificity, to speed up a process, or to simply create new products.

Mandatory requirements for large-scale enzyme application in industry are (1) safety, (2) effectiveness, (3) low cost of production and purification for both enzyme and cofactors if needed, and (4) stability to wide ranges of temperature, pH, and organic solvents and thus compatibility to productive processes. Native enzymes

usually do not respond to each unique requirement of a perfect industrial enzyme, while these features can be achieved via molecular engineering and structure-function modifications by mutagenesis. The most important limitation related to the application of degradation enzymes against mycotoxins is the reduced effectiveness of the process due to matrix effects. The physicochemical properties of food, such as the moisture, fat content, acidity, and texture, greatly influence the outcome of detoxification. Moreover, inhibitory compounds might be present in raw materials, and mycotoxins can occur in masked or other modified forms in plants. Their presence would reduce the enzyme catalysis at some degree.

For these cases, the implications might require pretreatments, additional time, and costs. Despite these limitations, the potentialities of the degradation enzymes used in the food and feed industries remain widespread. Their application is versatile since they can be used both in free or immobilized form and easily applied to well-established industrial processes of fermentation, ripening, brewing, or feed manufacturing (Loi et al. 2017).

7.6 Why Is Biotransformation of Mycotoxin by Enzyme More Superior than Other Control Methods?

Importance of biotransformation enzyme against mycotoxins ensuring food safety:

- They can be used as alternatives or even replacements to traditional chemical-based technology, with lower energy consumption and biodegradability.
- They are more specific in their actions than the applicable synthetic chemicals, with the fewer side reactions and waste by-products, resulting in higher-quality purified products.
- Especially for cereal-derived foods and feed, the wide application will minimize crop loss and maximize the levels of ensuring food safety.

7.7 Future of Biotransformation Enzyme Technology

The discovery of novel degradation enzymes for mycotoxins is becoming more interesting and stimulating worldwide.

One big challenge is how to find the applicable degradation enzymes targeting mycotoxin co-occurrence.

7.8 Conclusion

In conclusion, a standard biocontrolling and decontamination technology must have the following criteria: (1) a rapid and well-planned degradation, (2) of a broad spectrum of mycotoxins, (3) into nontoxic end products, (4) by an original nonpathogenic strain or consortium, and (5) under conditions that are relevant for the matrix in which the mycotoxin problem occurs. In order to attain these objectives, we urge to look beyond the disappearance of the mother compound and to explore strange new worlds and seek out new organisms and new metabolic pathways. Several conventional physical and chemical approaches have been used to remove mycotoxins from contaminated grains, but the loss of nutritional values or potential safety problems should not be ignored. Therefore, detoxifying mycotoxins by enzymatic reactions could be a more attractive approach for controlling mycotoxin contamination to the safe levels.

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Chapter 8
Confrontation of Microbes with Mycotoxin-Producing Strains

Ye Tian and Aibo Wu

Abstract Mycotoxins, as secondary microbial metabolites, frequently contaminate cereal grains and pose a serious threat to human and animal health at the global levels. Except for physical separation and chemical treatments, biological control with functional agents has been proved to more realistically manage mycotoxin contaminations, especially from the view of the whole food chain. In general, functional biological control agents (BCAs) cover the scope of antagonistic microbes, natural fungicides derived from plants, and detoxification enzymes. In this chapter, we summarize the developed BCAs against various agro-important mycotoxins (DON, ZEN, FB1, AFB1, etc.) on cereal grains and fruits, with more emphasis on its significance on the inhibition or degradation of mycotoxin contaminations, concerning food security and food safety.

Keywords Biological control agents (BCAs) · Degradation · Detoxification · Contamination mycotoxins

8.1 Introduction

It is well-known that some plant pathogens are responsible for crop diseases and mycotoxin contaminations in the whole food and feed production chain. Trichothecenes, zearalenone, and fumonisins are the major mycotoxins produced by various *Fusarium* species collected in different regions (Bertero et al. 2018). In the past several years, a few of lab or field experiments investigated the potential of beneficial microbes to manage plant diseases. The diseases caused by various species of toxigenic fungi not only cause yield losses of cereal grains but also lead to mycotoxin contamination, posing a great risk to the health of humans and animals. The use of the traditional chemical fungicides to manage pathogenic fungi is

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effective, but it may bring some adverse effects, such as environment pollution, causing fungal genetic variation, which is not good for long-term use (Yuen and Schoneweis 2007). Meanwhile, biological control of plant diseases caused by toxins with beneficial microbes is an emerging alternative method, which is environment-friendly and fit the requirements of sustainable agricultural development (Alberts et al. 2016).

Here, we will discuss new well-studied biological control agents (BCAs) against toxigenic fungi and mycotoxin contamination (Table 8.1). We will focus on various antagonistic actions of the BCAs on pathogenic fungi growth and their ability to inhibit mycotoxin production, which would be beneficial to have more understanding on the antagonistic potentials of BCAs on mycotoxin contamination with respect to the theme of food safety.

The well-studied antagonists mainly consist of *Trichoderma*, *Clonostachys rosea* (Schoneberg et al. 2015), *Cladosporium cladosporioides*, *Aureobasidium pullulans*, *Bacillus* and *Pseudomonas* genera, and yeast (Tian et al. 2016a). These antagonistic microbes can be used directly for inhibition of growth and mycotoxin production of fungi in pre-harvest stage or applied on crop residuals to inhibit spore production after harvest.

8.2 Antagonistic Fungi

Of the abovementioned BCAs, *Trichoderma* genus has been widely investigated in both lab and field experiments, because *Trichoderma* is a nonpathogenic genus for crops, which could produce a series of antibiotics against plant pathogens (Mukherjee et al. 2013). In addition, they grow faster than competitors which could inhibit other fungal growth. Another important mechanism for managing toxicogenic fungi is mycoparasitism mediated by production of the cell wall-degrading enzymes including cellulases, chitinase, and glucanases (Vinale et al. 2008). Consequently, *Trichoderma* isolates are potential candidates to control pathogenic fungi. For instance, an antagonistic strain *T. gamsii* 6085 was tested on its potentials against *F. culmorum* and *F. graminearum* (Matarese et al. 2012). This *Trichoderma* strain could suppress DON production by the two *Fusarium* pathogens up to 92%. Another study indicated that a *Trichoderma* strain, T-22, was able to decrease the perithecia formation of *F. graminearum* by 70% in a field experiment (Inch et al. 2007). Moreover, it is also very important to understand details in the interplay between antagonistic *Trichoderma* and toxigenic *Fusarium*. Recently, we demonstrated the potentials of *Trichoderma* genus for control of deoxynivalenol (DON) and zearalenone (ZEN) producers by dual culture on PDA medium. Also, we investigated the metabolic activity of the *Trichoderma* isolates on DON and ZEN. The achieved data suggested that *Trichoderma* isolates were effective antagonists to manage the growth and mycotoxin production of the tested DON- or ZEN-producing

Table 8.1 The mentioned antagonistic microbes for control of mycotoxin contamination

	Functional BCAs	Mechanism	References
Antagonistic fungi	*Trichoderma* strains	Inhibiting sporulation, growth and/or mycotoxin	Schoneberg et al. (2015)
	Clonostachys rosea	production of pathogens, or	Schoneberg et al. (2015)
	Cladosporium cladosporioides	bio-transform mycotoxin into less toxic compounds	Schoneberg et al. (2015)
	Aureobasidium pullulans		Wachowska and Głowacka (2014)
	Trichoderma strains		Schoneberg et al. (2015)
	Trichoderma strains		Matarese et al. (2012)
	Trichoderma T-22		Inch et al. (2007)
	Trichoderma atroviride P1		Lutz et al. (2003)
	Trichoderma strains		Tian et al. (2016b)
	Trichoderma strains		Tian et al. (2018)
	Trichoderma strains		Ferrigo et al. (2014)
	Trichoderma strains		Mukherjee et al. (2012)
	Panax notoginseng		Zheng et al. (2017)
Antagonistic bacterial	*Bacillus subtilis* SG6		Zhao et al., (2014)
	Bacillus subtilis RC 218 and *Brevibacillus* sp. RC 263		Palazzini et al., (2016)
	Bacillus amyloliquefaciens		Shi et al. (2014)
	Shewanella algae strain YM8		Gong et al. (2015)
	P. fluorescens		Palumbo et al. (2007)
	Piriformospora indica		Mousa et al. (2015)
	Pseudomonas and *Bacillus* genera		Figueroa-López et al. (2016)
	B. amyloliquefaciens		Pereira et al. (2007)
Antagonistic yeast	*Cryptococcus*		Khan et al. (2004)
	Candida parapsilosis IP1698		Niknejad et al. (2012)
	Cryptococcus spp., *Kluyveromyces* sp,., and *Saccharomyces* spp.		El-Tarabily and Sivasithamparam (2006)

