Chapter 2
Sample Preparation and Chromatographic Analysis

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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).

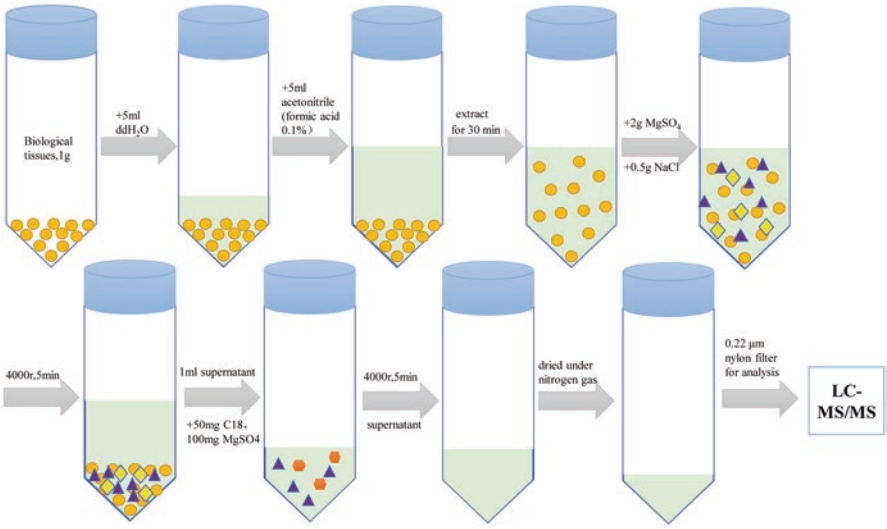

Fig. 2.1 Schematic diagrams of quick, easy, cheap, effective, rugged, and safe (QuEChERS) method for extracting mycotoxins

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.

References

Almeida MI, Almeida NG, Carvalho KL, Gonçalves GA, Silva CN, Santos EA, Garcia JC, Vargas EA (2012) Co-occurrence of aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A, zearalenone, deoxynivalenol, and citreoviridin in rice in Brazil. Food Addit Contam 29(4):694–703

Al-Taher F, Cappozzo J, Zweigenbaum J, Lee HJ, Jackson L, Ryu D (2017) Detection and quantitation of mycotoxins in infant cereals in the U.S. market by LC-MS/MS using a stable isotope dilution assay. Food Control 72:27–35. https://doi.org/10.1016/j.foodcont.2016.07.027

Andrade PD, Dantas RR, Caldas ED (2017) Determination of multi-mycotoxins in cereals and of total fumonisins in maize products using isotope labeled internal standard and liquid chromatography/tandem mass spectrometry with positive ionization. J Chromatogr A 1490:138–147

Arroyo-Manzanares N, Huertas-Pérez JF, Gámiz-Gracia L, García-Campaña AM (2015) Simple and efficient methodology to determine mycotoxins in cereal syrups. Food Chem 177:274–279

Bennett JW, Klich M (2009) Mycotoxins. Encycl Microbiol 16(3):559–565

Berthiller F, Schuhmacher R, Buttinger G, Krska R (2005) Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize by high performance liquid chromatography–tandem mass spectrometry. J Chromatogr A 1062(2):209–216

Blesa J, Moltó JC, Akhdari SE, Mañes J, Zinedine A (2014) Simultaneous determination of Fusarium mycotoxins in wheat grain from Morocco by liquid chromatography coupled to triple quadrupole mass spectrometry. Food Control 46:1–5

Cassandra MC, Paul T, Li-Sheng D, Xun L, Yi-Ming L (2014) Extraction of aflatoxins from liquid foodstuff samples with polydopamine-coated superparamagnetic nanoparticles for HPLC-MS/MS analysis. J Agric Food Chem 62(19):4261–4267

Chia-Ding L, Wong JW, Kai Z, Hayward DG, Lee NS, Trucksess MW (2013) Multi-mycotoxin analysis of finished grain and nut products using high-performance liquid chromatography-triple-quadrupole mass spectrometry. J Agric Food Chem 61(20):4771–4782

da Luz SR, Pazdiora PC, Dallagnol LJ, Dors GC, Chaves FC (2017) Mycotoxin and fungicide residues in wheat grains from fungicide-treated plants measured by a validated LC-MS method. Food Chem 220:510–516. https://doi.org/10.1016/j.foodchem.2016.09.180

Dashti B, Al-Hamli S, Alomirah H, Al-Zenki S, Abbas AB, Sawaya W (2009) Levels of aflatoxin M1 in milk, cheese consumed in Kuwait and occurrence of total aflatoxin in local and imported animal feed. Food Control 20(7):0–690

de Oliveira GB, de Castro Gomes Vieira CM, Orlando RM, Faria AF (2017) Simultaneous determination of fumonisins B1 and B2 in different types of maize by matrix solid phase dispersion and HPLC-MS/MS. Food Chem 233:11–19. https://doi.org/10.1016/j.foodchem.2017.04.091

