Mycotoxins detections

MYCOTOXIN DETECTION

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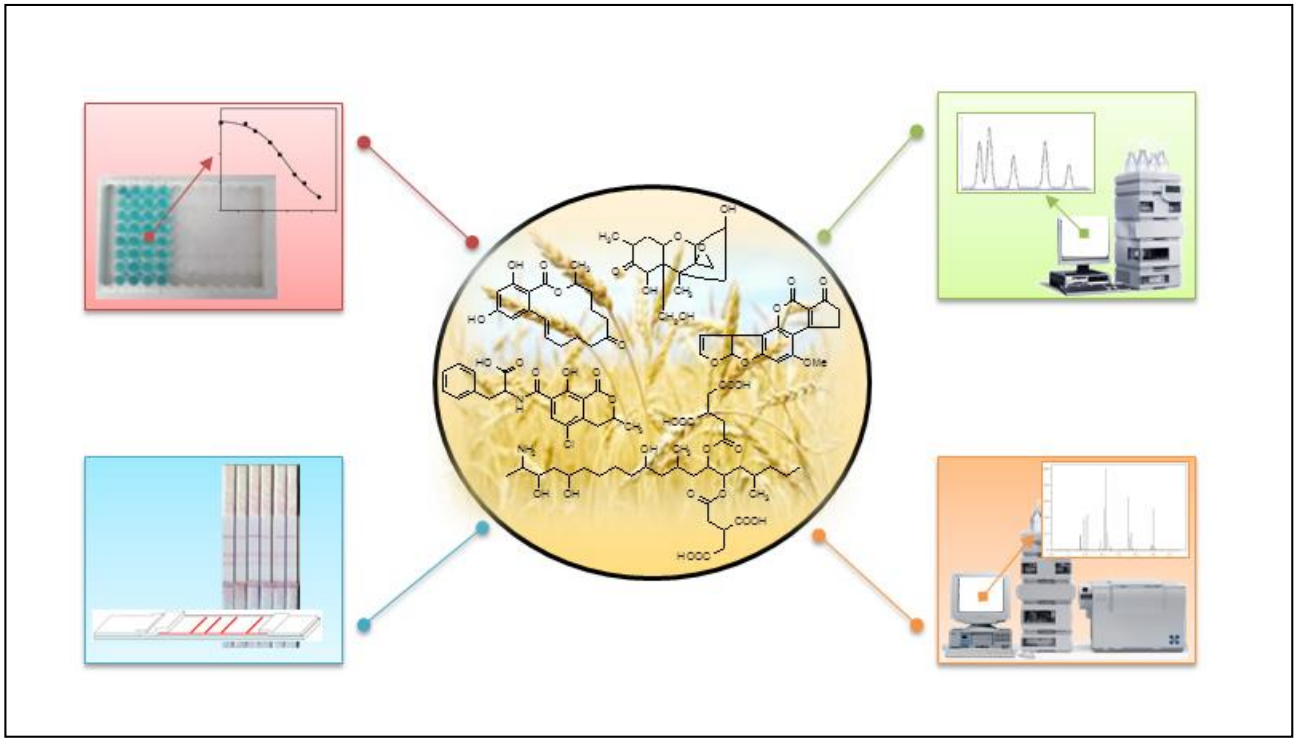
Highlights

- Mycotoxins are toxic metabolites of fungi that contaminate several basic foods
- LC-MS/MS allow simplify analytical strategies and develop multiresidue methods
- Emerging and masked mycotoxins are identified and detected by LC-MS/MS and LC-HRMS
- Immunochromatographic tests provide rapid, cheap, portable, and multiplex analyses
- Exposure to mycotoxins should be assessed by measuring suitable biomarkers



Mycotoxin detection for assuring food safety: (i) rapid, portable, ready-to-use devices; (ii) ELISA-based assays for routinely and extensive controls; (iii) chromatographic-based techniques for accurate determination of known mycotoxins; (iv) liquid chromatography coupled to tandem mass spectrometric detectors for multiresidue analysis and identification of new or modified compounds

Abstract

Mycotoxins are toxic metabolites of certain fungi that growth on a variety of crops, pre-, during and post-harvest. Due to their toxicity, maximum admissible levels of mycotoxins are regulated worldwide and monitoring of their occurrence in several commodities is mandatory for assuring food safety and consumers' health protection.