Fusarium strains, and results demonstrated that *Trichoderma* isolates could bio-transform DON into D3G via glycosylation (Tian et al. 2016b) and bio-transform ZEN into ZEN-S via sulfation (Fig. 8.1) (Tian et al. 2018). Interestingly, except for the obvious inhibition effects on the fungal growth of *F. graminearum* via confrontation, the masked form D3G appeared at various levels, highly co-related to the

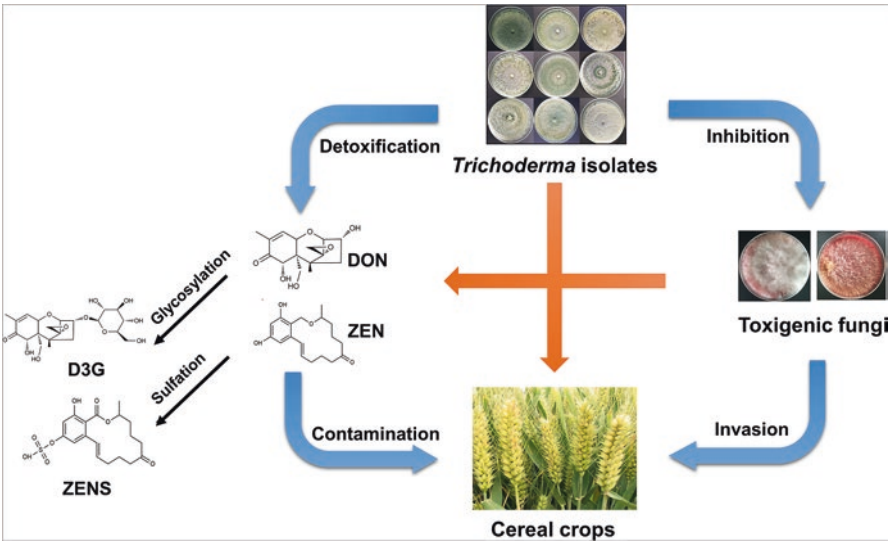

Fig. 8.1 *Trichoderma* isolates were effective BCAs to manage the growth and mycotoxin production of DON- or ZEN-producing fungi. In addition, we provided evidences that *Trichoderma* isolates were capable of bio-transforming DON into D3G via glycosylation and bio-transforming ZEN into ZEN-S via sulfation. (Tian et al. 2018)

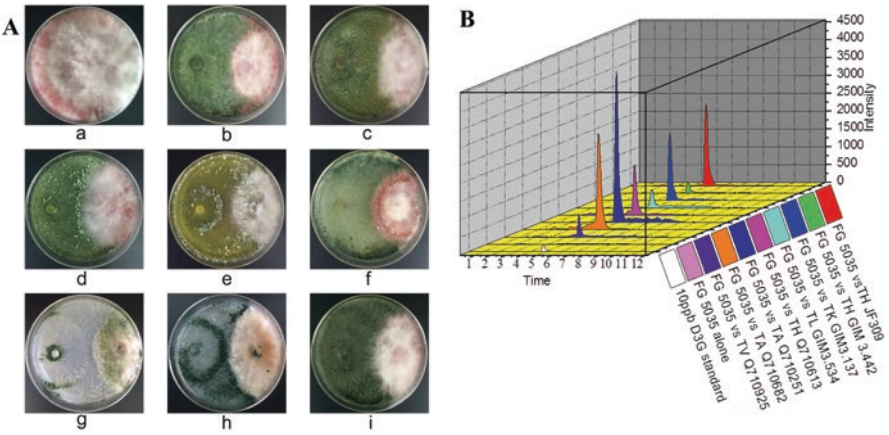

Fig. 8.2 Different trichoderma were co-cultured with *F. graminearum* 5035; (**a**) colony morphology of *F. graminearum* 5035 in dual-culture tests after incubation on the potato dextrose agar (PDA) medium; (**b**) the concentration of the D3G. (Tian et al. 2016a, b)

inhibition efficiencies of different *Trichoderma* isolates (Fig. 8.2). On the other side, some chitinase-encoding genes were upregulated in mycoparasitic *Trichoderma* spp. when confronted with *Fusarium*, while another study demonstrated that mycotoxin DON production could suppress one chitinase gene (nag1) expression in a *T.*

atroviride strain P1 as a negative signal in interaction of *Trichoderma* and *Fusarium* species (Lutz et al. 2003).

Furthermore, it has been proved that *T. harzianum* could promote plant growth and could enhance crop resistance against pathogenic fungi. Results of the dual culture of *Trichoderma* spp. with *F. verticillioides*, *F. graminearum*, and *A. flavus* showed that *T. harzianum* could inhibit the pathogen *F. verticillioides* in maize by inducing resistance though inducing signaling pathways (Ferrigo et al. 2014). A recent work identified some new endophytic *Trichoderma* strains capable of protecting plants against diseases by invading plant tissue and then inducing transcriptomic changes (Mukherjee et al. 2012). It is said that endophytic fungi could balance the system and promote host growth. Also, the endophytic fungi diversity of *Panax notoginseng* was investigated and then antagonistic potentials of endophytic fungi on phytopathogens causing root rot evaluated. Their results suggested that endophytic fungi would be a source for screening new natural compounds for biocontrol of plant root rot disease (Zheng et al. 2017).

8.3 Antagonistic Bacterial

The nonpathogenic bacteria are also being widely studied as antagonists against plant diseases recently (Shi et al. 2014). Antagonistic bacterial strains are usually endophytes which inhabit the rhizosphere or anthers of crops, which could not cause adverse effects on their host. For example, a *Bacillus subtilis* strain SG6 from anthers of wheat was proved that it could inhibit the mycelial growth, sporulation of conidia, and DON production of *F. graminearum* (Zhao et al. 2014). In addition, two bacterial strains of *B. subtilis* RC 218 and *Brevibacillus* RC 263 from wheat anthers could remarkably decrease the incidence of FHB diseases and mycotoxin DON contamination (Palazzini et al. 2016). This research work was done in semi-controlled field conditions. In another study, *B. amyloliquefaciens* isolated from peanut shells exhibited strong inhibitory effects on the mycelium growth and DON production of *F. graminearum* (Shi et al. 2014). Interestingly, a strain YM8 of *Shewanella algae* isolated from sediment, producing volatile organic compounds with inhibition effects against nine different important plant pathogens. This research work which indicates that the bacteria from marine is a potential and promising resource for screening effective BCAs against the growth and mycotoxin production of plant pathogens (Gong et al. 2015). Another work reported that *P. fluorescens* could produce antifungal compounds and chitinase, which had inhibitory effects on *A. flavus* and *F. verticillioides* growth (Palumbo et al. 2007).

Recently, it has been reported that *Piriformospora indica* was able to reduce both the severity the crop disease caused by *F. graminearum* and extended by DON contamination (Rabiey and Shaw 2016). In addition, some novel endophytes (*Piriformospora indica*) were predicted that they were able to detoxify DON in vitro, but the performance of the strains has not verified under field conditions (Mousa et al. 2015).

The rhizobacterial *Pseudomonas* and *Bacillus* genera could significantly inhibit the mycotoxin produced by *F. verticillioides* up to 70% (Figueroa-López et al. 2016). In another study, the authors treated the seed with B. *amyloliquefaciens*, and the amount of fumonisins was reduced in field trails (Pereira et al. 2007). Next, the results were confirmed in a 2-year field trials with the same *B. amyloliquefaciens* (Pereira et al. 2007).

8.4 Antagonistic Yeast

Besides the antagonistic fungal strains and bacteria, yeast are also promising candidates for mycotoxin control. It was reported that the yeast *Cryptococcus* spp. could control plant FHB disease by 50–60% on susceptible wheat in field tests (Khan et al. 2004). The yeast *Candidaparapsilosis* IP1698 could inhibit aflatoxin production up to 90% at various conditions of pH and temperatures (Niknejad et al. 2012). Yeasts such as *Cryptococcus* spp., *Kluyveromyces* spp., and *Saccharomyces* spp. were reported that they could produce bioactive metabolites targeting various pathogens for management of their growth (El-Tarabily and Sivasithamparam 2006).

8.5 Conclusion

Managing toxicogenic fungi and mycotoxin contamination in both pre-harvest and post-harvest stages is very important for ensuring food safety (Wegulo et al. 2015). It is still challenging to find stable and efficient BCAs to control the growth of phytopathogenic fungi and mycotoxin production in different stages of food production and storage. We know that a pathogenic fungus could produce different mycotoxins because of the biosynthetic pathways, and the next research work should focus on the management of multi-mycotoxin contamination with effective BCAs. Consequently, it will be more practical to select a biocontrol agent which is capable of suppressing the production of different mycotoxins at the same time. Another point is that the BCAs should be much more tolerant of different mycotoxins, which would guarantee efficiency when confronted with different toxicogenic fungi.

