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Review

Analytical methods for determination of mycotoxins: An update (2009–2014)

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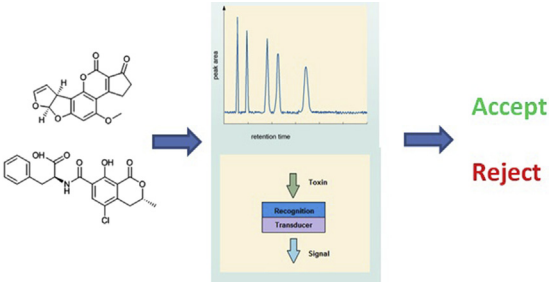
HIGHLIGHTS

- Discussion of sampling strategies for mycotoxin analysis.
- Comprehensive analysis of analytical techniques for mycotoxin analysis.
- Comparison of laboratory versus in-field techniques.
- Novel biosensors methods presented and discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

Mycotoxins are a problematic and toxic group of small organic molecules that are produced as secondary metabolites by several fungal species that colonise crops. They lead to contamination at both the field and postharvest stages of food production with a considerable range of foodstuffs affected, from coffee and cereals, to dried fruit and spices. With wide ranging structural diversity of mycotoxins, severe toxic effects caused by these molecules and their high chemical stability the requirement for robust and effective detection methods is clear.

This paper builds on our previous review and summarises the most recent advances in this field, in the years 2009–2014 inclusive. This review summarises traditional methods such as chromatographic and immunochemical techniques, as well as newer approaches such as biosensors, and optical techniques which are becoming more prevalent. A section on sampling and sample treatment has been prepared to highlight the importance of this step in the analytical methods. We close with a look at emerging technologies that will bring effective and rapid analysis out of the laboratory and into the field.

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Abbreviations

AF	Aflatoxin
AF B1	Aflatoxin B1
AF B2	Aflatoxin B2
AF G1	Aflatoxin G1
AF G2	Aflatoxin G2
AF M1	Aflatoxin M1
BEA	Beauvericin
CIT	Citrinin
DON	Deoxynivalenol
FB1	Fumonisin B1
FB2	Fumonisin B2
NIV	Nivalenol
OTA	Ochratoxin A
OTB	Ochratoxin B
PAT	Patulin
T2	T-2 Toxin (Insariotoxin)
TRC	trichothecenes
VOM	Vomitoxin
ZEA	zearalone
ZEL	zearalenol
ZON	Zearalenone
AOAC	Association of Analytical Communities
BR	Buyer's Risk
C_{18}	octadecylsilane column
CE	capillary electrophoresis
DAD	diode array detection
DaS	“Dilute and Shoot”
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
ESI	electrospray ionisation
FAO	Food and Agricultural Organisation
FID	flame ionisation detector
FD	fluorescence detection
FTIR	fourier transform infrared spectroscopy
GC	gas chromatography
LC	liquid chromatography
HACCP	Hazard Analysis & Critical Control Points
HPLC	high performance liquid chromatography
IA	immunoaffinity
IARC	International Agency for Research on Cancer
LIF	laser induced fluorescence
LLC	liquid–liquid chromatography
LLE	liquid–liquid extraction
LOD	limits of detection
LOQ	limits of quantification
MIP	molecularly imprinted polymer
MS	mass spectrometry
MW	molecular weight
NIP	non-imprinted polymer
OC	operating characteristics
PCA	principal component analysis
ppb	parts per billion
ppm	parts per million
ppt	parts per trillion
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
QCM	quartz crystal microbalance
RNA	ribonucleic acid
RP	reversed phase
SFE	supercritical fluid extraction
SPE	solid phase extraction
SPME	solid phase microextraction
SPR	surface plasmon resonance
SR	seller's risk
TLC	thin layer chromatography
TOF	Time of Flight
UHPLC	ultra-high pressure liquid chromatography
WHO	World Health Organisation

1. Introduction

Mycotoxins are notoriously toxic, are thermally stable and demonstrate high levels of bioaccumulation. The term "mycotoxin" is usually reserved for small compounds that are approximately 300–700 Da which are produced both pre- and post-harvest as secondary metabolites by several different species of fungus. Their production is genotypically specific, but is not limited to one species, or one toxin per species [1]. It is clear to differentiate between compounds that are indeed toxic to vertebrates (proven toxicity), and those that are just metabolites of biochemical reactions within the fungus. This is not helped by the diverse chemical structures and biosynthetic origins. Classification often depends on the training of the person performing the task [2].

There are several classes of mycotoxins with the aflatoxins, ochratoxins, fumonisins, trichothecenes the major compounds of interest. Other such as patulin and citrinin fall outside these families but are still of significant import [3–5]. While approximately 400 different compounds have been identified falling into the class of mycotoxins, however in reality only about 10–15 are considered to be of commercial interest. These are the major compounds in their families and are those most commonly found (Fig. 1). As such, the commercial and academic research fields associated with the detection of mycotoxins is considerable.

In general, their toxic effects range from severe toxic effects on the liver, kidney, hematopoetic, immune system, foetal and reproductive systems, as well as significant contribution to carcinogenic and mutagenic developments [1,6]. The International Agency for Cancer Research (IARC) has classified a number of mycotoxins formally as agents that are proven (Group 1), probably (Group 2A), and possibly (Group 2B) carcinogenic to humans [7]. For example four aflatoxins are classified in Group 1 (AF B1, AF B2, AF G1 and AF G2) while OTA is classified in Group 2B [8]. Numerous studies on other animals, including those in the human food chain have been carried out [9].

Several core food commodities are affected by contamination. Mainly cereals, nuts, dried fruit, coffee, cocoa, spices, pulses and some fruits, however they can also enter the food chain via bioaccumulation in eggs, milk and meat from animals eating contaminated feeds. Other processed foodstuffs (breads, wines, beers etc.) are also found to contain accumulated toxins though use of contaminated ingredients [1]. The occurrence of these compounds is a worldwide issue. Regional variations do exist however common compounds such as OTA and AF B1 are found worldwide.

The mycotoxins "problem" therefore requires robust analytical methods; however this is not an easy task. The detection and identification of these compounds is often used as a prime example of the complexity of environmental trace compound analysis. The varied nature of the environment, target, matrix, detection levels, time requirements and availability of suitable technology are considered to be particular challenges.

Of consideration for the analytical process are:

- **Multiple targets of interest**: while production of these compounds is genotypically specific it is observed that the same toxin can be produced by differing species. Likewise while crop-specific toxins (patulin on apples for example) are found, it is not a guarantee that this will be the only compound present as it is dependent on the species of fungal contamination [10,11]. Likewise regional variations in occurrence are observed.
- **Varied chemistry**: Often a particular fungal species will be favoured (based on region, type of crop and other environmental factors) therefore the particular target toxin will be known, however the vastly different chemistry (Fig. 1) observed for these toxins makes a generic extraction and detection method almost impossible [6,10,12].
- **Multiple matrices**: the development of a successful strategy for analysis of a compound is often dependent on the ability to separate this compound from the complex matrix. Given that the same compound could be found contaminating vastly different crops (e.g. wheat vs. cocoa beans) which have different ratios of carbohydrates, proteins, lipids etc., this can interfere with separation and measurements [1,10]. The effect of different matrices is commented on in Section 3.2.3.
- **Timing of testing**: the timing of the analysis is vitally important. Contamination can occur pre-harvest (in the field) and post-harvest (during harvest, transport and subsequent storage). Analysis *in-field* cannot rely on the use of complex high-throughput equipment (HPLC-MS for example) that might be found in a large storage facility in a European country. The question of what, when and where to test is important.
- **Geographical and financial limitations:** Limited resources to carry out analysis can play a major factor in what is possible. Small local farmers would not expect to have access to complex and expensive lab based equipment, simply due to cost to scale requirements. Likewise financial limitations in developing countries limit what is possible. Consumables are costly and can also be affected by environment (solvents in warm climate for example).
- **Speed of testing:** the speed of a testing is of great importance it governs the number of tests that can be performed. The large number of tests that need to be carried out to make results statistically valid means that each test should be performed as quickly as possible. While *in-situ* analysis can be carried out while crops are growing at a potentially more leisurely pace, the timing of a harvest is critical and this can lead to situation where multiple tests are required to be completed in a short time frame. If you picture a line of trucks each containing produce arriving at a storage facility, each requiring testing before acceptance to the facility, you can imagine the difficulty with performing required numbers of tests on time.
- **Sampling:** Given the potential bulk of a total harvest, ensuring that a representative sample is obtained is key to the whole analytical strategy. Toxin producing moulds will not be found uniformly throughout the sample but have the ability to ruin an entire sample (for example if missed before post-harvest storage). Any test that is ran needs to be statistically relevant, and account for the potential of false positives and negatives. Further information is given below.
- **Limits of Detection/Quantification.** While varying significantly depending of geographical location, type of produce and final use of produce, the required limits of detection are still extremely low, often in the lower parts per billion (ppb) levels (ng mL^{-1}) [13,14]. Any successful detection method must be able to detect this level of contamination with acceptable levels of false negatives and false positives.
  Likewise a number of regulatory limits are high (e.g. fumonisins in raw maize), or levels determined between that for feed, and that for human consumption. This can potentially pose high demands on the linearity of an analytical method, especially with a potentially complex matrix.
- **Nature of required measurements.** It is natural to think of the obvious "field to fork" analytical pathways, but we must also consider the requirement for mycotoxin detection in other research fields such as genomic research, where scientists are looking at development of fungal resistant crops. Here the emphasis isn't based on mass sampling and analysis, but the use of focused methodologies specific to developed matrices and singular targets.

