Chapter 10

Analysis of Multiple Mycotoxins in Food

Jana Hajslova, Milena Zachariasova, and Tomas Cajka

Abstract

Mycotoxins are secondary metabolites of microscopic filamentous fungi. With regard to the widespread distribution of fungi in the environment, mycotoxins are considered to be one of the most important natural contaminants in foods and feeds. To protect consumers' health and reduce economic losses, surveillance and control of mycotoxins in food and feed has become a major objective for producers, regulatory authorities, and researchers worldwide. In this context, availability of reliable analytical methods applicable for this purpose is essential. Since the variety of chemical structures of mycotoxins makes impossible to use one single technique for their analysis, a vast number of analytical methods has been developed and validated. Both a large variability of food matrices and growing demands for a fast, cost-saving and accurate determination of multiple mycotoxins by a single method outline new challenges for analytical research. This strong effort is facilitated by technical developments in mass spectrometry allowing decreasing the influence of matrix effects in spite of omitting sample clean-up step. The current state-of-the-art together with future trends is presented in this chapter. Attention is focused mainly on instrumental method; advances in biosensors and other screening bionanalytical approaches enabling analysis of multiple mycotoxins are not discussed in detail.

Key words: Mycotoxins, Liquid chromatography, Mass spectrometry, Food

1. Introduction

Mycotoxins are natural toxic secondary metabolites produced by microscopic filamentous fungi, which grow on various agricultural commodities in the field, and/or during post-harvest period (transport, processing, and storage). The toxinogenic fungi belong mainly to genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria* (1–4). Currently, more than 500 different mycotoxins are known; however, sufficient knowledge has been collected only for a limited number of them. With regard to the health hazard posed by mycotoxins to the end consumers (and farm animals), many countries have set up regulations for their control in food chain. In Table 1,

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Table 1
Overview of most common mycotoxins together with their producers, typical food commodities, major health adverse effects, and current maximal legislative limits (reproduced and updated from (19) with permission from Springer)

Mycotoxins	Main producers/origin	Food commodity	Maximum level (EC 1881/2006 amended by EC 1126/2007)
Fumonisins			
Fumonisins A1, A2, A3, B1, B2, B3, C1, C2, C3, P1, P2, P3	*Fusarium verticillioides*, *F. proliferatum*, *F. anthophilum*, *F. moniliforme*, *F. dlamini*, *F. napiforme*, *F. nygamai*, *Alternaria alternata*	Maize, maize based products, sorghum, sorghum, asparagus, rice	Sum of fumonisins B1 and B2: 200–4,000 μg/kg (infant foods, processed maize-based foods, unprocessed maize)
Hydrolyzed and partially hydrolyzed fumonisins	Product of food processing		
Trichothecenes			
Type A trichothecenes: T-2 toxin, HT-2 toxin, diacetoxyscirpenol, neosolaniol, verrucarol	*Fusarium sporotrichioides*, *F. poae*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. moniliforme*, *Cephalosporium sp.*, *Myrothecium sp.*, *Trichodermasp.*, *Trichothecium sp.*, *Phomopsis sp.*, *Stachybotrys sp.*, *Verticimonosporium sp.*	Cereals, cereal based products	In discussion for T-2 and HT-2 toxin
Type B trichothecenes–nivalenol, deoxynivalenol, 3-acetylDON, 15-acetylDON, fusarenon-X	*Fusarium graminearum*, *F. culmorum*, *F. sporotrichioides*, *F. cerealis*, *F. lunulosporum*	Cereals, cereal based products	Deoxynivalenol: 200–1,750 μg/kg (infant food, processed cereal-based foods, unprocessed cereals)
Deoxynivalenol-3-glucoside	Metabolite of deoxynivalenol		
Zearalenones			
Zearalenone	*F. graminearum*,*F. culmorum*, *F. crookwellense*, *F. equiseti*, *F. sporotrichioides*	Barley, oats, wheat rice, sorghum, sesame, soy beans, cereal based products	20–400 μg/kg (maize-based infant food, processed cereal-based and maize-based foods, unprocessed maize, refined maize oil)
α- and β-zearalenol, α- and β-zearalanol	Metabolites of zearalenone		

(continued)

Table 1 (continued)

Mycotoxins	Main producers/origin	Food commodity	Maximum level (EC 1881/2006 amended by EC 1126/2007)
Ochratoxins			
Ochratoxins A, B, C	*Aspergillus ochraceus*, *A. niger*, *A. melleus*, *A. alutaceus*, *A. alliaceus*, *A. albertensis*, *A. citricus*, *Neopetromyces muricatus*, *Penicillium viridicatum*, *P. verrucosum*, *P. cyclopium*, *P. carbonarius*	Cereals, dried fruit, raisins, wine, coffee, oats, spices, rye	Ochratoxin A: 0.5–10 μg/kg (infant foods, processed cereal-based foods, unprocessed cereals, dried vine fruits and instant coffee)
Ochratoxin α	Metabolite of ochratoxin A		
Aflatoxins			
Aflatoxins B1, G1, B2, G2	*Aspergillus flavus*, *A. nomius*, *A. parasiticus*, *A. arachidicola*, *Emericella astellata*, *E. venezuelensis*, *E. olivicola*	Maize, wheat, rice, spices, almonds, oilseeds, dried fruits, cheese	Sum of aflatoxins B1, B2, G1 and G2: 4–15 μg/kg, aflatoxin B1: 0.1–8 μg/kg; (nuts, ground nuts, dried fruits, cereals, maize)
Aflatoxins M1 and M2	Metabolites of aflatoxin B1 and B2	Milk, eggs, meat	Aflatoxin M1: 0.025–0.05 μg/kg (infant and dietary foods, milk)
Ergot alkaloids			
Ergocornine/inine, ergocristine/inine, ergocryptine/inine, ergosine/inine, ergotamine/inine	*Claviceps purpurea*, *C. africanana*, *C. fusiformis*, *C. fusiformis*, *C. paspali*, *Neotyphodium coenophialum*	Wheat, rye, hay, barley, millet, oats, sorghum, triticale	
Alternaria toxins			
Altenuene, alternariol, alternariolmonomethyl ether, altertoxin I, altertoxin II, altertoxin III, tenuazonic acid	*A. alternata*, *A. dauci*, *A. cucumerina*, *A. solani*, *A. tenuissima*, *A. citri*	Wheat, rice, rye, olives, sorghum, tobacco, apples, peppers, sunflower seeds, oilseed rape, pecan nuts, tomatoes, mandarins	

(continued)

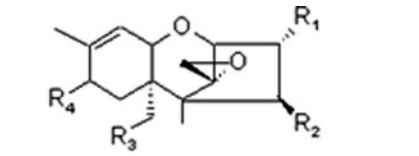
Table 1 (continued)

Mycotoxins	Main producers/origin	Food commodity	Maximum level (EC 1881/2006 amended by EC 1126/2007)
Enniatins			
Enniatin A, enniatin A1, enniatin B, enniatin B1	*Fusarium Avenaceum*, *F. orthoceras*, *some Alternaria*, *Halosarpheia*, *Verticillium ssp.*	Wheat, corn, barley, bread mill, oat flour, rice	
Patulin	*Aspergillus clavatus*, *A. longivesica*, *A. terreus*, *P. expansum*, *Penicillium griseofulvum*, *Byssochlamys sp.*	Apples, apple juice, cherries, cereal grains, grapes, pears, bilberries	10–50 μg/kg (infant foods, apple juice, solid apple, spirit drinks derived from apples or containing apple juice, fruit juices)
Beauvericin	*F. bulbicola*, *F. denticulatum*, *F. lactis*, *F. phyllophilum*, *F. pseudocircinatum*, *F. succisae*	Wheat, corn, barley, bread mill, oat flour, rice	
Fusaroproliferin	*Fusarium proliferatum*, *F. concentricum*, *F. antophilum*, *F. begoniae*, *F. succisae*, *F. bulbicola*, *F. circinatum*, *F. udum*, *F. subglutinans*	Wheat, corn, barley, bread mill, oat flour, rice	

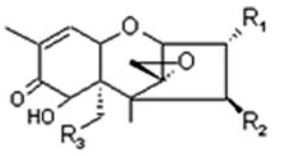
there is presented an overview of the major mycotoxins, which are currently under focus (5–7). Those, for which maximum limits based on exposure and toxicity data have been established by the European Union, are indicated by asterisk (8, 9). Aflatoxins, patulin, deoxynivalenol, fumonisins, and ochratoxin A are also included by the Food and Drug Administration Compliance program guidance manual (10).

