Mycotoxins in food and feed: Extraction, analysis and emerging technologies for rapid and on-field detection

(Article begins on next page)

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ABSTRACT

Mycotoxins are toxic compounds produced by various moulds, which can affect a variety of crops. Due to their high toxicity and wide diffusion, mycotoxins constitute a severe risk for human health, therefore maximum tolerance levels in food and feed products have been set up all over the world and analytical controls are mandatory for many commodities. Despite validated analytical methods for assessing mycotoxin contamination already existing, a number of papers describing new methods of extraction, identification or measurement appear daily in literature. Nevertheless, the extraction and determination of such contaminants in food and feed is a topic of constant and increasing interest, as also attested by the number of related patents which have been applied for in the last few years.

Scientific papers dealing with mycotoxin occurrence, potential risk and determination have recently been reviewed. Nevertheless, the objective of this review is to focus on patent activity rather than on the scientific breakthroughs on this subject. Therefore, the most recent patents regarding the whole analytical protocol to measure mycotoxins in food, starting from 2006 to date are presented and discussed. The possibility of a technology transfer for the various innovations presented is also discussed as are future developments in the field.

SHORT RUNNING TITLE

Rapid detection of mycotoxins in food and feed

INTRODUCTION
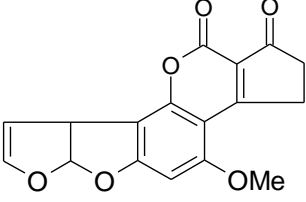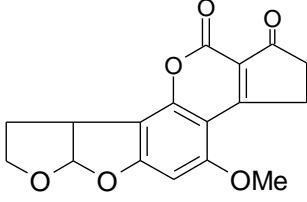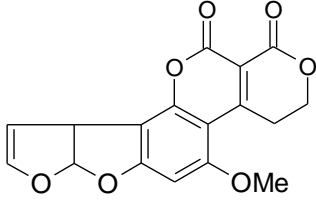
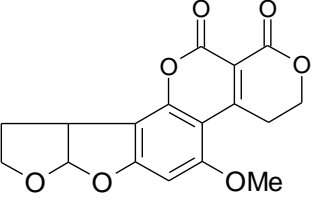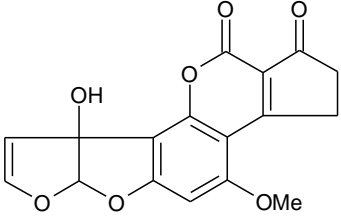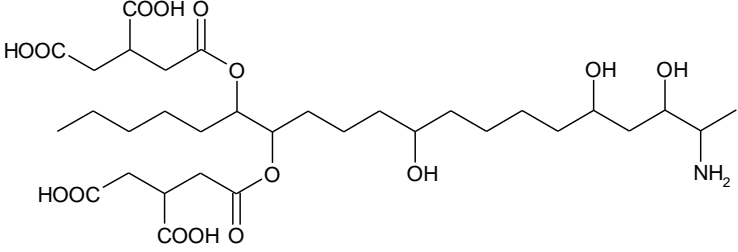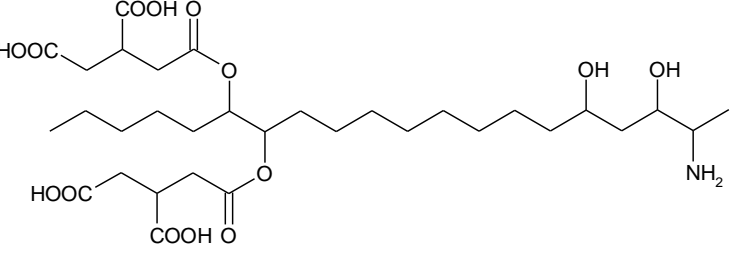Mycotoxins are naturally occurring secondary metabolites produced by certain species of moulds which develop at high temperatures and humidity levels and may be present in a large number of foods. The mould may occur on the growing crop or after harvesting during storage or processing. Toxins include a number of heterogeneous compounds of varying toxicity and frequency in food. There are currently more than 400 mycotoxins known. Among them, there are six major classes of mycotoxins that frequently occur: aflatoxins, trichothecenes, fumonisins, zearalenone, ochratoxin and patulin Table **1**.
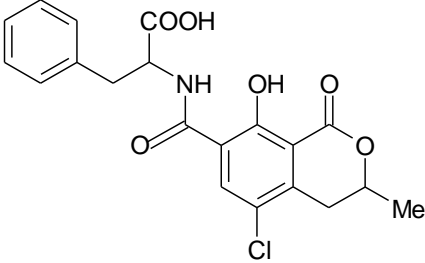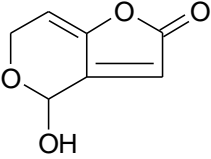
Mycotoxins differ in structure, which explains the great variation of symptoms. The main toxic effects are carcinogenicity, genotoxicity, nephrotoxicity, hepatotoxicity, oestrogenicity, reproductive disorders, immunosuppression or dermal effects. Each plant can be affected by more than one fungus and each of them can produce more than one mycotoxin. Consequently, there is a great probability that many mycotoxins are present in one feed, thus increasing the odds of interaction between mycotoxins and the occurrence of synergistic effects, which are of great concern to livestock health and productivity.
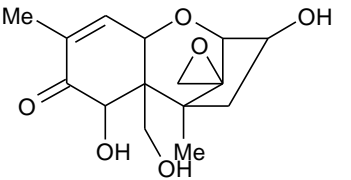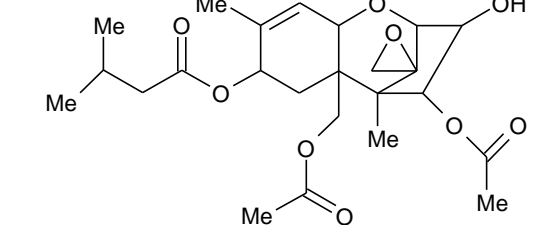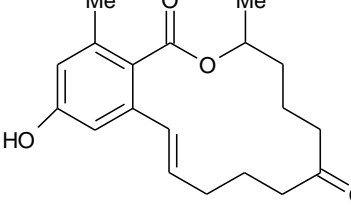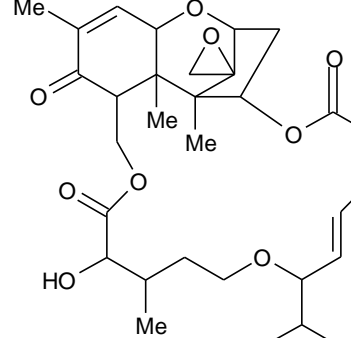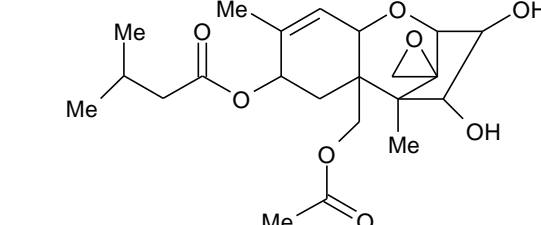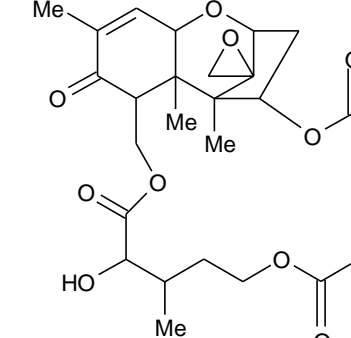
Food and feed involved in mycotoxin contamination include both raw materials and processed feed and foodstuffs, because mycotoxins are chemically stable under conditions used during the usual food and feed manufacturing processes. In addition, they are resistant to high temperatures and long-term storage.