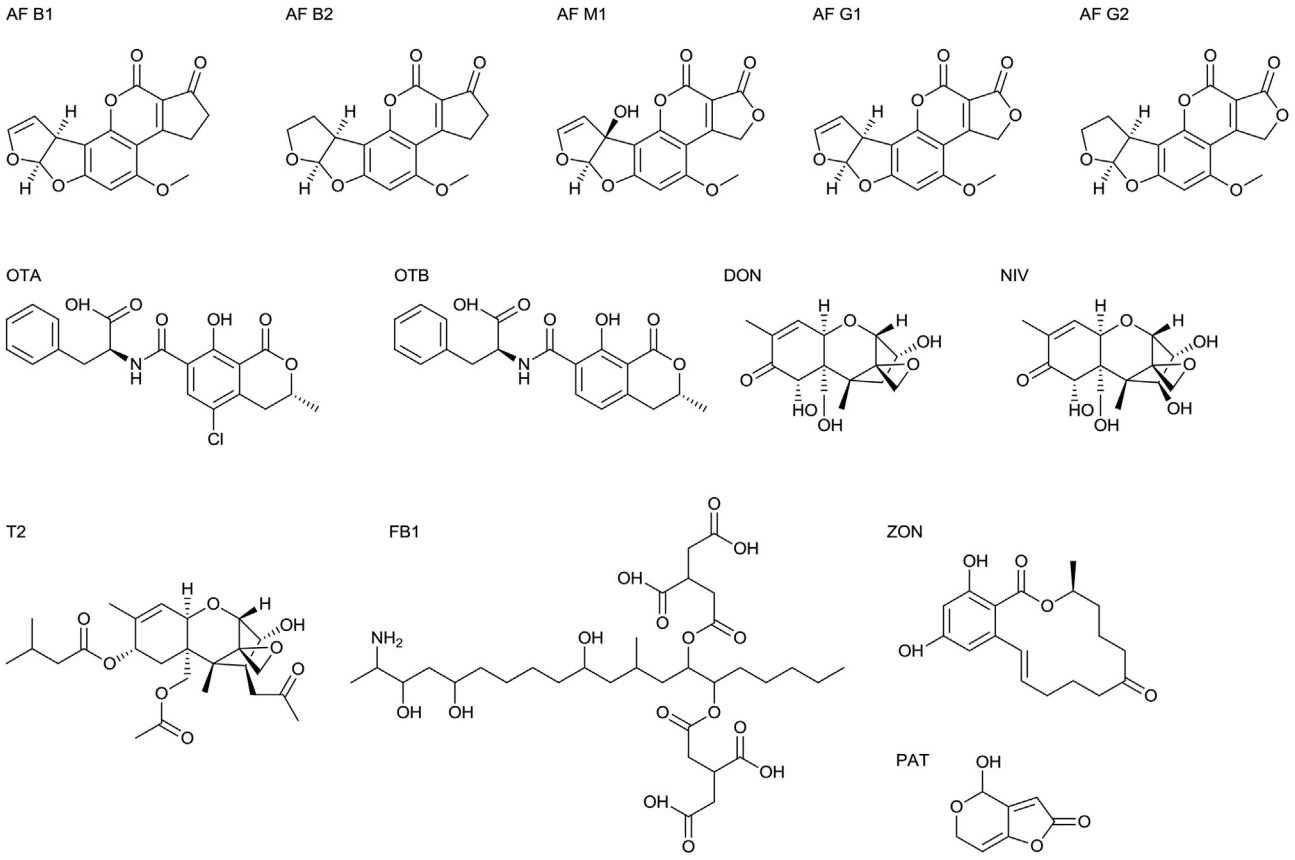

Fig. 1. Examples of common mycotoxins, demonstrating the variety in structure and functionality. Minor modifications (i.e. AF B1 and AF B2; or OTA and OTB) can lead to significant differences in toxicity and prevalence.

The principles of QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) analysis is particularly relevant to this kind of analysis as it embodies the core principles of what is required in in-field analysis [15,16]. It would be a nigh-impossible task to analyse every methodology developed towards the identification of mycotoxins, or to state which is the "best" method. In fact, there cannot be a best method due to the variance in requirements discussed above. This review seeks to build on the previous review by our group [1] and that of others [3,17–19]; and focuses on the advances in mycotoxin detection made between the years 2009 and 2014. Sampling methods are briefly covered as well as consideration for the future of mycotoxin analysis.

2. Sampling and sample preparation methods

2.1. Sampling plans

The control of mycotoxins in foods and feeds includes the implementation of regulations and quality assurance procedures (including Hazard Analysis & Critical Control Points, HACCP, Plans) and surveillance programmes. Each of these control activities involves the collection of representative samples and their conversion to laboratory samples which can then be subjected to mycotoxin detection by determination [20].

Whichever mycotoxin control measure is being employed, the related *sampling plan* will be characterised by clearly identified *sampling points, batches (lots) of food or feed, and a sample collection and sample preparation procedure*. The absence of a well-designed sampling plan will result in the collection of unrepresentative samples which will invalidate the results of subsequent mycotoxin determination [21].

The *sampling points*, at which samples are collected, may be on-farm, within a store or processing plant, at export and import locations, and within retail outlets.

The populations of food and feed located at the sampling points will be organised into readily definable batches, either as bag-stacks (Fig. 2) or as bulk material. Fig. 2 is typical of a small scale harvest; however a truck load could be several tons of grain loose loaded on a tractor trailer or truck.

In order to generate representative *laboratory samples* from a specific sampling point, the following steps will be followed [5,20]:

(i) The selection of one or more representative batches (i.e. a batch or batches which are characteristic of the total population of food or feed);
(ii) The collection of one or more representative *aggregate samples* from each batch, by combining a large number of *incremental samples;*
(iii) The collection of a representative *subsample* from each aggregate sample, and;
(iv) The preparation of a representative *laboratory sample* from each subsample.

It is essential that the method employed for the collection of aggregate samples recognises the heterogeneous distribution of mycotoxins, especially the aflatoxins, in food and feeds [22,23]. The incremental samples should be taken from a large number of points throughout the batch, such that the resultant aggregate sample is representative of the whole batch. Whenever possible, the aggregate samples should be collected whilst the batch is mobile; for

Fig. 2. A vehicle loaded with bags of groundnuts may be defined as a single batch for aflatoxin testing – Gudjurat province, India. Photograph courtesy of M. Szabo-Vezse.

example, during the construction or dismantling of a bag-stack, or from a conveyor belt during processing (Fig. 3) [12].

2.2. Established sampling plans

International and national agencies, including the European Union (EU), Codex Alimentarius, and the United States Dept. Agriculture (USDA) have established sampling plans for a variety of commodities.

Each sampling plan specifies the batch size (in the case of very large batches, a representative sub-batch is specified), the size of the aggregate sample, and the minimum number of incremental samples. While each of these is different, they are aimed at obtaining the same information. As an example we have collated the analysis of AFs in groundnut (peanut) kernels to highlight the type of sampling that is considered when dealing with a large sample size. These are found and discussed in the Supplementary Information. While each method has its own benefits they are each designed to ensure that an even spread of sample is taken and the amount % w/w is statistically adequate.

2.3. Sample preparation

Once a representative aggregate sample has been collected, an appropriate sample preparation procedure must be employed in order to convert the aggregate sample into a representative subsample and, subsequently, into a representative laboratory sample [24,25].

Fig. 3. Groundnut kernels passing along a conveyor belt during hand picking – Gudjurat province, India. Photograph courtesy of M. Szabo-Vezse.

There are two broad approaches in core preparation, illustrated below:

(a) Using a subsampling mill:

The aggregate sample of, say, 20 kg groundnut kernels is converted into a 1 kg representative, comminuted subsample by employing a subsampling mill [20].

Or:

(b) Using a conventional grinder:

The aggregate sample is comminuted using a mill which is capable of converting whole kernels into a free-flowing, ground sample. A variety of mills are available including hammer, disc and vertical cutter mills. The conversion of high oil content commodities, such as groundnuts, into a free-flowing material is especially difficult.

The comminuted aggregate sample is then subjected to riffle division employing, for example, a rotary cascade divider [20], which "divides" the aggregate sample into representative, comminuted subsamples, nominally 1 kg.

From here the 1 kg subsample is then mixed with water, in a blender, to produce an homogenous aqueous slurry. Replicate 100 g aliquots of slurry (laboratory samples) are finally collected and subjected to the chosen mycotoxin analysis, using an appropriate quantitative procedure. It is highly recommended that the laboratory sample should take the form of aqueous slurry, to reduce effects of solvent loss, handling ease and cost. The significant reduction in particle size, and the homogeneity of the mixture, results in a significantly higher level of precision than that afforded by the mixing of dry-milled material [26]. Aliquots of each can be stored for further replicate testing if required.

2.4. Sampling error

All sampling plans will possess a sampling error, which is expressed as the *Seller's (Producer's) Risk (SR)* and the *Buyer's (Consumer's) Risk (BR)* associated with the related Operating Characteristic (OC) Curve. The latter describes the probability of a defined sampling plan accepting a batch, when the batch has a specific level of AF contamination [27].

The *SR* represents the risk of a "good" batch (i.e. a batch with an acceptable level of contamination) being rejected by the sampling plan, whereas the *BR* represents the risk of a "bad" batch (i.e. a batch with an unacceptable level of toxins) being accepted [28]. Fig. 4a is an illustration of the OC curve for an error-free sampling plan, where the SR and BR are zero; and Fig. 4b illustrates a typical OC curve with associated seller's and buyer's risks.

The *total testing error* is the sum of the sampling, sample preparation and analysis errors associated with the "analytical sequence"; and it is widely recognised that the sampling error is by far the largest contributor [28]. The relative contributions of the sampling, sample preparation and analytical errors are illustrated in Fig. 5.

3. Analytical techniques

Once a sample is prepared into subjective aliquots, analysis can be performed. Factors that are of import to the type of analysis; and what should be considered are discussed in the introduction; however this section seeks to discuss the different potential methods available to the end user. Initially we discuss advances in

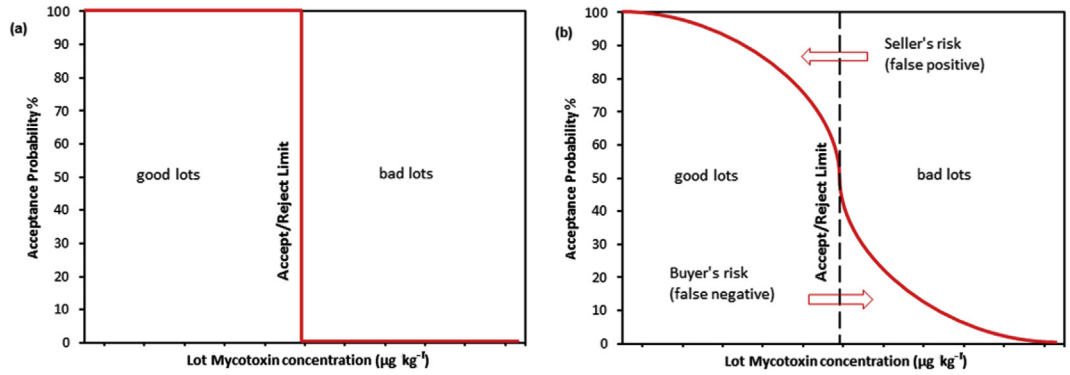

Fig. 4. (a) Operating Characteristic (OC) curve for an ideal (risk-free) sampling plan. (b) Operating Characteristic curve for a typical sampling plan, with identifiable Seller's and Buyer's Risks.

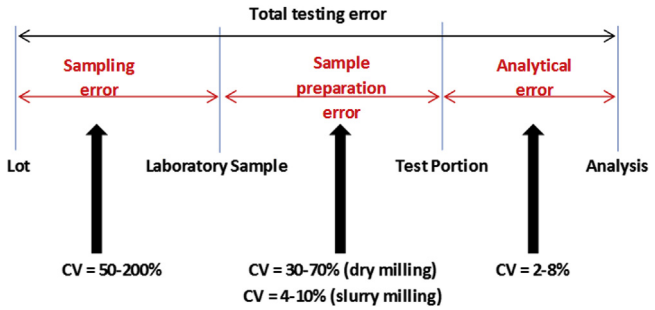

Fig. 5. Predicted total testing error in a complete method. Error can be found at all stages of the analytical procedure, however the error found within the actual analytical method (laboratory test) is low in comparison to that found in sampling and sample preparation. CV = Cross Variation.

sample preparation methods; followed by several sections each focussing on a different analytical *measurement* methods. These are namely chromatographic based; immunological based; sensor based; and other systems. For each we have tabulated methods in order of target toxin (or toxin family) for ease of browsing.

