8.1 INTRODUCTION

Mycotoxins are well-known and abundant toxins that are widely considered to be the most important natural contaminants found in food and feed. Mycotoxins represent the low molecular weight organic compounds formed as secondary metabolites of microscopic, mostly saprophytic, filamentous fungi species, frequently referred to as molds. Under favorable environmental conditions, that is, when temperature and moisture are conducive, these fungi proliferate and may produce mycotoxins. Mycotoxins represent a group of compounds with diverse chemical structures, various biosynthetic origins, and a myriad of biological effects. An overview of selected mycotoxins is provided in Table 8.1. Although the definition of mycotoxins is relatively easy, their classification represents a more difficult and challenging task, especially because of the high number of different fungal species producing these natural toxins. Among many others, the most prominent fungal producers are toxicogenic molds of *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps*, and *Alternaria* fungi genera [1,2].

Because of the ubiquity of molds in the environment, abundance of soil and plant debris, and their dispersion by wind currents, insects, and rain, both these pathogenic organisms and their toxic secondary metabolites can be frequently found in foods and feeds. Mycotoxins are practically unavoidable because the growth of toxicogenic strains of molds cannot be completely eliminated under real-life conditions. However, it is important to minimize the conditions under which mycotoxins are formed, although this is not always feasible within common agricultural, market, and household practice. Mycotoxins are notoriously difficult to remove and the best method of control is prevention [3,4].

8.1.1 Legislation and Regulatory Limits

General public awareness of health risks related to mycotoxins is steadily growing. When present in foods or feeds at sufficiently high concentrations, toxic fungal metabolites can induce both acute and chronic adverse health effects in humans and
<table>
<thead>
<tr>
<th>Fungi Producer Species</th>
<th>Mycotoxins Group</th>
<th>Structure</th>
<th>Name of Mycotoxins (Abbreviation)</th>
<th>R-Structures</th>
<th>Molecular Formula</th>
<th>Relative Molecular Mass</th>
</tr>
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<tbody>
<tr>
<td>Trichothecenes A</td>
<td></td>
<td></td>
<td>HT2 toxin (HT2)</td>
<td>R1=R2=OH; R3=OAc; R4=OCOCH3-Bu</td>
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<td>15-Acetoxyscirpenol (MAS)</td>
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<td>Neosanhol (NFO)</td>
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<td>Vernacrol (VER)</td>
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<td>Fusarenon-X (FUS-X)</td>
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<td>3-Acetyldeoxyxovalenol (3ADON)</td>
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<td>Beauvericin (BEA)</td>
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<td>Fumonisin B1 (FB1)</td>
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<td>$\text{C}<em>3\text{H}</em>{10}\text{NO}_3$</td>
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<td>Fumonisin B2 (FB2)</td>
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<td>Fumonisin B3 (FB3)</td>
<td>$R_1=\text{H}; R_2=\text{OH}$</td>
<td>$\text{C}<em>3\text{H}</em>{10}\text{NO}_3$</td>
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<td></td>
<td>Others</td>
<td>Zearalenone (ZON)</td>
<td>$\text{C}_{10}\text{H}_2\text{O}_3$</td>
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<th>R-Structures</th>
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<th>Relative Molecular Mass</th>
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<td>Ochratoxin α (OTA)</td>
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<td>Others</td>
<td>Patulin (PAT)</td>
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<td>C_{6}H_{12}O_{6}</td>
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<td>Alternatoxin (ALT)</td>
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Table 8.1 (Continued)

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<th>Fungus Producer Species</th>
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<td>Ergot alkaloids</td>
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<td>Ergosine</td>
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<td>Ergotaminine</td>
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<td>C_{23}H_{32}N_{2}O_{8}</td>
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<td>Ergoxonine</td>
<td>R_{1}=R_{2}=CH(CH)_{3}</td>
<td>C_{23}H_{32}N_{2}O_{8}</td>
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<td>Ergocristine</td>
<td>R_{1}=CH(CH)<em>{3}; R</em>{2}=CH_{2}C_{6}H_{5}</td>
<td>C_{23}H_{32}N_{2}O_{8}</td>
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<td>Ergosinine</td>
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<td>C_{23}H_{32}N_{2}O_{8}</td>
<td>547.3</td>
</tr>
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<td></td>
<td>Ergotaminine</td>
<td>R_{1}=CH_{3}; R_{2}=CH_{3}C_{6}H_{5}</td>
<td>C_{23}H_{32}N_{2}O_{8}</td>
<td>581.3</td>
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<td>Ergoxonine</td>
<td>R_{1}=R_{2}=CH(CH)_{3}</td>
<td>C_{23}H_{32}N_{2}O_{8}</td>
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<td>Ergocristine</td>
<td>R_{1}=CH(CH)<em>{3}; R</em>{2}=CH_{2}C_{6}H_{5}</td>
<td>C_{23}H_{32}N_{2}O_{8}</td>
<td>609.3</td>
</tr>
</tbody>
</table>
animals. While acute exposure to high concentrations can cause liver or kidney deterioration, chronic effects include carcinogenicity, cytotoxicity, nephrotoxicity, neurotoxicity, mutagenicity, teratogenicity, estrogenicity, and immune suppression [4,5].

Many international and governmental organizations, such as the World Health Organization (WHO), the Food and Agricultural Organization (FAO), the European Commission (EC), and the U.S. Food and Drug Administration (FDA), have recognized the occurrence of mycotoxins in food and feed as a serious health risk and have worked to establish and/or update respective maximum concentrations for these compounds [5]. In the past, numerous monitoring and toxicological studies were conducted to cope with problems related to occurrence of mycotoxins. Based on occurrence results, hygienic limits and/or tolerable daily intake (TDI) values were adopted for some related groups or individual mycotoxins. Out of \(\sim 500\) currently known mycotoxins [6], only a few are recognized as major food safety hazards. From the food safety viewpoint, the most significant, the most often discussed, and thus the most frequently studied and controlled mycotoxins are aflatoxins, deoxynivalenol (DON), T2 and HT2 toxins, zearalenone (ZON), ochratoxin A (OTA), fumonisins, and patulin (PAT) [3]. Despite the serious acute and/or chronic toxic effects, there are relatively large gaps in legislation for different foodstuffs, especially compared with those for other toxicants such as pesticide residues, veterinary drugs (also referred to veterinary medicinal product residues (VMPRs)) and environmental contaminants. Additionally, the available legislation is not harmonized worldwide and varies significantly among respective countries [7]. It is noteworthy that the standardization of regulatory limits for mycotoxins is an extremely difficult task, as many factors have to be considered when making such decisions. In addition to scientific factors, such as risk assessment and analytical accuracy, economic and political factors arising from the commercial interests of each country and the constant need for an adequate food supply also play a role in the decision-making process.

When critically assessing the current regulatory systems, the EU probably has the most comprehensive, well-developed, and stringent legal limits worldwide. The EU regulation covers a wide range of various foodstuffs and raw materials (\(\sim 50\)) intended for food production and direct consumption [8]. In addition to the EU, the following countries have at least partly established regulations for mycotoxins: Argentina, Australia, Bosnia and Herzegovina, Brazil, Canada, China, India, Japan, Mexico, New Zealand, Nigeria, Russia, South Africa, Switzerland, Turkey, and the United States. Aflatoxins are the only group of mycotoxins that are regulated in all of the above-mentioned countries. A summary of the worldwide regulatory limits on mycotoxins is available online [9]. Guidance on concentrations of representative mycotoxins (DON, ZON, OTA, fumonisins, and aflatoxins) in feedstuffs is also provided on the Internet [10].