De SB, De EP, Montevecchi G, Garbini D, Masino F, Antonelli A, Melucci D (2018) Determination of four *Alternaria alternata* mycotoxins by QuEChERS approach coupled with liquid chromatography-tandem mass spectrometry in tomato-based and fruit-based products. Food Res Int 106:677–685

Degen GH, Partosch F, Muñoz K, Gundert-Remy U (2017) Daily uptake of mycotoxins - TDI might not be protective for nursed infants. Toxicol Lett 277:S0378427417302278

Dong H, Xian Y, Xiao K, Wu Y, Zhu L, He J (2019) Development and comparison of single-step solid phase extraction and QuEChERS clean-up for the analysis of 7 mycotoxins in fruits and vegetables during storage by UHPLC-MS/MS. Food Chem 274:471–479

Du LJ, Chu C, Warner E, Wang QY, Hu YH, Chai KJ, Cao J, Peng LQ, Chen YB, Yang J (2018) Rapid microwave-assisted dispersive micro-solid phase extraction of mycotoxins in food using zirconia nanoparticles. J Chromatogr A 1561:S0021967318306149

Ediage EN, Poucke CV, Saeger SD (2015) A multi-analyte LC–MS/MS method for the analysis of 23 mycotoxins in different sorghum varieties: the forgotten sample matrix. Food Chem 177:397–404

Fan K, Xu J, Jiang K, Liu X, Meng J, Di Mavungu JD, Guo W, Zhang Z, Jing J, Li H, Yao B, Li H, Zhao Z, Han Z (2019) Determination of multiple mycotoxins in paired plasma and urine samples to assess human exposure in Nanjing, China. Environ Pollut 248:865–873. https://doi.org/10.1016/j.envpol.2019.02.091

Flores-Flores ME, González-Peñas E (2015) Development and validation of a high performance liquid chromatographic–mass spectrometry method for the simultaneous quantification of 10 trichothecenes in ultra-high temperature processed cow milk. J Chromatogr A 1419:37–44

Frenich AG, Vidal J, Romero R (2009) Simple and high-throughput method for the multimycotoxin analysis in cereals and related foods by ultra-high performance liquid chromatography/tandem mass spectrometry. Food Chem 117(4):705–712

García-Moraleja A, Font G, Mañes J, Ferrer E (2015a) Development of a new method for the simultaneous determination of 21 mycotoxins in coffee beverages by liquid chromatography tandem mass spectrometry. Food Res Int 72:247–255

García-Moraleja A, Font G, Mañes J, Ferrer E (2015b) Simultaneous determination of mycotoxin in commercial coffee. Food Control 57:282–292

Gerding J, Ali N, Schwartzbord J, Cramer B, Dan LB, Degen GH, Humpf HU (2015) A comparative study of the human urinary mycotoxin excretion patterns in Bangladesh, Germany, and Haiti using a rapid and sensitive LC-MS/MS approach. Mycotoxin Res 31(3):127–136

Girolamo AD, Ciasca B, Stroka J, Bratinova S, Pascale M, Visconti A, Lattanzio VMT (2016) Performance evaluation of LC–MS/MS methods for multi-mycotoxin determination in maize and wheat by means of international proficiency testing. TrAC Trends Anal Chem 86:222–234

González-Sálamo J, Socas-Rodríguez B, Hernández-Borges J, Rodríguez-Delgado MÁ (2017) Core-shell poly(dopamine) magnetic nanoparticles for the extraction of estrogenic mycotoxins from milk and yogurt prior to LC–MS analysis. Food Chem 215:362–368

Gutzwiller A, Gafner JL, Silacci P (2014) Urinary zearalenone measured with ELISA as a biomarker of zearalenone exposure in pigs. Mycotoxin Res 30(4):187–190

Hashemi M, Taherimaslak Z, Rashidi S (2014) Application of magnetic solid phase extraction for separation and determination of aflatoxins B 1 and B 2 in cereal products by high performance liquid chromatography-fluorescence detection. J Chromatogr B Anal Technol Biomed Life Sci 960(960C):200–208

Huang LC, Zheng N, Zheng BQ, Wen F, Cheng JB, Han RW, Xu XM, Li SL, Wang JQ (2014) Simultaneous determination of aflatoxin M 1, ochratoxin A, zearalenone and α-zearalenol in milk by UHPLC–MS/MS. Food Chem 146:242–249