3.1. Sample pre-analysis methods

The requirements for preparation are clearly known and understood. Given the complex matrices that the toxins are generally found it is generally accepted that for most chromatographic methods a pre-analysis step is required, whereas for some biosensors and/or immunological methods it may not be needed. This step can add extra cost and time to an analytical method but the improvements in sensitivity, as well as benefits to robustness of a technique (e.g. reducing column blockage and contamination) is important. Due to the wide range of properties of the different mycotoxins, the sample preparation method still remains a major bottleneck in an entire analytical protocol.

Commonly the two favoured methods are liquid–liquid extraction (LLE) and solid phase extraction (SPE). In our prior review we describe the basic principles and technology that has become common place. Here we update on the recent progress made in these methods as summarised in Table 1.

Table 1
Examples of clean-up methods used in the extraction and preparation of mycotoxins for further analysis.

Toxin	Matrix	Preparative method	Detection method	Recovery limits (RL)/limits of detection (LOD)	Reference
AFs	Olives and drupes	High throughput automated SPE	LIF screening/ HPLC-MS determination	Screening LOD 0.7 µg kg^{-1} Determination LOD 0.01–0.03 µg kg^{-1}	[29]
AFs	Peanuts, figs, paprika	IA-SPE	HPLC-MS	LOD 0.14–0.32 ng mL^{-1}	[30]
AFs	Peanut, Tea, vegetable oil	IA-SPE	HPLC-FD	LOD 0.03–0.09 µg kg^{-1}	[31]
AFs	Peanuts, spices	IA-SPE	HPLC-FD	0.1 ng mL^{-1}	[32]
AFs	Nuts, cereals, dried fruits and spices	in-tube SPME	HPLC-MS	2.1–2.8 ng L^{-1}	[33]
AFs	Chilli	IA-SPE	HPLC-FD	0.1 µg kg^{-1}	[34]
AFs	Cereal flours	In-tube SPME	HPLC-FD	0.035–0.2 µg kg^{-1}	[35]
AF B1	Cereals	Homogenous LLE	HPLC-FD	0.003 ng g^{-1}	[36]
DON	Processed foods	IA-SPE	HPLC-UV	RL 0.5 g g^{-1}	[37]
Fusarin C	Corn	Dispersive SPE	HPLC-MS	LOD 2 µg kg^{-1}	[38]
OTA	Wheat	MIP-SPE IA-SPE	HPLC-FD	RL 2.5 µg kg^{-1}	[39]
OTA	Wine	Dispersive LLE	HPLC-LIF	LOD 5.5 ng L^{-1}	[40]
OTA	Wine	Dispersive LLE	HPLC-LIF	LOD 5 ng L^{-1}	[41]
OTA	Coffee	IA-SPE/LLE	HPLC-FD	0.025 µg kg^{-1}	[42]
OTA	Wheat	Aptamer - SPE	HPLC-FD	LOD 23 ng kg^{-1}	[43]
OTA/T-2	Alcoholic beverages	Hollow fibre Liquid microextraction	UHPLC-MS	LOQ 0.02–0.09 µg L^{-1}	[44]
Patulin	Fruit and Fruit Juice	In-tube SPME	HPLC-MS	LOD 23 pg mL^{-1}	[45]
Various	Rice	Salt-out LLE - SPE	HPLC-MS	LOD 0.5–15 µg kg^{-1}	[15]
Various	Flours and breads	HLB - SPE	HPLC-MS	LOD 0.1–59.2 µg kg^{-1}	[46]
Various	Urine	Salt-out LLE	HPLC-MS	LOQ 0.07–3.3 µg L^{-1}	[47]
Various	Various	IA-SPE	Isotope dilution LC-MS	LOQs 0.05–0.25 µg kg^{-1}	[48]
Various	Beer	C_{18} SPE	UHPLC-MS	LOQ <0.05 µg L^{-1}	[49]
Various	Urine	DaS	HPLC-MS	0.005–40 mg L^{-1}	[50]

3.1.1. Solvent extraction methods

Liquid extraction or partitioning is a common method for purifying a sample ready for further analysis. It relies on the solubility of the target compounds in a particular solvent, and the insolubility of competing or interfering compounds in the same solvent. It is arguably the simplest method of pre-treatment available. Numerous methods exist in the literature, and exemplar being that of Sulyok [51].

Arroyo–Manzanares [40] demonstrated the use of a microextraction procedure for OTA in wine samples. Within a ternary component solvent system made up from water from the wine, chloroform (organic phase) and acetonitrile (disperser) with a total combined volume of only a few microlitres) a stable emulsion is formed. NaCl is added to the wine prior to this step. The large surface area and subsequent phase separation (by centrifugation) allows for the polar organics to enter the organic phase for further analysis by LC-LIF (laser induced fluorescence) gave an LOD of 5.5 ng L^{-1} well within the required limits. Likewise, Campone used a similar method but with a different detection method to achieve a comparable LOD from wine [41].

In LLE, traditional solvent with a low dielectric constant (those that tend to be immiscible with water) are poor at extracting polar compounds, such as most mycotoxins. The suitable solvents such as methanol or acetonitrile should be mixed with water in the presence of salts to reduce the mutual miscibility. The polar analytes selectively move into the polar organic phase from the aqueous. Both Song [47] and Koesukwiwat [15] demonstrate use of this technique in the preparation of samples from urine and rice respectively for multiple mycotoxin analysis. The achieved cleanup allowed for low levels of detection <1 μg kg^{-1} using LC-MS analysis. The group of Yousefi used a similar method on a larger scale, for AF analysis from cereals [36].

A potentially simpler method has been demonstrated by the group of Krska [50], who used a "Dilute and Shoot" (DaS) method for multiple analyte detection in urine. This is not technically a sample preparation method and relies on a sample dilution followed by a direct injection, working on the principle that some samples are inherently clean enough (with dilution) and the costs of doing a full sample preparation outweigh column replacement. This type of method is gaining ground given the improved sensitivity and robustness of modern equipment.

3.1.2. Solid phase extraction methods

Solid phase extraction (SPE) is a mainstay of sample preparation in mycotoxin analysis. A wide variety of solid phases exists ranging from ion exchange [46], hollow microfibers [44] and C_{18} materials [49] to more targeted immunoadsorbent materials.

Most common in the targeted solid phases are those based on immunoaffinity recognition, where the solid phase has a targeted antibody bound to it and the target mycotoxin acts as the antigen. The development of antibody specific solid phase materials has become a large and well-studied area [52–54]. Examples of recent studies include those of Ma et al. [31], who focused on the use of a familial antibody for the extraction of four different AFs from a variety of matrices; and Trucksess [37] who used a DON specific IA column combined with a PEGylated extraction protocol for further analysis by LC-MS. Likewise Jinap [30] used an in-line IA column for detection of various AFs. O'Riordan performed a comparison of IA methods, combined with detection techniques (ELISA and HPLC-FD) with or without post cleanup derivitisation of the chilli sample. His results favoured HPLC as a post clean up quantification method [34]. Similarly Tozlovanu compared an IA column with an LLE method for OTA analysis of coffee beverages. Both methods performed in a similar manner. However while OTA was bound to the IA column the antibody detection was affected by chemical changes in OTA during coffee roasting and by the presence of OTB. This could lead to underestimation of the toxin content in the sample [42]. Particularly exciting is development of an organic stable IAC for AFs clean-up which are tolerant a range of solvents and provide, when linked to an accepted LC-FD protocol, excellent levels of detection [32].

The Mottier group used a relatively underused method of isotope dilution LC (where ^{13}C labelled mycotoxins are used as internal standards to support an IA clean up method for various compounds. Good levels of quantifications (under regulatory levels) were demonstrated [48].

Molecular imprinting, a method by which specific recognition sites are created in a cross-linked polymer matrix, to in essence creating artificial IA columns, has seen resurgence in recent years. The group of Pichon employed a molecularly imprinted polymer (MIP) specific for OTA as a method for sample clean up and compared it to a commercial IA column [39]. The MIP provided effective clean-up and higher capacity demonstrating the potential effectiveness of the technique. Szumski developed an imprinted material for AFs for use in an SPE microextraction (SPME) coupled with a small scale LC-LIF analysis [55]. MIPs are discussed further in section 3.5.

Another potential antibody replacement, aptamers, have been used by the group of Visconti [43]. Aptamers are short (~40–80 bases) single strands of DNA/RNA that demonstrate molecular recognition properties. An OTA specific strand was coupled, via the 5′ end, to a diaminodipropylamine gel. The resultant material was used for SPE of OTA from durum wheat samples for analysis via LC-FD.

The traditional materials loan themselves well to automation, as they are commonly based on traditional chromatographic materials [29,46]. The development of viable automation in analysis means that the traditional bottleneck of sample preparation can be incorporated into a single process. Nonaka [33] and Quinto [35] demonstrates this with an in-tube method which uses a C8 sorbent for a microextraction of AFs in nuts and cereals. This method utilises an open tubular fused-silica capillary column with an inner surface coating which enables easy coupling to a LC system. Kataoka used a similar method for patulin detection from juices and dried fruits achieving pg mL^{-1} level detection [45].

A rapid dispersive-SPE method has been demonstrated by the group of Humpf for detection of Fusarin C in corn [38]. Similar to the dispersive LLE methods a tube containing sorbent is used to bind the target, followed by centrifugation, collection and elution and LC-MS analysis.

These methods discussed above were all effective and comparable in cleaning up the samples for further analysis and the stated detection limits of the methods were reliant on the analytical quantification methods used.

3.1.3. Considerations in sample preparation

The selection of a suitable sample preparation step is key to most analytical techniques. A typical step could include an LLE step, which is effective, but solvent intensive, and relatively slow. Alternatively SPE or SPME is used after some form of solvent extraction, with a variety of solid phases available, as discussed above and in Ref. [1]. These serve to lower the matrix effect, therefore lower detection limits, and increase analyte recovery. They are also suited to automation, and solvent reduction. However, disadvantages exist including complex methods (multiple steps) potential cross-reactivity, and high costs of one use materials.

Recently two further methods have come to the fore, using the strengths of the exceptional sensitivity and separation capabilities of modern LC-MS equipment. These are namely "Dilute and Shoot"

(DaS) methods; and modification of QuEChERS protocols from pesticide analysis [56]. These have the benefits of working with multiple analytes and are rapid.

The benefits of a DaS method lie in its lack of pre-treatment and its rapidity. It also limits the potential loss of analytes during a cleanup stage, but risks of excessive and unpredictable interference from matrix can limit this technique, as it can potentially overwhelm the sensitivity of the instrument. Ideally this method is used with instruments of high sensitivity and methods of high selectivity where the negative factors can be discounted. QuEChERS protocol based on pesticide analysis have also been used for mycotoxin analysis, (for example [48,57–59]) and have appeared to be relatively successful. The process is effectively a modified solvent clean-up that uses extraction in acetonitrile, followed by a salting out step, and a quick dispersive SPE step with simple materials (for example $MgSO_4$, primary secondary amine (PSA) materials, C_{18}, and alumina) to remove matrix. Then a direct injection is performed.

This has benefits in that it can be performed relatively cheaply, and in our view have been used effectively in mycotoxin analysis, however again, similar to DaS the choice of method is affected by chosen target and matrix. For example mycotoxins such as the fumonisins can bind to PSA, but removal of PSA's from a method will significantly affect matrix cleanup. Likewise the requirement of removing the polar matrix could also lead to the removal of polar compounds (NIV for example) resulting in analyte loss.