### 8.1.2 Emerging Mycotoxins

In addition to mycotoxins already mentioned, there are many other compounds such as ergot alkaloids, alternaria toxins, beauvericin and enniatins, moniliformin, diacetoxyscirpenol, nivalenol (NIV), citrinin, sterigmatocystin, and phomopsin that occur
in food and feed. Because both the evidence for toxicity and the occurrence of these compounds are increasing, they are currently the subjects of intensive research and monitoring studies. This occurrence trend is most probably linked to changes in climate conditions [11].

Mycotoxins occur in not only their native forms but also conjugated with peptides, carbohydrates, and/or sulfates. These so-called “masked mycotoxins” are formed after the metabolization of the original mycotoxins by plants, fungi, and mammals or during food processing. DON-3-glucoside (D3G), ZON-4-glucoside (Z4G), or masked fumonisins are in the forefront of conjugated mycotoxins research. In general, comprehensive information on the occurrence and toxicity of masked mycotoxins is not yet available. The main drawback of research on masked mycotoxins is the lack of both pure analytical standards and analytical methods needed for their accurate determination. The available data indicate that the native (parent) mycotoxins can be, at least to some extent, released from masked conjugates in the digestive tract, thus contributing to the overall exposure of both humans and animals to these toxins [12]. The issues related to masked mycotoxins were recently compiled in a comprehensive review by Berthiller et al. [13].

8.1.3 Analysis of Mycotoxins in High-Throughput Environment

Various analytical approaches have been developed and optimized to determine mycotoxins in food and feed; an overview is shown in Figure 8.1. Because the regulatory limits for certain compounds are set at very low (trace) concentrations, there is a need for highly sensitive analytical methods that are capable of detection, quantification, and confirmation of mycotoxins in complex matrices. Currently, the most frequently used technique for analysis of mycotoxins is liquid chromatography–mass spectrometry (LC–MS) utilizing various types of mass analyzers. Additionally, screening methods based on immunochemical techniques (e.g., enzyme-linked immunosorbent assay (ELISA)) or biosensors (e.g., protein chips and antibody/protein-coated electrodes) are also employed [14].

The term high-throughput analysis refers to an analytical procedure with minimal time requirements, which can be performed within minutes or which screens for a large number of compounds per unit time. As discussed in Chapter 1, high-throughput methods have to employ either no or only very simple sample preparation protocols followed by rapid, sensitive, and reliable detection and/or quantification steps suitable for all analytes of interest [15]. Keeping in mind certain limitations of analytical strategies applicable to mycotoxins, both biological (immunological) and instrumental methods can be effectively used for high-throughput analysis [5]. With the immunological methods, although the results may be obtained in minutes, the main drawbacks of their use are narrow scope in terms of number of analytes and typically poor accuracy. On the other hand, LC–MS-based analyses can provide accurate quantitative data for many target compounds. The number of analytes integrated in particular LC–MS-based methods can range from several (regulated) toxins to over 100 mycotoxins. The measurement of throughput of LC–MS methods is largely determined by the
complexity of the sample, and thus the extent of sample preparation needed prior to instrumental analysis can vary. Other important factors affecting the throughput of analysis are the time for chromatographic analyses followed by data processing and data evaluation.

In the following sections, topics relevant to high-throughput analysis of mycotoxins in food and feed, including sampling and sample preparation, are discussed. This chapter focuses primarily on workflows employing LC–MS, rapid immunological methods, and some nonchromatographic MS-based techniques, as these approaches represent the primary tools in this field.

### 8.2 SAMPLE PREPARATION

Sample preparation is a crucial step in the analysis of mycotoxins and creates a bottleneck in most analytical procedures [16]. Numerous sample preparation protocols that largely differ in overall time requirements have been described already, depending on the nature of the sample matrix and the type of detection and quantification. These processes typically involve (i) homogenization of the sample, (ii) extraction of target mycotoxins from the sample matrix, and (iii) cleanup of the crude sample extract with simultaneous preconcentration or dilution of analytes. In addition to sample preparation, sample collection (sampling) is another critical factor strongly influencing the results of a particular assay [4,5,17,18].

![Figure 8.1. The scheme and overview of analytical methods used in mycotoxins analysis.](image)
8.2.1 Sampling

The collection of representative samples is an important but often underappreciated phase in the analysis of mycotoxins. Because the distribution of mycotoxins in agricultural commodities is usually not homogeneous, an incorrect sampling procedure can cause extensive bias when determining the contamination of a particular commodity [5]. Whereas in the case of liquids it is often assumed that mycotoxins are evenly distributed, in fungus-contaminated solid samples (e.g., grains, nuts, or dried fruits) mycotoxins can occur in a few highly contaminated hot spots. The selection of an optimal strategy that enables proper collection of a representative sample is dependent on several factors, such as the properties of the sample matrix, type of packaging, and size of the sampling lot [16]. Sampling and homogeneity of the matrix become critical and extremely time-demanding, especially with regard to large samples. If sampling is performed in an improper way, low amounts of sample (1–5 g) that are being frequently used in rapid sample preparation procedures may lead to false negative results because local hot spots were missed or undersampled. In recent years, the design of official sampling procedures has become a significant concern to many national and international authorities, including the FDA, USDA, EC, and FAO [19,20]. This effort has resulted in the establishment of sampling methods that are believed to allow an objective assessment of contamination with regard to mycotoxins. Worldwide evaluation of these sampling protocols is still in progress.

8.2.2 Matrices of Interest

The studies dealing with monitoring mycotoxins and validating analytical methods for their determination are typically focused on matrices with the highest incidences of legislatively regulated compounds. Cereals, nuts, fruits, vegetables, and related products are in the forefront of interest due to their relatively high susceptibility to infestation by molds. According to the European Rapid Alert System for Food and Feed (RASFF), the greatest numbers of alerts are reported for the occurrence of aflatoxins and OTA in spices, nuts, cereals, and fruits. The presence of these toxins in named matrices is presumable and under strict control of producers, traders, and control authorities.

The monitoring studies are also performed for nonregulated compounds to fill gaps in knowledge on their occurrence in certain matrices to enable their eventual regulation. Examples are the lack of incidence data on T2 and HT2 toxins in oats and other cereals and on OTA in green coffee beans or licorice. The incidence of emerging mycotoxins in food, feed, and raw materials used for their production is also of significant concern. Recently, increased attention has been paid to dietary supplements that have gained high popularity among consumers. Dietary supplement products often contain extracts of various herbs and botanicals susceptible to fungal attack and may represent a significant source of consumers’ exposure to mycotoxins [21].

In addition to food, feedstuffs represent an important matrix in the control of mycotoxins. Significant concerns are for silage, because raw materials employed for
its production and by-products of food technologies are frequently contaminated. For instance, the occurrence of *Fusarium* mycotoxins was reported at high concentrations in samples of dried distiller’s grains with solubles (DDGS). This material represents a valuable by-product of the ethanol production process and is used for feeding livestock. Last but not least, mycotoxins are also analyzed in nonfood/feed biological matrices, such as urine, blood, and feces to monitor their occurrence and metabolic transformations *in vivo* [22,23]. Given this range of target analytes and matrices, it is clear that no single extraction process will be optimal for all analytes in all matrices. Therefore, a range of extraction techniques have been developed, differing in their specificity, complexity, and speed. In Section 2.3, we discuss several common extraction protocols.

