Current methods of analysis for the determination of trichothecene mycotoxins in food

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This article describes the trends in analytical techniques for the determination of trichothecene mycotoxins, namely deoxynivalenol, and T-2 and HT-2 toxins in cereals and cereal products with particular emphasis on screening and rapid approaches. The driving force behind the changing methodologies is mainly attributed to legislative demands. However, for commercial and governmental testing laboratories, the need to use validated official methods is ever increasing to ensure quality assurance of results.

Much research has been undertaken to improve screening assays, highlighted by the number of new methods using a variety of formats and platforms, including optical and electrochemical biosensors. Significant advances in the traditional reference methods have also been demonstrated in addition to the emergence of a variety of commercial immunoaffinity and solid-phase extraction columns for clean up. The use of liquid chromatography coupled to tandem mass spectrometry for mycotoxin detection is ever increasing, allowing simultaneous determination of many toxins in various sample matrices.
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1. Introduction

The trichothecene mycotoxins form a large chemically diverse group of compounds produced as secondary metabolites of fungi that have been linked to human and animal diseases [1,2] through the consumption of contaminated grains (e.g., wheat, oats, barley, maize and rice) [1–3]. Trichothecenes are mainly produced by *Fusarium* species, and they are globally distributed, even in more extreme environments [4]. While *Fusaria* produce a great range of trichothecenes [5], other fungal genera known to produce these toxins are *Trichoderma*, *Stachybotrys* and *Myrothecium* to name a few [1,4]. The trichothecenes are categorized as non-macrocyclic or macrocyclic, depending on their structures, and the focus of this review will be on the non-macrocyclic compounds that are further sub-divided into type A or type B trichothecenes. The type A trichothecenes are so classified due to the presence of a hydrogen or an ester group at the C-8 position and include T-2 toxin, HT-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS), while the type B trichothecenes [e.g., deoxynivalenol (DON), nivalenol (NIV), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON) and fusarenone-X] contain a ketone group at this position [2]. Fig. 1 highlights the structures of the type A and type B trichothecenes of interest.

Obvious clinical signs of trichothecene infection in animals include feed refusal and vomiting, growth retardation, reproductive disorders, blood disorders, dermatitis, oral lesions and depression of the immune response [1,6]. While all species are affected by these toxins, some are much more sensitive (e.g., swine and poultry). By contrast, ruminants are less affected, due to their ability to metabolize the trichothecenes into less toxic metabolites [7,8].
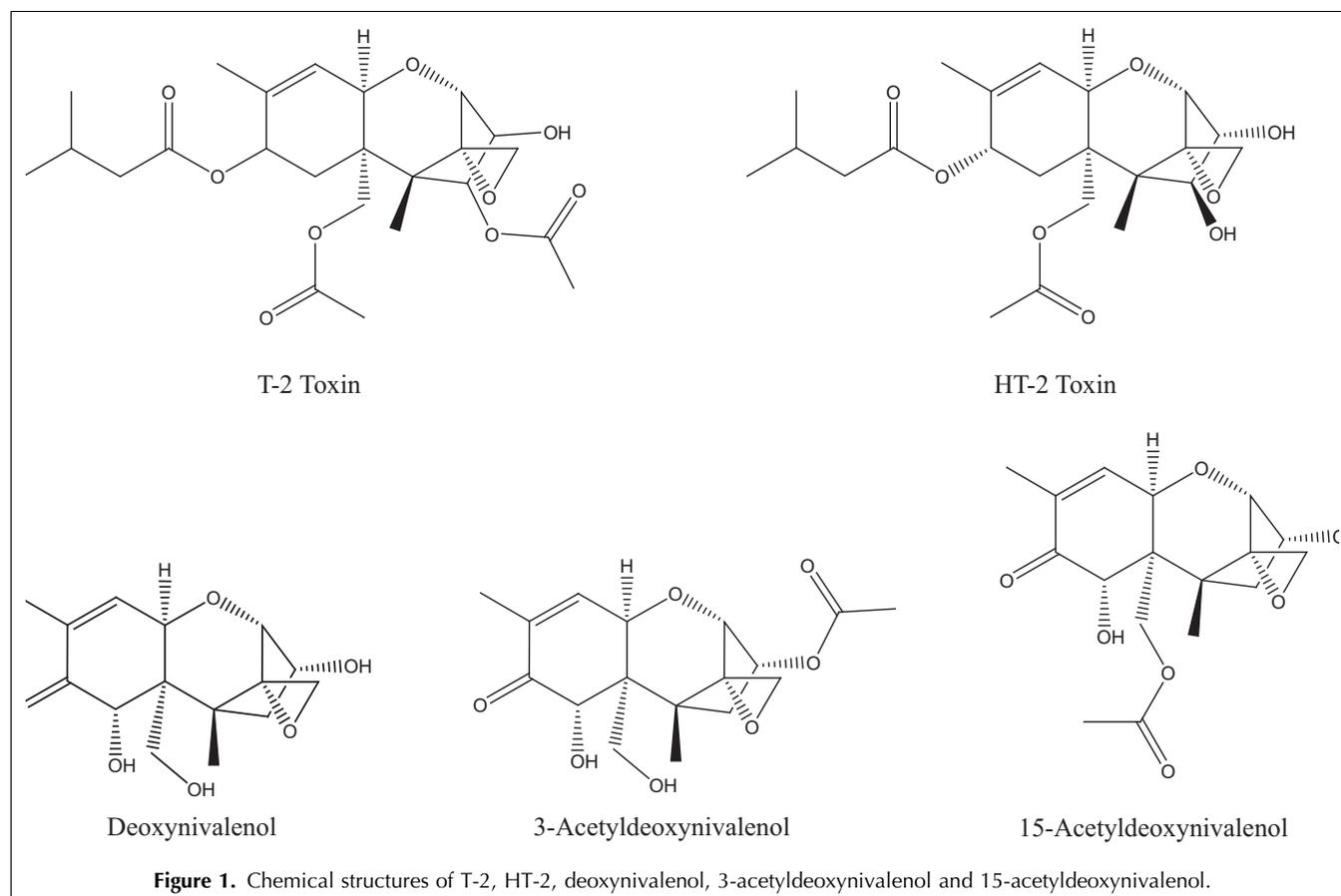
Not only are these compounds pathogenic to humans and animals, their phytotoxic nature is also of great importance in the global trade of cereal crops, as low