Huybrechts B, Martins JC, Debongnie P, Uhlig S, Callebaut A (2015) Fast and sensitive LC–MS/MS method measuring human mycotoxin exposure using biomarkers in urine. Arch Toxicol 89(11):1993–2005

Irena Kralj C, Helena P (2009) An overview of conventional and emerging analytical methods for the determination of mycotoxins. Int J Mol Sci 10(1):62–115

Jiang K, Huang Q, Fan K, Wu L, Nie D, Guo W, Wu Y, Han Z (2018) Reduced graphene oxide and gold nanoparticle composite-based solid-phase extraction coupled with ultra-high-performance liquid chromatography-tandem mass spectrometry for the determination of 9 mycotoxins in milk. Food Chem 264:218–225

Juan C, Mañes J, Font G, Juan-García A (2017a) Determination of mycotoxins in fruit berry by-products using QuEChERS extraction method. Lwt 86:344–351. https://doi.org/10.1016/j.lwt.2017.08.020

Juan C, Mañes J, Font G, Juan-García A (2017b) Determination of mycotoxins in fruit berry by-products using QuEChERS extraction method. LWT Food Sci Technol 86:344–351

Koesukwiwat U, Sanguankaew K, Leepipatpiboon N (2014) Evaluation of a modified QuEChERS method for analysis of mycotoxins in rice. Food Chem 153(9):44–51

Krska R, Pettersson H, Josephs RD, Lemmens M, Donald S, Mac WE (2003) Zearalenone in maize: stability testing and matrix characterisation of a certified reference material. Food Addit Contam 20(12):1141–1152

Liao CD, Wong JW, Zhang K, Yang P, Wittenberg JB, Trucksess MW, Hayward DG, Lee NS, Chang JS (2015) Multi-mycotoxin analysis of finished grain and nut products using ultrahigh-performance liquid chromatography and positive electrospray ionization-quadrupole orbital ion trap high-resolution mass spectrometry. J Agric Food Chem 63(37):8314–8332

Llorent-Martínez EJ, Fernández-Poyatos MP, Ruiz-Medina A (2019) Automated fluorimetric sensor for the determination of zearalenone mycotoxin in maize and cereals feedstuff [J]. Talanta 191:89–93

Mao J, Nan Z, Fang W, Guo L, Fu C, Ouyang H, Zhong L, Wang J, Lei S (2017) Multi-mycotoxins analysis in raw milk by ultra high performance liquid chromatography coupled to quadrupole orbitrap mass spectrometry. Food Control 84:S0956713517304036

Meerpoel C, Vidal A, Di Mavungu JD, Huybrechts B, Tangni EK, Devreese M, Croubels S, De Saeger S (2018) Development and validation of an LC-MS/MS method for the simultaneous determination of citrinin and ochratoxin A in a variety of feed and foodstuffs. J Chromatogr A 1580:100–109

Miró-Abella E, Herrero P, Canela N, Arola L, Borrull F, Ras R, Fontanals N (2017) Determination of mycotoxins in plant-based beverages using QuEChERS and liquid chromatography–tandem mass spectrometry. Food Chem 229:366–372

Monbaliu S, Poucke KV, Heungens K (2010) Production and migration of mycotoxins in sweet pepper analyzed by multimycotoxin LC-MS/MS. J Agric Food Chem 58(19):10475–10479

Myresiotis CK, Testempasis S, Vryzas Z, Karaoglanidis GS, Papadopoulou-Mourkidou E (2015) Determination of mycotoxins in pomegranate fruits and juices using a QuEChERS-based method. Food Chem 182:81–88

Natalia AM, Huertas-Pérez JF, Laura GG, García-Campaña AM (2013) A new approach in sample treatment combined with UHPLC-MS/MS for the determination of multiclass mycotoxins in edible nuts and seeds. Talanta 115(17):61–67

Nicholson P, Magan N, Olsen M (2004) Rapid detection of mycotoxigenic fungi in plants. In: Mycotoxins in food: detection and control. Elsevier Science, Burlington

Oliveira GBD, Menezes C, Orlando RM, Faria AF (2017) Simultaneous determination of fumonisins B1 and B2 in different types of maize by matrix solid phase dispersion and HPLC-MS/MS. Food Chem 233:11

Prandini A, Sigolo S, Filippi L, Battilani P, Piva G (2009) Review of predictive models for Fusarium head blight and related mycotoxin contamination in wheat. Food Chem Toxicol Int J Published Br Ind Biol Res Assoc 47(5):927–931

Ramirez ML, Chulze S, Magan N (2004) Impact of environmental factors and fungicides on growth and deoxinivalenol production by *Fusarium graminearum* isolates from Argentinian wheat. Crop Prot 23(2):117–125