### 8.2.3 Extraction of Mycotoxins

The use of optimal extraction procedures is dictated by the physicochemical properties of the target mycotoxins and the matrix they are in. Similar to other contaminants discussed in this book, solid–liquid extraction (SLE) is the most frequently applied approach to extract mycotoxins from sample matrices. The choice of suitable extraction solvents is crucial to ensure sufficient recoveries, and thus accurate quantification. In procedures that aim to isolate only a single analyte or a small group of related mycotoxins, the composition of the extraction mixture can be adjusted for optimum recovery. Nevertheless, with regard to current trends aimed at the simultaneous determination of numerous mycotoxins, which largely differ in physicochemical properties, solvent mixtures allowing generic extraction of analytes are required. A number of extraction solvents, including methanol, chloroform, acetone, ethyl acetate, and acetonitrile, and their mixtures have already been employed for the extraction of mycotoxins. Among various solvent combinations, the mixture of acetonitrile and water in ratios ranging from 84:16 to 75:25 (v/v) represents the most efficient extraction solvent commonly used. Additionally, in order to improve recoveries of some acidic mycotoxins, formic or acetic acid is frequently added to the extraction mixture [4,5,17,18]. The generic extraction strategy called “dilute-and-shoot,” which uses only pure solvents without any further purification, is nowadays commonly applied for the extraction of a wide range of mycotoxins [24]. The efficiency of extraction is usually improved by integrating shaking, sonication, or mixing into the extraction procedure. Alternatively, a combination of the above techniques is used.

Among various methods for the extraction of mycotoxins described in the literature, the QuEChERS (i.e., quick, easy, cheap, effective, rugged, and safe) protocol is probably one of the most relevant to high-throughput analysis. Since its original introduction for pesticide residues analysis [25], QuEChERS has already been used in numerous modifications to extract other chemical contaminants from various food matrices [26]. The QuEChERS procedure combines sample extraction from a mixture of an organic solvent (usually acetonitrile) with water and transfer of analytes into an organic layer with simultaneous separation of aqueous and organic phases induced by the addition of salts. The crude organic extract can be subsequently purified with the use of dispersive solid-phase extraction (dSPE) to remove undesired
coextracts (e.g., sugars and/or fatty acids). The dSPE is based on the addition of the sorbent material to an aliquot of the sample extract to remove matrix interferences. The sorbent is subsequently separated from the extract bulk by centrifugation. Various sorbents, such as primary–secondary amine (PSA), silica gel, octadecylsilane-bonded silica gel (C18), graphitized carbon black (GCB), or their combinations, are used for this purpose [26,27]. It is noteworthy that in the case of multitarget methods, the dSPE step is often omitted as it might otherwise decrease recoveries of some analytes [28]. The main advantages of QuEChERS over traditional extraction techniques are high sample throughput (15 versus 60 min per sample), use of small amounts of organic solvents (10 versus 25–100 ml), less glassware, and employment of relatively inexpensive laboratory equipment [26,27].

The use of QuEChERS in mycotoxin analysis was reported by several authors who applied this protocol mainly to cereals and cereal-derived products. In addition to these types of samples, wine, eggs, beer, fruits and vegetables, spices, oilseed, silage, milk, and meat were also matrices extracted for mycotoxins by employing a QuEChERS-type procedure (Table 8.2). Regarding the target analytes, the majority of studies focused on legislatively regulated mycotoxins and/or Fusarium mycotoxins. Several papers reported methods with broader scope, which in addition to the above analytes also included ergot alkaloids, alternaria toxins, and other mycotoxins produced by Penicillium and Aspergillus species. QuEChERS was also employed for simultaneous multiclass extraction of mycotoxins with other contaminants such as pesticides and veterinary drugs [24,29–31]. Table 8.2 provides an up-to-date overview of publications dealing with applications of QuEChERS to mycotoxin analysis and summarizes time demands of the extraction step [24,28–45].

The optimal QuEChERS-based extraction protocol largely depends on the type of matrix to be examined. Therefore, many modifications of the original QuEChERS design have been developed to fit particular sample types. The most important parameters of the QuEChERS method, which have a significant impact on recovery and other performance characteristics of the method, are the composition of extraction mixture, extraction time, type and amount of salts added, and the ratio between organic solvent volume and sample weight (matrix dilution factor, ml/g) [26]. Cereals and cereal-based products represent typical dry matrices that are frequently extracted for mycotoxins using QuEChERS. The matrix dilution factors applied to such samples are usually either 2.0 or 2.5 (i.e., 4 or 5 g of test sample and 10 ml of organic solvent). The volume of water used in published studies varied significantly and was in the range of 2–10 ml. The soaking of the sample matrix and/or prolonged extraction times were shown to be crucial in achieving sufficiently high recoveries of mycotoxins using QuEChERS-based extraction of cereals and similar dry samples [24,29]. However, longer extraction times ultimately result in diminished sample throughput. On the other hand, matrices with naturally high water content, such as vegetables, fruit, milk, beer, and wine, do not require soaking and can be processed at much higher throughput even without the addition of water (see Table 8.2). Regardless of the type of matrix, the extraction efficacy should always be assessed based on naturally contaminated reference materials rather than with the use of spiked samples. Improvement in recoveries of some problematic (acidic) analytes can be
Table 8.2. Overview of Recent QuEChERS Applications in the Analysis of Mycotoxins

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Analytes</th>
<th>Sample Amount</th>
<th>Extraction Mixture/ Extraction Time (min/Sample)</th>
<th>Salts</th>
<th>Cleanup Procedure</th>
<th>Detection Technique/Run Time (min/Sample)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed, maize, honey, meat, egg, and milk</td>
<td>Multiple mycotoxins (n = 23), plant toxins (n = 13), pesticides (n = 136), and veterinary drugs (n = 86)</td>
<td>2.5 g</td>
<td>Water (7.5 ml) and acetoneitrile with 1.0% acetic acid (10 ml)</td>
<td>MgSO₄ (4 g) and NaCl (1 g)</td>
<td>–</td>
<td>UPLC–MS/MS/20 HPLC–MS/MS/20</td>
<td>[29]</td>
</tr>
<tr>
<td>Wheat, maize, and millet</td>
<td>ADON, AO4, ALT, AME, DAS, DOM, DON, FUS-X, NIV, STC, and ZEA</td>
<td>2 g</td>
<td>Water (7.5 ml) and acetoneitrile (10 ml)/15</td>
<td>MgSO₄ (4 g) and NaCl (1 g)</td>
<td>dSPE of 4 ml with PSA (200 mg) and MgSO₄ (600 mg)</td>
<td>DART–Orbitrap/MS/0.2</td>
<td>[32]</td>
</tr>
<tr>
<td>Cereal-based commodities</td>
<td>3-ADON, 15-ADON, AFH1, AFH2, AFH2, AFG1, AFG2, DAS, DON, FB1, FB2, FUS-X, HT2, NIV, NIV, OTA, T2, and ZON</td>
<td>5 g</td>
<td>Water (10 ml) and 0.5% acetic acid in acetoneitrile (10 ml)/20</td>
<td>MgSO₄ (4 g) and NaCl (1 g)</td>
<td>Defatting by shaking with n-hexane</td>
<td>HPLC–MS/MS/24.5</td>
<td>[33]</td>
</tr>
<tr>
<td>Maize silage</td>
<td>Multiple mycotoxins (n = 27)</td>
<td>10 g</td>
<td>Water (5 ml) with sodium acetate trihydrate (1.67 g) and 1% acetic acid in acetoneitrile/13</td>
<td>MgSO₄ (4 g)</td>
<td>–</td>
<td>HPLC–MS/MS/44</td>
<td>[34]</td>
</tr>
<tr>
<td>Breakfast cereals and flour</td>
<td>15-ADON, DON, FUS-X, NIV, and ZON</td>
<td>5 g</td>
<td>Water (25 ml for cereals, 10 ml for flour) and acetoneitrile (10 ml)/20</td>
<td>MgSO₄ (4 g) and NaCl (1 g)</td>
<td>dSPE of 6 ml with C₁₈ (300 mg) and MgSO₄ (600 mg)</td>
<td>GC–MS (derivatization)/8</td>
<td>[35]</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>DAS, DON, HT2, NIV, and T2</td>
<td>5 g</td>
<td>Methanol (8.5 ml) and acetoneitrile (1.5 ml)/12</td>
<td>MgSO₄ (2 g) and NaCl (1 g)</td>
<td>–</td>
<td>HPLC–MS/25</td>
<td>[36]</td>
</tr>
<tr>
<td>Cereals</td>
<td>3-ADON, D3G, DON, FB1, FB2, FB3, FUS-X, HT2, NIV, T2, and ZON</td>
<td>4 g</td>
<td>Water (7.5 ml) with 0.1% formic acid and acetoneitrile (10 ml)/8</td>
<td>MgSO₄ (4 g) and NaCl (1 g)</td>
<td>–</td>
<td>UHPLC–TOF/MS/18</td>
<td>[37]</td>
</tr>
<tr>
<td>Flour, breakfast cereals, snacks, and bread</td>
<td>3-ADON, 15-ADON, ALT, AME, AO4, D3G, DAS, DON, enniatins, ergot alkaloids, FUS-X, HT2, NIV, NIV, T2, VOL, and ZON</td>
<td>4 g</td>
<td>Water (7.5 ml) and acetoneitrile (10 ml)/11</td>
<td>MgSO₄ (4 g) and NaCl (1 g)</td>
<td>–</td>
<td>UHPLC–Orbitrap MS/18</td>
<td>[38]</td>
</tr>
<tr>
<td>Bread</td>
<td>DON, HT2, and T2</td>
<td>4 g</td>
<td>Water (7.5 ml) with 0.1% formic acid and acetoneitrile (12.5 ml)/21</td>
<td>MgSO₄ (4 g) and NaCl (1 g)</td>
<td>–</td>
<td>UHPLC–Orbitrap MS/19</td>
<td>[39]</td>
</tr>
</tbody>
</table>