While relatively extensive information is available on occurrence of regulated mycotoxins, the requirements for more comprehensive information on food crops contamination by toxins such as ergot alkaloids, beauvericin, or enniatins have been raised only recently. In addition to free mycotoxins, also occurrence of mycotoxin conjugates in cereals represents an emerging issue in food safety. Nowadays, most attention has been paid to deoxynivalenol-3-glucoside and zearalenone-4-glucoside originating in food plants as a result of detoxification process (11–13). Supposing such compounds are, at least partly bioavailable, then, dietary exposure might be underestimated.

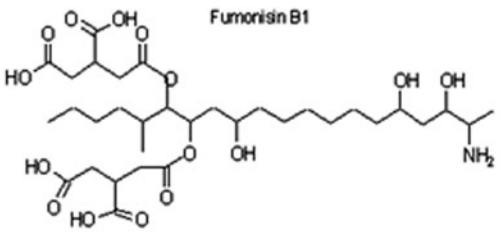
As Fig. 1 documents, mycotoxins introduced in Table 1 represent largely differing structure classes, and consequently, their

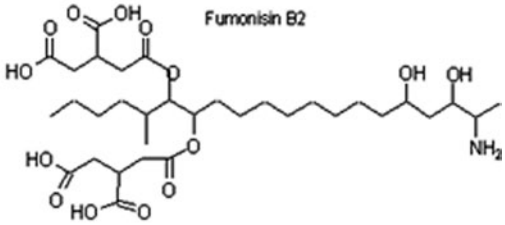


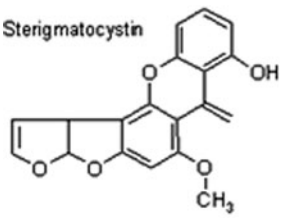
Verrucarol R_1=R_4=H, R_2=R_3=OH
Neosolaniol R_1=OH, R_2=R_3=R_4=OAc
Diacetoxyscirpenol R_1=OH, R_2=R_3=OAc, R_4=H
HT_2 toxin R_1=R_2=OH, R_3=OAc, R_4=OCOi-Bu
T_2 toxin R_1=OH, R_2=R_3=OAc, R_4=OCOi-Bu

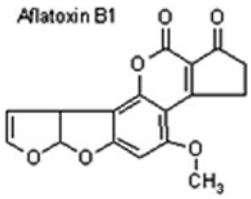


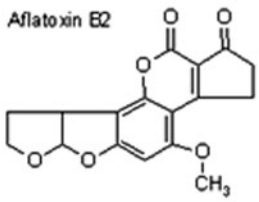
Deoxynivalenol R_1=R_3=OH, R_2=H
Nivalenol R_1=R_2=R_3=OH
Fusarenon-X R_1=R_3=OH, R_2=OAc
3-Acetyldeoxynivalenol R_1=OAc, R_2=H, R_3=OH
15-Acetyldeoxynivalenol R_1=OH, R_2=H, R_3=OAc

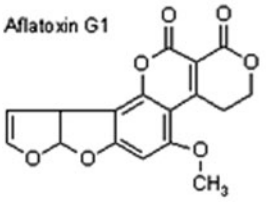


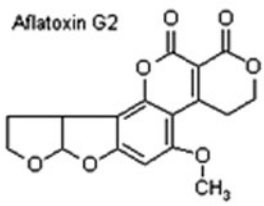


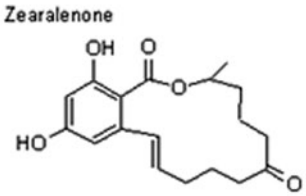


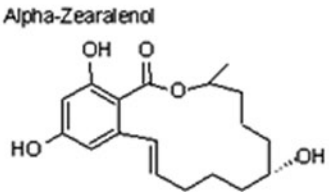


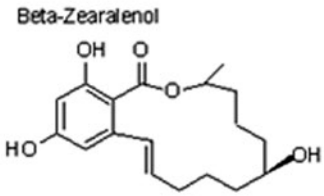












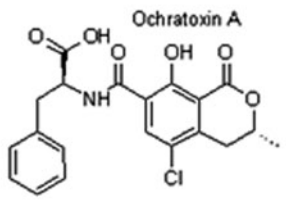
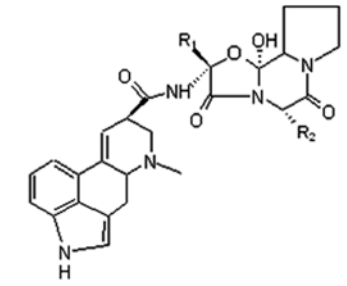
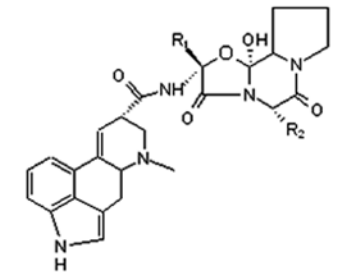


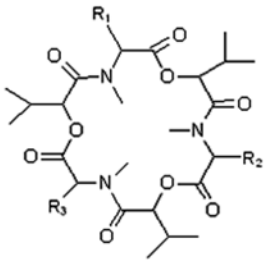
Fig. 1. Structures of selected mycotoxins.



Ergosine	R_1=CH_3, R_2=$CH_2CH(CH_3)_2$
Ergotamin	R_1=CH_3, R_2=$CH_2C_6H_5$
Ergocornin	R_1=$CH(CH_3)_2$, R_2=$CH(CH_3)_2$
Ergocristin	R_1=$CH(CH_3)_2$, R_2=$CH_2C_6H_5$



Ergosinine	R_1=CH_3, R_2=$CH_2CH(CH_3)_2$
Ergotaminine	R_1=CH_3, R_2=$CH_2C_6H_5$
Ergocorninine	R_1=$CH(CH_3)_2$, R_2=$CH(CH_3)_2$
Ergocristinine	R_1=$CH(CH_3)_2$, R_2=$CH_2C_6H_5$



Enniatin A	R_1=R_2=R_3=$CH_2C_6H_5$
Enniatin A_1	R_1=R_2=R_3=$CH(CH_3)CH_2CH_3$
Enniatin B	R_1=$CH(CH_3)CH_2CH_3$, R_2=R_3=$CH(CH_3)_2$
Enniatin B_1	R_1=R_2=R_3=$CH(CH_3)_2$