Though several BCAs have been well investigated on their activities against various toxigenic fungi in the lab experiments, their potentials on toxigenic fungi against pathogens in field experiments have not been further validated in depth. The performance of BCAs for managing pathogens in vivo or in field trails might be diffident, because there are more related factors affecting BCA's performance in field conditions, such as the nutrient differences in soil and the different microbial community. Other important factors which may affect the activities of BCA are the delivery method of BCAs to the crops, the delivery form of BCAs, and the time and route to apply BCAs against plant pathogens. Consequently, it is important to consider all the different factors in field trails which may affect the antagonistic results,

and field experiments should be comprehensively carried out to assess BCA's potentials against toxigenic fungi.

Future work needs to be done to comprehensively elucidate the biological control mechanisms and there are few novel enzymes responsible for mycotoxin transformation, both RNA-seq analysis for identification of key genes and metabolomic analysis by HRMS for screening unknown mycotoxin metabolism need to be done. In total, investigations on the interaction between BCAs and toxicogenic fungi, especially the inside mechanisms of BCAs on mycotoxin production of fungi, which will provide more new insights on applicable biocontrol practices in food safety and agricultural protection.

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Chapter 9
Chemical and Physical Treatments for Reducing Mycotoxin Contaminations

Dongqiang Hu and Aibo Wu

Abstract In most cases, enzymes, antifungal microbial, and various bio-sourced methods were effective for the control of mycotoxin contaminations, while it has a common deficiency that not all of the mycotoxins were desirably controlled when treating with these abovementioned means. Herein, we concluded multiple physical and chemical agents to control mycotoxins and also the most widely used way in the factory production. All of the physical and part of the chemical treatments aimed at the reduction of the mycotoxins themselves, which means destroying or transformation of their molecular structures. Most chemical reagents were used in the field experiments where they aimed at the resources of these mycotoxins, i.e., fungal pathogens. Of course, some antifungal reagents are especially absorbed or combined with these mycotoxins just like multiple bio-resourced agents. After all, these two methods played an important role in the front of food processing procedures, due to their low cost, simplicity, high efficiency, and sufficient universality.

Keywords Chemical treatment · Physical treatment · Contaminations · Reduction · Mycotoxins

9.1 Physical Treatments for Reducing Mycotoxin Contaminations

Before the appearance of variable chemical reagents and specific biogenic enzymes, physical processing was the most effective way to remove mycotoxins during the production practice, and it is still under extensive uses till now. Among these, thermo-treatment is seemed to be the simplest and operable method. Gbashi et al. (2019) suggested parameters of 210.85 °C/54.71 min for mycotoxin-contaminated maize flour as the optimum degradation conditions. Siciliano et al. (2017) also

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tested the heat exposure of 140 °C for 40 min, and it was effective for detoxification of aflatoxin with no effects on lipid profile and the nutritional quality in foodstuffs. Furthermore, during food processing at high temperature, ochratoxin A (OTA) could be turned to its thermal isomerization product 2′R-Ochratoxin A, showing different metabolic characteristics in human body (Sueck et al. 2018) (Fig. 9.1).

Besides heat, plasma and various waves were also attempted to be widely applied for mycotoxin control. The treatment of *Penicillium verrucosum* on barley after an incubation of 5 days using a $CO_2 + O_2$ plasma and air plasma resulted in a reduction of the ochratoxin A (OTA) content from 49.0 (untreated) to 27.5 (1 min) and 25.7 (1 min), respectively (Durek et al. 2018). In addition, Shi et al. (Shi 2017) confirmed the disappearance of the C8═C9 double bond in the furofuran ring of aflatoxin B1 (AFB1) after treatment of high-voltage atmospheric cold plasma (Shi 2017). Very recently, the cold atmospheric pressure plasma may overcome multiple weaknesses associated with the classical methods, and most mycotoxins exposed to this plasma were degraded almost completely within 60 s (Hojnik et al. 2017; Ten Bosch et al. 2017). Hernández et al. compared three treatments during milk storage, non-treatment, pasteurization, and thermoultrasound (Hernandez-Falcon et al. 2018), suggesting that 10 min-thermoultrasound unhomogenized milk 1 day after storage kept the lowest aflatoxin M1 (AFM1) level of 0.15 ± 0.05 pg/mL. Similarly, oven roasting and microwaving of chicken breast muscles can reduce the content of ten prevalent mycotoxins, reducing their increased bioaccessibility (Sobral et al. 2019) (Fig. 9.2).

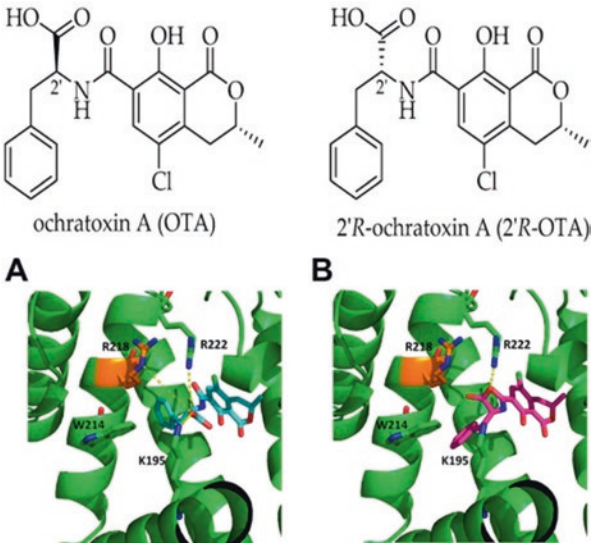

Fig. 9.1 The hypothetical molecular structures of ochratoxin A (OTA) and its thermal isomer 2′R-OTA. Important interacting residues are shown as sticks, OTA can form salt-bridge interactions with R218, R222, and K195; 2′R-OTA can only form these interactions with R222. (Sueck F et al. 2018)

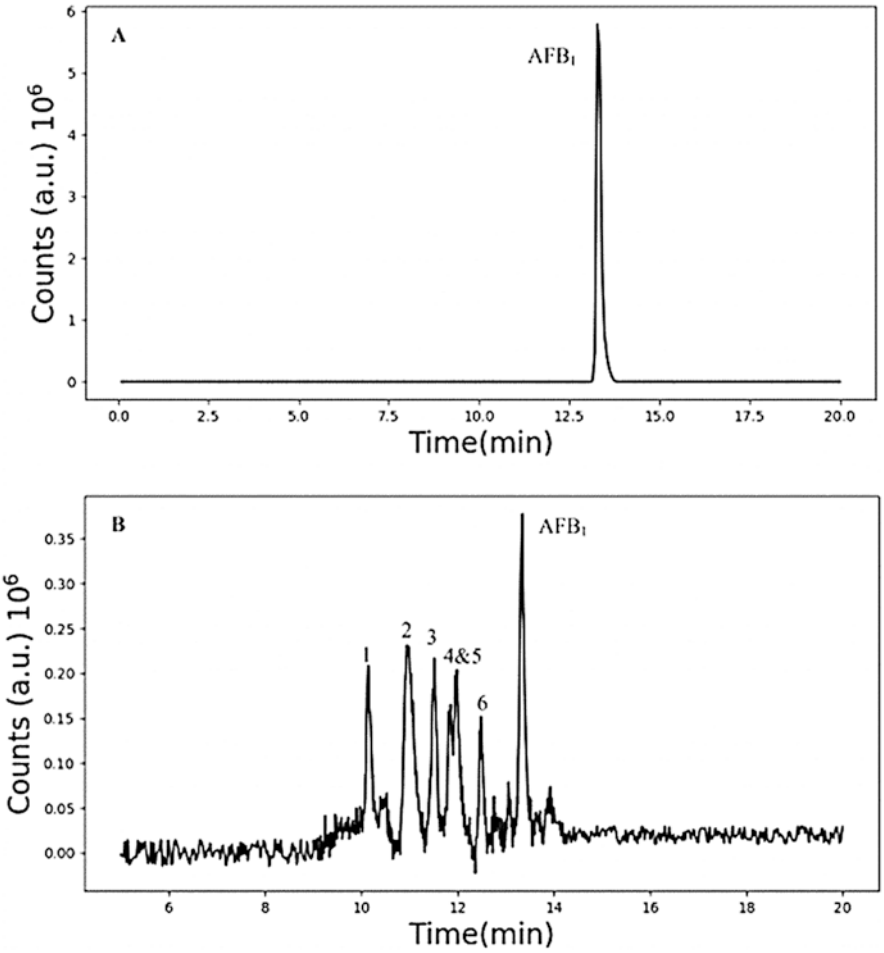

Fig. 9.2 Chromatograms of AFB1 untreated (5 μg/mL) in 50% ethanol solution (**a**) and AFB1 sample treated by HVACP in ambient air for 5 min (**b**). (Shi et al. 2017)

Interestingly, the light might be connected with mycotoxin production. As demonstrated, the optimized combinational treatments of radiation and essential oil were very effective in reducing the fungal growth and mycotoxins content (Kalagatur et al. 2018a). Also, Wang et al. found that high intensity of blue light decreased the accumulation of citrinin (CIT) production in *Monascus ruber*, providing a unique mentality of mycotoxin reduction in the field production (Wang et al. 2016) (Fig. 9.3).