(continued)
<table>
<thead>
<tr>
<th>Matrix</th>
<th>Analytes</th>
<th>Sample Amount</th>
<th>Extraction Medium/ Extraction Time (min/sample)</th>
<th>Salts</th>
<th>Cleanup Procedure</th>
<th>Detection Technique/Run Time (min/sample)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noodles</td>
<td>AFB1, AFB2, AFG1, and AFG2</td>
<td>2 g</td>
<td>Water (4 ml), methanol (5.1 ml), and acetonitrile (6.9 ml)/7</td>
<td>MgSO₄ (1.5 g) and NaCl (0.5 g)</td>
<td>–</td>
<td>HPLC-fluorescence detection (FLDV/23)</td>
<td>[40]</td>
</tr>
<tr>
<td>Wine</td>
<td>OTA</td>
<td>3 ml</td>
<td>30 mM NaH₂PO₄ buffer (pH 7.8 ml) and 5% formic acid in acetonitrile (10 ml)/5.3</td>
<td>MgSO₄ (4 g), NaCl (1 g), sodium citrate (1 g), and disodium citrate sesquihydrate (0.5 g)</td>
<td>–</td>
<td>HPLC-laser-induced fluorescence detection (LIF/4)</td>
<td>[41]</td>
</tr>
<tr>
<td>Eggs</td>
<td>AFB1, AFB2, AFG1, AFG2, BEA, CIT, emimizot, and OTA</td>
<td>2 g</td>
<td>Water with 1% acetic acid (2 ml) and methanol with 1% acetic acid (8 ml)</td>
<td>Na₂SO₄ (4 g) and sodium acetate (1 g)</td>
<td>–</td>
<td>UHPLC-MS/MS/6.5</td>
<td>[42]</td>
</tr>
<tr>
<td>Beer-based drinks</td>
<td>AFB1, AFB2, AFG1, AFG2, DON, FB1, FB2, FB3, HT2, NIV, OTA, PAT, T2, and ZON</td>
<td>10 ml</td>
<td>Acetonitrile (10 ml)/5.3</td>
<td>MgSO₄ (4 g), NaCl (1 g), sodium citrate (1 g), and disodium citrate sesquihydrate (0.5 g)</td>
<td>SPE cartridge (C₁₈)</td>
<td>UHPLC-MS/MS/6.5</td>
<td>[27]</td>
</tr>
<tr>
<td>Cereals, cereal-based foods, cucumber, wine</td>
<td>AFB1, AFB2, AFG1, AFG2, HT2, OTA, T2, and pesticides (n = 83)</td>
<td>10 ml/5 g</td>
<td>Water (5 ml, cereals only) and 1% acetic acid in acetonitrile (10 ml)/60 min soaking (cereals only), 7</td>
<td>MgSO₄ (4 g) and NaCl (1.5 g)</td>
<td>–</td>
<td>UHPLC-MS/MS/13</td>
<td>[31]</td>
</tr>
<tr>
<td>Milk</td>
<td>AFB1, AFB2, AFG1, AFG2, AFM1, HT2, OTA, T2, and pesticides (n = 42)</td>
<td>10 ml</td>
<td>Acetonitrile with 1% acetic acid (10 ml)/7</td>
<td>MgSO₄ (4 g), NaCl (1 g), sodium citrate (1 g), and disodium citrate sesquihydrate (0.5 g)</td>
<td>–</td>
<td>UHPLC-MS/MS/6.5</td>
<td>[30]</td>
</tr>
<tr>
<td>Product Type</td>
<td>Method</td>
<td>Recovery %</td>
<td>Reference</td>
<td></td>
<td></td>
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<tr>
<td>---------------------------</td>
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<td></td>
</tr>
<tr>
<td>Pear- and apple-based foods</td>
<td>PAT</td>
<td>10 g</td>
<td>HPLC-MS/MS/17.5 [43]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Popcorn</td>
<td>15-ADON, DON, FUS-X, NIV, and ZON</td>
<td>5 g</td>
<td>GC-MS (derivatization)/6 [44]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat, corn, rice, and noodles</td>
<td>3-ADON, 15-ADON, DAS, DON, FUS-X, HT2, NIV, and T2</td>
<td>1 g</td>
<td>HPLC-QTOFMS/23 [26]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>CIT and OTA</td>
<td>10 g</td>
<td>HPLC-photodiode array detection (PDA)/20 [45]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit, cereals, spices, and oilseeds</td>
<td>Multiple mycotoxins (n = 38) and pesticides (n = 288)</td>
<td>2.5 g/10 g</td>
<td>UHPLC-MS/MS/15.5 [24]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley</td>
<td>Multiple mycotoxins (n = 32)</td>
<td>2 g</td>
<td>UHPLC-Orbi-trapMS/18 [28]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

achieved by addition of various buffers and/or acids into the extraction mixture in order to adjust the pH and support the transfer of analytes into the organic layer. Regarding the type and optimal amount of salts, magnesium sulfate (MgSO₄) and sodium chloride (NaCl) at a ratio of 1:4 (w/w) have been used in most of applications.

8.2.4 Purification of Sample Extracts

The main goals of the cleanup step are to remove undesirable sample coextracts that may interfere with the analytes and to preconcentrate target analytes to allow acceptable sensitivity and selectivity to be achieved [5,17,18,27]. The most frequently employed sample purification approaches in the analysis of mycotoxins are using either solid-phase extraction (SPE) or immunoaffinity column (IAC) cleanup. Additionally, molecularly imprinted polymers (MIP) cleanup, matrix solid-phase dispersion (MSPD), or liquid–liquid partitioning of extract with n-hexane, acetone, or ethyl acetate have been used in this field, but to a limited extent [14].