Analytical methods for mycotoxins include immunochemical-based techniques that principally apply for routinely controls and rapid, on-site detection, and chromatographic-based techniques that provide sensitive, accurate and selective determination of known mycotoxins, besides identification of new or modified compounds through tandem mass spectrometric detectors.

Introduction

Mycotoxins are toxic compounds produced by the metabolism of certain fungi that affect a variety of crops, including commodities largely consumed by humans and animals. Although fungal growth depends on favorable environmental conditions and, therefore, the occurrence of mycotoxins varies among geographical areas, exposure to mycotoxins is a worldwide concern due to the globalization of food trade.

The most prominent mycotoxins are produced by *Aspergillus*, *Fusarium*, *Penicillium,* and *Alternaria* fungi and belong to the classes of: aflatoxins (AFs), ochratoxins (OTA), patulin (PAT), and *fusarium* toxins. *Fusarium* toxins include: tricothecenes (deoxynivalenol, DON, nivalenol, 3-acetyl-DON, 15-acetyl-DON, T-2 toxin, HT-2 toxin and chemically related compounds), fumonisins (FMs), and zearalenone (ZON) and zearalenone derivative (ZONs) s.

The consumption of food contaminated by mycotoxins rarely determines acute toxicity; however severe chronic effects have been demonstrated for several of them, including mutagenicity, induction of hormonal, gastrointestinal or kidney disorders, and immunosuppression. Most mycotoxins are suspected human carcinogenic agents, such as FMs, OTA , and AFs and their tumor-inducing activity has been confirmed in experimental animals. Instead, aflatoxin B1 (AFB1)has proven to be a potent human carcinogen, and has been classified as the strongest hepatocarcinogenic agent known [1].

Mycotoxins can be produced during the growth and storage of crops and are chemically and thermally stable, thus they are carried over into processed foods. Furthermore, they can enter the food chain through animals fed by contaminated feed, as, for example, is the case of AFB1 and its metabolite (aflatoxin M1), which are transferred into milk by dairy cattle exposed to AFB1.

Despite the risk poses for human health by mycotoxins, it is impossible to impose a total ban for these contaminants because mycotoxins occur naturally; however, maximum admissible levels have been established worldwide for most prevalent and toxic members of the group in certain commodities, which

are more prone to fungal proliferation and represent a source of repeated exposure (Table 1). Consumers protection is also pursued through keeping mycotoxin levels as low as reasonably achieved following good agricultural, storage and processing practices. Regulated mycotoxins and commodities, and maximum admissible levels vary significantly in different countries (Table 1, European Mycotoxin Awareness network; URL: http://services.leatherheadfood.com/eman/FactSheet.aspx?ID=79). However, the request for analytical methods to assess compliance to regulations and to monitor the occurrence of such contaminants in food and feed is a worldwide priority.

A sketch on the geographical provenience of the literature concerning both the development of new analytical strategies and the conduction of survey studies testifies a worldwide interest in mycotoxin detection topic. Likewise, the timely distribution of devoted papers attests a constantly growing scientific production in the last decade. Interestingly, though, also profiting from technical advance and availability of new analytical platforms, the scenario of mycotoxin detection is right now changing: in the last few years, the gold standard has been the availability of sensitive, rapid, cheap and easy-to-operate analytical tools to permit diffuse and continuous monitoring of these hazardous substances to assure safety of food and feed; therefore the development of so-defined screening methods was predominant. However, recent findings highlighted new concerns to be addressed. Primarily, co-occurrence of several toxins has been assessed because different metabolites are produced by the same fungus or because different fungi can affect the same crop [2], and possible additional risks for consumers' health have been suggested due to synergistic effects. The number of newly identified mycotoxins is growing day by day [3-4]; the so-called "emerging mycotoxins" have unknown toxicity and demand for dedicated analytical methods to be developed. Plant metabolism can intrude and produce modified compounds (masked or hidden mycotoxins) whose fate over human or animal metabolism has so far not been investigated. Masked mycotoxin determination requires rethinking the whole analytical procedure, because modified compounds are often not-extractable by the extraction media employed for their parent compounds [5-9]). Food and feed matrices potentially involved in mycotoxin contamination are exponentially increasing, each bringing its specific interference in the analytical protocols.