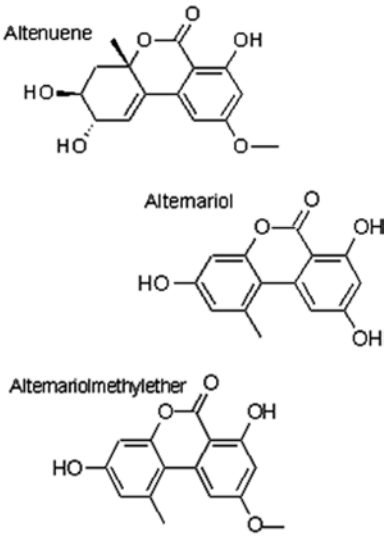


Fig. 1. (continued)

physicochemical properties vary in a wide range. Most of mycotoxins are relatively stable compounds thus surviving under various conditions employed in food processing including thermal treatment. On this account, they can be, at least in some extent, transferred from contaminated raw material into final product (14, 15). Some of them, like fumonisins, might be transformed into bound forms (to starch or to proteins) after thermal processing (16), or can be partially and/or fully hydrolyzed when alkaline treatment is performed (17). Another interesting finding of the recent years is a significant increase of deoxynivalenol-3-glucoside in fermented cereal-based products, such as malt and beer (15).

To support extensive preventive efforts made by establishing simultaneous restriction of the allowed amounts of certain mycotoxins in foods (and feedstuffs), but also to enable collecting

information on other, until now not regulated mycotoxins, reliable and accurate analytical methods, which allow their unambiguous identification and confirmation, as well as an accurate quantification at very low concentration levels in various matrices, have to be available. In following paragraphs, strategies to control multiple mycotoxins within a single analytical run will be discussed. In addition to meeting desired performance criteria (18), also laboratory throughput and workload are taken into consideration.

2. Methods for Mycotoxins Analysis

Analysis of mycotoxins in food is generally a multistep process comprised of (1) sampling, (2) extraction of analytes from the matrix (usually with mixtures of water and polar organic solvents) possibly followed by an extract purification, and (3) final detection and quantitative determination. Due to a large diversity of extraction, clean-up procedures, and respective detection steps available within analysis of mycotoxins and their conjugated forms, a comprehensive discussion of all existing methods would exceed the scope of this chapter. For this reason, we will focus just on the most common trends and recent advances in mycotoxins analysis.

2.1. Sampling

Distribution of mycotoxins in most of agricultural commodities is very heterogeneous; in most cases, the microscopic filamentary fungi and their secondary metabolites occur in so-called "hot spots." Thus, sampling is the largest source of variability associated with the mycotoxins analysis procedure, and the most crucial step in obtaining reliable results (19). In the past, a lot of papers related to sampling of aflatoxins were published (20–22). Recently, the sampling strategies have been set-up also for other mycotoxins, e.g. ochratoxin A, patulin, and *Fusarium* toxins. The European Commission issued the Commission Regulation (EC) 401/2006 laying out the sampling methods and the performance criteria for the methods of analysis to be used for the official control of mycotoxins in foodstuff (18). This Regulation provides sampling plans for groups of food commodities taking into account the heterogeneous distribution of mycotoxins. Different sampling plans were also established in other countries, e.g. in the USA for aflatoxins in peanuts (23).

Generally, it is possible to recommend that the most effective way to reduce the overall variability of results is to increase the size of the laboratory sample, ensure the proper milling, and homogenization (19, 24, 25).

2.2. Extraction and Crude Extract Purification

Mycotoxins are usually extracted from ground solid matrices by shaking with aqueous acetonitrile (*liquid–solid extraction*). Aqueous methanol or ethyl acetate has also been used to a lesser extent.

The most extensively used extraction mixture for a simultaneous co-isolation of a wide range of mycotoxins, is an acetonitrile:water (84:16, *v/v*) mixture. Rarely, mycotoxins can be isolated from samples by employing *accelerated solvent extraction* (ASE), in which the extraction efficiency is increased by enhanced pressure and temperature (26, 27). However, in spite of its automation, this technique may become rather laborious and time-consuming, since obstruction of extraction cells due swelling of starches in cereals often occurs. Moreover, performing a thorough clean-up of ASE extract is typically needed due to more co-extracted impurities compared to traditional shaking.

The choice of the extraction medium is closely related to the selected clean-up procedure. In mycotoxins analysis, purification of extracts is important, especially in case of their determination at trace levels. Commonly, procedures for mycotoxins clean-up are based either on *solid-phase extraction* (SPE) or use *immunoaffinity columns* (IACs). Among commercially available SPE columns, MycoSep cartridges are the most frequently used (28–30). Currently, multifunctional columns containing, e.g. charcoal, celite, and alumina are available for trichothecenes, zearalenone, aflatoxins, ochratoxins, moniliformin, fumonisins, and ergot alkaloids analysis. Employing SPE based on polymeric reversed-phase columns (*N*-vinylpyrrolidone/divinylbenzene columns Oasis HLB) is also possible obtaining good recoveries for both type-A and type-B trichothecenes (31).

Regarding the IAC-based clean-up, its main advantage includes, in addition to the purification effect, also the possibility of analytes pre-concentration what results in decreasing of detection limits. Another advantage is its applicability for complex matrices and reduced usage of organic solvents. The highly appreciated feature of this type of purification approach is its specificity, which is, however, limiting for simultaneous determination of different groups of analytes (32). Depending on the type of antibody, some cross-reactivity may be encountered potentially leading to results overestimation. This phenomenon can be successfully exploited in analysis of masked mycotoxins since, in addition to the target compound, also structurally related metabolites can be bound. For instance, thanks to cross-reactivity of DON dedicated DONprep columns also deoxynivalenol-3-glucoside, the major "masked" *Fusarium* toxin, can be isolated together with free DON (33). Additionally to DON, the IACs are commercially available for T-2 and HT-2 toxins, fumonisins, zearalenone, aflatoxins, and ochratoxin A. It is worth mentioning that combined multimycotoxin immunoaffinity columns capable to purify a broader range of mycotoxins, in particular HT-2 and T-2 toxins, deoxynivalenol, and zearalenone, (34), are currently available in the market.

Since all mycotoxins vary considerably in their polarities, in the case of multi-mycotoxin analysis, an optimal extraction and

purification step for each group of analytes is not possible to perform, and, unavoidably, some compromises have to be made. An example of very fast generic extraction/purification is the QuEChERS approach (Quick, Easy, Cheap, Effective, Rugged, and Safe), currently widely used in pesticide residue analysis. The key principle is partitioning of an acetonitrile:water mixture induced by addition of inorganic salts. While the analytes are transferred into an organic phase, more polar matrix impurities are left in an aqueous layer. As in the case of pesticides, the residual impurities in acetonitrile (some sugars and fatty acids) can be removed by dispersive SPE realized by addition of primary secondary amine (PSA) sorbent. However, due to the acidic nature of some mycotoxins (e.g. fumonisins) and the risk of their binding on the sorbent, this approach is not recommended (35).

2.3. Examination of Sample Extracts

Depending on the purpose of analysis, either simple semiquantitative (immunochemical) screening assays, or accurate instrumental methods, namely when compliance with legislation is to be checked, are used. Figure 2 shows trends in the mycotoxins analysis area during the last 50 years. A growing employment of bioanalytical methods from the beginning of the 1990s such as Enzyme Linked Immunosorbent Assay (ELISA) as well as the biosensors in the subsequent decade was noticed. Concerning the instrumental analysis, liquid chromatography coupled with mass spectrometry (LC–MS) revolutionizes the mycotoxins analysis area, enabling quantitative and confirmatory analysis of multiple mycotoxins, independent of their chemical structure or biological activity.