The SPE cleanup technique is based on the partitioning of analytes and the sample matrix between mobile and stationary phases. SPE is performed by passing the sample extract through a disposable cartridge containing sorbents with bound phases (with C₁₈ being the most used) and various adsorbents, such as charcoal, Florisil, or Celite. SPE in three different modes can be generally applied in food analysis: (i) selective extraction, (ii) selective washing, and (iii) selective elution [4,46]. In the selective extraction mode, the SPE cartridge retains mycotoxins and allows impurities to pass through the cartridge. In a subsequent step, target analytes are released from the SPE stationary phase using a suitable solvent. In the sample washing mode, both analytes and impurities are first retained on the SPE sorbent bed, the interfering matrix components are further rinsed out using strong enough solvent, and analytes are finely eluted with other but stronger solvents. In the selective elution mode, interfering impurities are retained by the stationary phase of the SPE cartridge and the target mycotoxins are allowed to pass through the column. No washing and elution steps are further required. Retention and elution of analytes and impurities are strongly dependent on properties of the stationary bed and elution/wash solvents, which are commonly designed for specific usage of analyte–matrix–solvent combinations. The description of sorbent and solvent selectivity is thoroughly described in a review by Lucci et al. [46]. The respective SPE modes are illustrated in Figure 8.2. It is apparent that for achieving the highest possible sample throughput, the matrix removal SPE mode is the most desirable as it enables the sample extract cleanup to be performed in a single step. It is worth noting that the MycoSep SPE cartridges, which are currently the most frequently used in mycotoxin analysis, are operated in the matrix removal mode [18].

While SPE offers cleanup and preconcentration for a broad range of analytes, IAC provides a higher selectivity and specificity to target analyte(s). After application of the sample extract to the IAC, mycotoxins are selectively bound to antibodies (either monoclonal or polyclonal) that are immobilized in the cyanobromide-activated sepharose gel [47] present in the cartridge. IAC combines the sample cleanup and sample concentration modes. Components of the sample matrix that do not interact with antibodies are gradually eluted from the column during application of the extract.
The analytes are then eluted by a small volume of pure organic solvent (methanol or acetonitrile), which disrupts mycotoxin–antibody bonds by protein denaturation. The most comprehensive studies on this topic have been published by Turner et al. [18] and Rahmani et al. [4]. Although both multifunctional IAC and SPE columns are commercially available for all of the main regulatory significant mycotoxins, these columns are still not developed for a wide range of mycotoxin groups. Moreover, the high cost and relatively long time of analysis caused by large loading and elution solvent volumes make these cleanup strategies poorly suited for rapid sample preparation within a high-throughput environment. In addition to the dSPE cleanup strategy that has already been described and that fulfills the requirements needed for high throughput, the dilute-and-shoot strategy has also been employed in conjunction with LC–MS for high-throughput multimycotoxin analysis. In this case, the poorer sensitivity caused by higher amounts of coextracted matrix compounds, which hamper the ionization of target analytes, is the price paid for generic extraction, no purification steps, and improved sample throughput [24,37,44,48].

### 8.3 SEPARATION AND DETECTION OF MYCOTOXINS

There are several chromatographic techniques available for the analysis of mycotoxins, including gas chromatography (GC), thin layer chromatography (TLC), and high- or
ultrahigh-performance liquid chromatography (HPLC and UHPLC). GC was frequently used for this purpose in the 1990s. However, the obvious drawbacks of GC-based methods relate to the need for time-consuming sample preparation and derivatization of analytes, which have led to their reduced use in mycotoxin analysis [49]. On the other hand, LC coupled to either conventional detectors [ultraviolet (UV) detector, diode array detector (DAD), fluorescence detector (FLD), and photodiode array detector (PDA)] or mass spectrometers is currently the most frequently applied separation technique. Note that conventional detectors are selective only for a limited number of toxins, and are thus less versatile than MS detection. LC-based methods are also used to confirm results of novel rapid screening techniques [47,50].

### 8.3.1 Liquid Chromatography–Mass Spectrometry-Based Methods

Currently, the analysis of mycotoxins relies largely on LC separation employing reversed-phase (RP) columns in combination with MS using different mass analyzers. Such methods represent the reference and definitive protocols for mycotoxin analysis [18]. Although most of the published LC–MS-based workflows have focused on simultaneous determination of structurally related mycotoxins in single food/feed matrices, several studies have described successful integration of analysis of multiple nonrelated mycotoxins into a single determinative LC–MS method. This was made possible by substantial advances in MS instrumentation that resulted in sufficiently sensitive and selective high-throughput broad-scope mycotoxin analysis. In these LC–MS methods, various combinations of LC operated in either high-pressure (HP) or ultrahigh-pressure (UHP) mode with low-resolution (LR) tandem MS or high-resolution (HR) MS have been used.

The most recently published studies aiming at rapid analysis of multiple mycotoxins using LC–MS are Refs [24,28,29,48,51–53]. In some of these studies, mycotoxins were analyzed simultaneously with other food contaminants or natural toxins, such as pesticides, plant toxins, marine toxins, and/or veterinary drugs [24,29,52]. The average number of mycotoxins analyzed by these methods was between 30 and 40. Typical groups of mycotoxins for which analytical standards are commercially available (e.g., trichothecenes, enniatins, fumonisins, aflatoxins, ergot alkaloids, alternaria toxins, ZON, OTA, and PAT) were tested. The only exceptions were multimycotoxin methods described by Sulyok et al. [48], Abia et al. [51], and Varga et al. [53], who developed procedures capable of simultaneous analysis of 106, 320, and 191 mycotoxins and other toxic or potentially toxic fungal secondary metabolites, respectively. Not only cereals, nuts, and related products were used for evaluation of the method recoveries, but other important matrices such as baby foods, fruits, seeds, spices, honey, milk, eggs, meat, alcoholic and nonalcoholic beverages, soybeans, and cheese were also included.

Regarding the separation step, (U)HPLC systems are usually applied for multimycotoxins analysis. An ongoing development in UHPLC instrumentation allows separation to be performed under substantially higher pressures using chromatographic columns with a sub-2 μm stationary-phase particle size, which generally result in narrower chromatographic peaks and lower overall run times. The effective LC
separation of multiple analytes requires the proper selection of both chromatographic columns and composition of mobile phases and a careful optimization of chromatographic parameters such as temperature, mobile-phase gradient, pH, and composition of buffers. In practice, chromatographic columns using RP-C18 stationary phases are almost universally applied. The most comprehensive multitarget LC–MS method dealing with the analysis of 320 toxic and potentially toxic mycotoxins was developed and published by Abia et al. [51]. This methodology amends methods published previously by Sulyok et al. [48,54]. For the chromatographic separations used here [51,53], an HPLC system employing an RP-C18 column was used. For sufficiently sensitive analyses for all compounds, two separate chromatographic runs had to be performed in positive (ESI(+)) and negative (ESI(−)) electrospray modes using a triple quadrupole linear ion trap (QLIT) MS instrument. The time needed for LC–MS analysis of all 320 analytes was 41 min per sample. A similar strategy was also employed in a study by Lacina et al. [24], who analyzed 38 mycotoxins together with 288 pesticides. This analysis was also subdivided into two consecutive runs with run times of 15.5 min each. In a study by Mol et al. [29], two 20 min UHPLC–MS/MS methods were applied for the determination of mycotoxins and natural toxins (n = 36), pesticides (n = 136), and veterinary drugs (n = 86) in both positive and negative ionization modes. Herrmann et al. [52] performed simultaneous analysis of 36 mycotoxins together with some drugs, pesticides, and other chemical contaminants, representing in total 127 target analytes. This analysis was again subdivided into two separate runs. Each was accomplished within 22 min and resulted in a total analysis time of 44 min per sample. Two separate runs in ESI (+) and ESI(−) modes are commonly applied in multitarget analyses where triple quadrupole (QqQ) or QLIT are used as mass analyzers. This is necessary because of the high number of simultaneously eluted analytes that differ in terms of their optimal ionization modes. Because the polarity switching is not rapid enough to enable simultaneous acquisition in both ionization modes when employing common LR-MS instruments, the only viable solution to achieve acceptable LODs is to separate the analytes into positive and negative ionization mode runs. For example, aflatoxins (ionizing in ESI(+)) and trichothecenes (ionizing in ESI(−)), which represent highly important regulated toxins, cannot be easily separated with C18 columns and therefore typically overlap or coelute.