The combination of the above mentioned issues is shifting the objective of mycotoxin detection towards multi-target methods, which prevalently exploit advanced instrumental techniques for assuring selectivity, sensitivity and also permit the identification of non-target compounds.

Traditionally analytical approaches for determining mycotoxins have been divided into two categories: reference methods for quantitative analysis and rapid methods for first-level screening of numerous samples. Although convenient, this classification is outdated and a more general classification based on the analytical technique would be adopted in this review.

Independently from the detection technique employed, the analytical workflow implies 5-7 common steps; however the time consumed for each step varies significantly (Figure 1).

Methods based on chromatographic techniques

Methods belonging to this group are aimed at quantitatively determine mycotoxins and involve liquid chromatography (LC) or gas chromatography (GC) coupled to ultraviolet (UV), fluorescence (FLD) or mass spectrometric (MS) detection. The use of sophisticated instrumental configuration combined to extensive sample preparation allows the largest range of mycotoxins to be determined with the highest sensitivity.

Chromatographic techniques coupled to UV and FLD detection are mainly devoted to confirmatory analyses, i.e.: confirm or not the non-compliancy to regulations previously assessed by a screening test. Methods are developed for a single compound or for few related chemicals usually belonging to the same class of mycotoxins. Occasionally, they serve as the reference method to validate immunochemical-based tests. Typically, covering all regulated mycotoxins for all regulated commodities require tens of protocols. Otherwise, instruments equipped with tandem mass spectrometry (MS/MS) detectors allow modifying the analytical strategy in mycotoxin determination and to respond to most analytical challenges above discussed (Figure 2). Mass spectrometry indisputable advantages (including high sensitivity, selectivity, accuracy, and throughput) make it the technique of choice for multiresidue analysis [10-13]. Furthermore, thanks to the inherent selectivity achieved by MS/MS detectors, extraction protocols with limited or no sample clean-up could be successfully developed. The QuEChERS (quick, easy, cheap, effective, rugged and safe) sample preparation approach applied in this context strongly simplified analytical procedures and, mostly, allowed the simultaneous extraction of impressive numbers of mycotoxins, even belonging to very different classes [4,9, 14-16]. However, usual QuEChERS protocols, being based on compromises between optimal extraction conditions for very different chemicals are inherently inefficient and reduce the sensitivity of the analytical method. Therefore, dedicated procedures including pre-concentration steps were employed, when ultrahigh sensitivity is mandatory (such as, for example, in the determination of AFs and OTA at levels required by EU regulations on baby food) [17]. Alternatively, the ultrahigh sensitivity achieved by the isotope dilution quantification method permitted compensating for low extraction rates and directly measuring without pre-concentration [14,16,18]. High-resolution MS (HRMS) and tandem MS detectors provide structure information and possible identification of unknown compounds. Coupling non selective extraction protocols and mass screening through HRMS or MS/MS allowed identification of new masked mycotoxins and of new members of the group [9,19]. Rapid and multiresidue LC-MS/MS methods have been applied to assess mycotoxin occurrence in food and feed [4,9,11,15,20-23]. Most authors confirmed that regulated mycotoxins are frequently recovered at levels suggesting health implications and emphasized the needs for further surveys. GC- MS(/MS) applications are almost exclusively confined to *Fusarium* toxins and patulin detection [13-14, 23].