Table 1. Chemical structure of major mycotoxins and raw material particularly involved in their determination.

Mycotoxin class	Chemical formula of major compounds

| Aflatoxins, AFs |
Aflatoxin B1, AF B1 Aflatoxin B2, AF B2
  Aflatoxin G1, AF G1 Aflatoxin G2, AF G2 |
| |
Aflatoxin M1, AF M1 |
| Fumonisins |
Fumonisin B1, FmB1
Fumonisin B2, FmB2 |

| Ochratoxins | Ochratoxin A, OTA |
| Patulin | Patulin |

Tricothecenes

Deoxynivalenol, DON

T2 toxin, T2

Roridin A

HT2 toxin, HT2

Verrucarin A |
| Zearalenones |

Zearalenone, ZON |

Due to all these reasons, mycotoxin contamination is one of the major concerns in agricultural and food analysis, as demonstrated by the fact that, as in previous years, 2008's mycotoxins represent the hazard category with the highest number of notifications received by the Rapid Alert System for Food and Feed (RASFF) of the European Union [1]. In addition, according to the FAO (Food and Agriculture Organization) 25% of the world's crop harvests are contaminated with mycotoxins [2]. The mycotoxin contamination of crops not only poses a severe risk for animal and human health, but also involves economic losses at all levels of food and feed production, including crop and animal production, processing and distribution [3].

Among the six major classes of mycotoxins aflatoxins still cover a prevalent role in terms of incidence in contaminated materials [1]. However, depending on the climatic conditions, widespread contamination of other mycotoxins could occur year by year [4-6].

Due to their toxicity, mycotoxin legal limits for mycotoxins in crops intended for human or animal consumption have been established in various countries worldwide; figures which strongly varied depending on the mycotoxin and on the country involved Table 2, [7-13]. The European Union has established a comprehensive regulatory framework, which is constantly being updated [10] and particularly strict limits have been set up for baby food [9].

Table 2. Range of legal limits for major mycotoxins in various countries: values vary depending on the commodities and from raw materials to processed food.

Mycotoxin	Toxic effects	potentially contaminated crops and commodities	European MRL ($\mu g\ kg^{-1}$) [a]	USA and Canada MRL ($\mu g\ kg^{-1}$) [b]
Aflatoxin B1	Hepatitis, nephritis, carcinogenesis, genotoxicity	Cereals, oilseeds, spices, fresh and dried fruit, cotton, nuts	2.0 – 8.0	
Total aflatoxin (sum of B1+B2+G1+G2)		Cereals, oilseeds, spices, fresh and dried fruit, cotton, nuts	4.0 – 15.0	15 - 300
Aflatoxin M1		Milk	0.05	0.5
Ochratoxin A	Hepatitis, nephritis, neurotoxicity,	Cereals, spices, cocoa, coffee, dried	2.0 – 10.0	200 – 2,000

	genotoxicity	vine fruit, wine, pork and chicken meat		
Patulin		Apple	10 - 50	
Tricothecenes	Gastroenteritis, intestinal haemorrhage, immunosuppression, dermatotoxicity	Cereals	DON: 200 – 1,750 [c]	DON: 1000 - 10,000 T-2: 25-100
Zearalenone	Oestrogenic action, hypofertility	Cereals, maize oil	50 – 400	1,000 – 10,000
Fumonisins (sum of B1+B2)	Carcinogenesis, neurotoxicity	Maize	200 – 4,000	2,000 – 4,000

[a] from references 7-10

[b] from references 11-12

[c] till now, legal limits have been set only for DON

Several methods for mycotoxin determination have been developed (for an overview of analytical methods for mycotoxins and associated literature see: http://www.mycotoxins.org/ and Krska *et al*. [14]). A lot of validated analytical methods already exist [15], however, a large number of papers are published daily on new methods for measuring such contaminants.

Generally speaking, analytical methods for mycotoxins can be divided into two major categories: screening methods and instrumental methods. The first category includes methods which are rapid, require limited or no sample treatment and no skilled personnel. In addition, they should be economical and truly applicable in crop fields or use very simple and cheap instruments elsewhere. A non-exhaustive list of such methods includes: thin layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA), flow through membrane based immunoassay; immunochromatographic assay (LFIA); and fluorescence polarization method (FPIA) [15-17].

As regards instrumental methods (which are usually employed to confirm positive samples resulting from the screening methods), these are based on liquid chromatography coupled with a variety of

detectors (also depending on the chemical-physical properties of the analyte): UV-visible, fluorescence, mass spectrometry, and tandem mass spectrometry [14, 18-19].