Figure 1. Chemical structures of T-2, HT-2, deoxynivalenol, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol.

yields may have devastating economical implications. In plants, the effects exhibited include growth retardation, wilting, chlorosis, necrosis and inhibition of germination [6]. However, as with ruminants, plants possess mechanisms by which they can reduce the toxicity of trichothecene mycotoxins by incorporating the toxin into the plant matrix or chemical modification. An example of chemical detoxification is illustrated by DON which has the ability to bind to glucose to form the less toxic DON-3-glucoside [9]. While it was commonly known that trichothecene mycotoxins inhibit protein synthesis in eukaryote cells [1–3], it is now widely accepted that DNA and RNA synthesis are inhibited, as is mitochondrial function, and that cell-membrane alterations and apoptosis can occur [6].

As trichothecenes are a global problem, many reviews reporting their occurrence in foods have been published. The CAST report [1] summarized early reports detailing the presence of trichothecenes in wheat, flour, bread, cornmeal, peanuts, pecans and moldy supermarket foods. Later reviews highlighted the presence of deoxynivalenol and nivalenol in wheat, barley, maize, oats, rice and rye, with some of the levels detected being well above the regulatory limits agreed [10].

In 2004, results from the SCOOP task 3.2.10 Report [11] detailing the occurrence of *Fusarium* toxins in food in member states of the European Union (EU) was published. Most of the occurrence data available for deoxynivalenol was for wheat, but data for barley, oats, rye and corn was included, indicating that corn had the highest levels of contamination of the toxin with 57% of the 11,022 samples proving positive. For T-2 toxin, 3490 samples (wheat, barley, oats, rye and corn) were analyzed, of which 20% were positive, while the analysis of 3032 samples for HT-2 toxin reported that 14% were positive.

Those samples found positive contained concentrations above the limits of detection (LODs) – for DON, these varied in the range 2–250 μg/kg; for T-2 toxin, they were 2–50 μg/kg; and, for HT-2 toxin, they were 10–40 μg/kg. The report also examined dietary intake and it was highlighted that, for DON in the most part, consumption fell below the tolerable daily intake (TDI) (see next section) of 1 μg/kg body weight. However, for infants and young children, the figures were very close to, or in some instances exceeded, this value. In the case of T-2 and HT-2 toxins, the estimated dietary intake was greater than the TDI of 0.06 μg/kg body weight, although the concentrations calculated were strongly influenced by the LODs of the analytical methods employed. In this instance, the concept of reducing levels of contaminants to as low as reasonably achievable (ALARA) should be applied. Producers are encouraged

to introduce measures to minimize the occurrence of such toxins to protect public health, and Commission Recommendation 2006/583/EC outlines these [12].

Desjardins presented a comprehensive review [13], looking at the geographical regions of North America, South America, Europe, Africa, the Middle East and South Asia, East Asia, and finally Southeast Asia, Australia and New Zealand. Deoxynivalenol was the predominant trichothecene found in wheat, maize and other cereals in Canada and the USA and again in South America, although occasionally other trichothecenes were detected. In Europe, deoxynivalenol is certainly the most significant trichothecene contaminant, but regular contamination of grains with T-2 toxin and HT-2 toxins has been reported [13], and food and feed inevitably may contain trichothecenes, leading to the exposure of adults and children to these toxins. In The Netherlands, for example, a study was carried out on the occurrence of various mycotoxins in duplicate diets of adults and children. In practically all children's duplicate diets investigated (123 samples) DON could be quantified [13], although exposure was in all cases below the TDI, as established by Joint Expert Committee on Food Additives, (JECFA) (see next section). In Africa, although data is limited, deoxynivalenol and nivalenol seem to be the most prevalent, and, similarly, in the Middle East and South Asia, although T-2 has also been detected at very high levels in wheat and flour. Both deoxynivalenol and nivalenol have been reported in cereal grain samples in Japan, South Korea and China and it is a similar story for Southeast Asia, Australia and New Zealand [14].

This occurrence data is extremely important in determining the risks posed by the trichothecenes both to humans and animals; however this forms the basis of only part of the risk assessment that leads to the establishment of regulations. Other factors pertaining to the inception of regulatory limits include toxicological data, knowledge of the distribution of toxin concentrations within a cereal lot, the availability of analytical methods for the determination of trichothecenes, knowledge of the legislation in the countries where trade contacts exist, and, of course, of paramount importance is the need for a sufficient food supply [15].

The development of international legislation for food and feeds lies with the Codex Alimentarius Committee (CAC) and is based on all the elements listed above, but the toxicological effects of these toxins are fundamental and provide the basis of all regulatory limits. JECFA performs these risk assessments by evaluating the scientific data available, in particular the determination of a No Observed Adverse Effect Level (NOAEL) in animal toxicological studies. An uncertainty or safety factor is then applied and the results presented as a figure representing the Acceptable Daily Intake (ADI, for avoidable substances), or the Tolerable Daily Intake (TDI, for unavoidable substances, e.g., mycotoxins) in

mg/kg bodyweight, of the food additive/contaminant [15,16]. For trichothecenes, JECFA has established provisional maximum TDIs for DON and T-2 and HT-2 toxins [17].