Immunochemical-based methods

Due to simplicity and cheapness coupled to sensitivity and selectivity, immunoassays are preferably employed for the first level screening and survey studies on mycotoxin contamination. ELISA-based kits are commercially available for all regulated mycotoxins and provide the most used analytical tool for

assuring food safety through the food chain [24]. Besides, immunochemical-based tests in diverse formats are continuously developed with the aim of providing rapid, portable and easy to operate systems [25-27]. Among these, the immunochromatographic test (ICT) technology plays the lead role and has been widely applied for the visual yes/no detection of mycotoxins and for their semi-quantification [28-31]. Strategies aimed at dealing with the intrinsic lack of sensitivity of this tests compared to traditional immunoassays have been reported, based on signal enhancement or combining the use of highly luminescent probes (quantum dots) [32-34]. Several biosensors that exploit the selectivity and affinity of antibodies coupled to disparate sensing devices have been described for most prevalent mycotoxins, which interest is currently limited to the research field [33-35]. In addition, biosensors based on synthetic ligands aimed at mimicking the binding capability of natural antibodies have been described [36-39].

Nonetheless, the immunochemical-based methods seems to suffer a potential limitation in the new scenario of mycotoxin investigation due to the extreme selectivity of the molecular recognition mechanism, which hamper the simultaneous determination of different compounds and the detection of unknown toxins as well of modified structures produced by plant metabolism (Figure 2). Strategies to face these emerging threats include designing analytical platform in the array format, in which several targets are separately detected in spatially defined zones [40-41]. The ICT approach is particularly suited for the purpose, because it is exactly conceived as a strip along which the sample flows and encounters diverse bio-reagents in different spatially confined zones. Indeed, multiplex ICT strips have been reported, in which up to 10 different mycotoxins could be detected simultaneously [344-45]. Alternatively, multi-target analysis could be achieved by using encoded signal reporters that responded differently to the presence of the target (i.e.: emitted fluorescence at different wavelengths) thus allowing the selective detection of each target based on the observed response [46-48].

Notwithstanding, immunochemical methods in the standard ELISA-based formats allow conducting large and frequent surveys, thus apply for monitoring mycotoxin occurrence and for assuring food safety also in developing countries. Furthermore, fungal and mycotoxin contaminations are expected to rise in the next years due to global changes of environment and climate [49]; therefore management of risks demands for routinely and efficient control programs to be carried out, which at the state-of-the art are assured by immunoassays.

Conclusions

Advances in mycotoxin analysis are highlighting current limitations in the comprehension of the effective impact on animal and human health due to their occurrence in foods. Especially, the demonstration of the co-occurrence of several toxic compounds in the same commodity and the identification of new compounds in the family of mycotoxins require new and dedicated toxicological investigations.

Moreover, international regulations are very variables (Table 1) and the connection between maximum tolerable limits and risk associated to the consumption of contaminated food is sometimes vague or based on precautionary estimations (such as for example, European limits for baby and infant foods). Therefore, the availability of effective exposure data could support in deciding more realistic maximum admissible levels for those contaminants In this context, analytical protocols aimed at the identification and measurement of specific biomarkers in biological fluids are increasingly made available [50-54].

A further hint that should deserve greater attention regards the exploration of mycotoxin diffusion in foods and beverage not included in the list of regulated commodities. Indeed, some authors investigated mycotoxin occurrence in medicinal plants and found alarming levels for principal mycotoxins [55]. The high-level of contamination found could be reasonably expected for commodities that undergo long storage in non-controlled conditions, even though these results should be brought more to the attention of consumers and authorities.

Likewise, foods derived from crops liable to fungal growth and from animals fed with contaminated feed have been clearly demonstrated to convey mycotoxins. Several regulations, primarily the one established by the European Union, partially recognized the risk of spreading mycotoxin contamination through the food chain. Nevertheless, recently, the occurrence of these hazardous substances has been reported for further derived foods [56-58] and, likely, the list of suspect food and beverage would be lengthening as a function of the availability of devoted analytical protocols.