The analytical protocol includes various steps: sample collection, extraction, clean-up and/or pre-concentration, and, lastly quantification by means of the screening or instrumental method (Fig 1).
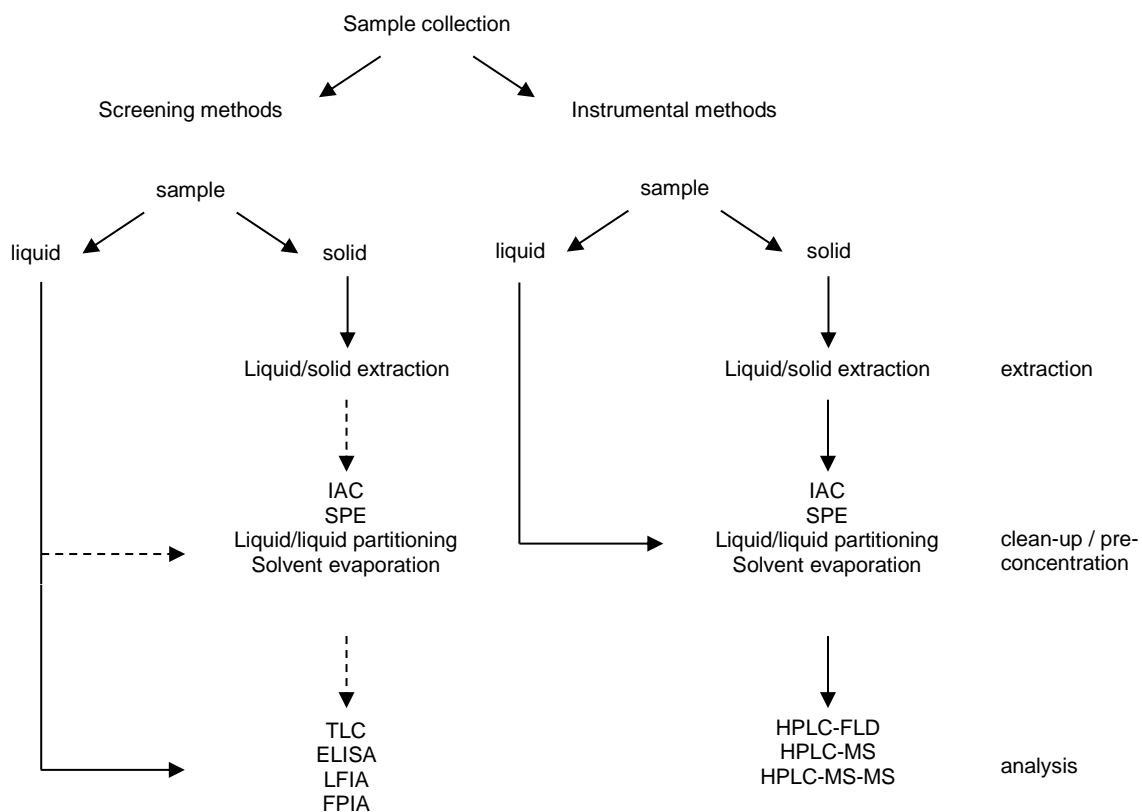
Fig (1). Analytical protocol for the measurements of mycotoxins in food and feed

The collection of representative samples is a critical aspect as attested by specific regulations [20] New issues regarding all the above cited steps appear day after day in literature (Figure 2) and in the form of patent applications. The main goals pursued are: an increase in the speed of analysis; the simplification of the protocol (reduction in the number of steps, in the need for instrumentation and experienced staff), the reduction in costs, the opportunity to complete the whole analytical protocol in the field or in situ. Therefore, research and patent activity are focused on three main developments which refer to: preparation of new materials for sample extraction and clean-up, design of portable and non-instrumental devices, strategies for obtaining multi-residue analysis.

Recent patents will be discussed and divided into two major groups depending on the step of the protocol which is referred to: sample extraction and clean-up or analyte detection.
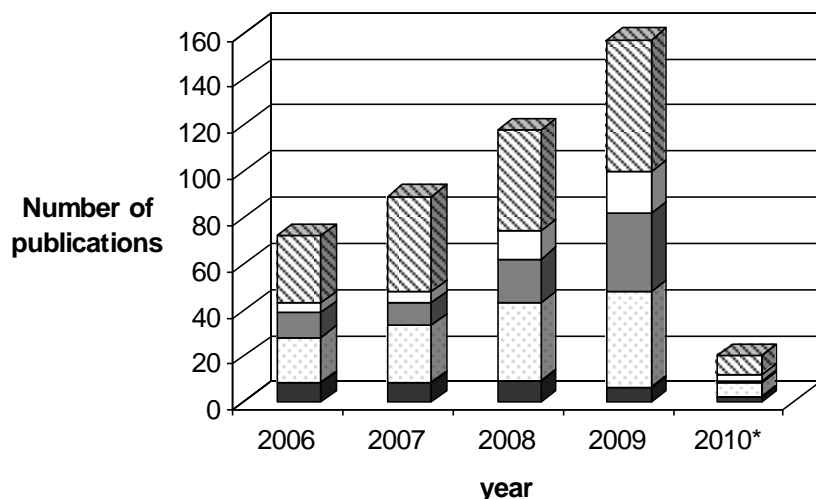
*Data collected in two months (January-February)

Fig (**1**). Number of scientific publications about new analytical methods for mycotoxins as a function of time: reviews and interlaboratory studies (black), instrumental and confirmatory methods of analysis (dotted), screening methods and sensors (grey), extraction and clean-up (white), survey and occurrence (dashed). Source: ISI web of Science.

EXTRACTION OF MYCOTOXIN FROM SAMPLES

Matrices involved in the mycotoxin analysis can be summarized as food and feed, which actually means a large number of very different materials Table 2. Moreover, not only raw materials, but also finished products passing through the various stages of processing should be checked for mycotoxin contamination. Therefore, the complexity and variability of the matrix is the initial and indeed major drawback in mycotoxin analysis. Most matrices are solid, non-homogeneous, composites. They usually contain all classes of potentially interfering substances: salts, proteins, lipids, sugars, coloured substances, acids,... In conclusions, the extraction and clean-up of the analyte from the matrix is the key-step of the entire analytical protocol, whatever method of analysis is to follow. However, when screening methods are employed, very simple and fast pre-treatment of the sample is strictly required. The use of non-hazardous chemicals is, in addition, preferable.