In the EU, a similar approach was taken by the Scientific Committee on Food (SCF) resulting in scientific opinions for DON, NIV and T-2/HT-2. Currently in the EU, TDIs of 1, 0.7 and 0.06 µg/kg body weight have been established for DON, NIV and the sum of T-2 and HT-2, respectively [18]. The TDIs for NIV and T-2/HT-2 were temporary, because of gaps in the data available. These risk assessments by SCF were the basis for the establishment of EU regulations for DON, for which maximum limits of 1750 1250, 750, 500 and 200 µg/kg have been set in unprocessed durum wheat, oats and maize, unprocessed cereals, cereals and pasta, bread and cereal-based foods and baby food, respectively [19]. For NIV, no regulations were established, in view of the relatively low occurrence of this toxin in the EU. Regulations are still being considered with respect to T-2 and HT-2 toxins. It is expected that the limits will refer to the sum of T-2 and HT-2 due to similar toxicities and the knowledge that T-2 toxin is rapidly hydrolyzed to HT-2 toxin *in vivo* [20]. Originally, the introduction of limits for T-2 and HT-2 in the EU were planned for July 2009, but, as yet, these have not been established. Discussion limits for the sum of T-2 and HT-2 were 100 µg/kg for unprocessed cereals and cereal products, 500 µg/kg for unprocessed oats, 200 µg/kg for oat products and 50 µg/kg for infant food [21]. At the time of writing, the European Food Safety Authority (EFSA; this organization was established in 2002 and took over the responsibilities of SCF) was starting to carry out a risk assessment of T-2/HT-2 toxins. For this purpose, recent data on occurrence of T-2/HT-2 in food was to be collected and reviewed in spring 2010. It is of interest to note that, within the framework of the MoniQA project (www.moniqa.org), a risk-benefit analysis is carried out focusing on the introduction of EU limits for T-2/HT-2 toxins. All this information will serve as scientific information, in support of the introduction of EU-limits for T-2/HT-2 toxins in the EU, which, most probably, will not be established until 2011.

2. Sampling, extraction and clean up

Trichothecenes are ubiquitous in cereals and as such various reasons exist for their determination (e.g., enforcement of legislation, quality control and monitoring the occurrence in order to determine consumer exposure). These methodologies are also a vital tool in research laboratories to investigate possible prevention and control, in toxicological studies, to assess decontamination and detoxification strategies, to gain further understanding of the relationship between the fungus

and its metabolites and to study their fate during grain processing [1,10].

Prior to any analysis, a representative (unbiased) sample must be provided, ground, and blended, and a sub-sample removed for testing. Sampling procedures have a significant effect on the reliability of analytical data generated, as not all mycotoxins are uniformly distributed throughout a lot. In 2006, Whitaker [22] investigated the important issue of sampling, and concluded that the true concentration of a bulk lot could not be determined with 100% certainty. There will always be some variability due to the sampling, the sample preparation used and the analytical technique applied. In particular, if small samples are selected, the variation in results will increase, so, to reduce the chances of incorrect decisions being made under regulatory control, increasing sample size and sub-sample size, ensuring even distribution of particle size (e.g., by blending and grinding), and having sufficient aliquots subjected to analysis will all serve to safeguard consumers and to protect against unacceptable economic loss or damage. Despite the problems associated with sampling, detailed sampling schemes specific for mycotoxins, including *Fusarium* toxins in foodstuffs, have been published in the EU [23] and by the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the U.S. Department of Agriculture (www.gipsa.usda.gov) which should be used for official purposes. It should be noted that many considered that these sampling schemes time-consuming and cumbersome, and that *Fusarium* mycotoxins do not present as great a source of variability, as seen in aflatoxin, ochratoxin A or fumonisin testing [1]. In addition, the EU regulation [23] includes guidelines on the performance criteria required for the testing of DON, T-2 and HT-2, and specifies that, where there is a lack of fully validated methods, a ''fitness-for-purpose'' approach may be used. In this instance, the measurement uncertainty associated with the method is calculated and the analytical result reported as x ± U (where x is the analytical result and U is the expanded measurement uncertainty). The entire measurement process must therefore be assessed and any standard uncertainties identified and quantified to enable calculation of the expanded uncertainty. Obvious sources include sample homogeneity, stability and recovery, instrument bias, the measurement conditions, reagent purity (calibrants) and operator skill.

Analytical procedures are classified and characterized by several criteria. Methods may be fully quantitative, semi-quantitative or qualitative. Other considerations include the simplicity of the test, the speed of analysis and the level of technical skill required to perform the assay. Generally, with quantitative methodologies, the simplicity and speed of analysis is compromised and greater technical expertise is required, thus impacting on the overall cost incurred. Regardless of whether the

analytical procedure is for screening or confirmatory purposes, the mycotoxins must first be efficiently extracted from the grains and subjected to a clean-up step to reduce any interfering compounds before determination is performed.

2.1. Extraction procedures
The majority of analytical techniques used for the determination of trichothecenes require their extraction from the food matrix into a solvent or liquid phase followed by some form of clean up and finally analysis. In general, organic-solvent extraction is used to allow further concentration of the analyte for accurate measurement at low levels and the choice of solvent or solvent/water mixture used is driven by the chemical characteristics of the toxin being analyzed, the sample type, health and safety issues and also the analytical technique being used. Another important consideration is extraction efficiency. It cannot be assumed that the toxins will be totally dispersed into the liquid phase, however the key issue is that the recovery values determined are consistent. As a result, during method development, scientists will investigate various solvents and solvent/water ratios to ascertain the most reproducible, efficient combinations for particular analytical methodologies.