The LC parameters of the above LC–MS methods were more or less similar. The dimensions of the most frequently employed UHPLC columns were 100 or 150 × 2.1 mm with 1.7 or 1.8 μm particle sizes [24,29,53] or 150 × 4.6 mm with 5 μm particle sizes for HPLC analysis [48,51]. In one case, a shorter column (50 × 2.1 mm, with 1.8 μm particle sizes) was applied in UPLC–MS/MS analysis [52]. The column temperatures ranged from 25 to 55 °C. The majority of methods employed acidified ammonium formate (1–5 mM) and acidified methanol in ESI (+) ionization mode for the mobile phase, while aqueous ammonium acetate (5 mM) and methanol were used in ESI(−) mode.

Multianalyte methods developed specifically for the determination of mycotoxins usually have quite similar parameters, as described in the above applications. These were recently summarized in a review by Hajslova et al. [55]. The state-of-the-art
trends focus on the development of high-throughput methods with generic sample preparation and low detection limits of a broad range of food contaminants. In all of these studies, the LR-MS represented by a QqQ is the most prevalent MS option in mycotoxin analysis for selective detection and confirmation of analytes. Using the detection/confirmation strategy based on monitoring two MRM transitions (one precursor ion → two product ions, or first precursor ion → one product ion and second precursor ion → one product ion) for each analyte, the requirements for analyte identification established by official documents such as Commission Decision 2002/657/EC [56] and the SANCO/12495/2011 document [57] can be fulfilled.

8.3.2 High-Resolution Mass Spectrometry in Mycotoxins Analysis

In addition to tandem MS, HR-MS analyzers have also been applied to quantitative, semiquantitative, and nontargeted screening analyses of multimycotoxins. Despite their ability to simultaneously detect and confirm multiple analytes, the HR-MS techniques have not yet been extensively used for multimycotoxin analysis. Additionally, current EU legislation requires certain conditions to be fulfilled when confirming positive findings with HR-MS. Confirmatory analysis must provide at least two characteristic masses (m/z) acquired at HR-MS conditions for a target analyte to fulfill the requirement for confirmation [56]. Unfortunately, achieving two ions with significant intensity is often difficult for certain analytes, especially when they are present at trace concentrations. From this perspective, hybrid HR-MS instruments capable of operating in the MS/MS mode to provide fragmentation mass spectra with accurate mass represent a new possibility for simultaneous analysis and confirmation of mycotoxins in food and feed.

The pioneering use of HR-MS techniques (utilizing a time-of-flight (TOF) analyzer) in mycotoxin analysis was described by Tanaka et al. [58], Mol et al. [29], and Zachariasova et al. [37]. Tanaka published an LC–TOFMS method with atmospheric pressure chemical ionization (APCI) for simultaneous determination of trichothecenes, aflatoxins, and ZON in corn, wheat, corn flakes, and biscuits. The disadvantage of this method was the additional SPE cleanup that had to be employed resulting in slightly decreased throughput of the entire workflow. Zachariasova et al. [37] employed UHPLC coupled to TOF and Orbitrap mass analyzers to examine 11 major Fusarium mycotoxins (fumonisins, DON, 3-ADON, NIV, HT2, T2, ZON, D3G, and fusarenon-X) in cereals. Two alternative sample preparation procedures based on either modified QuEChERS extraction or aqueous acetonitrile extraction were used prior to instrumental analysis. The UHPLC–TOFMS chromatograms of DON are shown in Figure 8.3. Based on these results, it was concluded that both technologies are applicable for mycotoxin detection, but the approach using TOFMS required some additional cleanup strategy to achieve sufficient sensitivity for the target analytes [37]. In a comparative study by Mol et al. [29], the UHPLC–TOFMS method was shown to be a generic tool in multiresidue and contaminants analysis compatible with the MS/MS approach regardless of sample preparation. Hybrid quadrupole/time-of-flight (QTOF) instrumentation was applied in a study by Sirhan et al. [26], who determined trichothecene mycotoxins in wheat, corn, rice, and
Figure 8.3. UHPLC-TOF/MS chromatograms of barley extract spiked with DON at levels 25, 125, 250, 500, and 1250 μg/kg; sample preparation: (a) QuEChERS-based method and (b) crude extract-based method; extraction window was 37 ppm [37]. Source: Ref. [37], Figure 1, p. 56. Reproduced with permission of Elsevier Science Ltd.
noodles. Another study by Polizzi et al. [59] investigated the occurrence of mycotoxins in air, dust, wallpaper, and silicone materials using both LC–MS/MS and LC–QTOFMS techniques. Application of QTOF technology was also described by Veprikova et al. [60], who used it for identification of masked glycosylated forms of T2 and HT2 toxins.

The most comprehensive HR-MS studies devoted to the application of UHPLC–Orbitrap MS technology in multimycotoxin analysis were published by Herebian et al. [61], Zachariasova et al. [37,62], Rubert et al. [28], and De Dominicis et al. [63]. All tested 32 mycotoxins as the main representatives of Fusarium, Claviceps, Aspergillus, Penicillium, and Alternaria fungi. In the study of Herebian et al. [61], the HPLC–ESI–MS/MS and microcapillary-HPLC–LTQ/Orbitrap MS instruments were critically assessed for their use in cereal examination. Based on analyses of the undiluted acetonitrile:water extracts, it was concluded that HR-MS is also a time-saving method useful for the suggested purpose. Zachariasova et al. [37,62] published the use of Orbitrap MS technology for the analysis of mycotoxins in cereals and beer. Both studies were focused on comparing two HR-MS instruments (TOF and Orbitrap MS) and their possible applicability for the fully validated screening and quantitative methods. In both cases, Orbitrap MS instrumentation was shown to offer superb sensitivity without the need for lengthy sample preparation protocols. This particular instrumentation was also used in a validation study aimed at regulated mycotoxins in wheat/barley flours, crisp bread, and other bakery ingredients [8,63]. The increasing interest in HR-MS for nontargeted screening of masked forms of mycotoxins and various metabolites was also demonstrated in several publications [64–66]. HR-MS was shown to be applicable as a detection tool for potentially harmful compounds, for which analytical standards were not available.

8.4 NO-SEPARATION MASS SPECTROMETRY-BASED METHODS

In addition to MS-based applications employing separation of the sample extract, some rapid no-separation techniques, such as matrix-assisted laser desorption MS, ambient ionization MS, and ion mobility spectrometry, have also been used to analyze mycotoxins. Examples of applications of these techniques are provided in the following sections.

8.4.1 Matrix-Assisted Laser Desorption Ionization–Mass Spectrometry

The principles of matrix-assisted laser desorption ionization–mass spectrometry (MALDI–MS) have been described in other chapters. Although not widely employed, several applications of MALDI–MS aimed at analysis of mycotoxins have been published. With regard to the need for internal standardization to allow quantification, MALDI–MS has been used mainly for qualitative analysis. MALDI is also a useful tool in characterization and classification of toxigenic fungi and mycotoxin-related proteomics.

In a study by Elosta et al. [67], a thorough optimization of positive-mode MALDI coupled to TOFMS was performed to allow sensitive determination of DON, NIV,
and ADONs in SPE-purified acetonitrile–water extracts of barley and malt. The use of sodium azide matrix provided good reproducibility and relatively low limits of detection ranging from 0.6 to 0.9 μg/ml. The authors also explored the capability of the method to quantify DON in naturally contaminated malt based on external calibration. The results for DON obtained by MALDI–TOFMS and the reference HPLC–MS/MS method were 507 ± 9 and 780 ± 124 μg/kg, respectively.