The simplest strategy to extract mycotoxin from food and feed involves a solid-liquid extraction, usually employing organic solvents such as methanol, acetonitrile (mixed at different levels with water) or dichloromethane [14-15,19,21], followed by various clean-up steps. According to the

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nature of the interferences, liquid-liquid partitioning [22,23], centrifugation / filtration [24,25], or solid-phase extraction are performed. Often, more than one of the above mentioned procedures are combined in order to remove matrix interference and, at the same time, pre-concentrate the analyte. This last goal is particularly important when some mycotoxins, such as aflatoxins and ochratoxin A, should be measured at very low concentrations (ppt-ppb) and/or in processed food.

Simple and rapid sample treatments are preferred for screening purposes, whereas instrumental methods usually require some clean-up and enrichment of the analyte. Solid-phase extraction using cartridges is the most widely employed strategy; the absorbent material could be, in order of selectivity: a reverse or an ion exchange phase [23, 26, 27], a molecularly imprinted polymer [28-31] or an immuno-reactive absorbent [25, 32-34].

The molecular imprinting technique consists of a radical co-polymerization of a mixture of monomers and cross-linking agents in present of a template molecule able to establish interactions with these monomers. The removal of the template leaves nanocavities in the polymeric structure, which have the same features of antibody binding sites (binding reversibility, specificity, high affinity constants). Due to these properties, they are suited as selective sorbents in solid-phase extraction, allowing selective clean-up of compounds prior to analysis. In recent years, molecular imprinted polymers (MIPs) have been used as sorbents in solid-phase extraction (MISPE, molecular imprinted solid-phase extraction) for various compounds. Generally speaking, the extraction protocol is based on three distinct steps: (1) column loading, in which analytes are retained and other substances with similar polarity can be also retained; (2) column washing, in which analytes are retained (because of the molecular recognition effect) whereas other substances that may have been retained during loading are eluted; (3) column elution, in which the analytes are recovered.MIPs able to selectively bind some mycotoxins have been described (for an exhaustive discussion regarding MIPs for mycotoxins see Baggiani *et al*. [31]). However, no commercially available columns based on MIP technology are currently available. Compared to immunoaffinity columns, MIPs have the advantages of limited costs and possibility of regeneration. On the other hand, lower performances such as pre-concentration factors and clean-up can be achieved.

A number of immunoaffinity columns (IACs) are commercially available. The analyte molecules (i.e. the mycotoxin) are bound selectively and highly efficiently to an antibody immobilized on the column. As matrix components do not interact with the antibody and the analyte is bound with high affinity, large loading of diluted solutions and rinsing steps aimed at removing most of the possible interferences can be carried out. The toxin is finally eluted by antibody denaturation, which is usually obtained by employing limited volumes of some organic solvent. In addition, ten to fifty fold pre-concentration of the analyte can be obtained.

Single analyte columns include those for major mycotoxins. Multifunctional columns for the simultaneous determination of two mycotoxins at the same time are becoming available. The fact that columns can only be used once and their relatively high costs are major disadvantages. Adsorbent media belonging to both categories (synthetic ligands and antibodies) have been recently patented Table 3.

Table 3. Patents concerning mycotoxin extraction

Inventors Patent n.	Title	Method	Mycotoxin concerned	Result	Tested matrices
Piletsky S. Piletsky O. Coker R. WO08125887	Solid phase extraction of ochratoxins	Preparation of a polymer containing amido or aminoalkyl moieties and acidic moieties.	OTA	The polymer can be used for clean-up purposes or direct detection in a fluorimeter.	Maize, peanuts
Coker R. Piletsky S. Piletsky O. WO08096179	Solid phase extraction of aflatoxins	Preparation of a polymer containing amido or aminoalkyl moieties and acidic moieties.	AF B1, B2, G1 and G2	The polymer can be used for clean-up purposes or direct detection in a fluorimeter.	Maize, peanuts
Tozzi C. Ferroglio C. Giraudi G. Anfossi L. Baggiani C. WO07072212	Synthetic ligands able to bind ochratoxin A and uses thereof	Adsorbent prepared with short synthetic peptides.	OTA	Clean-up of matrix interference from wine with performance comparable to those of IACs.	Wine
Zabe N. Basker C.J. US20070117218A1	AFOZ multi-analyte affinity column	Combination of proper amounts of various adsorbent layers, each functionalized with an antibody	AF B1, B2, G1 and G2, Fumonisin, OTA, ZON	Solid-phase extraction of four classes of mycotoxins at the same time. A 20-fold concentration	Spiked phosphate buffer, alcoholic beverages

		selective towards a different mycotoxin.		of analytes is reached, together with sample clean-up.	
Zabe N. Basker C.J. US20070117219A1	AOZD multi-analyte affinity column	Combination of proper amounts of various adsorbent layer, each functionalized with an antibody selective towards a different mycotoxin.	AF B1, B2, G1 and G2, OTA, ZON, DON	Solid-phase extraction of four classes of mycotoxins at the same time. A 20-fold concentration of analytes is reached, together with sample clean-up.	Spiked phosphate buffer, alcoholic beverages
Zabe N. Basker C.J. WO07059316	Multi-analyte affinity column	Combination of proper amounts of various adsorbent layer, each functionalized with an antibody selective towards a different mycotoxin.	AFs, DON, fumonisin, OTA, T-2, HT-2, and ZON	Solid-phase extraction of all major toxins at the same time. A 20-fold concentration of analytes is reached, together with sample clean-up.	Dried distiller's grain
Hooper D.G. US20080014582A1	Methods and compositions for detecting fungi and mycotoxins	Extraction of mycotoxins from tissues and body fluids coming from humans or animals. Mycotoxins quantification was obtained by commercial ELISA kits after	AF B1, B2, G1, and G2, OTA, T-2, HT-2, Roridin A, Verrucarin A, and other minor tricothecenes	Measurement of mycotoxins in patients which permits correlation between mycotoxin exposure and health effect to be observed.	Urine, nasal secretion, blood, spinal fluid, heart tissue, and liver tissue from humans.

		purification and pre-concentration by commercial immunoaffinity cartridges.			