The choice of preparative method depends, as many things in the analytical world, on the target, template and analytical instrumentation. However, the selection is key to how well a method will perform.

3.2. Chromatographic methods

The principle of chromatography is a key to the field of trace analysis. Ideally, a compound has to be separated into an individual entity from its surrounding matrix before identification. This technique has been at the core of mycotoxin analysis for over 50 years. The advent of highly sensitive detectors (fluorescence, mass spectrometry) has allowed the field to expand rapidly with at least 50% of all papers published each year in the field relying on some form of chromatography [60]. Commonly this is liquid chromatography (HPLC or UHPLC), though the use of TLC and GC, once common, are still considered. In the following sections we look at the recent studies in the area.

3.2.1. TLC and GC

TLC and GC analyses are often considered to be less practical, or to have insufficient sensitivity compared with LC methods; however a small number of researchers are investigating these techniques as they still have uses in certain situations (Table 2). Echarte produced a comprehensive study comparing LC with GC validated methods [61].

Welke have used a sensitive Charge-coupled device (CCD) detector to analyse patulin using its natural fluorescence via TLC. This method is far more sensitive than a visual inspection under a UV lamp and gave detection limits comparable to LC methods (low ppb) [62].

Klaric performed a study using both TLC and ELISA to analyse specific contamination during an outbreak of endemic neuropathy in Croatia. Analysis of food and feed was performed to isolate the source of the contaminants [63]. It was found that 29% of cereal food stuff had levels above the permissible limits, while the feedstock, which has a higher tolerance level were below. The TLC results correlated with the more sensitive ELISA methods used except in the case of OTA where the TLC was not sensitive enough. Caputo presented a study on the development of TLC detection for OTA where 0.2 ng of OTA deposited onto the TLC plate could be detected, when 2 μL was deposited onto a plate. With spiked beer and wine samples this is equivalent to 0.1 $\mu g\ mL^{-1}$ [64]

GC linked with MS is a method that delivers very high sensitivity in analysis, often down in the ppt ($pg\ mL^{-1}$ level, however it suffers from the risk of contamination and column blockage more than comparable LC methods. Cunha and Fernandes [58] utilised a so-called heart cutting GC–MS method to the analysis of various toxins. This involves transferring selected groups of compounds eluted from a GC column onto a second column and allowing everything else pass to waste, reducing risk of column damage. An extraction in acetonitrile followed by an SPE step and silane derivitisation before injection allowed for excellent LOD's to be achieved, lower than the stated EU limits for these toxins in foodstuffs. A similar method was described by the group of Mañes for use in semolina [65]. Both of these methods subscribe to the QuEChERS principles. The same group also developed a GC–MS method for analysis in human urine [66] and grain products [67].

3.2.2. LC

Liquid chromatographic methods are the mainstay of mycotoxin analysis, with the key AOAC (Association of Analytical Communities) methods based around HPLC as standard [68]. It is accepted that LC-MS, or LC Fluorescence are the gold standard against which all other methods are compared [60,69]. As such there is significant focus on the further development of HPLC protocol and related techniques. Therefore LC-MS acts as the standard reference method against which a novel method is compared; or as an analytical system for identification and quantification after compounds are isolated via other means. This review contains numerous examples of both. The former could be highlighted by Shen's work where an ELISA method for AF B1 and AF M1 was standardised against LC-MS [70] and the latter, the combined “method”- LC technique is the work of Aqui who used a magnetic bead flow cytometry based immunoassay to isolate the target toxins, combined with a small volume HPLC method. This miniaturised method offered excellent detection of OTA and OTB from naturally contaminated samples [71].

Table 3 summarises relevant papers that have been published in the recent years. Works in which LC is used as a standard method,

Table 2
TLC and GC methods used in the analysis of mycotoxins.

Toxin	Matrix	Protocol method	Detection method	Detection limits	Reference
OTA	Feeds, food and beverages	TLC	Photosensor	0.1 ng μL^{-1}	[64]
Various	Cereals and Feeds	TLC	Visual inspection	N/A	[63]
Patulin	Apple Juice	TLC	CCD	14 $\mu g\ L^{-1}$	[62]
Various	Human urine	GC	MS/MS	LOQ 0.25–8 $\mu g\ L^{-1}$	[66]
Various	Grain products	GC	MS/MS	LOQ <10 $\mu g\ kg^{-1}$	[67]
Various	Cereals and flours	GC	MS	2–15 $\mu g\ kg^{-1}$	[58]
Various	Wheat semolina	GC	MS	LOQ ~10 $\mu g\ kg^{-1}$	[65]

N/A = Not ascertained.

Table 3
LC methods used in the analysis of mycotoxins.

Toxin	Matrix	Protocol method	Detection method	LOD unless stated otherwise.	Reference
AFs	Pistachio nuts	HPLC	DAD	0.05 mg L^{-1}	[72]
AFs	Complex dietary product	HPLC	FD	1.6–5.2 μg kg^{-1}	[73]
AFs	Milk, egg, meat	HPLC	UV and FD	UV – 0.1 μg kg^{-1} Fluoro – 0.05 μg kg^{-1}	[74]
AFs	Animal feeds	HPLC with multifunctional column	FD	0.06–0.10 ng g^{-1}	[75]
Beauvericin/Enniatins	Human skin	UHPLC	MS/MS	10–17 pg mL^{-1}	[76]
DON	Barley	UHPLC	MS/MS	Qualitative study	[77]
DON/ZON/T-2/HT-2	Cereal and baby food	HPLC	MS/MS	10–60 μg kg^{-1}	[78]
AF M1/OTA	Human milk	HPLC	FD	0.8 ng L^{-1}	[79]
AF M1	Dairy products	HPLC	FD	3 ng kg^{-1}	[80]
AF M1	Milk	HPLC	MS/MS	0.02 μg kg^{-1}	[81]
AFs/OTA	Animal feed	UHPLC	MS/MS	LOQ 5 μg kg^{-1}	[82]
OTA	Grapes and wines	HPLC	MS/MS	<1 ng L^{-1}	[83]
OTA	Wine	Capillary HPLC	LIF	5.5 ng mL^{-1}	[40]
Trichothecenes	Wheat flour	HPLC	MS	1–30 μg kg^{-1}	[84]
Trichothecenes	Wheat and maize grains	HPLC	MS/MS	0.2–3.3 μg kg^{-1}	[85]
Various	Cereals	UHPLC	TOF-MS	5–500 μg kg^{-1}	[86]
Various	Urine	HPLC	ESI-MS/MS	0.005–40 μg L^{-1}	[50]
Various	Beer	UHPLC	MS/MS	LOQ 0.25–5.0 ng mL^{-1}	[87]
Various	Various household foods with moulds	HPLC	MS/MS	0.04–160 μg kg^{-1}	[88]
Various	Cereals	UHPLC	MS/MS	0.01–25 μg kg^{-1}	[89]
Various	Milk	UHPLC	MS/MS	LOQ 0.003–0.015 μg kg^{-1}	[90]
Various	Baby food and milk	UHPLC	MS/MS	Estimated 1–9 ng kg^{-1}	[91]
Various	Pig plasma	HPLC	MS/MS	LOQ 0.5–5 ng mL^{-1}	[92]
Various	Eggs	UHPLC	MS/MS	1–10 μg kg-1	[93]
Various	Cereal based products	UHPLC	MS/MS	0.01–2.1 μg kg^{-1}	[94]
Various	Wheat and maize	HPLC	ESI-MS/MS	1 to 30 μg kg^{-1}	[95]
Various	Wheat and maize	Micro-HPLC	LTQ - Orbitrap (triple quadrupole)	1 to 30 μg kg^{-1}	[95]
Various	Pseudocereals, spelt and rice	UHPLC	MS/MS	0.10–69.8 μg kg^{-1}	[16,96]
Various	Dried fruit	UHPLC	ESI-MS/MS	0.08–15.0 μg kg^{-1}	[57]
Various	Porcine serum and urine	HPLC	MS/MS	0.03–0.16 ng mL^{-1}	[97]
Various	Biscuit	HPLC	ESI-MS/MS	LOQ 0.04–80.2 μg kg^{-1}	[98]
Various	Various	HPLC	MS/MS	LOQ 0.05–0.25 μg kg^{-1}	[48]
Various	Apple, maize, pepper and hazelnuts	UHPLC	ESI-MS/MS	Qualitative	[99]
Various	Wines	UHPLC	ESI-MS/MS	0.4–200 μg L^{-1}	[100]
Various	Human breast milk	UHPLC	ESI-MS/MS	1 ng mL^{-1}	[101]
Various	Chilli powder and peanut	HPLC	ESI-MS/MS	0.15–80 ng g^{-1}	[102]
Various	*Puerariae lobatae radix*	UHPLC	ESI-MS/MS	0.751–1.10 μg kg^{-1}	[103]
Various	Milk and milk powder	UHPLC	ESI-MS/MS	0.05–2 ng L^{-1}	[104]
Various	Corn grits	UHPLC	ESI-MS/MS	0.01–0.71 μg kg^{-1}	[105]
Various	Feed and ruminant milks	UHPLC	ESI-MS/MS	LOQ 0.05–10 μg kg^{-1}	[106]
Various	Ginger	UHPLC	FD and MS/MS	0.005–0.2 μg kg^{-1}	[107]
Various	Palm kernel cake	UHPLC	ESI-MS/MS	0.02–17.5 μg kg^{-1}	[108]
Various	Human urine	HPLC	ESI-MS/MS	Qualitative	[109]
Various	Nutraceuticals	UHPLC	Orbitrap	4–300 μg kg^{-1}	[110]
Various	Bottled water	HPLC	MS/MS	0.2–2.0 ng mL^{-1}	[111]
Various	Milk	UHPLC	MS/MS	LOQ 0.003–0.015 μg kg^{-1}	[90]
Various	Feeds and spices	HPLC	FD	Qualitative	[112]
Various	Feeds	UHPLC	MS/MS	LOQ 0.5–250 μg kg^{-1}	[113]
Various	Small grain cereals	HPLC	ESI-MS/MS	LOQ 1 and 1250 μg kg^{-1}	[114]
Various	Maize silage	HPLC	MS/MS	1–739 μg kg^{-1}	[115]
Various	Maize silage	UHPLC	MS/MS	5–248 μg kg^{-1}	[116]
Various	Baby food	HPLC	MS/MS	0.05–50 μg kg^{-1}	[117]
Various	Urine	HPLC	MS/MS	0.5–10 ng mL^{-1}	[118]
Various	Sweet pepper	UHPLC	MS/MS	0.32–42.5 μg kg^{-1}	[119]
Various	Animal feed	UHPLC	MS/MS	0.7–60.6 μg kg^{-1}	[120]
Various (and pesticides)	Baby food, wheat flour, spices and seeds	UHPLC	MS/MS	8–500 μg kg^{-1}	[121]
Various (and pesticides)	"Organic" food products	UHPLC	MS/MS	LOQ <10 μg kg^{-1}	[122]

LOQ. Limits of Quantification.

or as a secondary analytical method linked with an alternative primary method have been excluded and are discussed in their relevant sections.