Specially for mycotoxins of *Alternaria* toxins, improved food processing methods, such as milling process (Janic Hajnal et al. 2019) and extrusion processing (Janic Hajnal et al. 2016), can affect the residue of tenuazonic acid (TeA), alternariol (AOH), and alternariol monomethyl ether (AME), suggesting the demands of

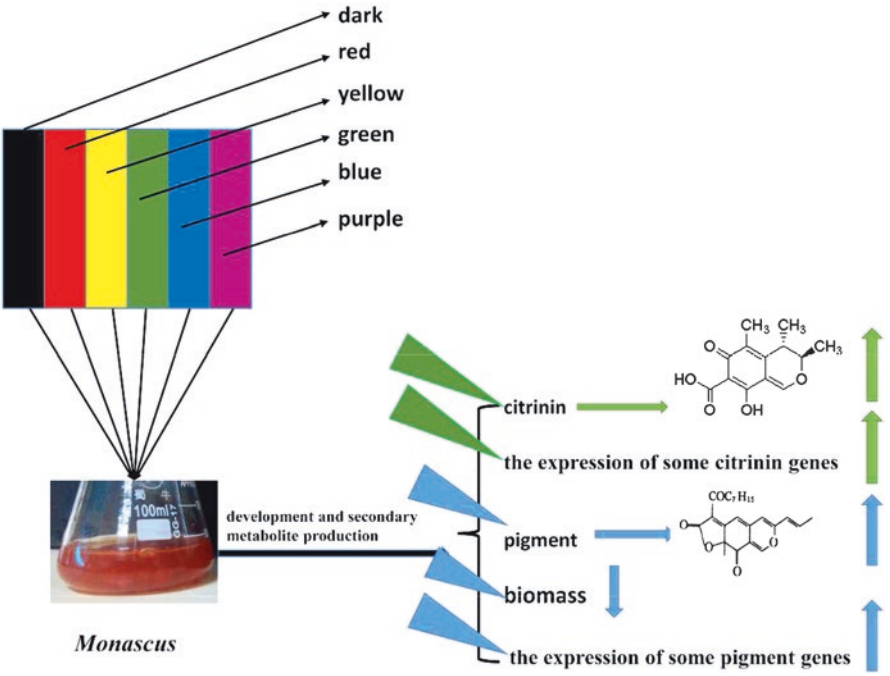

Fig. 9.3 The principles of light affect the production of citrinin (CIT). (Wang L et al. 2016)

high-grade raw-food material and optimized machine process. Not just *Alternaria*, various physical postharvest treatments were deadly for pathogenic fungi, which obviously cause oxidative injury, protein impairment, and cell wall degradation, thus reduced mycotoxin contaminations (Liu et al. 2018; Karlovsky et al. 2016), for instance, extrusion and alkaline cooking of corn kernels reduced the fumonisin toxicity for mice (Voss and Ryu 2017). Also, processing methods such as color sorting reduced the levels of aflatoxin and can be practically utilized to monitor the occurrence of aflatoxins in some commercial products (Zivoli et al. 2016) (Fig. 9.4).

9.2 Chemical Treatments for Reducing Mycotoxin Contaminations

With the rapid development of traditional inorganic and synthetic chemistry, structural biologists applied the known interplay between multiple mycotoxins and diverse structures, eliminating mycotoxins from the molecular levels. The common removers normally include various acids, fungicides, organic oils, and little parts of other materials, while all of the compounds can be divided into two parts, some are the inhibitors of fungal growth, especially the essential metabolic pathways of mycotoxin synthesis, and the others act on the mycotoxins themselves, removing them by modification or completely degradation.

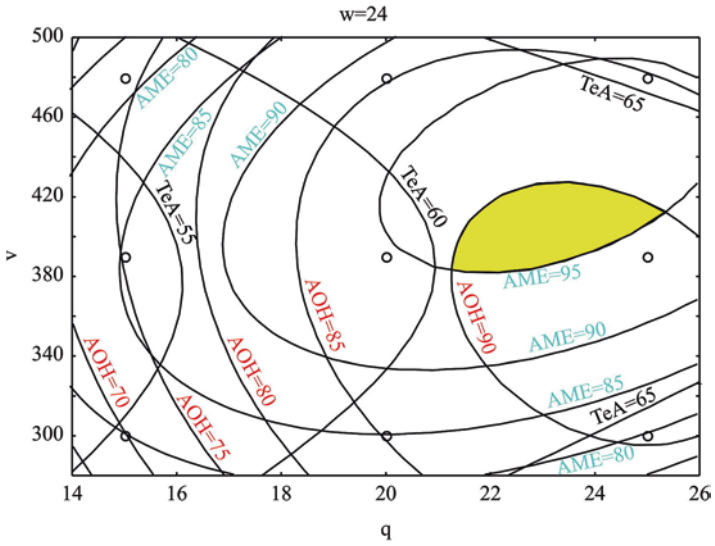

Fig. 9.4 Optimum region obtained after superimposing the contour plots of the system response. (Janic Hajnal et al. 2016)

Over the past decades, some scientists have found plenty of antifungal agents to control mycotoxin contamination. Moon et al. (2017) tested 38 coumarins and found that 4-hydroxy-7-methyl-3-phenyl coumarin was the most effective controller of aflatoxin for its downregulated function of aflD, aflK, aflQ, and aflR in the pathways of aflatoxin biosynthesis. Furthermore, coumarins can prevent the aflatoxin B_1 (AFB_1)-induced hepatotoxic effects via increasing antioxidant capacities and inhibiting the CYP450 isozyme-mediated activation (Zhang et al. 2016). One of the anthocyanins, delphinidin, showed great protection effects of gut from *Alternaria*-induced genotoxicity (Aichinger et al. 2018). Also, grape seed proanthocyanidin extract was found to be related to the activation of the Nrf2/ARE signaling pathway, which mRNA expression levels can be downregulated by zearalenone (ZEN), protecting the ZEN-induced hepatic injury (Long et al. 2016). Besides, more and more active substances, such as rutin and its derivatives (Huang et al. 2019), tannis (Peng et al. 2017) and validamycin (Li et al. 2019), were revealed to be connected with biocontrol efficiency and mycotoxin production of filamentous fungi. A similar group of biocontrol substances also includes piperine, and the main purpose was aimed at AFB1. Results showed that piperine inhibits AFB1 production while positively modulating fungal antioxidant status in *Aspergillus flavus* (Caceres et al. 2017), and thus it was demonstrated useful of its antigenotoxic effect in broiler chickens (da Silva Cardoso et al. 2016). Furthermore, researchers assessed several piperine-like synthetic compounds, in which 1-(2-methylpiperidin-1-yl)-3-phenylprop-2-en-1-one showed the most potential of antifungal and antiaflatoxigenic effects against *Aspergillus flavus* (Moon et al. 2016) (Fig. 9.5).

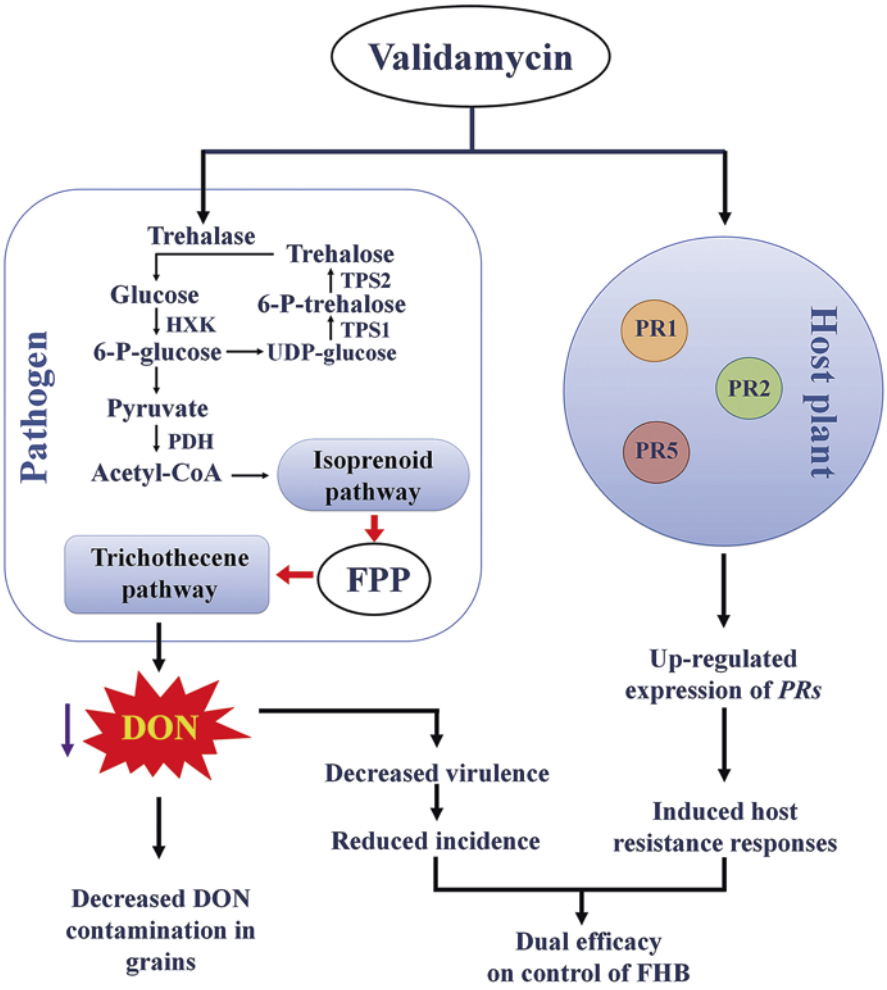

Fig. 9.5 The proposed working model of how validamycin affects the pathogen *F. graminearum* and the host wheat. (Li et al. 2019)