Piletski *et al*. [35, 36] describe two polymers able to selectively bind ochratoxins and aflatoxins respectively. Those polymers can be packed into cartridges and can be used as absorption media to selectively capture the mycotoxin from a complex solution (ie: a liquid extract from food or feed). The polymers described are not molecularly imprinted polymers, since no analyte or analogues were used as a template to prepare the polymers. The invention is based on the observation that polymers containing amido or aminoalkyl moieites together with acidic moieties show recognizing properties towards OTA and AFs. Various combinations of such monomers are described, together with different polymerization techniques. Some useful polymers, showing selectivity and affinity towards each of the two toxins could be identified, and their applicability as SPE media were demonstrated for the extraction of OTA and AFs in relevant matrices. An interesting advantage of this approach regards the fact that no templating molecule is required. On the contrary, the preparation of molecularly imprinted polymers involves the use of large amounts of the templating molecule. As far as toxic substances are concerned, this fact constitutes a severe risk for operators during the synthesizing procedure. Consequently, non or less toxic mimic of the analytes have been employed as a template to prepare MIPs for mycotoxins [37].

The same adsorbent media can be used to detect the mycotoxins. In this case, the toxin is selectively captured by the packed polymer; interferences are washed out and the presence of the toxin is directly measured exploiting the natural fluorescence of OTA or AFs. The suggestion could be extremely interesting, because in a single device extraction, clean-up and detection of the analyte would be obtained. It must be noticed, however, that the levels of contamination which could be measured in such a way would be decidedly higher than legal levels.

Synthetic polymers with molecular recognizing properties towards OTA have also been described in patent WO2007072212 (Tozzi *et al.,* [38]). The innovation proposed by our research group regards the use of short synthetic peptides (6 aminoacids) as ligands able to selectively bind the toxin. Peptides with binding properties are obtained by a combinatorial approach, which allows us to rapidly and easily select sequences of 4-8 aminoacids with the desired characteristics. [39-42]

The possibility of tailoring the sequence during the selection phase allows us to partially direct the final result at will, thus choosing if the higher affinity, the higher selectivity or a broad spectrum selectivity should be obtained [40].

These peptides are covalently bound onto a solid support and the resulting medium is usable exactly as a classic SPE absorption medium. Packed in cartridges it has been demonstrated to allow the extraction and clean-up of OTA from various wines, with recoveries higher than 75%, measured at legally relevant OTA concentrations.

Major advantages and drawbacks of using such adsorption media are the same as those showed by molecular imprinted polymers. In addition, the synthetic procedures do not require the use of any toxic templates and hazardous organic compounds. Peptides are also perfectly compatible with water and common polar organic solvent, which allows to develop varying extraction protocols, according to the needs of the following detection technique.

Adsorbent media based on the use of antibodies are described in three patents of Zabe and Basker [43-45]. The object of the patent is, in all cases, the simultaneous extraction of more than one mycotoxin. In particular, patents describe immunoaffinity columns for the extraction of: AFs, OTA, ZON and DON the first patent; AFs, OTA, ZON and fumonisins the second; and AFs, OTA, ZON, fumonisins, DON, T-2, and HT-2 the third. The multi-analyte extraction is achieved by mixing the immunosorbent directed towards each separate analyte in the correct ratios. These ratios should be determined by the combination of the affinity of the antibodies used and of the relative amount of toxins which have to be captured. Apparently, there is no correlation between immunosorbent quantity for each toxin and level of contamination of the same toxin in food and feed. Therefore, the determining factor is likely to be the affinity of the antibodies. As far as single analyte IACs are concerned, a strong pre-concentration of the analyte is obtained, however, the most interesting aspect is the real reduction of analysis time for those matrices which can be contaminated by various toxins at the same time, such as cereals. In these cases, samples can be treated in one step and then analysed together or individually. Recovery values for mycotoxins measured in the part per billion and part per million range were between 70 and 90%, with good precision (RSD <=10%).

Belonging to the category of classical immunoassay, the enzyme immunoassay patented by Hooper [46] owes its novelty to the matrices involved. In fact, Hooper describes the use of commercially available ELISA kits for the determination of aflatoxins, ochratoxin A, T-2 toxin, HT-2 toxin, roridin A. verrucarin A and other minor tricothecenes in body fluids and tissues from humans and

animals. Even if labelled as a method for detecting mycotoxins, the interesting step of the protocol is that of treatments to extract mycotoxins from non-food matrices. Moreover, the determination of mycotoxin presence and levels in human or animal subjects allows us to be able to verify correlations between mycotxin intakes from diet and their levels in the various body fluids or tissues. At the same time, studies aimed at establish correlation between mycotoxin exposure and development of diseases, which are supposed to be related, could be of major interest. Extractions have been demonstrated out on various human body fluids and tissues, which have been fortified at low level (ppb).

ANALYTE DETECTION

As previously discussed, the method of analysis for mycotoxins can be divided into two groups: screening methods and confirmatory methods Fig. (1). Confirmatory methods are based on instrumental techniques, mainly liquid chromatography coupled with different detection procedures [14, 19], such as UV [47], fluorescence (exploiting the natural fluorescence of same toxins [48, 49] or using derivatization [50-52]) and mass spectrometry [18, 22]. Even if much literature exists on this matter and is updated daily, no recent patents have been focused on any aspects of the instrumental detection of mycotoxins. Contrarily, methods and devices mainly aimed at measuring such contaminants very rapidly and in the crop fields themselves have been recently patented Table 4.