Due to the inherent complexity of food matrices and the impossibility to get samples free of co-extracts, most common

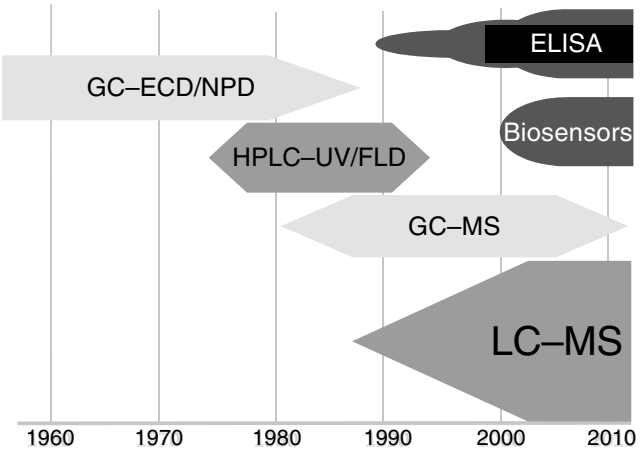


Fig. 2. Trends in the analysis of mycotoxins from the time perspective (years 1960–2010).

instrumental analytical procedures involve some sample separation step prior to identification/quantification of the analytes to reduce interferences within these processes. However, recent availability of highly selective detection tools opened the door to applications in which the separation step is eliminated. In the following paragraphs, a brief introduction of conceivable analytical approaches and their development during the time is summarized.

2.3.1. Gas Chromatography

In the past, methods based on a gas chromatographic (GC) approach were routinely used for determination of trichothecenes, zearalenone, ochratoxin A, patulin, and citrinin (36–40). However, GC-based methods suffer from some significant drawbacks; the major one is the need to carry out derivatization of analytes prior to sample analysis. Most of the mycotoxins are small nonvolatile and polar molecules, which require breaking of hydrogen bridges to become amenable to GC–MS analysis. For this purpose, silylation and acylation agents are preferably employed. Moreover, for detection of mycotoxins with the electron capture detector (GC–ECD), brominating or fluoroacylating agents have to be used to take advantage of detector specificity (28, 30, 41, 42). In addition to labor and time demands of these procedures, problems such as double peaks of analytes caused by the incomplete derivatization can appear (43). Other analytical problems encountered with procedures employing GC included non-linearity of calibration curves, over-estimation of results due to matrix effects (when using pure standards for calibration), poor repeatability, and memory effects from previous sample injections (30, 44). Except of the study of Jelen and Wasowicz reporting the use of comprehensive two-dimensional GC with time-of-flight mass spectrometry (GC×GC–TOFMS) for the trichothecene analysis in wheat (45), no other advances in the GC area have been recently published. LC–MS is becoming the most effective tool for the mycotoxins analysis.

2.3.2. Liquid Chromatography with Conventional Detectors

Liquid chromatography (LC) represents the dominating separation strategy in mycotoxins analysis. Current "classic" procedures are based on high performance LC coupled to the conventional detectors such as fluorescence detector (FLD), UV detector, diode-array detector (DAD), or photodiode array detector (PDA). In any case, sample pretreatment for minimizing matrix interferences, thus unbiased results, is a task of major importance (it should be noted that, contrary to mass spectrometric detection mentioned below, correction of results by using isotopically labeled internal standards is not feasible for optical detection). An overview of the latest methods for mycotoxins analysis using conventional detectors is presented in Table 2. Fluorescence detection is often employed for the analysis of ochratoxin A, aflatoxins, and zearalenone. However, in the absence of natural

Table 2
Overview of latest analytical methods for mycotoxins determination employing liquid chromatography coupled with conventional detectors

References	(46)				(47)	(48)	(49)	(50)	(51)	(52)		(53)	(54)	(55)	(56)	(57)	(58)	(59)
Matrices	Cereals, cereal products				Rice	Medical plants	Red paprika, black pepper	Wheat flour	Milk	Paprika		Honey, natural sweetners, vinegars, apple juice	Rye, rye products	Dried apple rings	Apple juice	Oats, cereal foods	Green coffee, roasted coffee	Blue cheese
Analyte	FB1, FB2	OTA	ZEA	DON	Aflatoxins	OTA	OTA	DON	Aflatoxin M1	Aflatoxins	OTA	PAT	Ergot alkaloids	PAT	PAT	HT2, T2	OTA	OTA
LODs (μg/kg)	41 (FB1); 31 (FB2)	0.014	5.5	30–55	0.1–0.16	0.3	3	0.03[a]	0.01	0.23–0.45[b]	0.8[b]	0.09 (μg/L)	up to 3.3	n.p.	0.23 (μg/L)	8 (HT2); 8 (T2)	0.032	0.02
Extraction solvent	MeCN: MeOH: water	MeCN: water	MeCN: water	MeCN: water	MeOH: water	MeOH: water	MeCN: water	MeCN: water/ water/ MeOH: water	None	MeOH: water	MeOH: $NaHCO_3$	Water: MeCN: perchloric acid	Ethyl acetate: MeOH: aqueous ammonia	Water	Ethyl acetate: Na_2CO_3 solution	MeOH: water	MeOH:water containing sodium hydrogen carbonate and PEG	$CHCl_3$, NaCl, H_3PO_4
Purification	IAC	IAC	IAC	IAC	IAC	IAC	Mycosep	MycoSep/ IAC/Oasis HLB	IAC	IAC	IAC	no	SPF with basic alumina	Silica gel SPE	no	IAC	IAC	IAC
Detection method	FLD[c]	FLD	FLD	DAD	FLD	FLD	FLD	PDA	FLD	FLD	FLD	DAD	FLD	DAD	DAD	FLD[b]	FLD	FLD

Abbreviations of analytes: *FB1* fumonisin B1, *FB2* fumonisin B2, *OTA* ochratoxin A, *ZEA* zearalenone, *DON* deoxynivalenol, *PAT* patulin, *HT2* HT-2 toxin, *T2* T-2 toxin
n.p. – information not provided
[a]LOD presented in μg/L
[b]LOQ (limit of quantification)
[c]Pre-column derivatization employed

fluorescence for trichothecenes and patulin, UV/DAD detection is available.

2.3.3. Liquid Chromatography–Mass Spectrometry

Considering the need for efficient food safety control, the speed of analysis and the applicability to as wide as possible range of mycotoxin/matrix combinations are nowadays obviously the driving forces in multi-mycotoxin analysis development. Robustness, selectivity, sensitivity, as well as flexibility regarding the method scope, are the key criteria for selection of optimal detection methods enabling identification/quantification following sample separation. In this context, mass spectrometry is currently the only powerful detection tool providing satisfactory solutions for accurate analysis including confirmation of target, and, in some cases, nontarget analytes.

High and ultra-high performance liquid chromatography (HPLC/U-HPLC) coupled with various mass spectrometric platforms are outstandingly qualified for multi-toxin analyses. Triple-quadrupole (QqQ) tandem mass spectrometry (MS/MS) is currently considered as a "gold standard," although the benefits of other mass analyzers mentioned below have been recognized by many laboratories concerned with control of natural toxicants in the food chain.

Besides its high sensitivity, MS/MS also provides a high degree of certainty in analytes identification (especially in the case of poor chromatographic resolution). Under common conditions, obtaining a sufficient number of identification points, in accordance with the EU guidelines for obtaining unambiguous data (60), is easily possible. Confirmation of target analytes is usually achieved by recording at least two mass transitions in selected reaction monitoring (SRM) mode.