When we compare information presented in Table 3, (and Table 1); and that presented in our last review on this subject (Table 4 in Ref. [1]), there are three noticeable changes.

Firstly, there is a significant increase in development of multi-mycotoxin techniques instead of targeting single species. In some methodologies mycotoxins are analysed alongside other compounds such as pesticides [121,122]. This is an obvious step change in toxin analysis as it allows for a "one size fits all" method for a particular matrix which reduces costs and time for analysis.

Table 4
Traditional ELISA methods used in the analysis of mycotoxins.

Toxin	Matrix	Method	Detection method	LOD	Reference
AF B1	Peanuts	Competitive FLISA (quantum dot linked)	FD	0.016 ng mL^{-1}	[127]
AF B1 and fumonisin B1	Model samples	Competitive ELISA transferred to microarray	UV absorbance	AF B1 3 ng mL^{-1} Fumonisin B1 43 ng mL^{-1}	[128]
AF B1 and M1	Various foodstuffs	Indirect Competitive ELISA	UV absorbance	0.13–0.16 μg L^{-1}	[70]
AF M1	Milk	Indirect competitive ELISA	UV absorbance	0.04 ng mL^{-1}	[129]
AF M1	Milk and milk products	Competitive ELISA	UV absorbance	3–6 ng L^{-1}	[130]
AF M1	Milk	Competitive ELISA	Chemiluminescence	0.001 ng mL^{-1}	[131]
AF M1	Milk	Competitive ELISA	UV absorbance	N/A	[132]
AFs	Chilli	Competitive ELISA	UV absorbance	0.1 μg kg^{-1}	[34]
AF B1	Feed	Indirect competitive ELISA	UV absorbance	10.5 ng g^{-1}	[133]
AF B1	Corn	ELIME-array	Electrochemical	0.6 ng mL^{-1}	[134]
AF B1	Peanuts	cFLISA	FD	0.016 ng mL^{-1}	[135]
AF B1 and OTA	Wine and Beer	Enhanced indirect competitive ELISA	Optical	0.19–0.035 ng mL^{-1}	[136]
Citreoviridin	Rice powder	Indirect competitive ELISA	UV Absorbance	0.56 μg mL^{-1}	[137]
Fumonisin B1	Model samples	Immunomagnetic bead based indirect competitive ELISA	Optical	0.24 ng mL^{-1}	[138]
OTA	Various	Direct competitive ELISA	Chemiluminescence and UV absorbance	0.01–2.2 ng mL^{-1}	[139]
Sterigmatocystin	Cereal and Oil	Competitive ELISA	UV absorbance	0.06 ng g^{-1}	[140]
Various	Cereals and feed	Competitive ELISA	UV absorbance	N/A	[63]
Various	Poultry and corn	Competitive ELISA	UV Absorbance	0.24–3.0 ng g^{-1}	[141]
ZON/AF B1	Cereals	FLISA (quantum dot linked)	FD	0.2–10 μg kg^{-1}	[142]
ZON	Corn	biotin–avidin amplified ELISA	UV absorbance	0.35 ng mL^{-1}	[143]
ZON	Maize	Indirect competitive ELISA	UV absorbance	0.02 μg L^{-1}	[144]

The second noticeable point is linked to the first. A significant proportion of the mycotoxin group are naturally fluorescent and as such fluorescence has been a common method of detection. However, as we can observe the favoured method of detection has moved from this to tandem mass spectrometry which has greater flexibility in detecting and identifying various fluorescent and non-fluorescent toxins. The important contributing factor in this success was reduced cost of mass spectrometer technology.

The third point is the positive move from traditional HPLC to UHPLC. The use of smaller particles (2 μm over 5 μm) allows for greater surface area and higher pressures to be used, leading to better separation by increasing the theoretical plate numbers (as calculated by the van Deemter equation) [123]. The rate of separation is also increased improving cost/time/analysis ratio.

The de Saeger group developed a method for simultaneous determination of 23 mycotoxins in feed [120]. Following a solvent extraction and C_{18} SPE clean-up, UHPLC separation was performed followed by electrospray ionisation (ESI) MS/MS detection. This method fulfilled the criteria set by the EU on detection levels of all 23 compounds, with a range of 0.7–60.6 μg kg^{-1}. A similar method was applied in analysis of sweet peppers for the same range of toxins. The achieved LOD's were slightly lower due to less complex matrix [119]. Rasmussen used a solvent extraction followed by HPLC-ESI-MS/MS for determining 27 different toxins in silage with excellent inter lab reproducibility and repeatability [115]. In a similar work vein Van Pamel [116] used a UHPLC based method to achieve low LOD/LOQ, and high reproducibility and repeatability.

Grain, flour and cereal based products are important commodities prompt to mycotoxins contamination and as such are an obvious target for method development. Kokkenen obtained excellent results within the EU commission recommended levels for 31 targeted toxins using a solvent extraction followed by UHPLC-MS/MS [114]. Frenich used a UHPLC method linked with tandem mass spectrometry for the determination of 12 toxins in cereals [94], a method which was then turned to egg analysis with similar success [93]. Herebian performed a comparative analysis of different mass spectrometer detection, from a direct injection of crude extract resulting from a simple acetonitrile water extraction, looking to determine 32 toxins [95]. Both MS instruments exhibited very high sensitivity and repeatability in positive ionization mode; however, coupling of micro-LC to Orbitrap technology was not applicable to the negatively ionizable compounds. Zachariasova determined the presence of 11 *Fusarium* toxins using a UHPLC- TOF-MS method, which also relied on ^{13}C labelled standards [86]. Despite having a wide range of LOD's, and not being as sensitive as other published methods, this paper offered good scope for detecting the notoriously difficult targets such as *Fusarium* compounds. Similar results were demonstrated also by Santini [85].

Specialist foodstuffs, such as baby food, or "organically grown foods" are an important targets as they are a source of public concern and attention [78,90,91,117,121]. Beltrán published a UHPLC-MS/MS method for AFs and ochratoxins that was fast, and estimated to be extremely sensitive giving low ppt level detection [91]. An interesting study by Sulyok targeted foodstuffs that exhibited mould growth generated not from production, but post preparation contamination (i.e. mouldy food samples from private households). In this prospective study they found nearly 50 different analytes many of which had never been reported [88]. Tamura analysed beer using UHPLC and tandem mass spectrometry. As beer is produced from grains it is a potential source of toxins. Using a very rapid methodology Tamura was able to detect 15 toxins in 6.5 min at detection limits well below the legal levels [87].

Lacina developed a method of multiple mycotoxin determination that worked in several different matrices [121]., Rubert demonstrated a method for multiple analyte analysis in baby food [117], that was also transferred to the analysis of body fluids, in this case urine [118]. It should be noted that a 10 × improvement of LOD's (0.05–50 μg kg^{-1} in baby food vs. 0.5–10 ng mL^{-1}) in urine was obtained due to differences in matrix complexity. Other studies into blood plasma [92] and urine [50] yielded similar levels of compound detection.

While focus has moved to more generic methods capable of detecting a wide range of compounds, specific compound or family focussed studies are still important. These often rely on more generic detection methods such as fluorescence and UV, instead of

the gold standard mass spectrometry. A QuEChERS principle method for trichothecene determination in wheat flour has been published by Sospedra [84]. Here the study was focussed on solvent extraction followed by HPLC-MS to match the directive LOD's. Arroyo–Manzanares used laser induced fluorescence linked to HPLC for OTA detection. A simple liquid–liquid microextraction was used to reduce solvent requirements and concentrate the toxin at low ng L^{-1} level [40].

The AFs are arguably the most common dangerous toxins found and as such as are subject to strict regulations. Familial determinations of AFs were demonstrated by Zahn [73], Herzallah [74] and Khayoon [75]. These used traditional C_{18} reverse phase or multifunctional column HPLC followed by either UV, or fluorescence detection. All of these methods were able to detect the key toxins within the legal limits (0.5 ppb). Vosugh applied a simple SPE linked with HPLC-DAD to generate a second-order calibration using a bioinformatic model [72]. This allowed for the simplified analytical protocol to be used in complex samples. López-Grío used a highly sensitive UHPLC-MS for familial determination, while also detecting OTA [82].

AF M_1 is a lesser known AF that is not traditionally considered, however in certain matrices, such as milk and other dairy products it is a significant risk. Wang developed an effective protocol for detection of this specific toxin and chloramphenicol using HPLC – MS/MS [81], while Iha used a fluorescence detection method linked to HPLC for AF M_1 and OTA detection in dairy products [80] and human milk [79].

3.2.3. Considerations to using LC-MS based systems

The level of sensitivity gained by modern chromatographic and mass spectrometry equipment has revolutionised the field of trace analyte detection; even compared to equipment of 10 years ago. For example, Pizzutti [100] demonstrates how sensitive this instrumentation is, showing that a complete method (extraction, clean-up and analysis) doesn't reach the limit of potential sensitivity of the instrumentation. Key to the description of how well a method works is the final presented LOD; and in particular whether this is a method derived LOD compared to the instrumental LOD. That is how much the method used affects the sensitivity of a method. In all cases in this review we have tried to present the method limits, as the reviews and discussion in this paper is based around method analysis.

This takes into account of the matrix effect which can supress (or enhance in a few rare cases) the signal obtained from determination of a toxin. The type and nature of a matrix can strongly affect the suitability of a method. The review of Caprotti [68] discusses this in considerable detail.

It is also known that the matrix effect doesn't just influence sensitivity but also affects the choice of calibration. Ideally a matrix-matched calibration will be used in LC-MS protocols, where a sample of "clean" matrix is used with spiked samples to produce a calibration curve as this gives superior results when compared to an external calibration curve generated without matrix. This is preferable to a standard addition method where further error can be introduced into the system, and doesn't require extra analyses to be performed, reducing costs. Another potential method is to use a known standard of an isotope labelled compound for direct comparison within a sample, with the MS being able to generate the ion comparisons. This is an ideal method as it requires no need for extra preparation, or the generation of calibration curves that other methods do. In the works discussed here the use of matrix-matching is slightly more common, especially within determination methods for a wide range of toxins, (for example [108]) though isotope labelling methods do feature [83]. Validation of the LC-MS/MS methods are described by EU commission regulations (EC 657/2002), and many papers follow these guidelines. These suggest basic required performance characteristics included LODs, LOQs, linearity, recovery and precision. The sensitivity of a methodology also depends on its preparation. See discussion in Section 3.1.3.

In summary, chromatographic techniques will always be the gold standard against which all other techniques are compared. The extreme sensitivity and flexibility observed in published analyses, as highlighted by the ability to detect multiple analytes (sometimes over 50) at ppb levels in complex samples cannot be matched. However, LC methods will always suffer from portability, cost and practical issues, based around the sample type, the matrix effect, choice of calibration, sample preparation hence there is significant focus on other analytical methodologies, such as the development of biosensors, and the use of immunological techniques, as this are often designed to measure directly with minimal preparation.

3.3. Immunological methods (ELISA)

The antibody–antigen binding relationship is a critical molecular recognition event that is exploited for numerous scientific purposes. The ability to generate highly specific antibodies, be they mono-or polyclonal is relatively easy and well understood [124]. Immunochemical detection methods vary from simple lateral flow immunoassay and ELISA to highly sophisticated immunosensors. In her 2012 review Meulenberg gives an excellent explanation of how these antibodies are generated and incorporated within these assays [125,126]. It should be noted that given the size of most toxins they often require conjugating to a protein (often bovine serum albumin- BSA).