Table 4. Patents concerning mycotoxin detection

Inventors Patent n.	Title	Method	Mycotoxin concerned	Result	Limit of detection	Tested matrices
Dhar T.K. Pal A. US200670 87389	Highly cost-effective analytical device for performing immunoassays with ultra high sensitivity	Immobilization of reactives onto a membrane (as spot) for performing an immunoassay with visual detection	AF B1	Semi-quantitative determination of AF B1. Fast analysis (3-10 min), limited sensitivity.	So called "conventional assay": 0.8 µg l^{-1} "Ultra-sensitive assay": 0.02 µg l^{-1} (in an aqueous sample extract)	Seeds

Schneider T. Lawton J.H. Bandea M. Ferguson B.S. WO07072212	Test device for analyte detection	Preparation of the components to produce lateral flow strips.	AF B1	Semi-quantitative determination of AF B1. Fast (5 min) and sensitive analysis.	ND [a]	Ground corn
Danks C. Flint J.R. US200802 89068A1	Analyte detection system	Preparation of the components to produce lateral flow strips. Strips can be read by a reflectance reader and line intensity can be semi-quantitatively correlated to mycotoxin concentration.	OTA	Semi-quantitative determination of OTA in 15 min.	ND [a]	Grain
Sibanda L. De Saeger S.M. Van Peteghem C. US200701 17222A1	Device and method for detecting the presence of an analyte	On-line clean-up of liquid food extracts and detection of OTA and/or AF B1. Detection is achieved by a competitive immunoassay	OTA, AF B1	Simultaneous extraction and qualitative detection of OTA and AF B1	Cut-off levels: 5 μg kg^{-1} for AF B1, 10 μg kg^{-1} for OTA	Spices, roasted coffee

		performed in a column packed with an immunoaffinity medium.				
Coker R. Nagler M.J. Andreau M.P. US200801 98379	Device for detection and measurement of a target compound such as a food toxin	Use of a selective adsorbent material to capture the mycotoxin. The cartridge which contains the entrapped toxin is inserted in a device equipped with a UV source and a fluorescence detector	AF B1, B2, G1, and G2	Direct quantification of the toxin exploiting its native fluorescence	ND [a]	Maize meal
Burmeister J. Dorn I. Rabe U. Hauser-Hahn I. US200900 81808	Device and method for identifying mycotoxins	Immobilization of reactives onto a wave-guiding layer, which is put into contact with a second wave-guiding layer which has a different refractive index. Binding	ZON, DON	Portable chip able to quantitatively measure mycotoxins in 10 to 20 min.	Aqueous solutions of ZON: 0.4 μg l^{-1}, DON: 15 μg l^{-1}	Feed cereal

		to the immobilized reactive determines a variation in the optical properties at the interface and can be measured.				
Nasir M.S. Jolley M.E. US2008022 7221 Patent application	Fluorescen ce polarizatio n-based homogene ous assay for aflatoxins	Development of a homogeneous competitive immunoassay which exploits the variation of the fluorescence polarization of a low-molecular-mass fluorophore (the tracer in the assay) when it binds to an antibody.	AF B1, B2, G1, and G2	Quantitative and rapid measurements of mycotoxins in food and feed extracts.	ND [a]	Corn and pop-corn

[a] ND means that no limit of detection nor cut-off levels are not claimed in the patent

Historically, screening methods employed to identify the presence of and to measure the level of contamination of mycotoxins in food and feed were Thin Liquid Chromatographic determinations [7,19]. Nowadays, even if TLC methods still remain as validated and official analytical methods for

screening of some mycotoxins, immunoassays have become the most commonly employed and most widespread technique for screening analyses, due to their simplicity, low costs, relative rapidity and ability to process several samples at the same time. In addition to more classical assay formats, such as direct and indirect ELISAs, which are commercially available for the majority of analytes and matrices, immunochromatographic assays (also named lateral flow immunoassay, LFIA) have been very recently developed [53-57]. Qualitative LFIAs have become commercially available in the last few years and some quantitative assays are beginning to appear on the market [53, 58]. The main goal of the applied research in this field is to obtain very rapid, portable, cheap devices to allow their use in the crop fields by farmers, in the factories by workers, at the customs checkpoints by customs officers, etc... The nature of the samples involved, which are perishable, the extent of the contamination and the requirement of surveillance of the entire food chain determine the fact that a very large number of analyses should be carried out at the screening level. Therefore, there is a great economic boost in the development of new devices for screening purposes.

Two of the more recent patents concerning mycotoxin detection regard the development of immunochemical assays in the new LFIA format (Schneider *et al.* [59] and Danks and Flint [60]). The principle of the competitive lateral flow assay relies on the competition for antibody binding sites on gold nanoparticles. Antibodies that are raised to the analyte (i.e: the mycotoxin) are bound to gold particles, which are then applied onto a release pad. Two lines of reagent are immobilized onto a membrane, which allow the lateral flow to develop. The test line comprises a protein conjugate of the target to be identified. The control line is a line of anti species antibodies. The release pad and the membrane are assembled together with an absorbent pad to form the so-called lateral flow strip.

The strip is posed in contact with a liquid sample, thus releasing the gold particles, which then begin to flow across the membrane. If some analyte molecules are present in the sample, antibody binding will occur. Any gold particles that fail to bind to the analyte will attach to the immobilized test line as they traverse the membrane; thus producing a visible line of deposited gold. The anti species antibody on the control line captures any excess gold particles, bound or unbound, to produce a control line as a visible confirmation of particle flow. The decrease of the test line is directly correlated to the increase of analyte amount in the sample extract. Analyte detection can be obtained by visual reading of the strip or by measuring the colour intensity of the two lines.

Although not introducing new material from a scientific perspective, these patents will surely have a positive impact on the market. Dipstick format, in fact, has all the characteristics required of the screening methods discussed above.

Scheneider *et al*. have described the preparation of lateral flow strips to detect aflatoxin B1. Measurements are visual, and semi-quantitative, however the response is very fast (5 min) and the cut-off is decidedly low (< 20 μg kg^{-1}), in accordance with legal requirements.

Danks and Flint have described the preparation of lateral flow strips to detect OTA. After the development, strips are read by a reflectance reader thus allowing a correlation between line intensity and OTA concentration to be established. The system is slower than the one of Schneider *et al*. (15 min for each analysis) and less portable, because of the need to have a reflectance reader. On the other hand, quantification or semi-quantification of OTA is possible, even if no validation data have been reported. The device has been only tested to detect OTA in grain samples contaminated at 50 and 100 μg kg^{-1} confirming that contaminated crops could be clearly distinguished from non-contaminated materials.