Work published by Catharino et al. [68] described the MALDI–TOFMS protocol for screening aflatoxins (AFB1, AFB2, AFG1, and AFG2) in peanuts at concentrations as low as 50 fmol. The use of an ionic liquid matrix (triethylamine–α-cyano-4-hydroxycinnamic acid solution in methanol) enabled the acquisition of interference-free mass spectra. The target mycotoxins were isolated from the samples by a procedure based on extraction with an aqueous–methanol solution containing potassium chloride and chloroform and purified with the use of CuSO4 and diatomaceous earth (Celite).

An interesting MALDI–TOFMS approach to qualitative analysis of gliotoxin was reported by Davis et al. [69], who developed a single-pot derivatization strategy using sodium borohydride-mediated reduction of gliotoxin followed by immediate alkylation of exposed thiols by reaction with 5'-iodoacetamido fluorescein to yield a stable product, diacetamido fluorescein-gliotoxin, of molecular mass 1103.931 Da ([M+H]+ ion). Unlike free gliotoxin, this product was readily detectable by MALDI–TOFMS at concentrations above 530 fmol. Although demonstrated only for the analysis of Aspergillus fumigatus culture supernatants, the above strategy may also be applicable to analysis of extracts of food and feed.

Marchetti-Deschmann et al. [70] used MALDI–TOFMS to classify closely related Fusarium species responsible for Fusarium head blight disease of crops based on the analysis of intact spores. The spore suspensions were directly embedded into a MALDI matrix without laborious sample cleanup or enrichment steps and the surface-associated compounds were analyzed by MALDI–TOFMS. These mass spectra were used to develop partial least-squares discriminant analysis (PLSDA) models for sample classification. The authors demonstrated the potential to build a database for accurate Fusarium species identification and for fast response in the case of infection in the cornfield. In another study, MALDI–TOFMS was used to identify resistance-associated proteins in response to Aspergillus flavus infection under drought stress [71]. MALDI–MS is also frequently used for detection and identification of conjugates of mycotoxins with proteins, which can also be used in analytical applications, such as immunogens for production of selective antibodies [72,73] or as biomarkers of intoxications with mycotoxins [74].

### 8.4.2 Ambient Ionization Mass Spectrometry

Novel ambient desorption ionization techniques such as direct analysis in real time (DART) and desorption electrospray ionization (DESI) hold great potential in high-throughput analysis of food. Various techniques and principles have been described in other chapters. Only a few applications were described for the analysis of mycotoxins in food and feed with the use of ambient ionization MS.
The most comprehensive study dealing with the high-throughput ambient MS analysis of mycotoxins in cereals was performed by Vaclavik et al. [32], who used a DART ion source coupled with an Orbitrap mass spectrometer. In the first step, the DART ionization efficiency of various mycotoxins was investigated. Of the 24 tested mycotoxins, 11 target analytes could be efficiently ionized by the DART technology. Only poor ionization of major trichotheccenes A (T2 and HT2) and some aflatoxins (AFB1 and AFB2) was achieved by DART. The ionization of OTA and other mycotoxins such as ergot alkaloids, fumonisins, and D3G was not possible under the experimental conditions employed. The samples of test cereals were processed by a modified QuEChERS extraction procedure and, due to relatively high ion signal fluctuations, analytes were quantified by means of matrix-matched standards with addition of isotope-labeled internal standards (Figure 8.4). The data generated by DART–MS analysis of certified reference materials were in good agreement with those obtained by a UHPLC–TOFMS method. The method was shown to be applicable for high-throughput detection of DON and ZON at limits established in the EU for unprocessed wheat and maize.

Another study described rapid DART–TOFMS analysis of DON in beer samples following immunoaffinity cleanup and sample preconcentration [75]. In a paper focused on UHPLC–MS analysis of multiple mycotoxins in beer, DART–Orbitrap MS fingerprinting was employed to document the purification effect achieved by acetonitrile-induced precipitation of some matrix components [62]. The application of the DESI ionization technique coupled with an ion-trap mass spectrometer was demonstrated for the determination of mycotoxins in a review by Maragos et al. [76]. Fumonisin B1 (0.2 ng) was deposited on the surface of maize kernels and, after drying, was easily detected as [M+H]+ ion by DESI–MS. Moreover, after subjecting the DESI-analyzed kernels to a germination test, 9 of 10 were found viable. Such results document the nondestructive nature of the DESI technique.

8.4.3 Ion Mobility Spectrometry

Ion mobility spectrometry (IMS) is an analytical technique that has gained widespread use in many applications dealing with the detection of contaminants due to its excellent sensitivity and rapid operation. Its main advantages include low detection limits, rapid response, simplicity, portability, and relatively low cost. IMS is a gas-phase ion separation technique in which ion mobility measurement is based on the drift velocities of ions in an electric field at ambient pressure. The technique is similar to TOFMS except that it operates under atmospheric pressure [77,78].

The IMS approach has been applied to the detection of mycotoxins in only a few studies. The first study focused on determining aflatoxins B1 and B2 in pistachios by means of corona discharge IMS [78]. In another study, the mycotoxin ZON and its metabolites α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), and α-zearalanol (α-ZAL) were analyzed by means of a novel high-field asymmetric waveform ion mobility spectrometry (FAIMS) method coupled with electrospray ionization (ESI). In comparison with ordinary ESI–MS performance parameters, significantly lower detection limits were obtained [79]. Khalesi et al. [80] described the IMS determination of OTA...
Figure 8.4. Calibrations employing matrix-matched standards and isotope dilution. (a) Extracted target ion record: DON (m/z 331.0943 ± 4 ppm); concentration in the range of 50–2500 μg/kg. (b) Extracted target ion record: 15NClad DON (m/z 346.1456 ± 4 ppm); concentration 500 μg/kg. (c) External calibration curve. (d) Isotope dilution calibration curve. Error bars are standard deviations calculated from three repeated injections [32]. Source: Ref. [32], Figure 3, p. 1956. Reproduced with permission of Elsevier Science Ltd.
in licorice root after sodium bicarbonate (0.13 M) and methanol (9:1, v/v) extraction and immunoaffinity cleanup. A detection limit as low as 0.01 ng/g of OTA in matrix was reported.

### 8.4.4 Immunochemical Methods

Immunochemical screening assays represent an important group of high-throughput tools for analyzing mycotoxins in various biological matrices, including food and feed. These techniques are characterized by rapid sample preparation and minimal time of analysis [17]. Because of their high selectivity provided by specific antibodies, their relative simplicity, and field portability, immunochemical methods are widely employed in industry and for purposes of agricultural control to obtain instant information on contamination with mycotoxins [81,82]. The predominant immunochemical techniques are based on ELISA, lateral flow devices (LFD), and surface plasmon resonance (SPR) technology [83]. Similar to the previously discussed techniques, the trends in this field are toward the development of rapid multimycotoxin screening methods with improved detection limits, decreased matrix effects, and simplified operation [81,83]. Several comprehensive reviews have recently been published by Zheng et al. [15], Goryacheva et al. [81] and Maragos et al. [84]. The following sections provide an overview of current applications of and future trends in immunochemical methods.

#### 8.4.4.1 Enzyme-Linked Immunosorbent Assay

The microtiter plate ELISA is the most frequently applied rapid method for the analysis of mycotoxins. Both direct and indirect ELISA kits are commercially available for a variety of mycotoxins. The ELISA kits are usually intended for the analysis of aflatoxins, fumonisins, trichothecenes, OTA, and ZON in cereals (maize, wheat, and oats), nuts, milk or cheese (AFM1), and feed. The majority of studies employing ELISA are aimed at monitoring mycotoxins in raw materials and food products. Additionally, new synthetic antigens and monoclonal antibodies for other mycotoxins, such as citrinin, are continuously being developed [85]. Other new polyclonal antibodies and ELISA kits for determination of tenuazonic acid in flour [86], trichothecene mycotoxin verrucarin A in indoor environments [87], and aflatoxins in herbal medicine products [88] have been recently introduced.