Finally, the number of emerging mycotoxins and modified compounds in the family (not only produced by plant but also by microbial metabolism) is destined to increase together with the analytical advances.

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   Adopting a compromise extraction strategy combined to the use of the high selective and sensitive LC-MS/MS technique, Malachova et al. achieved the impressive performance of simultaneously detecting up to 295 contaminants in food, including all regulated mycotoxins, and several emerging and masked mycotoxins

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   Masked mycotoxins result undetectable by usual analytical methods causing underestimation of total content. Undetectability could depend on the analytical method (i.e.: according to selectivity of bio-reagents, immunochemical-based method can also detect masked mycotoxins) or on the extraction protocol. Since masked mycotoxins are more polar than parent compounds, most extraction media are unable to extract them

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Authors developed an ICT device that comprehended three test lines, each responsive for a different class of mycotoxins (AFs, DON and its analogues, and ZON and its analogues). The overall system was able to simultaneously detect at least 10 different toxins; among these 6 were regulated toxins. The ICT device was conceived as a visual test and was sensitive enough to be used for discriminate sample compliance to current regulation. Semi-quantitative detection was also demonstrated by recording line intensity, which was related to mycotoxin concentration in samples.

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The proposed method was based on using four batches of polystyrene microspheres, each functionalized with a different bio-reagent (specific for a determined mycotoxin) that were distinguishable by the detecting system because characterized by unique spectral address. Microspheres were added as a suspension to the liquid sample and reacted in a usual competitive assay. Authors demonstrated the feasibility of the approach by optimizing the simultaneous sensitive detection of AFB1, DON, ZON and T-2 toxin in peanuts.

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The investigation of the presence of the parent mycotoxin and its metabolites in urine from 32 volunteers showed that consumers are exposed to mycotoxins. DON derivatives were found in 90% samples, while the parent compound was detected in 60% samples. In addition, OTA was present in 70% samples. However, citrinin was detected in 90% samples. No admissible levels have been set for citrinin yet, though it is produced by the same fungi as OTA and is nephrotoxic as the regulated OTA. These findings highlight the need of suitable biomarkers to assess human exposure to mycotoxins.

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Figures

Figure 1. Most common sequence of analytical steps and estimated time of accomplishment for chromatographic-based and immunochemical-based methods in mycotoxin detection.