Here we look at the latest developments in using ELISA detection for toxin analysis and screening, as summarised in Table 4. The use of antibodies (and ELISA principles) as recognition elements in biosensors are discussed in Section 3.4.

Many ELISA kits for mycotoxin detection have been successfully commercialised in the past two decades. The portability of a test, and opportunity for minimal use (i.e. a customer requiring a single screening test instead of purchasing expensive analytical equipment) has meant that studies into ELISA methodology are still popular. Given the nature of the antibody–antigen complex multiple (familial) toxin analysis as well as single toxin analysis are possible [129,130,132]. Jiang has used an antibody which showed familial recognition to detect AFs B1 and M1 [70]. Rossi produced a protocol for AF B1 in feed [133] while O'Riordan used ELISA in a comparative nature in a study looking at HPLC preparative methods for AF analysis [34]. Klarić performed significant study concerning ELISA determination of 9 different toxins [63]. Tang et al. demonstrated an indirect method for ZON detection with an IAC sample preparation using the same antibody. This method proved to be highly sensitive with an LOD of 0.02 μg L^{-1}.

A number of research groups have attempted to modify the traditional ELISA methodology to improve sensitivity. Lamberti miniaturised the process into a microassay to reduce the required quantity of the sample/reagents and hence cost [128], while Yu utilised a chemiluminescent method instead of the traditional colorimetric techniques to increase the sensitivity of a method for detection of Ochratoxin A in soybeans [139]. A similar method was employed by Vdovenko et al. for AF M1 in milk [131].

Zhang replaced the enzyme conjugate that is traditionally linked to the antibody with a fluorescent quantum dot, which allowed for direct detection in a competitive style assay. This competitive fluorescence-linked immunosorbent assay (FLISA) gave excellent LODs for AF B1 in peanuts [135]. A similar method was also developed for ZON [142].

In a slightly different method presented by Piermarini an enzyme linked immunomagnetic electrochemical-array (ELIME-

array) was employed to screen for AFs in corn. The system is based on an indirect competitive ELISA format using magnetic beads as immobilisation support and magnetised screen-printed electrodes as electrochemical transducers [134]. Wang developed a similar method for Fumonisin B1 [138].

Soares developed a method using an aqueous two phase (salt-polymer) system to improve resolution for detection of OTA and AF B1 [136], while Huang used the biotin–avidin interaction to increase sensitivity instead of a second antibody reporter [143] In a similar vein, Urusov developed a three toxin ELISA based on the biotin – streptavidin reporter mechanism [141].

ELISA and ELISA formats are known to be reliable and excellent for screening, but the method is quite time consuming, requires specialist plate readers, if you wish to gain more information beyond simple screening, and is not suitable for field testing. Therefore the integration of suitable molecular recognition elements (immunochemical for example) directly with transduction systems is favoured for portable, non-laboratory analysis.

3.4. Biosensors

Since the development of the portable glucose sensor, the field of biosensors has been rapidly growing. Advances in fabrication techniques, decreased cost, increased sensitivity of transducers, and the desire to bring analyses out of the lab have led to significant interest in the development of such sensors [145]. Mycotoxins provide excellent targets for such studies, not only from the academic angle, but also from a commercial viewpoint. Interestingly, the development of analytical methods that would class as biosensors has considerably increased since the publication of our last paper. The number of articles presented in this section almost equals in number the other analytical studies presented here, and outweigh the chromatographic development significantly.

For ease of presentation we have elected to split these into sections based around the molecular recognition elements used in the presented methodology.

3.4.1. Immunological linked sensor systems

As discussed in section 3.3, the antibody–antigen interaction is a key system used for the detection of mycotoxins. In that section what would be considered traditional ELISA methods are discussed. The ubiquity of the relevant chemistry and biology is well understood and has led to the use of antibodies as toxin capture agents for electrochemical and optical analytical methods [146]. Table 5 summarises the numerous different sensors that have been developed in the past five years.

The ELISA methodology is easily transferred to other transducers, instead of the common colorimetric methods discussed in the previous section. Specifically a number of direct and indirect competitive assays (or as they are often known “sandwich” assays) have been developed for use with a number of different transducers.

Direct competitive methods using an electrochemical determination have been shown by Kadir for fumonisins [176], and Khan for OTA [191]. The former was based on a screen printed gold electrode while the latter was based on a conducting polymer surface. Neagu used a 96-well plate format to create an electrochemical sensor for AF M1. Transduction was based on an intermittent pulse amperometry technique [167]. Olcer developed a profiling technique via direct electrochemical ELISA for DON [173]. The enzymatic signal amplification allowed for real-time determination.

Panini used anti-ZEA monoclonal antibodies immobilised on magnetic microspheres to compete with a horseradish peroxidase conjugation. The enzyme was used to catalyse the oxidation of a substrate 4-tert-butylcatechol which could be measured electrochemically [217]. Peters used a multi component coloured microsphere array for colour-coded detection of multiple analytes based on direct competition [199]. These paramagnetic spheres allowed for capture and targeted analysis.

Mirasoli created an optical system for determination of fumonisins based on enzyme linked chemi-luminescence detection after a direct competitive immunochemical system, measured directly by a CCD camera [177]. Chun used a specific fluorescent conjugate label for the detection of ZON. The fluoro-label was bound to the analytes which was directly captured and measured using fluorescence polarisation [216].

Beizaei created a microarray format ELISA for AF B1 which relied on a classic colorimetric change [157]. He modified the reported phage developed nanobody for use as part of a sandwich assay for AF B1 [156]. The Taitt group developed a flow-cytometry method for measurement of fumonisin B1 and OTA, based on an indirect competitive method linked with labelled tracers [181]. The Visconti group produced similar sensors for DON [172] and OTA [187].

Tan developed a complex methodology for AF B1, where an alkaline phosphatase conjugated secondary antibody was used to catalyse hydrolysis of ascorbic acid-2-phosphate into ascorbic acid. This in turn reduced silver ions in solution to silver which deposited onto an electrode that was measured electrochemically [161]. Bonel demonstrated OTA determination using a nanostructured electrochemical device and α-naphtyl phosphate as the substrate [193]. Vig et al. used an impedometric analysis of AFs M1 in milk by measuring silver deposition on electrode [170]. Parker and Tothill have used cheap screen printed electrode based systems for AFs M1 and OTA [166,189],. A magnetoresistivity sensor for several toxins was developed by Mak, who used magnetic nanotags as labels on the secondary antibody in a sandwich format [203].

Jin et al. have labelled antibody for AF B1 with a gold nanoparticles. Upon binding the change of mass was detected by a piezoelectric sensor (QCM) [158]. A similar method was also shown by Urusov [190] for OTA using SPR as a transducer.

Kadota used SPR detection for simultaneous detection of DON and NIV based on a cross-reacting antibody [183] SPR is an effective, flexible and sensitive transducer that has been used by several groups for a variety of toxins [168,188,197,215] The Saiki group developed a classic indirect ELISA for OTA but then expanded the study to a flow immunoassay based around kinetic exclusion and fluorescence measurements [192]. Both systems were shown to detect OTA excellently in cereals and wines.

Indirect competitive immunoassays have also been developed to analysis multiple toxins simultaneously. Wang used an immunochip, bearing a variety of antibodies to detect AF B1, OTA, DON, ZEA and T-2 toxin in one compound analysis [208]. The same group also developed a suspension based indirect competitive immunoassay on antibody coated microspheres for detection of 4 toxins [206]. In a similar system to the immunochip work presented by Wang, Lattanzio generated a multiplex dipstick sensor for fusarium toxins [207]. The de Saeger group created flow through assays (gel and membrane based) for detection of OTA and other toxins [191,200,210,196], Raman spectroscopy offers a sensitive platform for transduction by linking a gold particle to the immunochemical recognition element (signal antibody). A simple sandwich ELISA can be performed bringing the Au particle close to the surface allowing for measurement. This is shown by Liu for zearalenone [212] and Ko for AF B1 [155].

A QCM method based on direct binding of an AF B1 – protein conjugate was shown by Vidal where the antibodies were fixed straight to the piezoelectric surface and the mass changes measured [185]. Funari used this approach for patulin [198] and Chauhan for AF B1 from groundnuts [153].

Table 5
Biosensor systems that utilise the antibody–antigen immunochemical relationship for toxin-specific molecular recognition.