One of the first quantitative LC–MS/MS methods for multi-mycotoxin analysis allowing simultaneous determination of mycophenolic acid, griseofulvin, roquefortine C, chaetoglobosin B, verruculogen, and penitrem A in food and feed matrices was published by Rundberget and Wilkins in 2002. The extraction step performed by an acetonitrile:water:formic acid mixture (900:99:1, *v/v/v*) was followed by defatting with hexane. The atmospheric pressure chemical ionization (APCI) was used for quantification by an ion trap MS instrument (61). Another method for determination of type A trichothecenes (T-2 and HT-2 toxin, acetyl T-2 toxin, diacetoxyscirpenol, monoacetoxyscirpenol (15-acetoxyscirpenol), and neosolaniol) in oats after MycoSep purification was published in 2002. Analytes were separated on a reversed-phase narrow-bore column and detected in positive APCI-MS/MS (62). Other tandem MS method for the determination of four trichothecenes type B in maize was published in 2003 by Lagana et al. Nivalenol, deoxynivalenol, fusarenon-X, and 3-acetyl deoxynivalenol were determined under negative electrospray

ionization and multiple reaction monitoring mode (MRM) of a triple-quadrupole mass spectrometer (63). One year later, Royer et al. reported a method for the determination of deoxynivalenol, fumonisin B1, and zearalenone in maize. ASE was used for sample extraction. For quantification, isotopically labeled internal standards were employed for obtaining accurate results. Detection of target analytes was carried out by APCI-ion trap-MS/MS (26). LC–ESI-MS/MS method for detection and quantification of beauvericin, enniatins, and moniliformin in grain-based foods was published (64). Similar triple quadrupole LC–MS/MS methods for the quantification of trichothecenes and zearalenone in cereals were presented in 2005. After extraction with an acetonitrile:water (84:16, *v/v*) mixture and MycoSep clean-up, analytes were detected by using of ESI (65) and APCI (66) interfaces. The MycoSep purification was enabled also by Tanaka et al. who, additionally to trichothecenes and zearalenone, included aflatoxins B1, B2, G1, and G2 into their method. The APCI-TOFMS ionization/detection was found to be suitable for the screening of multiple mycotoxins in cereals and cereal-based products (67). In 2005, Cavaliere et al. presented the method for the determination of trichothecenes, fumonisins, zearalenone, and α-zearalenol in corn, and the ESI-MS/MS technique was employed for detection (68). Furthermore, the LC–ESI-MS/MS method for the determination of mycotoxins and their metabolites in milk was introduced by Sorensen et al. in 2005. Aflatoxin M1, deoxynivalenol, deepoxynivalenol, 3- and 15-acetyldeoxynivalenol, HT-2 and T-2 toxins, T-2 triol, diacetoxyscirpenol, monoacetoxyscirpenol, fumonisins B1 and B2, ochratoxin A, zearalenone, and its α- and β- metabolites (zearalenols and zearalanols) were extracted with an acetonitrile:hexane mixture (16:10, *v/v*), and purified by employing *N*-vinylpyrrolidone/divinylbenzene co-polymer columns (69). Kokkonen et al. published an MS/MS method for the determination of aflatoxins, ochratoxin A, mycophenolic acid, penicillic acid, and roquefortine C in blue cheese by triple quadrupole with ESI ionization. For fats removal, hexane was added to an acetonitrile:formic acid extract (70).

Continuous advances in technical parameters of modern LC–MS instrumentation offered new possibilities to increase sample throughput and expand methods scope. Both introduction of U-HPLC and improving MS detection sensitivity (modification of ion sources and mass analyzers performance) enabled, approximately in mid of first decade of this century, application of the "dilute-and-shoot" approach.

In 2006, Spanjer et al. presented an ESI triple-quadrupole MS/MS method for the simultaneous determination of aflatoxins B1, B2, G1, and G2, ochratoxin A, DON, 3-acetyl-DON, 15-acetyl-DON, fumonisins B1 and B2, diacetoxyscirpenol, ZON, T2-toxin, HT2-toxin, roquefortine, and sterigmatocystin

in various foodstuffs like peanuts, cornflakes, wheat, and figs. An acetonitrile:water extract was diluted in a ratio of 1:3 and analyzed directly, without any clean-up (71). Further ESI-MS/MS method omitting the clean-up step was published by Sulyok et al. in 2006. Altogether 39 mycotoxins (in addition to common mycotoxins represented by A- and B-trichothecenes, zearalenone, patulin, fumonisins, aflatoxins, ochratoxins, and their metabolites along with the ergot alkaloids, *Alternaria* toxins, enniatins, and moniliformin) potentially occurring in cereals were determined in diluted an acetonitrile:water:acetic acid extract (72). The LC–ESI(+)-MS/MS chromatogram of diluted wheat extract was spiked with a multi-mycotoxin mixture is presented in Fig. 3

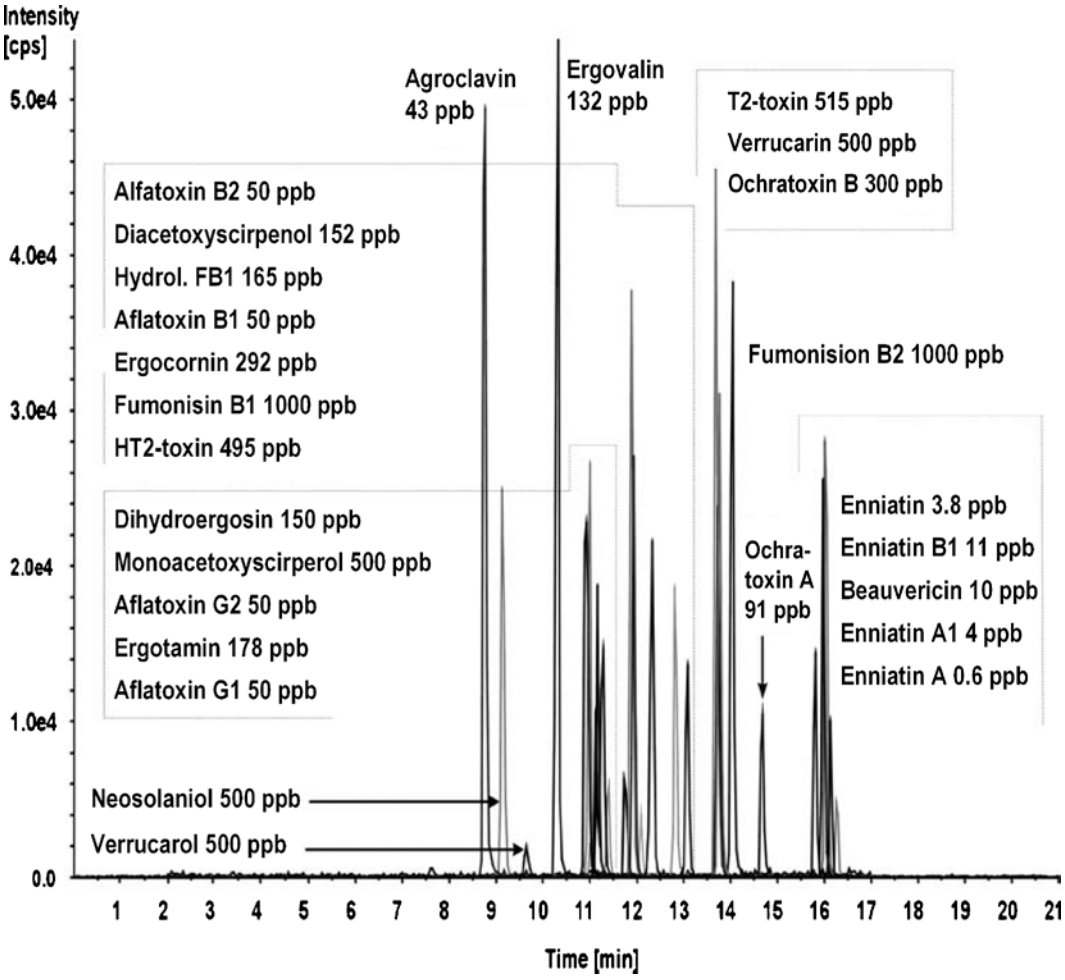


Fig. 3. The LC–ESI(+)-MS/MS total ion current chromatogram (sum of all MRM transitions) of a mixture of mycotoxins. The diluted wheat extract was spiked with a multi-mycotoxin standard and injected directly (reproduced from (73) with permission from Springer).