According to the scientific literature, the most common extraction solvents employed are methanol/water and acetonitrile/water, although acidified acetone and ethyl acetate/acetonitrile/water have been reported to a lesser extent [24–27]. The incorporation of water into the solvent encourages the grains to swell, assisting the release of mycotoxins [10], thereby increasing extraction efficiency, which is crucial when quantifying toxin concentration in naturally-contaminated samples. Many immunochemical methods report the use of deionized or distilled water for extraction of deoxynivalenol due to its high polarity, which is particularly advantageous in that other interfering substances are unlikely to be co-extracted, leading to more accurate, reliable results [28].

Various physical techniques have been reported for mixing sample and extraction solvent (e.g., shakers or blenders). Blending tends to offer a more rapid approach, Ueno et al. [29] described a 5-min procedure for type A and B trichothecenes, while sample shaking invariably involves lengthier time periods of 60–90 min. [30,31], but Kolosova [32] recently reported accurate, reliable results after shaking for only 10 min.

2.2. Sample clean up
Following extraction, the resulting solution is often further processed to remove any impurities/interfering materials in addition to concentrating the extract prior to analysis and quantification. While this step is a common requirement for many confirmatory analytical methods, it is not required for many screening assays

[e.g., enzyme-linked immunosorbent assays (ELISAs), lateral flow devices (LFDs) and biosensor assays. If detection capability is impaired in such rapid tests, then some form of clean up and perhaps concentration will be necessary to ensure the test remains fit for purpose.

A number of clean-up strategies have been employed to facilitate the accurate measurement of trichothecenes in cereal products including solid-phase extraction (SPE) columns, immunoaffinity columns (IACs), ion-exchange columns and the more traditional liquid–liquid partitioning [1,10]. The latter is now close to redundant, due to the large volumes of organic solvents necessary for the procedure, the time required to perform such separations and indeed the mediocre recovery rates demonstrated [33].

SPE columns are one of the most commonly applied methods of clean up. Various column packings are now commercially available and include silica, charcoal, Florisil, C_8, C_{18} reversed phase and aluminum oxide [25,33]. These surfaces are chemically modified to allow the adsorption of the analyte of interest or the impurities. In summary, the sample extract is applied to a pre-conditioned SPE column, the analyte is retained on the column, which is washed to remove impurities, then eluted by means of a solvent wash. In the majority of cases, the eluate is evaporated to dryness and reconstituted in a particular solvent or buffer depending on the analytical technique being used. Langseth et al. [25] provided a comprehensive review of a variety of SPE-column packings available. The disadvantage of using such columns for the trichothecenes is that these toxins often differ greatly with respect to polarity and solubility, so recovery for some may be compromised. With this in mind, commercial companies have sought to overcome this problem [e.g., Varian (now Agilent Technologies, Inc. USA)], which produced a Bond Elut Mycotoxin cartridge designed for the simultaneous clean up of 12 type A and B trichothecenes in addition to zearalenone, another important *Fusarium* mycotoxin food contaminant. This will significantly reduce the cost of analysis in addition to making it more rapid and straightforward. Another well-established method for the preparation of trichothecenes is the use of MycoSep columns, (Romer Lab, Inc.) [25,33]. Three types of columns are available, depending on the types of samples being analyzed and also the amount of resultant extract required for the analytical procedure, including:

- MycoSep/MultiSep 227 Trich+, applicable to complex food and feed matrices;
- MycoSep/MultiSep 225 Trich, for grain and simple sample matrices; and,
- MycoSep 113 Trich, employed for analytical procedures requiring small amounts of extracts.

These columns comprise different adsorbents (e.g., charcoal and ion-exchange resins). A plastics tube is packed with the desired adsorbent between filter discs and contains a rubber flange at the lower end of the tube, which contains a porous frit and a one-way valve. The plastics column is inserted into the sample extract (in a test tube), the rubber flange provides a strong seal and the extract is forced through the packing into the top of the column resulting in a clean extract ready for analysis. This method is more advantageous in terms of time, in that there is no column pre-conditioning or rinsing required [33], but it should be recognized that no sample concentration is possible with the use of these products.

Another format increasingly exploited for the clean up of trichothecene-containing extract is the IAC. Antibodies are attached to an inert support and will specifically bind the analyte of interest while allowing interfering components to pass through the column. As with other SPE formats, pre-conditioning, rinsing and elution of the toxin are required and, in this instance, the extract must be an aqueous solution containing little or no organic solvent that will have a detrimental effect on the antibody and subsequently the antibody-antigen binding event. A major advantage in this approach is the specific interaction between the antibody and the analyte in question, but, as with all antibody-based methods, non-specific interactions, due to cross reactivities with other trichothecenes, may impair the results achieved.

A detailed review was published by Senyuva et al. [34] on the use of IACs for food analysis, in relation to mycotoxins, veterinary drugs, pesticides, phycotoxins, process and environmental contaminants and vitamins. The authors concluded that IAC clean up has made a huge impact in the analysis of trichothecenes, however one drawback mentioned was the cost of the commercial columns available. They are designed for single use only, although it has been demonstrated that many IACs produced in-house by testing laboratories may be re-used up to 100 times before any deterioration is observed. What is advantageous is the fact that many of the commercial producers are developing multi-analyte IACs to meet the technical demands of research and enforcement laboratories. Several commercial companies have produced IACs for deoxynivalenol, T-2 and HT-2 toxin.