An alternative system to develop some kind of membrane immunoassay is proposed by Dhar and Pal [61]. In their patent, authors describe the immobilization of a protein conjugate of the target (i.e.: AF B1) onto the membrane, in a defined micro-area, to create spots. Then an antibody selective to AF B1 is mixed together with the sample (a liquid extract) which could contain the target, and then put into contact with the membrane. The antibody binds to the analyte when present in the sample, or to the immobilized target. A labelled reagent (typically an anti-species antibody) is added onto the membrane and the excess of reagents is washed out. Finally a colour development is carried out by adding some suitable substrate, which allows visual detection of the colour intensity of the spot. The decrease of the colour intensity of the spot is directly correlated to the increase of the amount of analyte in the sample extract. Results are semi-quantitative and sensitivity is poor, mainly because the declared sensitivity has been measured in aqueous solutions, therefore methanol sample extracts need to be strongly diluted before being analyzed. The major advantage of the proposed method is its rapidity, while the need to perform numerous steps strongly limits its application in the crop fields.

A rapid immunoassay is also proposed by Nasir and Jolley. [62] In their patent, authors describe an homogeneous immunoassay. Compared to heterogeneous immunoassays, such as direct and indirect ELISAs, which are usually employed as screening methods to determine mycotoxins, the homogeneous assays are potentially swifter and easy to automatize. [63-65] Therefore, homogeneous assays are more easily transferred into on-field portable devices. Nevertheless, they are usually less sensitive than heterogeneous IAs [66].

Several homogeneous IA formats have been described, based on the use of tracers which exploit fluorescence polarization. [65-69] In these assays small fluorescent probes are conjugated to an homologous of the analyte and used as a tracer. The analyte and the tracer compete for a limited number of antibody sites. When the tracer is free, its polarization value is low, because of its fast rotation, due to its low molecular mass. The polarization value strongly increases when the tracer is bound to the antibody, which is a large molecule, and thus has a slow rotation. Since the measured propriety (ie: polarization value) is modulated by the binding, the free and the bound forms can be distinguished without separation. The elimination of separation and washing steps determines the rapidity and the potential transfer into portable devices. The patented assay is affected by limited sensitivity, however, it allows the quantification of aflatoxins in raw and processed food in a range near to the legal limits for those contaminants. The method has been validated by comparison with a HPLC reference method, showing a good correlation between data.

The detection method patented by Sibanda *et al*. [70] is once again based on the use of antibodies, even if it exploits a very different technology. Authors propose a device composed of two cartridges connected to each other. The first cartridge is packed with an adsorbent material aimed at cleaning-up the sample extracts, whereas the second cartridge contains an immunosorbent material, which is exploited to carry out an immunoassay in the column. The "in-column immunoassay" has been already described in the literature [16,26,71,72] In fact, a specific antibody is immobilized onto a solid support, which is used to pack the cartridge. When a liquid extract flows through the column, the mycotoxin, which might be present, binds to the anchored antibody. Subsequently, the column is flushed with (in the following order): some washing solutions; a solution of an enzymatic tracer (which binds to the free antibody sites of the immunosorbent, if present); some washing solutions; a substrate of the enzyme which develops some kind of colour. The final result is a visual detection of the toxin: if the detection cartridge is coloured no mycotoxin was present in the sample (negative sample), if the detection cartridge is not coloured the mycotoxin was in the sample (positive sample). The device has been demonstrated to allow the simultaneous detection of both AF B1 and OTA, with acceptable recovery values (70-85%) and repeatability of obtained results. There are two major points of interest in the described system. The first is the on-line clean-up of the sample, which strongly simplifies handling. The other is the observation that the detection cartridge could accommodate more than one immunosorbent layer, functionalized with antibodies directed towards different mycotoxins, thus allowing the multi-analyte detection to be carried out. On the other hand, the detection protocol, which involves a series of tedious and time-consuming steps could limit the possible application of this device.

Mycotoxin detection by using a selective adsorbent material packed into a cartridge is also the object of the patent of Coker and co-worker. [73] They propose a device to measure fluorescence emitted by a cartridge packed with a suitable adsorbent media. An useful absorbent material should be able to selectively capture the mycotoxin, while a contaminated sample extract flows through the cartridge, and "pack" or concentrate the mycotoxin in the smallest layer. At the same time, the adsorbent material should be non-fluorescent and should provide a background with very low fluorescence, because the captured mycotoxin is revealed by exposing the cartridges to UV radiation and observing fluorescence, if present. Therefore, only mycotoxins which show natural fluorescence such as aflatoxins, ochratoxin, and zearalenone could be detected. The device allows quantitative measurements to be obtained, because it comprises an UV source, a fluorescence detector and a cartridge housing. The cartridge is mounted in a holder which can rotate so that the emission can be detected from substantially 360° around the sample. In this way, fluorescence could always be measured, even if the immobilized mycotoxin is unevenly distributed in the immobilized band. Additional features of the device could be the interfacing with some processing systems which rather than merely convert fluorescence readings into some kind of numbers, could transform readings into quantitative results, by means of a proper calibration. Alternatively, authors also propose to add more than one adsorbent layers, each selective for a different mycotoxin to exploit multi-analyte detection. The patented device would preferentially use the polymers described in the patents of Piletski and co-workers [35-36] and which has been discussed above. Such polymers are particularly suitable, because they are selective towards aflatoxins and ochratoxin, have very low fluorescence background and allow both the clean-up and the detection of the mentioned mycotoxins. The apparatus for the fluorescence detection combined with the selective polymers forms a portable device for the effective and quantitative measurement of some mycotoxins in food and feed extracts. The major drawback of this system is the measurement of the native fluorescence of mycotoxins which limits its applicability and, above all, strongly affects sensitivity of measurements. In fact, no results of the measurement of a toxin content have been reported aimed at exemplify the performances of the invention.