The main disadvantage of ELISA tests is the existence of antibody cross-reactivity to matrix or structurally related mycotoxins, which can produce overestimation or false positive results. Therefore, LC–MS-based confirmation of positive results obtained by ELISA is often performed. Although there is good agreement between data generated by ELISA and instrumental techniques for some matrices (cereals and rice), this trend cannot be generalized. To provide more accurate results, each lot of ELISA kits should be characterized by the producer in terms of cross-reactivity and recovery and this respective information should be provided to the users and declared on the product [89]. Currently, no ELISA kits that enable simultaneous determination of multiple mycotoxins are available.
8.4.4.2 Membrane-Based Immunoassays

Noninstrumental immunoassays based on antimycotoxin antibody principles are LFD, dipstick tests, and flow-through assays. In these assays, antigens or antibodies are immobilized on carrier membranes prepared from polyvinylidene difluoride, nylon, or nitrocellulose. Based on the appearance of colored lines on analysis strips, qualitative, semiquantitative, and in some cases quantitative results can be obtained by membrane immunoassays. Concentrations of mycotoxins then correlate with the intensity of the color. Test kits enabling both qualitative and quantitative analyses are commercially available for routinely controlled analytes, such as *Fusarium* mycotoxins (DON, ZON, T2 toxin, and fumonisins), aflatoxins, and OTA in many matrices. Additionally, new antimycotoxin monoclonal antibodies have also been developed, such as those for ZON or total fumonisins [90,91].

To document that a particular assay is fit-for-purpose, several studies focused on comparison between data obtained and those generated by conventional ELISA or LC–MS techniques. Most recently, the concentrations of both DON and 3-ADON have been assessed by both ELISA and LFD assays [92]. Although the data obtained with LFDs were in agreement with ELISA at most of the concentrations tested, in some cases, the recoveries of LFDs were outside the range of EU requirements (70–120%). In a study by Liu J. et al. [93], the accuracy of a new quantitative LFD for DON determination in durum wheat, semolina, and pasta was verified by parallel LC–MS/MS analyses. The assay was shown to be capable of simple, rapid, cost-effective, and robust on-site screening or remote quantitative analysis for ZON at concentrations fulfilling the worldwide legislation requirements.

Great attention has also recently been paid to the development of reliable multi-target dipsticks. For instance, a study describing semiquantitative determination of multiple mycotoxins in wheat, oats, and maize by multiplex indirect dipstick immunoassay was published in 2012 [8]. In this study, two application reports on the use of commercial dipsticks for simultaneous determination of DON, ZON, T2/HT2 toxins and fumonisins FB1, FB2, and FB3 in cereals were described. A methanol and water mixture used for the extraction of samples demonstrated recoveries in the range of 73–109% for all tested mycotoxins in all examined matrices (wheat, oats, and maize). The complete sample preparation and extraction was performed within 10 min and the dipstick analysis was performed in ~30 min. The reliability of these assays was confirmed by LC–MS analysis. The rate of false positive results, which can be caused by cross-reactivity of structural analogs, was below 13%.

The development of these types of devices is still in progress. In particular, the use of nanotechnologies and nanomaterials for preparation and construction of assays has been applied and published. For instance, a quantitative LFD for measuring of OTA in maize and wheat was developed [94], in which a ready-to-use device with antibodies labeled with gold nanoparticles was applied. Similar establishment was also published for the detection of aflatoxins B1 in food [95], but in this particular case a monoclonal antibody immobilized on nanoparticles with a silver core and a gold shell as a detection reagent was used. The assay was evaluated with the use of naturally contaminated rice, wheat, sunflower, cotton, chili peppers, and almonds. A good correlation was obtained between
results obtained with a commercially available ELISA. Additionally, magnetic nanogold particles were also applied in microsphere-based lateral flow immune-dipsticks for the detection of AFB2 in food [96].

8.4.4.3 Surface Plasmon Resonance

SPR represents a relatively new analytical technique that has gained increasing popularity due to its rapid, real-time, and highly selective and sensitive determination of analytes. Various applications of SPR in biochemistry, clinical diagnosis, and food analysis have already been described and several reviews describing the principles and benefits have been published [97]. SPR is an optical phenomenon used to measure changes on the surface of thin metal films under conditions of total internal reflection [81]. It allows direct detection of analytes without any labeling of interactants. As in the case of other immunoassay-based methods, SPR sensors have also been developed exclusively for mycotoxins of regulatory interest such as aflatoxins, trichothecenes, ZON, fumonisins, and OTA. Some of these tests are also commercially available [97]. There is a trend in the use of SPR technique to develop and validate multisensors for detection and quantification of numerous mycotoxins in a single analysis. This was achieved by van der Gaag et al. [98], who introduced a multiple SPR sensor for simultaneous determination of AFB1, DON, ZON, and FB1. This unique device was constructed from four flow cells containing four types of antimycotoxin antibodies. The evaluation of a prototype of the multiplex microimmunoassay quantification sensor for DON and ZON was published by Dorokhin et al. [99]. The limits of detection achieved in this study were 84 and 68 μg/kg for DON and 64 and 40 μg/kg for ZON in maize and wheat, respectively.

8.4.4.4 Fluorescence Polarization Immunoassay

In fluorescence polarization (FP) immunoassays, an analyte labeled with fluorophore (fluorescein) competes with free analyte for specific antibody-binding sites in solution, while fluorescence polarization of the fluorescein label is measured. An FP immunoassay has been successfully used for the determination of DON, ZON, and OTA in wheat, corn, and some food samples [17]. In a study by Bondarenko et al. [100], the influence of various fluorescent-based tracers on sensitivity of the assay for the determination of ZON and OTA was examined. The LODs (15 and 10 μg/kg for ZON and OTA, respectively) and acceptable recoveries ranging from 84 to 97% were obtained. The development and application of new FP immunoassay has recently been published for simultaneous quantitative analysis of T2 and HT2 toxins in contaminated wheat samples [101]. In this particular study, the synthesis of four fluorescein-labeled T2 or HT2 toxin tracers was carried out and their binding responses with seven monoclonal antibodies were evaluated. Using extraction with a methanol:water mixture (90:10, v/v), it was possible to obtain an average recovery of 96% and a LOD as low as 8 μg/kg for the sum of the toxins. The assay allowed quantitation of target analytes within 10 min.
8.5 CONCLUSIONS

Monitoring, control, and prevention of occurrence of mycotoxins in agricultural raw materials, food, and feed represent an important task related to quality and safety of the technological production of food and feed and to human health. With increasing number of mycotoxins of interest, there is an ongoing need for developing rapid and robust analytical strategies for analysis of these hazardous compounds in a wide range of matrices. Both instrumental (LC–MS-based techniques) and immunochemical methods (ELISA, LFD, SRM, etc.) can fulfill requirements for detectability, selectivity, and throughput. These techniques ensure accurate and reliable data applicable for further food/feed risk assessments. Both types of procedures have their advantages and disadvantages. Generally speaking, the application of sophisticated UHPLC–MS/MS instrumentation is a cutting-edge methodology for the simultaneous multimycotoxin analysis in a wide range of matrices. Considering the sensitivity of MS coupled with rapid and simple sample preparation of dilute-and-shoot or QuEChERS strategies, this combination enables development of screening methods for rapid monitoring (several minutes) of a wide range of contaminants. On the other hand, the noninvasive and easy-to-handle methods such as immunoassays, dipsticks, and biosensors offer much less costly but still sufficiently accurate strategies, which are also able to determine mycotoxins in a relatively short time. Moreover, these approaches can potentially be used on-site in industrial or agricultural settings. In the case of MS-based techniques, future trends and challenges can be seen in the incorporation of HR-MS instrumentation into routine determination of mycotoxins and, in the case of immuno-based methods, in the increase in the number of matrices and target mycotoxin combinations.

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