3. Screening/rapid methods of analysis

Screening assays are very important tools for monitoring mycotoxins in food and feed. Generally, they are qualitative tests demonstrating presence or absence of the toxin in question, but there are also a variety of rapid tests that are semi-quantitative or quantitative. Advantages of screening tests include the speed at which the analysis is performed, the simplicity of the sample preparation and the low cost per analysis. There are also drawbacks to such procedures, the most notable being

reliability, as very often false-positive results are reported. These are acceptable provided the incidences are low (i.e., <5%), as these samples are also likely be sent for confirmation prior to acceptance or rejection of a lot. False-negative results must be minimized or totally avoided. Otherwise, it would be very difficult to implement such a test in a routine monitoring scheme.

The majority of screening assays employed for trichothecenes are immunochemical methods and include ELISAs, LFDs, dipstick tests, fluorescence polarization immunoassay (FPIA), immunofiltration assays and, more recently, biosensor assays, but thin-layer chromatography and bioassays have also been used.

Examples of immunochemical rapid methods are detailed in Tables 1 (published methods) and 2 (commercially-available kits), outlining the analytes of interest, the samples matrices they can be applied to, the extraction technique employed and some performance characteristics of the assays.

Table 2 highlights a variety of commercially-available test kits for the rapid screening of trichothecenes. Often, these are employed in monitoring laboratories, so it is vital that the data generated are accurate and precise. To this end, AOAC International runs a program whereby rapid test kits are reviewed by an independent third party to ascertain if the performances are acceptable and meet the specifications determined for the method. Generally, this involves an inter-laboratory validation study, and, if the results meet the criteria, then Performance Tested Method status is approved. Some of the manufacturers outlined in Table 2 have been successful in obtaining this status for rapid methods (e.g., Ridascreen Fast DON, AgraQuant DON ELISA Test Kit and Ridascreen Fast T-2 Toxin). This affords confidence in the results obtained and perhaps gives these manufacturers a more competitive edge in the market, but it should be noted that this process is very time consuming and expensive, so it is not a viable option for all.

One of the most fundamental requirements for an immunoassay is the specificity of the antibody. To date, many publications have described the successful production of monoclonal or polyclonal antibodies against the trichothecenes, but often the cross-reactivity profile is not ideal and can therefore lead to overestimation and increased measurement uncertainty. This is certainly true for deoxynivalenol, where most of the literature highlights that antibodies raised against deoxynivalenol have strong cross-reactivity to 3-acetyldeooxynivalenol, 15-acetyldeoxynivalenol or both [30,35]. Schneider et al. [31] reported the production of a more specific deoxynivalenol antibody isolated from chicken-egg yolk, the cross-reactivity with 3 and 15-acetyldeoxynivalenol was determined as 15% and 4%, respectively. For accurate screening of T-2 toxin and HT-2 toxin, the antibody should have a high specificity for both, based on the legislative measures that are soon to be imposed

in Europe. The majority of antibodies raised against T-2 toxin show little specificity with HT-2 toxin [36], but Yoshizawa et al. [37] and Meneely et al. [38] reported the use of a specific monoclonal antibody in the development of an ELISA kit and a surface-plasmon resonance (SPR) biosensor method for the detection of T-2 and HT-2 toxins in foods.

Probably the most commonly used antibody-based test is a competitive ELISA. One of the limiting factors of this format is that structurally-related toxins or matrix interference may modify the signal by either increasing or decreasing it and therefore lead to increased method uncertainty. Another problem is that usually the antibody-toxin interaction must be allowed to reach equilibrium and this can take anything up to 2 h, although there are several commercial kits (Table 2) that report results in 10 min.

LFDs or dipstick tests have been developed for the detection of deoxynivalenol and T-2 toxins [32,36]. The principle is much the same as ELISA, but, by contrast, these assays are qualitative and give a simple yes/no in relation to the presence of a particular contaminant; in addition, they are extremely rapid to perform and may be used in field conditions. Many commercial companies have exploited this method and are included in Table 2.

FPIAs have emerged for the determination of trichothecenes in cereals and cereal-based foods with assays for deoxynivalenol having been published [39]. This format measures interactions between a specific antibody and an antigen labeled with a fluorescent tag (tracer) and measures the polarization value (P). The mycotoxin in the sample extract competes with the tracer for the antibody-binding sites, so the more toxin in the sample, the less tracer is bound to the antibody. In this case, P would be low (since little tracer is bound) and so the value of P is inversely proportional to the mycotoxin concentration in the sample.

Biosensors are becoming more popular in many industrial sectors (e.g., pharmaceutical, environmental, agriculture, healthcare and food). They comprise a biological-recognition element (e.g., enzyme-substrate, antibody-antigen, or receptor-biospecific molecule) connected to a transducer or sensing device (e.g., electrical, optical, acoustic, or thermal).

Optical biosensors based on the principle of SPR have found successful application in the fields of drug discovery and development, biotherapeutics and life-science research, and, over the past few years, have emerged as an important analytical tool in food analysis with reference to veterinary drug residues, mycotoxins and phycotoxins [40–42]. Several papers have been published on the determination of deoxynivalenol by SPR-based immunoassays [43].