(73). In the follow-up study, the method was extended up to 87 analytes and fully validated (74).

It is worth to note that the generic sample preparation allows the simultaneous determination of mycotoxins with other food contaminants, including pesticides, plant toxins, and veterinary drugs (75). The detailed characterization of the most interesting multi-mycotoxin methods published in the recent 5 years is summarized in Table 3.

The key limitation of MS/MS-based methods is that due to monitoring only specific mass transitions, neither post acquisition data reprocessing nor screening of unidentified unknowns is possible. In this context, the growing interest in employing high-resolution mass analyzers is not surprising; they represent, indeed, a challenging option in the field of LC–MS mycotoxins analysis: full scan experiments make possible not only non target screening, but also retrospective data mining (35, 81, 82). The mass resolving power of currently marketed instruments with high performance time-of-flight (TOF) analyzers is around 15,000 FWHM (full width in half maximum). In some very recent instruments, the FWHM value might be up to 30,000, and mass accuracies are typically <5 ppm. A mass resolving power as high as 100,000 FWHM and higher is achievable by orbitrap MS technology. Figure 4 illustrates the benefits of high mass resolving power for the discrimination of isobaric interferences. The importance of an optimal choice of the extraction window width is demonstrated here. Employing a wide mass extraction window may result in worsened selectivity, however, too narrow mass extraction window, especially in case of employing lower mass resolving power, may imply the occurrence of false-negative results.

As mentioned above, in LC–MS analysis, the ionization efficiency can be affected by the presence of co-extracted matrix compounds co-eluting with the analyte of interest leading to the signal suppression or enhancement. For compensation of those matrix effects, matrix-matched standards are often used. Other possible strategy to ensure good trueness of generated data is employing of isotopically labeled internal standards. Nowadays, ^{13}C-labeled standards of deoxynivalenol, 3-acetyldeoxynivalenol, zearalenone, HT-2 and T-2 toxin, fumonisin B1 and B2, ochratoxin A, and aflatoxin B1 are commercially available (19, 83).

2.3.4. Ambient Ionization Mass Spectrometry

Another challenging development in the field of MS is associated with introduction of novel ambient desorption ionization techniques, represented mainly by (commercialized) desorption electrospray ionization (DESI) and direct analysis in real time (DART) (84). As in other ambient MS techniques, (chromatographic) sample separation is omitted.

Table 3
Overview of recent LC–MS based methods for multi-mycotoxin analysis with none of minimal sample clean-up

Reference	(74)	(75)	(76)	(77)		(27)	(78)	(79)	(35)	(80)	(81)
Matrices, which is method validated for	Wheat, maize, bread-crumbs	Maize, meet, eggs, milk, honey, horse feed	Peanut slurry, pistachio slurry, wheat slurry, maize slurry, dry-miller cornflakes, raisin slurry, fig slurry	Wheat, maize	Wheat, maize	Wheat, barley, oats	Maize, walnuts, biscuits, breakfast cereals	Maca, soy isoflavones, garlic, black radish, St John's wort, ginko biloba	Wheat, barley, maize	Beer	Beer
Example of LOD (µg/kg) for particular analyte/ matrix combination[a]	Bread-crumbs	Horse feed	Maize slurry	Wheat	Wheat	Wheat	Maize	Maca	Wheat	Beer[b]	Beer[b]
DON	20[c]	>250	50	10	2,000	35	1.1	6	25	0.14	3
HT2	20	50	25	8	100	100	1	1	12.5	0.06	4
T2	2	20	25	1	20	15	0.1	3	5	0.07	2
ZEA	0.4	250	10	4	100	20	1.5	6	5	0.1	1
FB1	8	50	100	35	80	20	0.1	1	10	0.07	n.d.
FB2	7	20	100	30	80	15	0.2	0.3	5	0.09	n.d.
OTA	1	50	1	4	12	10	0.3	1	n.d.	0.02	60
AFB1	0.8	10	0.5	0.5	20	10	0.02	6	n.d.	0.04	2
AFB2	0.7	10	1.0	30	20	10	0.1	6	n.d.	0.05	0.5
AFG1	0.5	10	1.0	1	20	10	0.2	6	n.d.	0.03	3
AFG2	1	20	0.5	10	20	20	0.2	6	n.d.	0.08	2
PAT	100	n.d.	n.d.	800	2,000	15	n.d.	n.d.	n.d.	n.d.	n.d.

Extraction solvent (purification)	MeCN: water: acetic acid	MeCN: water: formic acid	MeCN:water	MeCN: water: acetic acid	MeCN: water: acetic acid	MeCN: water	MeCN: water	Ethyl acetate: formic acid	MeCN: water: formic acid (NaCl, $MgSO_4$)	Sonication (C18 clean-up)	Sonication, (MeCN precipitation)
Total number of targeted mycotoxins	87	23	33	30	32	31	12	23	11	12	32
Run time/no of chromatographic runs necessary for determination	21 min/2	22 min/2	35 min/1	33 min/2	33 min/1	35 min/2	8.5 min/1	25 min/1	18 min/2	6.5 min/1	18 min/1
Matrix equivalent per 1 mL of injected sample/matrix equivalent in the injected volume	0.125/ 0.000625 g	0.125/ 0.000625 g	0.0625/ 0.00125 g	0.25/ 0.00125 g	0.25/ 0.0025 g	5/0.05 g	0.5/ 0.0025 g	5/0.1 g	0.2/0.001 g	5/0.025 mL	1/0.005 mL
Number of steps within the sample preparation[d]/timing estimate of the preparation of 1 sample	2/98 min	1/190 min	2/130 min	1/93 min	1/93 min	3/40 min	1/15 min	5/155 min	3/18 min	2/45 min	3/15 min
Type of MS detection	ESI-MS/MS (Qtrap)	ESI-MS/MS (triple quadrupole)	ESI-MS/MS (triple quadrupole)	ESI-MS/MS (triple quadrupole)	ESI-MS (orbitrap)	ESI-MS/ MS (triple quadrupole)	ESI-MS/ MS (tandem quadrupole)	ESI-MS/MS (triple quadrupole)	ESI-MS (time-of-flight)	MS/MS (tandem quadrupole)	APCI-MS (orbitrap)
MS detector	Qtrap 4000 (Applied Biosystems)	Quatro Premiere XE (Waters)	Quatro Ultima (Waters Micromass)	TSQ Quantum Ultra AM (Thermo-Finnigan)	LTQ Orbitrap (Thermo Scientific)	Micromass Quatro Micro (Waters)	Acquity TQD (Waters)	Micromass Quatro Micro (Waters)	LCT Premier XE (Waters)	Acquity TQD (Waters)	Exactive (Thermo Fisher Scientific)