Fortunately, we can also see some valuable investigations aimed at the detoxification of mycotoxin in vitro. With the development of new macromolecular inorganic carbon matters such as activated carbon (Kalagatur et al. 2017), activated carbon fibers (El Khoury et al. 2018) make the mycotoxin absorption available. Similar eliminated artificial adsorbents include varied clays or bentonites. Cetylpyridinium chloride ions modified around organozeolites prepared by clinoptilolite and phillipsite are responsible for ZEN absorption (Markovic et al. 2016). Moreover, it was identified from two human cells that bentonite modified with zinc enhanced the antiaflatoxigenic ability (Nones et al. 2017). Researchers also evaluated the detoxification potential of both ZEN and AFB via multiple montmorillon-

ites (Wang et al. 2018a, 2019b) and surfactants (Wang et al. 2018b), plus, Kang et al. (2016) used the combined batch experiment with computational models, indicating that adsorption affinity of mycotoxins is dependent on the surfaces of clay aggregates. With the aid of micropore structure in chitosan derivatives (Yang et al. 2017), shrimp shells (Assaf et al. 2018), natural zeolites (Eroglu et al. 2017), and bentonite clay (Shannon et al. 2016), small molecules of mycotoxins were all sedimented and aggregated to the fixed microporous structure, reducing their toxicities to variable target organisms (Fig. 9.6).

There is also one part of important mycotoxin absorbents or antifungal agents, nanocomposites. Gao et al. (2019) synthesized an organic-inorganic P(DMDAAC-AGE)/Ag/ZnO composite for the usage of antibacterial cotton fabrics with laundering durability, showing more than 99.00% of anti-mildew performance even after 11 washing cycles. A kind of surface active maghemite nanoparticles was made to bind citrinin; mass spectroscopy and magnetization measurements showed that the material was an efficient and reliable mean for citrinin removal (Magro et al. 2016). Ag doped hollow TiO_2 nanoparticles were identified to be effective in protecting farm products affected by *Fusarium solani* or other fungi, where the Ag promotes the formation of stable Ag-S and disulfide bonds (R-S-S-R) in cellular protein, leading to cell damage (Boxi et al. 2016). Furthermore, nanocomposites mixed with other agents can significantly increase their antifungal property, such as chitosan nanoparticles encapsulated with essential oil (Kalagatur et al. 2018b), pyraclostrobin solid nanodispersion by self-emulsifying technique (Wang et al. 2019a).

Ozone treatment was also effective for pathogen prevention and mycotoxin control. It has been concluded that ozone has great potential to improve the functional-

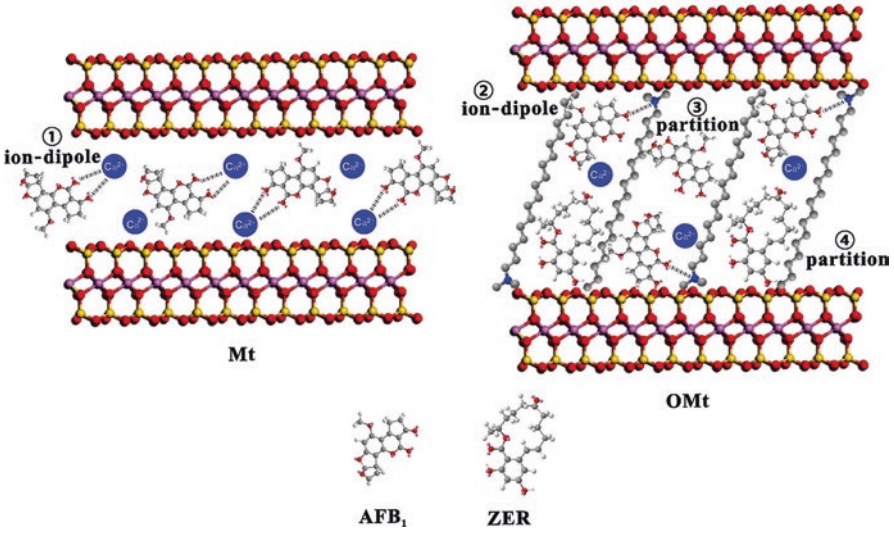

Fig. 9.6 Adsorption/partition mechanisms of adsorption of AFB1 to natural Mt. (Wang et al. 2018a, b)

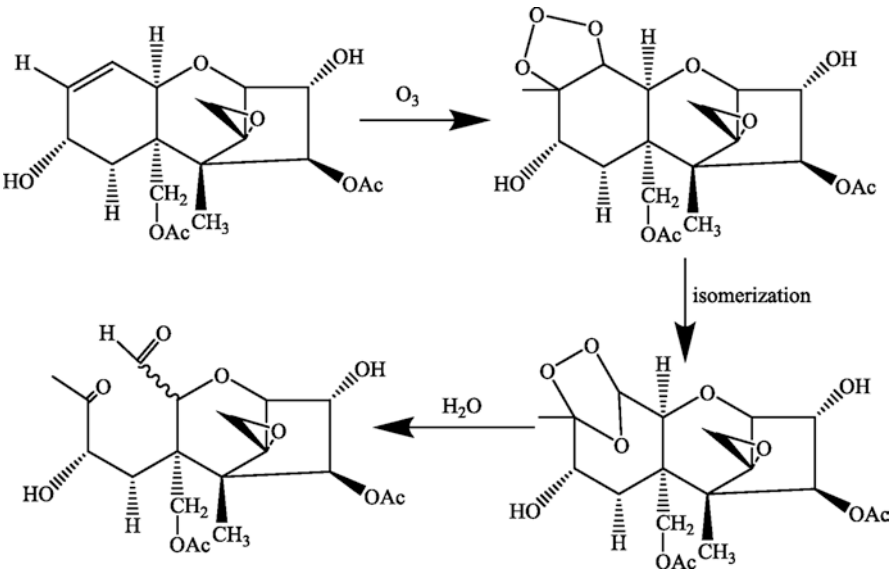

Fig. 9.7 The proposed mechanism of the reaction of ozone to the mycotoxin NEO. (Hua-Li et al. 2018)

ities of grain products while ensuring food safety (Zhu 2018). After treatment of ozone at 1.10 mg/L for 120 min, *Fusarium* rot development and neosolaniol accumulation in fruits were discriminately controlled (Hua-Li et al. 2018) (Fig. 9.7).

Interestingly, potassium may also relate to the antifungal attempt. *Penicillium expansum* under 5 mM potassium phosphite stress presented yield reduction of patulin and lower infectivity to apple fruit (Lai et al. 2017). Moreover, Chang et al. demonstrated that addition of triphenylphosphonium cation can enhance or at least restore compounds' antifungal activity, resulting in mitochondrial dysfunction and increased levels of intracellular reactive oxygen species (Chang et al. 2018).

Besides, other common chemical treatments to remove mycotoxins were varied organic active substances; among these organic acids, essential oils, aldehyde derivatives, organism extracts, and artificial fungicides were the substances with great representations.