More recently, a method has been developed for the determination of the sum of T-2 and HT-2 in cereals and maize-based baby food [38]. This work was performed

Table 1. Rapid immuno-chemistry-based methods for the determination of DON, and T-2 and HT-2 toxins in cereals and cereal products

Analyte	Sample	Format	Extraction	Clean up	Antibody cross-reactivity (%)	Limit of detection	Recovery (%)	Ref.
DON	Wheat	ELISA	Phosphate buffered saline	None	DON 100 3-AcDON 632 15-AcDON 3.3	0.2 µg/L	89	[35]
DON	Wheat	ELISA	Acetonitrile/water 10.5/89.5% v/v	None	DON 100 3-AcDON 15 15-AcDON 4	2 µg/L	80–125	[31]
DON	Maize, wheat	ELISA	Methanol/water 10/90% v/v	None	DON High 3-AcDON Low 15-AcDON High	50 µg/L	Not reported	[30]
T-2/HT-2	Wheat kernels	ELISA	Acetonitrile/water 85/15% v/v	None	T-2 100 HT-2 100 AcT-2 79	30 µg/kg	94–112	[37]
DON	Wheat kernels	ELISA	Acetonitrile/water 85/15% v/v	None	3,15-diacetylDON 100 DON > 10000 15AcDON 3 Nivalenol >10000	80 µg/kg	94–112	[37]
DON	Wheat	LFD	Methanol/water 80/20% v/v	None	DON 100 15-AcDON 429	Ranges of 250–500 µg/kg 1000–2000 µg/kg	N/A	[32]
T-2	Wheat, oat	LFD	Methanol/water 70/30% v/v	None	T-2 100 HT-2 5.4	Not reported	N/A	[36]
DON	Wheat	FPIA	Phosphate buffered saline	None	DON 100 3-AcDON 339 15-AcDON 8	Not reported	71	[39]
DON	Barley, wheat, oats, maize meal, maize flakes	NRL Array Biosensor	Methanol/water 75/25% v/v	None	Not reported	9, 6, 50, 1, 1 µg/kg	Not reported	[45]
DON	Wheat	SPR Biosensor	Acetonitrile/water 80/20% v/v	None	3 clones tested. All had high% CR with DON, 1 had high% CR with 3-AcDON, all had low% CR with 15-AcDON	Not reported	Not reported	[43]
T-2/HT-2	Wheat, wheat-based breakfast cereals, maize-based baby food	SPR Biosensor	Methanol/water 40/60% v/v	None	HT-2 100 T-2 92	26, 25, 25 µg/kg	95–125	[38]
DON	Wheat, wheat-based breakfast cereals, maize-based baby food	SPR Biosensor	Methanol/water 40/60% v/v	None	DON 100 3-AcDON 161 15-AcDON 1	57, 9, 6 µg/kg	92–107	[44]

under the remit of BioCop, the European Commission-funded project looking at the development of screening methods for food contaminants using new platforms (www.BioCop.org). A method was also developed for deoxynivalenol in cereals and maize-based baby food [44], and some performance characteristics of this procedure are given in Table 1.

Another recent optical-biosensor assay described for the detection of deoxynivalenol in foods is the array biosensor developed at the Naval Research Laboratory, USA [45]. This again is an example of a competitive immunoassay where a DON-biotin conjugate is immobilized onto a neutravidin-coated optical waveguide and competes with DON in the sample for the binding sites of a fluorescently-labeled DON monoclonal antibody.

One method recently investigated, which certainly showed promising results for the rapid screening of deoxynivalenol in wheat and barley, is Raman spectroscopy, a non-destructive approach requiring no sample-extraction steps. While the technique could distinguish between low and high levels in contaminated grain (by detecting changes in grain quality rather than the presence of trichothecenes), larger studies are essential to confirm these findings [46]. If further scrutiny of this analytical technique proves its applicability to analysis of cereal grains for such contaminants, then it could become an invaluable screening tool for not only trichothecene analysis but also other food pollutants.

Another non-invasive analysis technique to screen for DON in grain is based on acoustic waves, penetrating through and/or reflected by air-filled porous materials (e.g., unconsolidated solid beads of grain) [47]. The technique with in-line and on-line capabilities can be used to screen DON in contaminated wheat at the point of harvest. As the technique is fast, cheap and suitable for high-throughput analysis, it may solve some of the problems commonly associated with sampling and analysis. The acoustics method will be further explored in a EUREKA-ITEA project, running in 2010–2012 (www.eurekanetwork.org).

Due to the complex nature of food samples, there are often strong matrix interferences that can have a huge effect on the results reported. To overcome such difficulties, a range of electrochemical-detection schemes have been described for application in food analysis [48,49].

Electroactive interferents are relatively rare, suggesting that electrochemical approaches to monitoring for example antigen/antibody interactions might be of broad utility. Advantages of electrochemical measurements over those of spectrophotometric procedures include the possibility of increased speed, miniaturization and multiplexing [48]. The low cost of instrumentation, the possibility of in situ analysis and the insensitivity to turbid samples are other advantages of this technique. The use of screen-printed electrodes (ScPEs), which allow

the mass production of electrodes at a very low cost, is another significant development. For these reasons, electrochemical techniques, in particular, amperometric detection systems, have been demonstrated to be a suitable means for sensor construction in food-contaminant analysis.

Despite this, only few examples of electrochemical detection of trichothecenes have been reported to date [50,51]. One of these is particularly interesting and straightforward and is based on the disclosure that, after a hydrolysis step performed in basic solution [52], group B trichothecenes give rise to electroactive compounds. This entails a method to detect this important class of contaminants in a fast, sensitive mode.