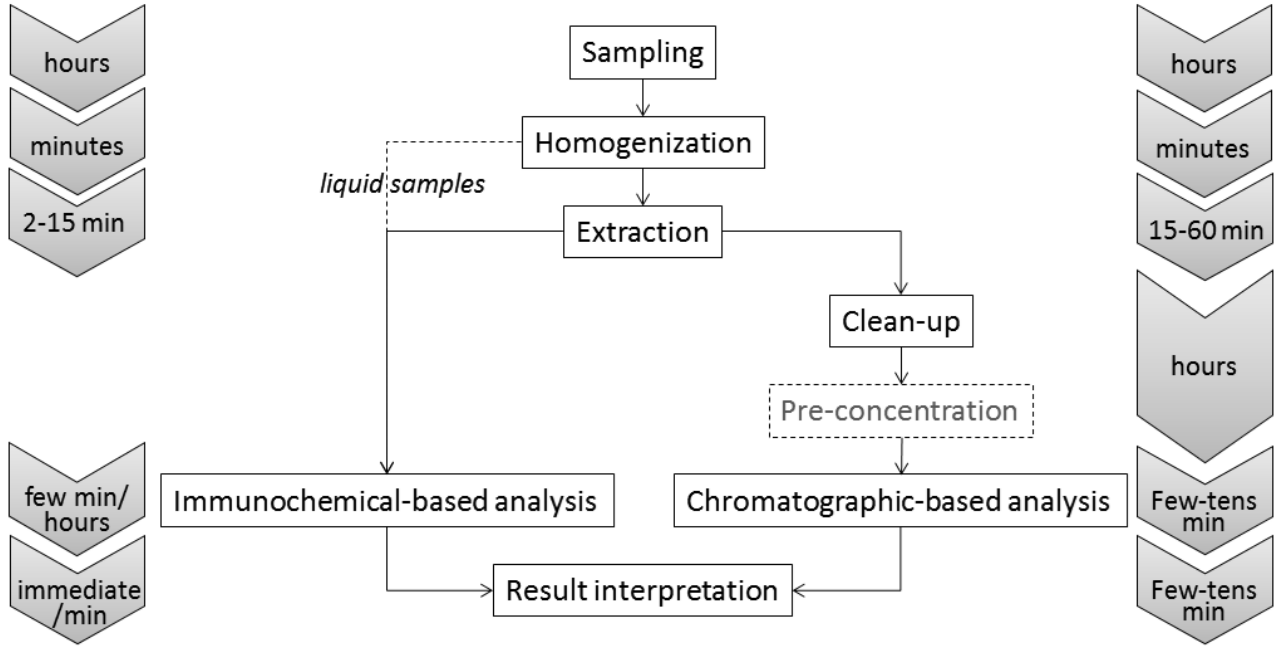

Figure 2. SWOT analysis for comparing chromatographic-based and immunochemical-based methods for mycotoxin detection

Chromatographic-based methods		Immunochemical-based methods
• Validation (in compliance to regulations) • Allows compound identification and structural elucidation of unknown • Multi-target	**Strength**	• Limited sample treatment • Simple, cheap, portable • Managing of large number of samples
• Expensive • Sophisticated (skilled personnel is required for operating and interpreting results) • Operated in laboratory	**Weakness**	• Excessively selective • Long time needed for the development (to obtain bioreagents, mainly antibodies)
• Simplified (QuEChERS) sample preparation for high-throughput and multiresidue analysis • Biomarkers in biological fluids	**Opportunities**	• Provide up-to-date information on occurrence • Provide epidemiologic data
• Emerging mycotoxins • Masked mycotoxins	**Threats**	• New matrices • Multiplex analysis

Tables

Table 1. Overview of the worldwide legislation on mycotoxins

Mycotoxin	Commodity	Country	Maximum Tolerable Levels[a] (µg/kg)
AFs	Oil seeds, nuts, dried fruits, cereals, spices	EU	4-15[a] (2-12[a] for AFB1)
		Australia, Canada, GCC, Nigeria, New Zeland, South Africa	(15 for AFB1)
		USA, Brazil, MERCOSUL	20
		India	30
AFM1	Milk and infant formula	EU, Turkey, South Africa	0.25-0.05[a]
		Argentina, China, GCC, India, Kenya, Mexico, Uruguay, USA	0.5
		Brazil, MERCOSUL	0.5-5[a]
DON	Cereals, bakery products	EU	500-1750[a]
		Brazil	750-3000[a]
		Russia	700-1000
		Canada, China, India, Japan, USA[b]	1000
FMs	Maize	EU, Turkey, Norway, Switzerland	800-4000[a]
		USA[b]	2000-4000[a]
		Brazil	2000-5000[a]
OTA	Cereals, dried fruits, coffee, cocoa, wine, beer, grape juice,	EU, Egypt	2-10[a]
		China, GCC, Kenya, Nigeria, Russia	5

	spices, liquorice, blood products	India Brazil Uruguay	20 2-30[a] 50
Patulin	Fruit juice, apple products	Brazil, China, EU, GCC, India, Japan, Kenya, Nigeria, Russia, South Africa, USA	50
T-2 and HT-2	cereals	EU Russia	Not permitted 50-100[a]
ZON	Cereals, bakery products, maize oil	EU Brazil China, Russia, Chile	75-400[a] 200-1000[a] 200,000

[a] depends on the commodity (lowest-highest MRL)

[b] advisory level