Burmeister and co-workers propose an optical biochemical sensor using a two-layer slab type of thin-film glass waveguide to measure mycotoxins [74]. The principle of measurement is based on the absorption of an evanescent wave of the guided laser light. Optical waveguides allow the detection of change in the optical properties of a medium bordering the wave-guide layer. When light is transported in a guided mode within the wave-guide layer, the light field does not decrease

abruptly at the interface between the waveguide layer and the surrounding medium. However, it decays exponentially as it moves away from the layer surface in the medium. Such decaying electromagnetic field is referred to as an evanescent field. A modification of the layer surface, which causes a variation of the refractive index at the interface between the waveguide layer and the medium, determines a variation of the evanescent field which can be detected using a suitable tool. The patented device is a thin-film waveguide formed by two layers, the first being an optically transparent wave-guiding layer and the second an optically transparent layer, which have a lower refractive index than the first. The method patented for the quantification of mycotoxins is based on the use of such a device and on systems which exploit molecular recognition, such as antibodies. A derivative of the mycotoxin (ie: a protein conjugate of the mycotoxin) is anchored onto the optically transparent layer. When antibodies bind to the immobilized mycotoxin, the optical properties of the medium change at the interface with the waveguide layers, thus determining a modification of the evanescent field which can be measured. If a liquid sample contaminated with the mycotoxin is mixed with the antibody and the mixture is put into contact with the thin-film waveguide layer, a competition is established between mycotoxins in the sample and those immobilized on the waveguide layer surface for the binding of antibodies. Therefore, changes in the evanescent field can be quantitatively correlated to the amount of the mycotoxin in the sample: as this amount increases, antibody binding to the waveguide layer surface decreases, and changes in the refractive index decrease accordingly, thus determining lower modification of the evanescent field. As such an optical waveguide may be used as a component in integrated optical circuits, a portable biosensor, characterized by high sensitivity and fast response, could be constructed exploiting the patented device. The system has been demonstrated to allow the quantification of zearalone in aqueous solutions and DON in a certified reference material (feed cereal) at levels of regulatory relevance.

CURRENT AND FUTURE DEVELOPMENTS

The evaluation of mycotoxin contamination in food and feed is a major concern in food safety controls. The spread of the contamination, the number and nutritional importance of the involved matrices, and the strict regulations induce the continuous updating of analytical methods. The objective is to develop methods with the widest possible applicability, from raw to complex matrices, with as limited a sample treatment as possible, no requirements of skill or instrumentation and which give answers in the shortest time. In this field, the accuracy of the answer is somewhat secondary. It is better to have an inexpensive rapid response, accepting a limited percentage of false

positive, rather than having very accurate results which are costly and time-consuming. In fact, the controls are carried out mainly by the producers themselves along the different stages of the food chain. Although official controls are compulsory and only confirmatory methods of analysis have official validity, the number of test carried out is necessarily limited compared to screening test. Consequently, there is less interest in those who patent methods and devices for the development of instrumental methods to be used as confirmatory analyses. Major interest dwells in the development of screening methods and sample treatments aimed at saving time and/or money.

Regarding sample treatments, a comprehensive review of patents published in the last few years has shed light in two main directions: the multi-analyte approach [43-45] and the search for innovative materials that can replace the use of antibodies and immunoaffinity columns [35-36, 38]. Certainly, the multi-residue approach will be one of the most promising lines of development, since the number of mycotoxins to be analyzed is steadily increasing. [6,75,76] However, the research and patent activity could also guide one to a quite different viewpoint. Till now, very few works have been carried out aimed at studying methods of extraction of mycotoxins from food samples that do not involve or reduce the need for subsequent treatments of the extracts. Almost all the published works use the same extraction protocols and are focused on the following clean-up or analysis [25,26, 29-34,40-42,47-48]. Nevertheless, the modification of conditions in the very first step of the analytical method could reduce co-extraction of interfering substances, allow direct measurements of the extracts without needing solvent exchange, dilution, or the decrease of the performance of the analytical method. For example, organic solvents are not compatible with the antibodies. Therefore, screening methods based on immunochemical methods such as ELISAs or purification with immunoaffinity columns are affected by the presence of the majority of organic solvents, which have to be eliminated or at least reduced. Alternatively, extraction protocols using aqueous media would allow the problem to be overcome [76, 77], thus increasing the sensitivity (no dilution required), the precision (decrease in the number of steps) and the safety (no use of hazardous materials). In the meantime, they would simplify and speed-up sample treatments and reduce costs of analysis.

Similarly, in reference to the methods of analysis, we can identify guidelines, even if there are a great diversity of approaches. The common denominator in this case is primarily the ability to carry out the analysis outside the laboratory by non-skilled personnel, looking for a semi-quantitative response. The objective is the development of even more compact integrated systems, which reduce the number of operations to be performed and provide a response within a few minutes, so that they can be used directly in production lines.

Generally speaking, most patents exemplify the use of such devices aimed at demonstrating their accuracy. However, limited validations have been performed in the majority of patents and, in particular, little data is generally presented about the precision of the results obtained.

A series of patents regards the development of membrane-based immunoassay (such as lateral flow immunoassays), with visual detection or with some kind of reading system. [59-60] This is definitely a trend in rapid development, as highlighted by the number of very recent publications on the subject. [53-57]

Another group of patents covers "in-column" methods of analysis, which in same cases exploit the same adsorbent materials patented as extraction media. Surprisingly, no molecular imprinted polymers have been patented nor employed as an affinity media in such in-column analysis, while a number of MIPs for mycotoxins have been described in the literature [28-30,37]. These approaches may have application as qualitative methods, while it is more difficult to find employment as quantitative methods. This would be due to the lack of established technologies which can be easily adapted. However, the major concern still remains the great sensitivity required, which cannot be reached by all the patented devices.

Patents on the development of a system based on fluorescence polarization [62] and the establishment of an optical biosensor [74] are potentially very interesting.

Immunoassays which exploit fluorescence polarization is a recent technique, anyway now well documented. [66-69] Moreover there are also instruments for the measurement of fluorescence polarization, even portable ones, which enable the effective application of the method described.

As regards the optical sensor, despite a certain amount of literature available on the subject [78] and literature quoted herein], yet there are no practical applications. One limitation to developing these systems could be the fact that the tools and devices for analysis in this field will have to be cheap, capable of processing many samples and easy to use as extensively discussed above.

CONFLICT OF INTEREST:

There are not conflict of interest.

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