Toxin	Matrix	Recognition method	Detection method	LOD	Reference
Total AF	Peanut	Immunochromatographic strip (ICS)	Colorimetric	0.03 ng mL^{-1}	[147]
Total AF and OTA	Model samples	Direct binding	Microcantilever	3–6 ng mL^{-1}	[148]
AF B1	Olive Oil	Direct binding	Electrochemical impedance spectroscopy	0.03 ng mL^{-1}	[149]
AF B1	Model samples	Direct binding onto antibody modified carbon nanotube	Electrochemical	0.08 ng mL^{-1}	[150]
AF B1	Maize	Direct binding onto immunochromatographic chip	FD	0.42 pg mL^{-1}	[151]
AF B1	Various foods	Direct binding	Electrochemical enhanced by ionic liquids	1 fM	[152]
AF B1	Groundnut	Direct binding	Piezoelectric	0.1 ng mL^{-1}	[153]
AF B1	Model samples	Direct binding	FD (intrinsic quenching)	0.35 ng mL^{-1}	[154]
AF B1	Model samples	Antibody coated nanoparticles and magnetic beads	Surface enhanced Raman spectroscopy	0.1 ng mL^{-1}	[155]+
AF B1	Various	Phage-probe linked immunoassay	Colorimetric	0.117 ng mL^{-1}	[156]
AF B1	Wheat	Indirect competitive immunoassay	Colorimetric	15 pg g^{-1}	[157]
AF B1	Model samples	Indirect competitive immunoassay	Piezoelectric	0.01 ng mL^{-1}	[158]
AF B1	Various	lateral-flow immunodipstick assay	Colorimetric	0.1 ng mL^{-1}	[159]
AF B1	Model samples	Lateral-flow assay	Colorimetric	10 μg mL^{-1}	[160]
AF B1	Rice	Indirect competitive ELISA	Electrochemical	0.06 ng mL^{-1}	[161]
AF B1	Model samples	Antibody coated RnNi nanoparticles immobilised to an indium-tin oxide surface	Electrochemical	32.7 ng dL^{-1}	[162]
AF B1	Model samples	antigen-modified magnetic nanoparticles	Antibody functionalized upconversion nanoparticle Signal probes.	0.01 ng mL^{-1}	[163]
AF B2	Nuts	lateral-flow immunodipstick assay	Colorimetric	0.9 ng mL^{-1}	[164]
AF M1	Milk	Impedimetric assay	Electrochemical	1 pg mL^{-1}	[165]
AF M1	Milk	Direct competitive ELISA	Electrochemical	39 ng L^{-1}	[166]
AF M1	Milk	Direct competitive ELISA	Electrochemical	5 pg mL^{-1}	[167]
AF M1	Milk	Direct binding	Surface Plasmon enhanced fluorescence	0.6 pg mL^{-1}	[168]
AF M1	Milk	Two-step lateral flow immunoassay	Colorimetric	0.02 μg mL^{-1}	[169]
AF M1	Powder Milk	Indirect competitive ELISA	Electrochemical	15 ng L^{-1}	[170]
DON	Wheat and Maize	Direct binding to polymer coated quantum dots	FD	220–500 μg kg^{-1}	[171]
DON	Wheat	Competitive immunoassay	Fluorescence polarisation	120 μg kg^{-1}	[172]
DON	Wheat	Direct binding	Electrochemical	6.25 ng mL^{-1}	[173]
DON	Wheat and maize	Immunochromatographic strip (ICS)	Colorimetric	50 ng mL^{-1}	[174]
DON/ZON	Maize and wheat	Competitive inhibition immunoassay	Surface Plasmon resonance	10–17 ng mL^{-1}	[175]
Fumonisins	Corn	Indirect competitive ELISA	Electrochemical	5 μg L^{-1}	[176]
Fumonisins	Maize	Direct competitive ELISA	Chemi-luminescence	2.5 μg L^{-1}	[177]
Fumonisins	Maize	Immunodipstick assay	Colorimetric	2.5 ng mL^{-1}	[178]
Fumonisins	Maize	Lateral flow immunoassay	Colorimetric	199 μg kg^{-1}	[179]
Fumonisins	Beer	Direct competitive magnetoimmunoassay	Electrochemical	0.33 μg L^{-1}	[180]
Fumonisin B1 and OTA	Grains	Microsphere linked indirect competitive fluid array	Fluorescent flow cytometry	10–100 ng g^{-1}	[181]
HT-2	Model Sample	signal transduction by ion nano-gating (STING)	Electrochemical	100 fg mL^{-1}	[182]
Nivalenol and DON	Wheat	Indirect competitive immunoassay	Surface plasmon resonance	0.05 mg kg^{-1}	[183]
OTA	Model samples	Label-free immunosensor	Electrochemical impedance spectroscopy SPR	0.01 ng mL^{-1} 1 ng mL^{-1}	[184]
OTA	Model samples	Direct immobilisation	Piezoelectric	10 ng mL^{-1}	[185]
OTA	Model Samples	Mimotope peptide antibody based lateral flow strip	Colorimetric	~10 ng mL^{-1}	[186]
OTA	Wheat	Competitive immunoassay	Fluorescent polarization	0.8 μg kg^{-1}	[187]
OTA	Cereal and beverages	Competitive immunoassay linked to gold nanoparticles	SPR	0.042 ng mL^{-1}	[188]
OTA	Wine	Indirect competitive immunoassay	Electrochemical	0.5 μg L^{-1}	[189]
OTA	Model samples	Colloidal gold- antibody conjugate	SPR	60 pg mL^{-1}	[190]
OTA	Model samples	Direct immunoassay	Electrochemical impedance spectroscopy	N/A	[191]
OTA	Wine and cereals	Indirect competitive immunoassay	Fluorescence	0.2 μg L^{-1}	[192]
OTA	Wine and cereals	Flow immunoassay	Fluorescence	0.01 μg L^{-1}	[192]
OTA	Wheat	Direct competitive ELISA	Electrochemical	0.86 ng mL^{-1}	[193]
OTA	Model Sample	Nanostructured ZnO supporting antibodies	Electrochemical	0.006 nM L^{-1}	[194]
OTA	Model Sample	Cerium oxide nanoparticles/ITO coated glass slide	Electrochemical	0.25 ng dL^{-1}	[195]
OTA	Red wine	Flow-through gel and membrane based direct competitive assays	Colorimetric	2 μg L^{-1}	[196]
Patulin	Model samples	Competitive immunoassay	SPR	0.1 nM	[197]
Patulin and parathion	Apple puree	Sandwich assay	Piezoelectric	50 -140 nM	[198]
Various	Barley	Direct inhibition assay	Colorimetric via targeted reporter microspheres	2–1000 μg kg^{-1}	[199]

Table 5 (*continued*)

Toxin	Matrix	Recognition method	Detection method	LOD	Reference
Various	Cereals grains and silage	Flow-through membrane based direct competitive assays	Colorimetric	2.5 μg L^{-1}	[200]
Various	Model samples	Lateral flow immunoassay	Colorimetric	0.05–3 μg kg^{-1}	[201]
Various	Maize	Indirect competitive lateral-flow immunoassay	Chemiluminescence via CCD	1.5–6 μg kg^{-1}	[202]
Various	Model samples	Direct binding	Magnetoresistivity	50 pg mL^{-1}	[203]
Various	Cereals	Multiplex photonic crystal microsphere suspension array	Optical	0.5 pg mL^{-1}	[204,205]
Various	Corn and peanut	Indirect competitive immunoassay	Colorimetric	0.22 pg g^{-1}	[206]
Various	Cereals	Indirect competitive immunoassay	Colorimetric	80% required cut off levels – EU directive	[207]
Various	Drinking water	Indirect competitive immunoassay	Colorimetric	0.04–35.6 mg mL^{-1}	[208]
Various	Cereals	polyvinylidene fluoride (PVDF) membrane-based dot immunoassay	Colorimetric	20–1000 μg kg^{-1}	[209]
Various	Various	Flow-through gel and membrane based assays	Colorimetric	3 μg kg^{-1}	[210]
Zeranol	Bovine tissue	Chemiluminescent enzyme immunoassay	Optical	0.05 μg kg^{-1}	[211]
ZON	Feed	Competitive immunoassay llinked to gold nanoparticles	Surface enhanced Raman Spectroscopy	1 pg mL^{-1}	[212]
ZON	Wheat	Non-competitive immunoassay	Fluorescence resonance energy transfer (FRET)	0.8 ng mL^{-1}	[213]
ZON	Corn	Immunochromatographic test strip	Colorimetric	3.4–20 μg kg^{-1}	[214]
ZON	Cereal	Phage-probe linked rapid-dot immunoassay	Colorimetric/SPR	50 μg kg^{-1}	[215]
ZON	Corn	fluorescence polarization immunoassay	FD	137 μg kg^{-1}	[216]
ZON	Feed	Direct competitive immunoassay via paramagnetic beads	Electrochemical	0.41 μg kg^{-1}	[217]
ZON	Corn	Indirect competitive immunoassay	Colorimetric	2.5 ng mL^{-1}	[218]
ZON	Model samples	Metal-oxide semiconductor field effect transistor	Electrochemical/SPR	0.1 μg mL^{-1}	[219]
ZON	Wheat	Flow-through gel and membrane based assays	Colorimetric	100 μg kg^{-1}	[220]

LOQ. Limits of Quantification.

Riccardi created a microcantilever sensor for AF and OTA [148]. Bacher developed an impedimetric sensor for direct detection of AF M1 [165] Other direct binding systems for AF B1 have been developed with a variety of transducers including electrochemical [149,150,152] and fluorescence [151,154,213]. For example Speranskaya used direct binding to fluorescent CdSe quantum dots as a reporter mechanism. Binding of the target toxin altered the fluorescent signal allowing for direct toxin quantification [171].

Zamfir developed a label –free OTA sensor based on antibody functionalised magnetic nanoparticles that were also bound to a gold surface [221]. Binding of OTA resulted in signal detected by both SPR and electrochemical means [184]. This was shown to be equal in sensitivity to a standard commercial competitive ELISA kit. In a similar protocol Dorokhin have used imaging SPR to detect DON and ZON [175].

A slightly different label-free method was shown by Xu et al. who employed gold nanorods coated with antibodies to detect AF B1. The nanorods under experimental conditions clump and the process of binding of the toxin causes dispersion which was measured by dynamic light scattering [222].

The use of magnetic nanoparticles has also being explored by Wu who had used a conjugation method between a magnetic capture, and a secondary sensing particle to independently detect and quantify two different toxins in solution. Each sensing particle is colour specific and set for an individual toxin allowing for independent measurements [163]. Jodra used a corresponding method to capture magnetically labelled particles carrying specific antibody–enzyme complex. Here fumonisin detection was demonstrated via electrochemical means [180]. Ansari used a nanostructured ZnO support to hold OTA specific antibodies for electrochemical detection. The large surface area increased the sensitivity of the surface to the impedimetric measurements resulting in a highly sensitive system [194]. Kalita used Ni nanoparticles immobilised in an ITO surface to support anti AF B1 antibodies for detection of that toxin [162]. This is similar to the work of the Malhotra group who used cerium oxide particles [195].

Actis et al. created an electrochemical system based on signal transduction by ion nano-gating (STING), which uses a functionalized quartz nanopipette as an electrochemical biosensor. Using a tailored nanopipette to bear antibodies for HT-2 they achieve detection limits of 100 fg mL^{-1} [182].

The more traditional lateral flow sensor, where the recognition element is held on a membrane and the presence is detected by a colour change is an obvious area that can be exploited [223]. This is similar to the common pregnancy test than you might find over the counter and is well understood. This technique has also been explored by various groups, each with slight variations on the chemistries used, including the incorporation of labelled nanoparticles or specific conjugated labels [104,147,159,160,164,169,174,178,179,202,209,214,218]. Of particular interest is the work of Lai who used a mimotope peptide to mimic OTA for development of a lateral flow system [186].

Another sensitive and practical method is demonstrated by the Toximet “Toxiquant” system which utilises a two stage process. A sample clean-up cartridge followed an immunoaffinity SPE style cartridge captures the toxin which is directly measured on the cartridge by a purpose designed fluorimeter. It is able to distinguish between different toxins based on their fluorescent spectra with LOD's demonstrated around 0.04–0.20 ng mL^{-1} [224]. This method has gained significant recognition in the markets where expensive and complex equipment is not practical due to its simplistic and direct practical methodology.

It is clear that the recognition afforded by an antibody is the most employed molecular recognition element in sensors. However, a wide range of other recognition elements exist which will be discussed in the following section.

3.4.2. Sensor systems linked with other biological recognition elements

The alternatives to antibodies used in sensing and analytical protocols are summarised in Table 6 and include enzymes, peptides and aptamers. The first two are summarised in Table 6 and the latter in Table 7.

The first are enzymes that are known to interact directly with the toxin substrate. The prime example here is that of aflatoxin–oxidase, which targets aflatoxins and related compounds. Yao

Table 6
Biosensor systems to incorporate enzyme or peptides as the recognition element.

Toxin	Matrix	Method	Detection method	LOD unless stated otherwise	Reference
AF B1	Model samples	Aflatoxin oxidase bound to carbon nanotube-gold electrode	Amperometry	1.6 nmol L^{-1}	[226]
OTA	Wine	Peptide Based ELISA	Colorimetric	2 μg L^{-1}	[228]
OTA	Beer and Coffee	Immobilised peroxidase on a screen printed electrode	Amperometry	0.1 ng mL^{-1}	[227]
OTA	Red wine	Synthetic peptide bound to chitosan support	Chemiluminescence	0.5 μg L^{-1}	[229]
Sterigmatocystin	Model samples	Aflatoxin oxidase bound to carbon nanotube-gold electrode	Amperometry	3 ng mL^{-1}	[225]

and Liu group have used this enzyme to generate an amperometric sensor for AF B1 and steragmatocystin [225,226]. In both cases the enzyme is fixed to the walls of carbon nanotubes which in turn are absorbed to an Au electrode surface. Oxidation of the toxin at the sensor surface provides a measurable response well within the required detection limits set by the EU. An alternative enzyme Horseradish peroxidase (HRP) was used by Alonso–Lomillo to detect OTA. The enzyme was fixed to a screen printed electrode via polypyrrole. Oxidation of OTA led to a reduction in HRP activity which was further measured [227].