Rodríguez-Carrasco Y, Moltó JC, Mañes J, Berrada H (2014) Development of a GC–MS/MS strategy to determine 15 mycotoxins and metabolites in human urine. Talanta 128:125–131

Rodríguez-Carrasco Y, Mañes J, Berrada H, Juan C (2016) Development and validation of a LC-ESI-MS/MS method for the determination of alternaria toxins alternariol, alternariol methyl-ether and tentoxin in tomato and tomato-based products. Toxins 8(11):328

Romera D, Mateo EM, Mateo-Castro R, Gómez JV, Gimeno-Adelantado JV, Jiménez M (2018) Determination of multiple mycotoxins in feedstuffs by combined use of UPLC−MS/MS and UPLC−QTOF-MS. Food Chem 267:S0308814617318538

Rychlik M, Humpf HU, Marko D, Dänicke S, Mally A, Berthiller F, Klaffke H, Lorenz N (2014) Proposal of a comprehensive definition of modified and other forms of mycotoxins including "masked" mycotoxins. Mycotoxin Res 30(4):197–205

Sanzani SM, Reverberi M, Geisen R (2016) Mycotoxins in harvested fruits and vegetables: insights in producing fungi, biological role, conducive conditions, and tools to manage postharvest contamination. Postharvest Biol Technol 122:95–105

Slobodchikova I, Vuckovic D (2018) Liquid chromatography – high resolution mass spectrometry method for monitoring of 17 mycotoxins in human plasma for exposure studies. J Chromatogr A 1548:S0021967318303236

Suzane RDL, Cesar Pazdiora P, José Dallagnol L, Carla Dors G, Clasen Chaves F (2017) Mycotoxin and fungicide residues in wheat grains from fungicide-treated plants measured by a validated LC-MS method. Food Chem 220:510–516

Tamura M, Mochizuki N, Nagatomi Y, Harayama K, Toriba A, Hayakawa K (2015) A method for simultaneous determination of 20 fusarium toxins in cereals by high-resolution liquid chromatography-orbitrap mass spectrometry with a pentafluorophenyl column. Toxins 7(5):1664–1682

Tolosa J, Font G, Mañes J, Ferrer E (2016) Multimycotoxin analysis in water and fish plasma by liquid chromatography-tandem mass spectrometry. Chemosphere 145:402–408

Wang X, Li P (2015) Rapid screening of mycotoxins in liquid milk and milk powder by automated size-exclusion SPE-UPLC–MS/MS and quantification of matrix effects over the whole chromatographic run. Food Chem 173:897–904

Wang M, Jiang N, Xian H, Wei D, Shi L, Feng X (2016) A single-step solid phase extraction for the simultaneous determination of 8 mycotoxins in fruits by ultra-high performance liquid chromatography tandem mass spectrometry. J Chromatogr A 1429:22–29

Wang L, Zhang Q, Yan Z, Tan Y, Zhu R, Yu D, Yang H, Wu A (2018) Occurrence and quantitative risk assessment of twelve mycotoxins in eggs and chicken tissues in China. Toxins 10(11):477

Xu JJ, Ye LH, Cao J, Cao W, Zhang QY (2015) Ultramicro chitosan-assisted in-syringe dispersive micro-solid-phase extraction for flavonols from healthcare tea by ultra-high performance liquid chromatography. J Chromatogr A 1409:11–18

Zhao Z, Rao Q, Song S, Liu N, Han Z, Hou J, Wu A (2014) Simultaneous determination of major type B trichothecenes and deoxynivalenol-3-glucoside in animal feed and raw materials using improved DSPE combined with LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 963:75–82

Zhao Z, Liu N, Yang L, Deng Y, Wang J, Song S, Lin S, Wu A, Zhou Z, Hou J (2015) Multimycotoxin analysis of animal feed and animal-derived food using LC–MS/MS system with timed and highly selective reaction monitoring. Anal Bioanal Chem 407(24):7359–7368

Zhao Y, Wan LH, Bai XL, Liu YM, Zhang FP, Liu YM, Liao X (2017) Quantification of mycotoxins in vegetable oil by UPLC-MS/MS after magnetic solid-phase extraction. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 34(7):1

Zhiyong Z, Qinxiong R, Suquan S, Na L, Zheng H, Jiafa H, Aibo W (2014) Simultaneous determination of major type B trichothecenes and deoxynivalenol-3-glucoside in animal feed and raw materials using improved DSPE combined with LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 963:75–82

Zhou J, Xu JJ, Cong JM, Cai ZX, Zhang JS, Wang JL, Ren YP (2018) Optimization for quick, easy, cheap, effective, rugged and safe extraction of mycotoxins and veterinary drugs by response surface methodology for application to egg and milk. J Chromatogr A 1532:20–29