9.3 Organic Acids

Acids play a great role in control of pathogens and mycotoxins; single or multiple combined acids were proved effective in the food or food products protection. Researchers purified cuminic acid from the seeds of *Cuminum cyminum* L., which significantly distorted the mycelial morphology of *Fusarium oxysporum*; more importantly, synthetic genes of bikaverin (Bike1, Bike2, and Bike3) and fusaric acid

(FUB1, FUB2, FUB3 and FUB4) both were downregulated (Sun et al. 2017). Also, p-aminobenzoic acid was present in the secretions of rhizobacterium *Lysobacter* to show a broad spectrum of antifungal activities, which inhibits septation during cell division (Laborda et al. 2019). Most acids targeted in the membrane of fungal hyphae, ethyl p-coumarate, can cause severe lipid peroxidation and heavy oxidative damage to the cellular membranes and organelles of pathogens (Li et al. 2018). Chlorogenic and caffeic acids were also groups of antifungal micromolecular acids; both can be degraded into protocatechuic acid in the *Fusarium graminearum* and dramatically enhanced the counteraction of the fungal ability of mycotoxin production (Gauthier et al. 2016). Similar compounds including I-pyroglutamic acid (Bilska et al. 2018), sinapic acid (Kulik et al. 2017a), fluorinated dihydroguaiaretic acid (Yamauchi et al. 2017), etc. were all with antifungal potential. Meanwhile, gene expression revealed that *Tri* genes responsible for trichothecene biosynthesis were downregulated after acid treatment (Fig. 9.8).

Combination of multiple or even unknown acids seems like to have synergistic effects for the antifungal property. For example, the cell-free supernatant of *Lactobacillus plantarum* inhibits the production of aflatoxins by 91%, where lactic acid, phenyllactic acid, hydroxyphenyllacetic acid, and indole lactic acid were detected in the metabolites and all were tested separately with antiaflatoxigenic property (Guimaraes et al. 2018). Combination of exogenous abscisic acid and gibberellic acid can also reduce head blight severity, as phytohormones, combining fungicidal agents and these acids may also be useful for the management of head blight infection (Buhrow et al. 2016). Phenolic acids were a group of organic matters with a phenolic hydroxyl group; caffeic, chlorogenic, ferulic, and p-coumaric

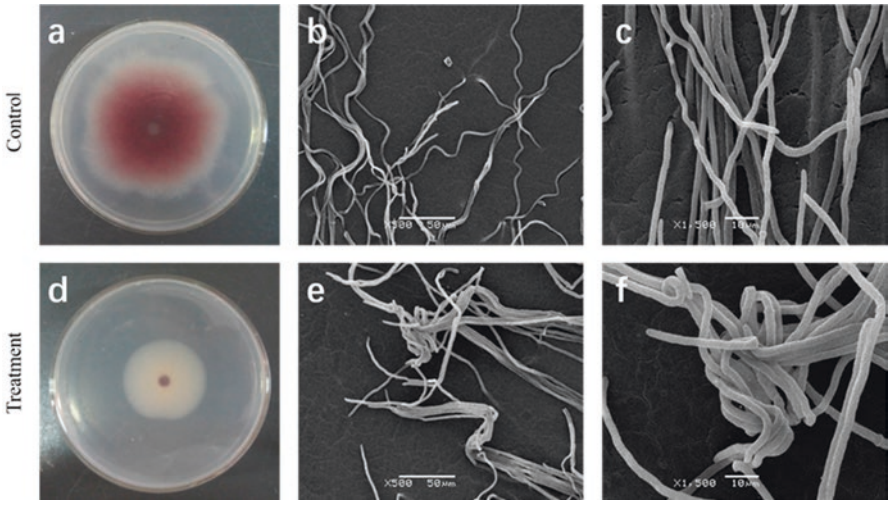

Fig. 9.8 Effect of cuminic acid on mycelia morphology of *Fusarium oxysporum* f. sp. *niveum*. (**d**–**f**) Untreated plates; (**a**–**c**) plates treated with cuminic acid at EC50 value (22.53 μg/mL). Values are means and standard errors. (Sun Y et al. 2017)

acid were all tested. All those have somewhat variable effects on fungal growth and mycotoxin production, depending on the host strain and the concentration (Ferruz et al. 2016). Moreover, Ferruz et al. also proved that phenolic acids have the antifungal property and upregulated ergosterol biosynthesis by *Fusaria* (Kulik et al. 2017b).

9.4 Fungicides

Traditional fungicides were mostly aimed at pathogen growth inhibition, thus leading to the reduction of multiple mycotoxins. Wayne et al. (Jurick et al. 2019) tested the sensitivity of *Penicillium* spp. to difenoconazole and suggested a minimum limitation of 5 μg/ml to fruit quality and mycotoxin control. A field experiment showed that prothioconazole could significantly reduce the total deoxynivalenol and zearalenone contents by applications at 18th leaf and R2 stages, while not visibly changing other mycotoxin contents (Limay-Rios and Schaafsma 2018).

And tebuconazole was also one efficient fungicide, the addition of tebuconazole significantly decreases the DON production, and meanwhile, TRI5 and TRI6 of biosynthetic genes were downregulated (Diao et al. 2018). Similarly, epoxiconazole (Duan et al. 2018), triticonazole (Zhang et al. 2018), arylpyrazole (Zhang et al. 2017), and varied triazoles (Hellin et al. 2017) were all verified to be effective to the pathogen prevention and mycotoxin control (Fig. 9.9).

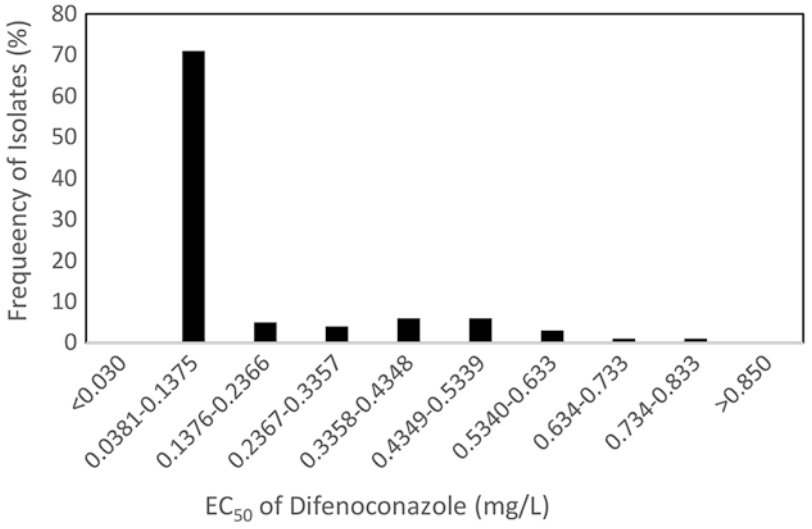

Fig. 9.9 Frequency distribution of isolates in fungicide concentration that inhibit mycelial growth by 50% for technical grade difenoconazole for *Penicillium* isolates. (Jurick et al. 2019)

As a powerful and simple method to control fungal pathogens, fungicides were always compared with other emerging antifungal agents, but no doubt, fungicides were still the most widely used field method. Scaglioni et al. (Scaglioni and Blandino 2018) compared the inhibition effects of fumonisin production between tebuconazole and microalgal phenolic extracts, and both showed antifungal potential, while either would directly control the fumonisin contents. Moreover, a direct comparison of the synthetic commercial antifungal compound and the antifungal protein PgAFP showed that both were with fungal growth inhibition. Furthermore, the presence of PgAFP produced a significant reduction in mycotoxins accumulation (da Cruz Cabral et al. 2019). Additionally, researchers investigated the effects of two fungicides, quinone outside inhibitors and demethylation inhibitors, on *Fusarium* head blight and deoxynivalenol production, and results suggested that the combination effect of two fungicides depended on the active ingredients and whether the two were applied as a mixture at heading or sequentially (Paul and Bradley 2018). Anyway, new emerging antifungal agents were always tested by making comparison with these commercial fungicides, which represents a dominance of fungicides in fungal pathogens and mycotoxins control in a very long period (Fig. 9.10).

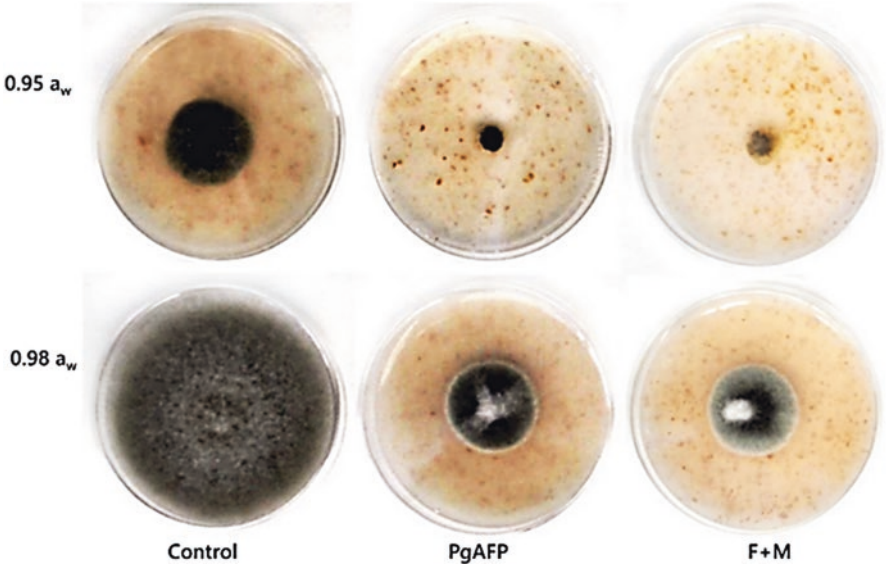

Fig. 9.10 The changes on the colonies of *Alternaria tenuissima* sp. grp. grown on wheat media in the presence of two antifungal treatments (PgAFP and F + M) at two water activity (aw) levels, 0.95 and 0.98 after 6 days of incubation at 25 °C. (da Cruz Cabral L et al. 2019)

9.5 Aldehydes, Essential Oils, and Organism Extracts

Besides organic acids, aldehydes and their derivatives were also popular for mycotoxin control, and most of them were derived from plants, especially herbs. Harohally et al. (2017) tried to enhanced the antiaflatoxigenic activity of 2-hydroxy-4-methoxybenzaldehyde, cinnamaldehyde and the molecules with similar chemical structures, and the productions, Schiff bases, which showed great commendable antiaflatoxigenic activity. Plus, the antiaflatoxigenic potential of cinnamaldehyde was attributed to alleviate oxidative stress, which was possibly induced by modifications of cellular structure (Sun et al. 2016). Meanwhile, cuminaldehyde thiosemicarbazone was structurally modified, and then it was testified to be more special for aflatoxin control via yeast experimental model (Degola et al. 2017).