Peptide-based sensors which use synthetic peptide sequences to mimic the antibody recognition have been demonstrated. Bazin used a peptide sequence that recognised OTA as a replacement in a direct ELISA based assay [228]. Soleri used a similar peptide targeting OTA but bound it to a chitosan foam support. Any OTA in a sample was conjugated with HRP which was used for a chemiluminescent reaction for measuring OTA presence [229].

Aptamers are a novel and particularly useful targeting tool with a unique ability to bind to a variety of targets including small molecules, peptides and proteins [230]. Aptamers are single stranded oligonucleotides that vary in size between 25 and 50 bases long and are derived from combinatorial libraries through selective targeting. They offer unique benefits compared to other targeting agents, in that they bind with high affinity and selectivity, are easily and quickly synthesised using *in vitro* techniques, and are relatively thermally stable and consistent [231]. These characteristics make them attractive alternatives to antibodies and peptides in diagnostic [232,233] and therapeutic agents [234·235],. Aptamers have been used extensively in a variety of assay and sensor formats [232,233] although these remain primarily at research and academic settings due to the early stage of the technology and issues associated with their susceptibility to enzymatic and chemical degradation. Table 7 highlights aptamer based assays and sensors targeting mycotoxins.

The flexibility of using an aptameric system has led to a number of different methods.

Examples of direct binding have been demonstrated by several groups using a wide range of transducers. Zhu [242] developed a SPR based assay for OTA The aptamer was attached to the sensor by using the streptavidin-biotin linker, and binding measured directly. An alternative method was demonstrated by Park [238] who bound a gold nanorod coated in aptamer to a glass surface creating a miniature SPR zone which could be directly interrogated, removing the need for a specialised chip. In a similar method using aptamer modified gold nanoparticles the Akkoca group used electrochemical impedance spectroscopy and cyclic voltammetry to measure interactions of these particles bound in a cystamine layer, detecting AF m1 in milk [241]. Castillo used the same analytical measurement techniques to study a dendrimer based support structure to which aptamers specific for AFB1 were attached [236].

Direct binding to other forms of nanoparticles have been shown. Both Wang [248] and Wu [252] used spectroscopic methods to detect OTA binding to modified fluorescent nanoparticles. Marty group used paramagnetic beads linked with a capture aptamer to develop electrochemical flow sensors for OTA in alcoholic beverages [246,247].

Table 7
Aptamer based biosensor for mycotoxins.

Toxin	Matrix	Method	Detection method	LOD unless stated otherwise	Reference
AF B1	Peanuts/corn	Dendrimer –linked aptameric capture	Electrochemical	0.40 nM	[236]
AF B1	Hay/rice	Competitive DNA interaction	PCR	25 fg mL^{-1}	[237]
AF B1	Corn	Direct binding	SPR	1 nM	[238]
AF B1	Corn	Competitive DNA interaction	Fluorescence	0.1 ng mL^{-1}	[239]
AF B1	Corn	Competitive assay	Chemiluminescence	0.1 ng mL^{-1}	[240]
AF M1	Milk	Direct binding	Electrochemical	1 ng mL^{-1}	[241]
OTA	Wine/peanut oil	Direct binding	SPR	0.094 ng mL^{-1}	[242]
OTA	Model samples	Enzyme mimic formation	Colorimetric	~1 nM	[243]
OTA	Corn	Structure switching	FD	0.8 ng mL^{-1}	[244]
OTA	Wheat	Structure switching	FD	2 pg mL^{-1}	[245]
OTA	Wine	Indirect competitive/direct competitive magnetic beads	Colorimetric	0.11 ng mL^{-1}	[246]
OTA	Beer	Indirect competitive/direct competitive magnetic beads	Colorimetric	0.05 μg L^{-1}	[247]
OTA	Model samples	Direct binding to quantum dot labelled magnetic beads	FD	5.4 pg mL^{-1}	[248]
OTA	Wheat	Competitive removal of DNA	Electrochemiluminescence	0.007 ng mL^{-1}	[249]
OTA	Wine	Competitive removal of DNA leading to exonuclease action	Electrochemiluminescence	0.64 pg mL^{-1}	[250]
OTA	Model samples	Binding leads to protection from endonuclease action	Colorimetric	0.4 pg mL^{-1}	[251]
OTA/Fumonisin B1	Model samples	Direct binding to fluorescent nanoparticles	Fluorescence resonance energy transfer (FRET)	0.05 ng mL^{-1}	[252]
OTA/Fumonisin B1	Cereals	Competitive removal of DNA from photonic crystal array	FD	0.25 pg mL^{-1}	[253]

Competitive DNA interactions have been used by several groups for sample detection. Here the mycotoxin competes with a complimentary strand of DNA, which generates a signal. The aptamer can be bound to a surface and allowed to interact with complimentary DNA strand. Mycotoxin binds to the aptamer and disrupts its interaction with complementary DNA sequence that can be measured. Examples of these assays rely on the signal derived from PCR amplification [237], measurement of the fluorescent intensity of attached particle labels which are quenched/excited by binding [239,244,245,253]; luminescence [156]; or enzyme labelling of one of the DNA strands [249,250]. These techniques are extremely sensitive with detection levels in the lower pg mL^{-1} (ppt) range.

Zhang developed method which relied on ability of OTA to protect DNA from endonuclease action; therefore a drop in DNA concentration generated signal proportional to mycotoxin concentration, which can be detected at low ppt levels [251].

Lee showed a conformational system where upon binding of OTA to a hairpin aptamer, structural changes occur to allow the formation of an active G-quadruplex which acts as a catalyst for a colorimetric reaction [243].

The use of aptamers as a recognition elements offers exceptional sensitivity of the detection (the sensors described here are some of the most sensitive presented in this paper); suggesting that these systems might be considered for commercialisation assuming that complexity of the methods can be improved. While they have superiority in terms of sensitivity, they rely on specialist analytical equipment, something that more traditional antibody based systems such as ELISA or lateral flow assay have overcome.

3.5. Other emerging detection technologies

Other potential replacements for antibodies are recognition elements that are not based around biological materials as discussed in the next section. There are situations where a biological recognition element is too unstable in unfavourable environmental conditions or a chromatographic method is too impractical. Therefore the development of artificial systems for recognition of mycotoxins is a key development area [254].

Molecularly imprinted polymers (MIPs) are leading the way in the development of biological molecular recognition mimics. They are prepared by polymerisation of appropriate functional monomers in the presence of a target molecule. After polymer formation the original template is removed, leaving cavities that are complementary to the shape and chemical profile of the template (and related compounds) (Fig. 1), allowing specific recognition and rebinding of the targeted analytes.

MIPs can be used in numerous formats, from the basic "crushed" particulates, to membranes, films, nanoparticles and supported coatings [255,256]. Mycotoxins have been long studied as targets for molecular imprinting and commercial examples of relevant polymers are available [257]. Table 8 gives some examples of imprinting polymers for mycotoxins in the past 5 years.

The most common method is using MIPs as a solid phase extraction material to selectively pre-concentrate the toxin while cleaning the sample for further analysis, often performed by chromatography [39,258,259]. However MIPs have an inherent flexibility in their use as shown by Fang, who created fluorescently active MIP-coated quantum dots sensitive to ZON [260]. Choi grafted an electropolymerised layer of imprinted polypyrrole onto the surface of an SPR sensor, which was used for direct binding and measurement of the target toxin [261].

Inorganic materials have also been used to create sensors for mycotoxins. Non-specific inorganic materials have been used as supports for antibodies. Hu used smectite (a group of clay minerals known to absorb AF B1) to produce a toxin-bound surface for direct fluorescent measurement of the aflatoxin ($\lambda_{max} = 430$ nm) [262].

Todescato studied the effects of metal enhanced fluorescence for OTA detection. OTA was labelled with a fluorescent probe and absorbed on Ag surface. Silver increases the intensity of the generated signal allowing for enhanced detection (0.5 μg kg^{-1}) [263]. Other spectroscopic methods include the use of NIR for detection of AF B1 in maize and barley [264]. By spectral pattern analysis the presence of the toxin was measured directly on the grain [265].

The Karp group created a whole-cell sensor based on the genetically modified *Saccharomyces cerevisiae* strain. The presence of the toxin, which is an estrogenic compound, affects activity of firefly luciferase-enzyme leading to enhanced light emission in the presence of the substrate D-luciferin [266]. The emissions were shown to be proportional to the concentration of AF B1.

Ricci used a hydrolysis method (via microwave) for detection of DON and nivalenol. The hydrolysis products could be measured directly using screen-printed electrodes as they were electroactive. A reasonable detection limit was observed (1.1 μg kg^{-1}) in cereals using extremely rapid detection protocol that can be performed in a minute [267].

4. Conclusions and future outlook

It is clear from the level of activity shown in this field that mycotoxin analysis continues to be critically important. The analytical field of mycotoxin detection serves not only to bolster the needs of the food industry, but also as a test bed for new analytical methodologies.

A broad range of detection and determination techniques used for practical analysis and detection of a wide spectrum of mycotoxins are available, with many showing very high levels of sensitivity. With chromatographic analysis and significant improvements in availability for mass spectrometry equipment, the trends have moved from determination of single compound or familial compound analysis to simultaneous detection of multiple targets, headed up by broad compound clean-up steps such as DaS and QuEChERS. However, while lab based methods will always be preferred for final determination of sample contamination, the need to take a method *in ager* will need to be accounted for. For more portable analysis, technologies based on antibody recognition are still proving popular, with numerous examples ranging from traditional ELISA and lateral flow devices to more esoteric ones. The latter group is rapidly expanding providing interesting analytical methodologies for the use in other areas. Finally new technologies,

Table 8
MIPs for mycotoxins.

Toxin	Matrix	Method	Detection method	LOD unless stated otherwise	Reference
OTA	Wine	SPE	HPLC-FD	0.075 ng mL^{-1}	[259]
OTA	Cereals	SPE	HPLC-FD	2.5 μg kg^{-1}	[39]
OTA	Ginger	SPE	UHPLC-FD	0.09 ng mL^{-1}	[258]
ZON	Corn, Rice, Wheat	Optosensing material based on ionic liquid (stabilized CdSe/ZnS quantum dots	SEM/FTIR	0.002 μmol L^{-1}	[260]
ZON	Corn	Electropolymerisation onto surface	SPR	0.3 ng mL^{-1}	[261]

such as MIPs, aptameric systems or novel spectroscopic and electrochemical methods are shown to be highly effective with detection limits in the sub ppt levels. In portable instrumentation, no one technique can be said to be superior to another so this leaves a lot of scope for continuing development in this field.

In summary we envisage that the field will continue to grow, due to the importance of mycotoxins detection in food and environment.

Appendix A. Supplementary information

Supplementary information related to this article can be found at http://dx.doi.org/10.1016/j.aca.2015.10.013.

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