Recently, an improved approach by using both ScPEs and a new 96-well electrochemical plate for the determination of DON and NIV trichothecenes in wheat samples was published [53]. ScPEs offer several advantages compared to the classic electrode surfaces usually adopted in analytical-chemistry applications [48]. They are inexpensive, easy to produce and highly reproducible. The cost allows the single use of these sensors and avoids problems that usually arise from electrode fouling. In addition, the use of ScPE allows the handling of a very limited sample volume, which is always desired when extraction procedures are employed for sample treatment. The use of a microwave-hydrolysis procedure for the production of electroactive compounds from DON and NIV was also introduced in the same work [53].

Again in the BioCop project, there was proposed the development of an enzyme-linked-immunomagnetic-electrochemical (ELIME) array for DON detection in food samples based on the use of magnetic beads and a 8-electrode strip of ScPEs [54]. In addition, an electrochemical immunosensor for the detection of T-2 and HT-2 toxins was successfully developed [55], allowing the determination of the sum of T-2 and HT-2 toxins in cereal samples.

Despite these advances, examples of electrochemical immunosensors can be found only as academic research tools and no commercial examples are based on this approach.

4. Confirmatory/reference methods of analysis

These methods are used to confirm samples deemed as positive during the screening process and to quantify accurately the concentrations within food or feed samples. Generally, physicochemical techniques [e.g., chromatographic separation using gas chromatography (GC) or high-performance liquid chromatography (HPLC) coupled with a specific detector] are employed to obtain highly-accurate results. The types of detectors normally used with such chromatographic techniques are flame-ionization detection (FID), ultraviolet (UV), UV

Table 2. Commercial immunochemical test kits for DON, and T-2 and HT-2 toxins

Analyte	Sample	Format	Extraction	Antibody Cross-reactivity	Limit of Detection	Brand Name	Manufacturer
T-2	Cereals, silage	EIA	Acetonitrile/water 84:16% v/v	T-2 100% Acetyl T-2 12.3% HT-2 3.4% Iso T-2 2.5%	30–55 µg/kg	T-2 Toxin EIA	Eurodiagnostica B.V. The Netherlands
T-2	Cereals, feed	EIA	Methanol/water 70/30% v/v	T-2 100% Acetyl T-2 114% HT-2 7% Iso T-2 2%	<5 µg/kg	RIDASCREEN T-2 Toxin	R-Biopharm AG, Germany
T-2	Grain, cereals	ELISA	Not reported	Not reported	35 µg/kg	AgraQuant T-2 Toxin	Romer Labs Diagnostica GmbH, Austria
T-2	Maize and derived products	ELISA	Methanol/water	T-2 100% HT-2 38% T-2 Triol 1.6%	25 µg/kg	ELISA kit for T2 Toxin	Tecna S.r.l. Italy
T-2/HT-2	Barley, maize, oats, rye, soy, wheat	ELISA	Methanol/water 70/30% v/v	T-2 100% HT-2 100%	Not reported. Limit of Quantification 25 µg/kg	Veratox for T-2/HT-2 Toxins	Neogen Corporation, U.S.A.
DON	Cereal, food, feed, beer	ELISA	Distilled water	DON 100% 3AcDON 96% Nivalenol 40%	10–30 µg/kg	DON EIA	Eurodiagnostica B.V. The Netherlands
DON	Grain, cereals	ELISA	Not reported	Not reported	0.2 mg/kg	AgraQuant DON	Romer Labs Diagnostica GmbH, Austria
DON	Barley, oats, wheat, wheat bran, flour, wheat midds	LFD	Distilled water	DON 100% & 3AcDON (not reported)	Semi-quantitative <0.5 mg/kg, >0.5 to <1 mg/kg, >1 to <2 mg/kg, >2 mg/kg	Reveal for DON SQ	Neogen Corporation, U.S.A.
DON	Wheat, wheat midds, wheat flour, wheat bran, barley, maize, maize meal, maize screenings, malted barley, oats	ELISA	Not reported	DON 100% & 3AcDON (not reported)	1 mg/kg	Agri-Screen for DON	Neogen Corporation, U.S.A.
DON	Cereals, malt, feed, beer, wort	EIA	Distilled water	DON 100% 3AcDON > 100% 15AcDON 19% NIV 4% Fusarenon X < 1% T-2 < 1%	Cereals, malt feed 18.5 µg/kg Beer 3.7 µg/kg Wort 3.7 µg/kg	RIDASCREEN DON	R-Biopharm AG, Germany
DON	Cereals, malt, feed	EIA	Distilled water	Not reported	<0.2 mg/kg	RIDASCREENFAST DON	R-Biopharm AG, Germany
DON	Grain	LFD	Distilled water	Not reported	0.5–1.25 mg/kg	RIDAQUICK DON	R-Biopharm AG, Germany

Table 3. Chromatographic methods for the determination of DON, and T-2 and HT-2 toxins in cereals and cereal products

Analyte	Sample	Detection method	Extraction	Clean up	Limit of detection	Recovery (%)	Ref.
T-2/HT-2	Wheat, maize, oats	HPLC-Fluorescence detection	Methanol/water 90/10% v/v	IAC	10, 6.3, 2 µg/kg T-2 6.3, 2.3, 2.8 µg/kg HT-2	Not reported	[62]
Type A & B trichothecenes	Wheat	GC-FID	Acetonitrile/water 84/16% v/v	MycoSep Columns	25 µg/kg	79–116	[57]
Trichothecenes	Cereals, cereal-based products	GC-MS	Acetonitrile/water	SPE	Type A 80 µg/kg (LOQ) Type B 60 µg/kg (LOQ)	Type A 61–90 Type B 56–93	[58]
Trichothecenes	Maize-based products	GC-MS	Acetonitrile/water	MycoSep Columns	20–120 µg/kg	71–155	[59]
DON	Wheat	HPLC-UV	Acetonitrile/water 82/18% v/v	SPE, MycoSep Columns	50 µg/kg	92	[63]
T-2, DON	Cereals, cereal-based products	LC-ESI-MS/MS	Acetonitrile/water 85/15% v/v	MycoSep Columns	10 µg/kg (LOQ)	54–93	[65]
Type A & B trichothecenes	Maize	LC-APCI-MS/MS	Acetonitrile/water 84/16% v/v	MycoSep Columns	0.3-3.8 µg/kg	82–98	[66]

diode array, fluorescence, electron capture, and, of course, mass spectrometry (MS). Examples of the types of chromatography, detection systems and some analytical parameters of these types of methods are outlined in Table 3.