Similarly, essential oils were mainly separated from medical plants, different from other antifungal agents; most of these oils were mixtures with complicated composition. As reported, the bioactive ethylene-vinyl alcohol copolymer (EVOH) films, containing cinnamaldehyde, linalool, isoeugenol, or citral, showed strong inhibition of OTA biosynthesis in corn at very low concentrations (Tarazona et al. 2018). And Mateo et al. (2017) compared several oils contained in EVOH films; the result showed that EVOH-cinnamaldehyde film showed the most inhibition of fungal growth and aflatoxin control. Natural essential oil derived from turmeric was used as an eco-friendly antifungal agent. Moreover, expression of mycotoxin genes in aflatoxin biosynthetic pathway was relatively downregulated (Hu et al. 2017). The essential oil of rosemary was also applied to the investigation of multiple pathogens, including *Alternaria* spp., *Fusarium* spp., *Colletotrichum* spp., and *Penicillium* spp., all with good control practice (Waithaka et al. 2017). Moreover, similar essential oils with antifungal properties were extracted from other plants, such as *Hedychium spicatum L.* (Kalagatur et al. 2018a), *Mentha cardiaca L.* (Dwivedy et al. 2017), and so on. Clove oil-in-water nanoemulsions were one new material with different oil composition, and addition of corn oil or triacylglycerol would reduce its antifungal potential to this material, while the inhibition activity would be enhanced when clove oil was encapsulated in nanoemulsions (Wan et al. 2018).

Actually, all the pure antifungal agents were from multiple organisms; as a result, extractions from these can also be of antifungal potential. Researchers investigated the reduction rate of grape pomace to varied mycotoxins, and it can reduce the gastrointestinal absorption of mycotoxins, as well as be a natural binder for multiple mycotoxins (Gambacorta et al. 2016). In the extraction of peanut, peanut stilbenoids could provide the new strategies for preventing plant invasion and production of aflatoxin (Sobolev et al. 2018). Furthermore, the fermentation liquid of *Bacillus subtilis* also showed great growth inhibition to varied fungal pathogens, for its volatile organic compounds in the metabolites (Gao et al. 2018) (Fig. 9.11).

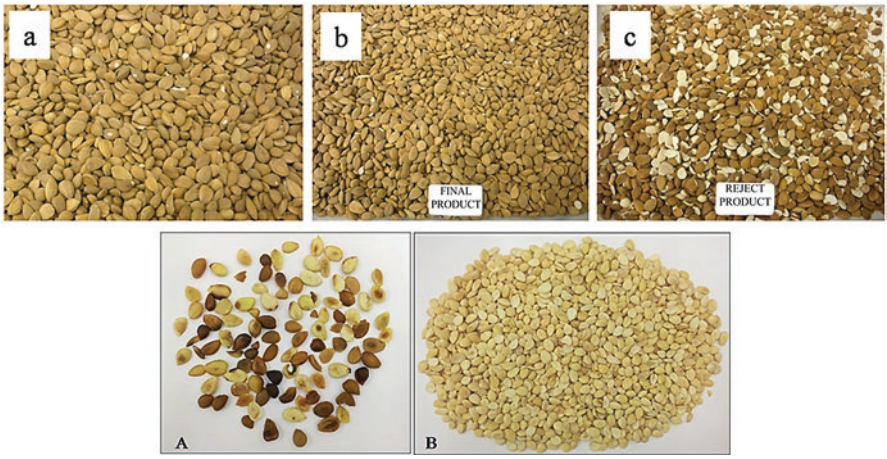

Fig. 9.11 Initial apricot kernels (**a**), final product (**b**) and reject product (**c**) obtained by e-sorting and discolored apricot kernel (**a**, **b**) and healthy apricot kernels. (Zivoli et al. 2016)

9.6 Conclusion

To sum up, physical treatments for control of mycotoxin contamination were aimed at the molecular structure of mycotoxins; change of multiple physical parameters leads to the destruction or modification of their carbon skeleton, resulting in the removal of origin mycotoxins. On the other hand, for chemical treatments, most detoxifiers were aimed at the antifungal property, thus leading to the reduction of mycotoxins. Chemical reagents are too large of a group; some are broad antifungal agents, and some are specific, also with little certain microstructures to absorb mycotoxins. Unlike other emerging bio-sourced enzymes, physical and chemical detoxifiers were always the direct, simple, and broad ways to remove mycotoxins, or we can conclude that pretreatment of foodstuffs would always be these traditional ways to remove mycotoxins and protect food safety. Industrial production should be a processing chain, from treatment to the early generation and then to the final package and delivery; among these, before treatment, either the physical or chemical pretreatments show their indispensability in practical uses.

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Part IV
Summary and Prospective

Chapter 10
Summary and Prospective

Aibo Wu

Abstract The key points related to three above aspects related to food safety and mycotoxins will be proposed. Also, the authors will predict some upcoming research activities in the future. As we all know, due to the increasingly agro-economical importance and highly frequent contamination in foods and feed at relatively high levels, together with the un-detoxifying characteristics in post-harvest strategies or procedures, mycotoxins are arousing more and more attentions in the fields of agriculture, food, and environment. Similar to other hazardous contaminants, analysis, risk assessment, and control of mycotoxins are faced with great challenges on complex matrices, trace levels, unknown forms, effective and practical prevention, or degradation approaches. According to the target mycotoxins focused in the group, this book is more concentrated on *Fusarium* mycotoxins in cereal grains, with more relevance to two aspects of food security and food safety. The overall ongoing perspectives are still to invent new venues to rapidly and sensitively recognize the presence of mycotoxins contamination, reasonably evaluate the risk levels and shifting ways, and ultimately dramatically reduce the existing hazards to the safe levels for human beings and animal health. The specifically proposed or ideal means for detection, risk assessment, and control of mycotoxins in foods related to food safety are concluded in details according to the future plans or designs for experimental research on mycotoxins.

Keywords Mycotoxins · Analysis · Risk assessment · Control · Food safety

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10.1 The Applicable Ideal Framework of Rapid Detection and Confirmed Lab Analysis

The analytical methodologies and achieved data are always the basis for monitoring and regulation systems of food safety in different geographic locations, either developed or developing countries. On the common sense, rapid detection is generally performed for large-scale analysis due to its quickness and low cost. Afterward, lab analysis would be operated for the small portion of positive or likely positive samples based on the above obtained results. According to this principle, the simplest sample preparation procedures would be first clarified with automatically operation for large-scale screening or confirmed analysis on lab equipments, against the multiple mycotoxins existed in nature at utmost volumes. Followed by the analysis, the data obtained from rapid detection or quantitation would be wirelessly transported to the platforms of smartphone-based app or office computers. Finally, the data with negative for non-menace or positive for warning would be appeared for subsequent decision-makers. Also, the continuous collected information for the detected samples would accurately reflect the contamination levels or risks, thus supporting to choose the appropriate approaches for control to the safety level as ultimate goals (Fig. 10.1).