Abbreviations of analytes: *DON* deoxynivalenol, *HT2* HT-2 toxin, *T2* T-2 toxin, *ZEA* zearalenone, *FB1* fumonisin B1, *FB2* fumonisin B2, *OTA* ochratoxin A, *AFB1* aflatoxin B1, *AFB2* aflatoxin B2, *AFG1* aflatoxin G1, *AFG2* aflatoxin G2, *PAT* patulin

n.d. not determined

[a]Examples of LODs (limits of detection) for selected mycotoxins (mostly regulated, maximum limits established by (EC) No 1881/2006 implemented by (EC) No 1126/2007) in selected matrices

[b]LOD in beer in µg/L

[c]LOD in the solvent standard due to the lack of blank

[d]Operations considered as the sample preparation step: sonication, extraction, dilution, evaporation, liquid–liquid extraction, solid phase extraction, and partitioning

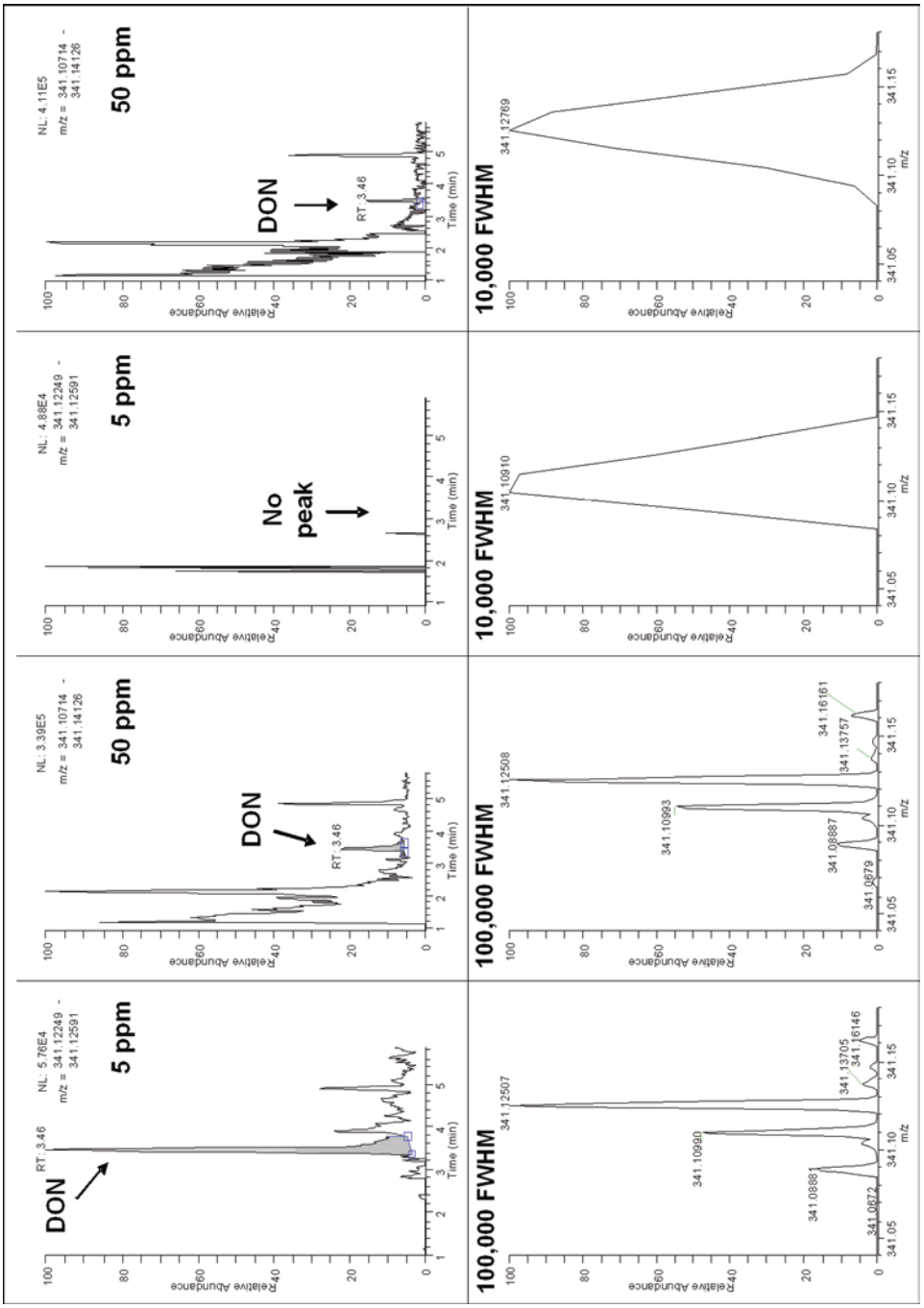


Fig. 4. Extracted ion chromatograms and mass spectra of deoxynivalenol (m/z 341.1242) in beer (10 µg/L) obtained at two different mass resolving power settings of orbitrap MS (10,000 and 100,000 FWHM) and two different mass extraction windows (±5 and ±50 ppm).

In the DESI source, ionization takes place by directing an electrically charged mist to the sample surface. Created ions travel through the atmospheric pressure interface into the mass spectrometer. The only, until now, reported study concerned with application of DESI (ionization resembles ESI) for mycotoxins analysis was focused on fumonisins in maize (85).

DART technology employs excited-state helium atoms to produce reactive species for APCI-like ionization of analytes that occurs in the vapor phase following their thermo-desorption from the sample (86). The first study concerned with analysis of multiple mycotoxins has been reported only recently. Vaclavik et al. demonstrated the potential of DART coupled to ultra high resolving power orbitrap MS to quantify selected trichothecenes, *Alternaria* toxins, zearalenone, and sterigmatocystin in a QuEChERS-based extract prepared from cereals (87). Figure 5 shows the DART–orbitrap MS spectra obtained for particular mycotoxins in wheat extract spiked at a level of 500 μg/kg. The lowest calibration levels (LCLs) ranged from 50 to 150 μg/kg. The method was shown to be applicable for high-throughput control of maximum limits of ZEA and DON established in EU regulation [(EC) 1126/2007] for unprocessed wheat/maize. Improved reproducibility of the measurement was achived by employing of matrix-matched calibration together with isotope dilution-based quantification.

2.3.5. Bio-Analytical Tools

Immunochemical techniques, represented by ELISA, are a widely established technology employed mainly for rapid and sensitive screening of mycotoxins in unprocessed commodities/raw materials. The most common microtitre-plate format has found a place in routine laboratories. It is easy to use, typically, no clean-up or analyte enrichment steps are required. In most cases, the endpoints are colorimetric or fluorimetric, hence only very simple devices are needed to run the assay. Also other formats (some of them portable) of bio-analytical tools have become available during this time; nevertheless, many of the biosensors, immunosensors, and test strips/dipsticks are essentially modifications of the two basic forms of ELISA where either the antigen or the (anti-toxin) antibody is immobilized. In recent years, membrane-based immunoassay methods, such as flow-through immunoassays and lateral flow devices (LFDs) have been introduced into the market. This innovative approach is of growing interest since it allows rapid on-site (pre)-screening. More detailed discussions of advantages and limitations is available in recent reviews (85, 88, 89).