For the determination of trichothecenes, GC has largely been the method of choice. While these methods provide sensitive, accurate results, the polar compounds require derivatization, which is often a lengthy exercise [56]. A variety of chemicals have been used to perform the derivatization and the choice depends on the trichothecene being analyzed and the method of detection employed [25].

Trimethylsilyation has been reported for both Type A and B trichothecenes. Schothorst et al. [57] and LeBlanc [58] used this approach with the derivatization procedure taking anything from 15 min to 2 h. The methods of detection include FID and MS.

Fluoroacetylation is another common approach taken when determining trichothecenes. Derivatives formed include trifluoroacetic acid anhydride (TFAA) [59], heptafluorobutyrate (HFB) [60] and pentafluoropropionic anhydride (PFPA) [61]. The detectors employed in these instances were MS and electron-capture detection (ECD).

HPLC is perhaps the most frequently and widely used method for the determination of mycotoxins [1], and many papers have been published in relation to the trichothecenes. The use of HPLC coupled with fluorescence detection has been applied to the determination of T-2 and HT-2 toxins [62] and involves pre-column derivatization of the compounds using 1-anthroylnitrile, coumarin-3-carbonyl chloride, 1-naphthoyl chloride, 2-naphthoyl chloride, or pyrene-1-carbonyl cyanide. UV detection is not generally a choice for Type A trichothecenes, as it is only applicable to samples containing very high concentrations [25], but it has been successfully reported for the detection of Type B trichothecenes [63].

LC-MS is now probably the most extensively used analytical method in the industrialized part of the world for not only trichothecenes but also mycotoxins in general. In recent years, there have been significant advances with reports of methods capable of detecting hundreds of fungal and bacterial metabolites [64]. The recent literature highlights the technical advances in this field, allowing the simultaneous determination of mycotoxins having great chemical diversity, something which is not achievable with the use of GC or HPLC with fluorescent or UV detection.

There are very few official methods adopted by AOAC International or the European Committee for Standardization (CEN) for the determination of trichothecenes. Currently, there is only one CEN standard for DON entitled ''Determination of Deoxynivalenol in animal feed – HPLC method with UV detection and

immunoaffinity column clean-up" (EN 15791:2009). No standardized method exists for DON or T-2/HT-2 in food; however, it is anticipated that a standard method for DON in cereals, cereal products and cereal-based foods for infants and young children will be adopted later this year, but it may take several years until we see any standard methods for T-2 and HT-2 in food or feed.

AOAC International has published two methods in its Official Methods of Analysis, both of which are for DON in grains or wheat. One is a thin layer chromatographic method while the other is a gas chromatographic method, the analytical ranges of which are >300 μg/kg and >350 μg/kg, respectively. While these support European legislation for wheat, they are not suitable for cereal-based foods designed for infants and young children, so more sensitive methods of analysis need to be validated and adopted by such organizations.

5. Conclusions

Rapid methods for trichothecene detection in feeds and foods have improved considerably over the years and many commercial companies now provide screening kits for these toxins. The scientific literature also contains many improved detection methods. Different formats exist (e.g., ELISA, LFD, FPIA, optical, array and electrochemical sensors), but there is still a lack of rapid methods that enable the simultaneous measurement of deoxynivalenol, T-2 and HT-2 and other trichothecenes commonly found in food for human consumption, or indeed other mycotoxins that contaminate cereal grains. In addition, many of the immunochemical methods available are limited by the specificity of the antibodies used, and this will become a very important issue especially with respect to T-2 and HT-2 toxins once the European Commission establishes the maximum limits that are expected to be for the sum of T-2 and HT-2.

With respect to traditional or reference methods, HPLC, coupled with fluorescence, UV or diode-array detection, is still used in many laboratories, as is GC coupled with ECD. The reason for this is the relative cost of the instrumentation compared with detection using MS and the technical expertise required, but there are an ever increasing number of publications on the subject of LC-MS^2 methods for the detection of trichothecenes and other mycotoxins. The advantages these methods have over conventional methods are that they:
- can measure a wide variety of mycotoxins simultaneously, displaying great chemical diversity; and,
- may require little or no sample clean up to produce accurate, precise results.

There is little doubt that significant technological advances in analytical methods for the determination of the trichothecenes have been made in the recent past. It is also clear that the driving forces for advances in

tricothecene detection are national and international legislative requirements.

There is still a lack of approved methodologies. Quality assurance of data generated is highly important to reference laboratories monitoring cereals in food or feed, as inaccurate results could have dire consequences for public health or the economy. Several approaches can be undertaken to make these improvements [e.g., interlaboratory collaborative validation studies or participation in the Food Analysis Performance Assessment Scheme (FAPAS), co-ordinated by the Food and Environment Research Agency (FERA), in the UK].

With developments in analytical capabilities, it is likely that many more mycotoxins will be isolated, characterized and proved to be potential health risks to humans and animals, resulting in new challenges to the research, enforcement and commercial community.

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