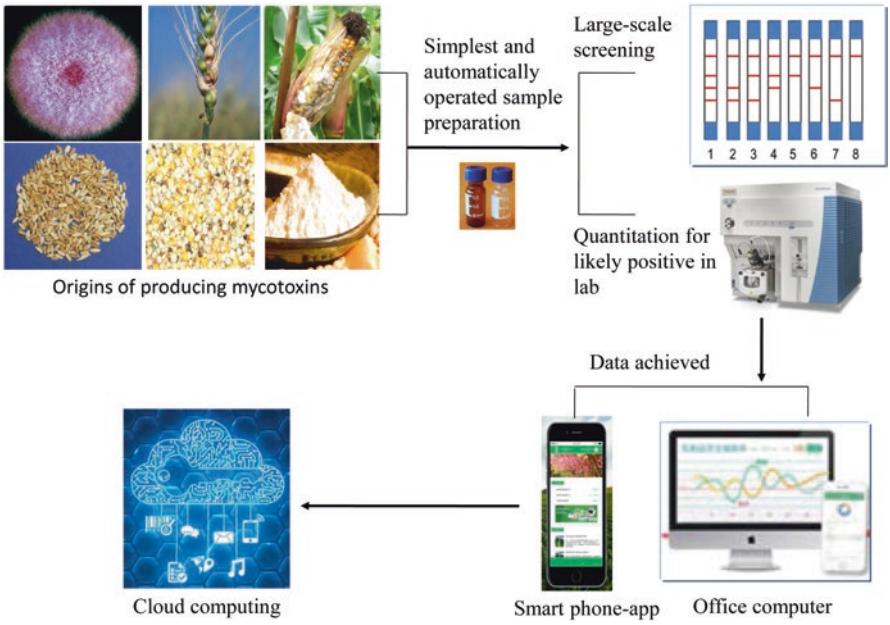

Fig. 10.1 The applicable ideal analytical model for practical detection of mycotoxins in various matrices

10.2 The Substantial Support of Risk Assessment for Updating the Thresholds of Co-occurring Mycotoxins and Other Hazards

Actually various countries and international organizations have already made the standards for the safe limits of main mycotoxins contaminated in different variety of foods and feeds, despite of some differences on the thresholds or sample types. However, multiple mycotoxins are always co-occurred in one single food or feed stuffs and always together with other harmful substances as hazards, such as pesticide or veterinary residues, as well as illegal additives, etc. The toxicity of one single mycotoxin is usually evaluated via cells or experimental animal-based methods, which are directly utilized as valuable references for supporting its legislations on the limits of the target mycotoxin in real samples. Unfortunately, these data achieved are not so persuasive and not so solid as toxicological evidences. On the one hand, with more understanding on the models of cells or animals as evaluation tools, more toxic phenomena would be observed, with efforts to lowering the limits for some purposes on economical reasons or safety concerns based on new toxicological proofs. Simultaneously, risk assessments on only one mycotoxin are not so consistent to the real situation of co-occurring mycotoxins or other hazards in real samples. Thus, the solutions for toxicological evaluations on the mixtures of various mycotoxins or other hazards would be the most tough tasks in the field to risk assessment of mycotoxins, especially considering the problems of food safety. Also, more importantly, the external and internal exposure assessments by analyses of consumed foods and human biological samples of urine and blood would be considered for large-scale food samples and diverse populations as representative data. Anyhow, there are key factors including large-scale sample analysis and comparative toxicological evaluation, as well as long-term investigation, which should be completed before the conclusion of risk assessment of the mentioned mixtures for updating the already existed standards (Fig. 10.2).

10.3 The Safe, High-Efficient, and Acceptable Control Approaches for Detoxifications in Pre- and Post-Harvest Practices

Realistically, to the best of our knowledge, there are no single solutions for various mycotoxins control in practice. On the principle, as shown in Fig. 10.3, from the beginning in the field of agriculture, the effective means is to inhibit or obviously reduce the growth of mycotoxin-producing fungal pathogens and then to cut off the original biomass for mycotoxin production in fields. As follows, after the cereal grains were harvested for storage and further sow. The enzymes for detoxification

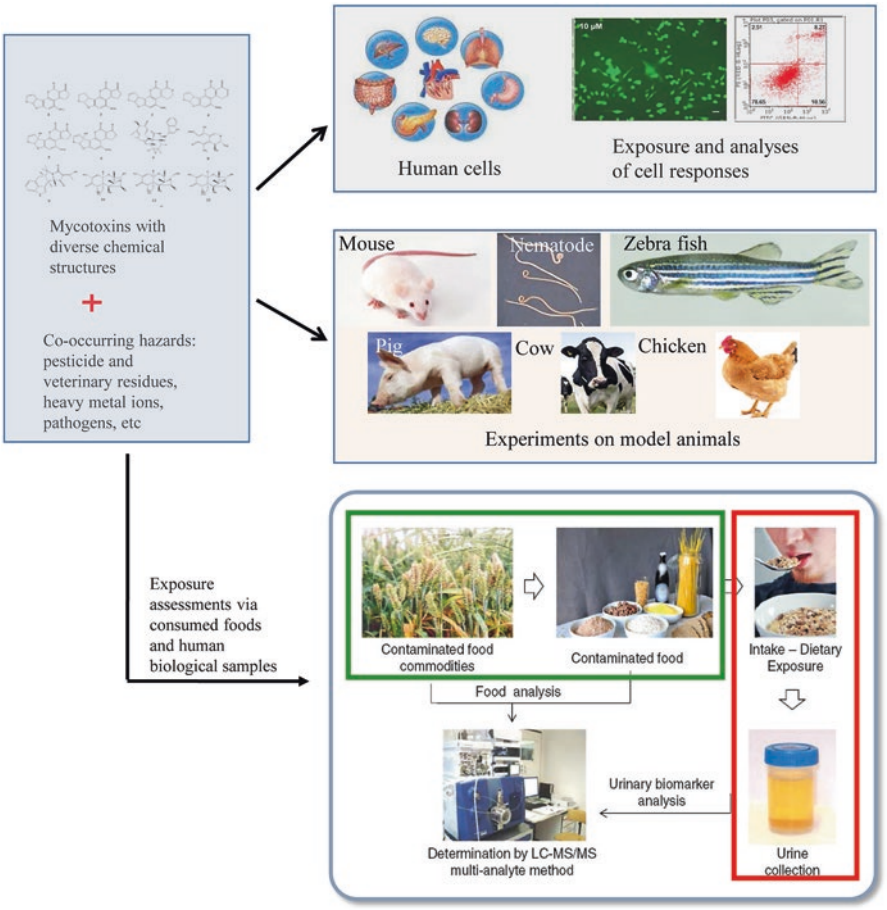

Fig. 10.2 Proposed risk assessments for the mixtures of co-occurring mycotoxins or other hazards by toxicological evaluations via human cells or model experimental animals and exposures via analyses of consumed foods and human biological samples of urine and blood

purposed would be more optimal for practicability, with the functionalities of degrading mycotoxin.

However, even so, the contamination of mycotoxins in foods and feeds are still not solved at this moment. Considering the long life cycle of mycotoxin-producing phytopathogens and non-clearance of contaminated mycotoxins in food chain, more appropriate approaches with safer and more efficient control are indeed needed in the whole food chain, either physical, chemical, or biological strategies.

Totally, to sum up, the solutions for strict regulation on mycotoxins contaminated in the whole food chain are not so easy to operate but solely counting on the basis of scientific knowledge and close collaborations from multidisciplines and diverse nationalities at global level. Also, as expected, the current knowledge and

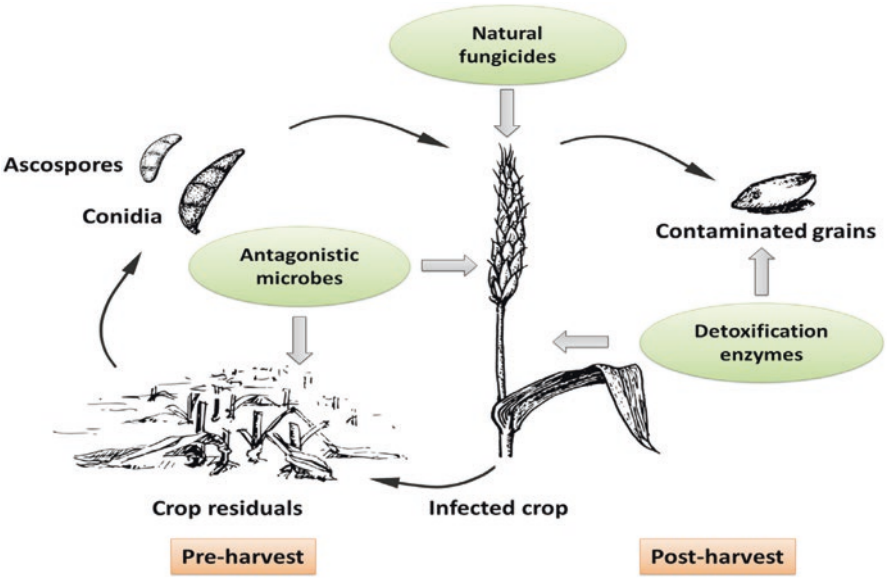

Fig. 10.3 Combinatorial strategies of mycotoxins control in pre- and post-harvest stages via various physical chemical and biological means. (Tian et al. 2016)

methodological advances plus suggestions mentioned in this book will somewhat contribute to graduate students, research scientists, and policy-makers in governmental agencies, as well as the industrial companies or even famers for practical uses.

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