Substantial developments reflecting the demand for multiple mycotoxins measurement have also occurred. Biosensor arrays employing parallel simultaneous assays, physically separated from one another, seem to be a very challenging option (90). The most pertinent for routine applications appear to be those based upon fluorescence or surface plasmon resonance (SPR). The later technique

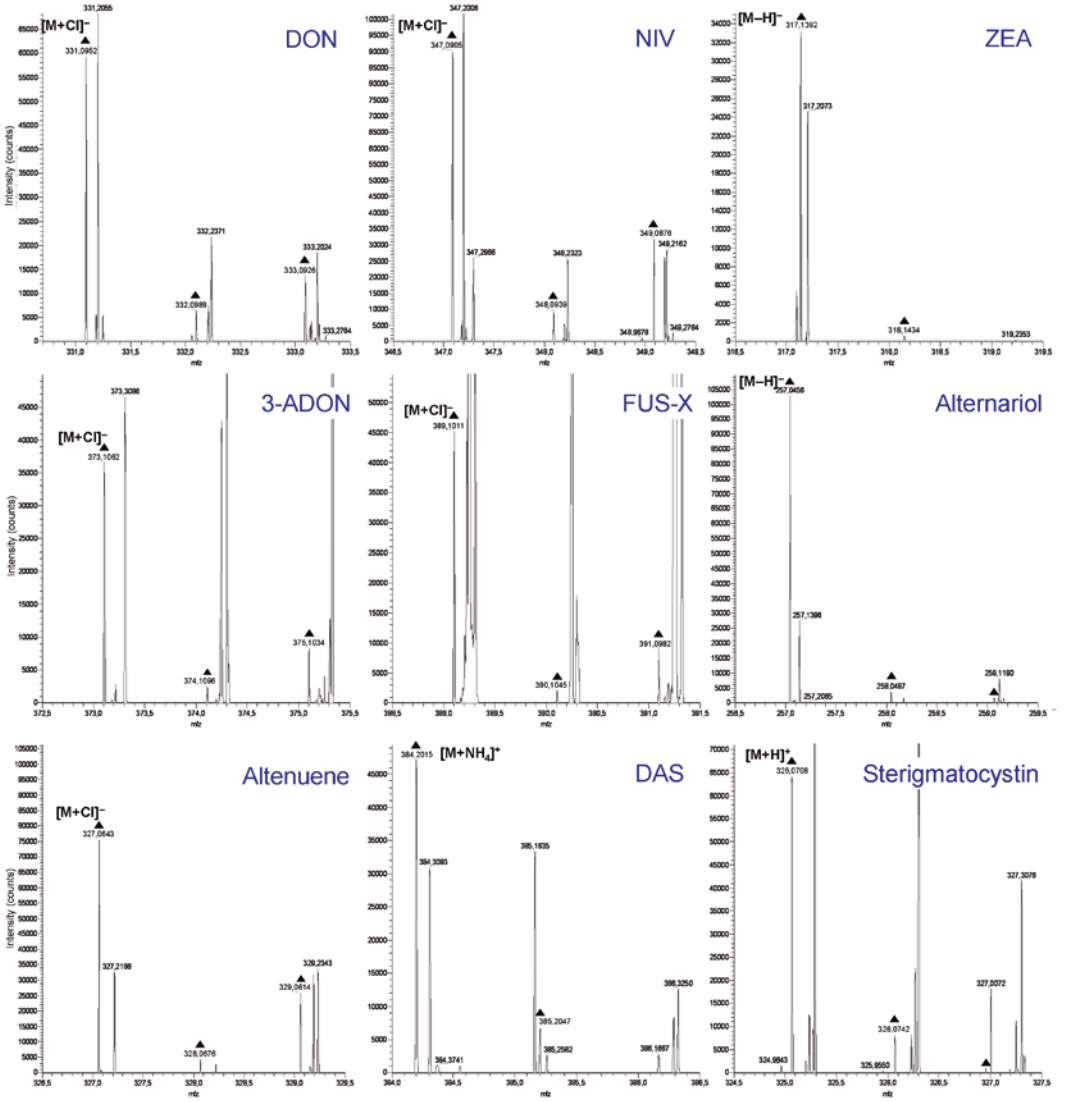


Fig. 5. Mass spectra of examined mycotoxins obtained by DART–orbitrap MS analyses of wheat extract (spike 500 μg/kg) at a mass resolving power setting of 50,000 FWHM. Ions yielded by target analytes (filled triangle) (Analytes abbreviations: DON deoxynivalenol, NIV nivalenol, ZEA zearalenone, 3-ADON 3-acetyl deoxynivalenol, FUS-X fusarenon-X, DAS diacetoxyscirpenol) (Reproduced from (87) with permission from Elsevier).

is based on measuring the impact of mass concentration changes on angle, or intensity, of internally reflected light at metal film liquid interface in respective flow chip where binding/dissociation event between analytes (mycotoxin) and (bio)recognition element (antibodies, molecular imprinted polymers, MIPs) takes place.

Only recently, challenges offered by RNA fingerprinting assay or Luminex xMAP technology (which comprise existing technologies – flow cytometry, microspheres, lasers, digital signal processing, and traditional chemistry) in the analysis of multiple mycotoxins have been addressed in EU funded projects Biocop (91) and Conffidence (92).

3. Notes

Following recommendations (Notes 1–5) should always be considered within multi-mycotoxin analysis:

1. *Blank matrix.* In mycotoxins analysis, for compensation of matrix induced ion suppression/enhancement, matrix-matched standards should be used whenever possible. Although the use of absolutely blank matrix is an ideal solution, unfortunately, obtaining it in practice is hardly achievable (most of wheat-based matrices contain at least traces of DON, similarly for maize-based matrices, presence of fumonisins traces is typical). Higher background mycotoxin concentration tends to increase the analytical bias of the results. Hence, samples with no or very low mycotoxins levels should be kept in the laboratory for analytical purposes (matrix-matched calibration for matrix effects correction, as well as spiking experiments for the recovery assessment).
2. *Internal standards.* As a general rule, internal standard employed for mycotoxins analysis must not be present in the sample, and should combine physiochemical properties chromatographically similar to those of target mycotoxins. Use of internal standards as surrogates (known amount of internal standard added at the beginning of the sample preparation procedure) is recommended for compensation of the analytes losses throughout the analytical procedure. During recent years, the number of internal standards available in mycotoxins analysis has rapidly increased, especially in case of ^{13}C-labeled mycotoxins, which are also often employed for matrix effects correction.
3. *Clean-up.* When immunoaffinity columns are used for purification of sample extract and/or pre-concentration of analytes, exceeding of the column capacity (this information, usually in nanograms of analyte, should be given by column producer) has to be avoided. Breakthrough of analytes may occur when antibodies binding sites are saturated.

4. *LC determinative steps.* For checking the signal stability during the sequence, running of analytical standards at the beginning and the end of each (longer) sequence is recommended. Analyses have to be performed within the linear range. In case of highly contaminated samples possibly exceeding the calibration range, they have to be diluted before the analysis, and the diluting factor has to be included in the results calculation.
5. *Instrument's maintenance.* When a significant decrease in signal of analytes is observed, instrument's maintenance including cleaning of the ion source and ion optic is required. As far as decreasing of the quality of chromatographic data is registered (poor peak shape), replacing of a pre-column or the LC column is recommended. The LC–MS analyses, especially the U-HPLC (ultra-high performance LC with sorbent particles less than 2 μm) should always include filtration of the final extract by a syringe filter (0.22 or 0.45 μm for U-HPLC or HPLC, respectively). This simple procedure significantly prolongs the lifetime of a particular LC column.

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