Mycotoxin control in cereals: safeguarding food

Technical Project Report

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Foreword and Acknowledgements

This research was undertaken by Professor Chris Elliott, Professor of Food Safety, Institute for Global Food Security at Queen's University Belfast, and his team of researchers, in particular Dr Julie Meneely, Dr Brett Greer, Dr Oluwatobi Kolawole and Dr Qiqi He. Dr Martin Danaher, Teagasc-Ashtown Food Research Centre, was the principal collaborator on this project.

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Executive Summary

Aims

The aim of this research was to review (and where possible improve/refine current rapid screening methods for the mycotoxin T-2 and its metabolites in cereals for human consumption.

Researchers engaged with the relevant sector of the cereal processing industry in Ireland and the UK, and the relevant primary production sector, to gain detailed, valuable information.

The research conducted was designed to clarify deficits in T-2 toxin control in the relevant processing and primary production sectors, evaluate the robustness of current analytical detection and control methods therein, augment same if deemed necessary, and implement workable T-2 toxin control solutions at sectoral level.

Objectives

The specific objectives of the research project were to:

- Conduct a literature review of the most up-to-date scientific and regulatory data for T-2 toxin and its metabolites, the specific environmental conditions required for their production, and the impact of climate change on their occurrence and distribution.
- Undertake a thorough analysis of the currently available commercial rapid analytical techniques for the screening of T-2 and HT-2 toxins in cereals for direct human consumption.
- Undertake a survey of the relevant processing and primary production sectors to determine what testing they currently conduct for mycotoxins, to fully understand the shortfalls in relation to mycotoxin monitoring and control within the industry.

- Conduct a survey of oats and barley (n≥100) using mass spectrometry to identify contamination levels of, not only the mycotoxins of interest, i.e., T-2 and HT-2, but also all other regulated mycotoxins including aflatoxins, deoxynivalenol, zearalenone, fumonisins and ochratoxin A.
- Perform a thorough evaluation of a selected number of rapid test kits to ascertain fitness for purpose and determine how to adapt the most robust methods for better performance. If required, undertake a training workshop on the use of the best performing rapid testing kits.
- Engage with stakeholders to discuss the implications of the findings of the project and produce a series of conclusions and recommendations for stakeholders on the island of Ireland.

Final outcomes

The systematic literature review on T-2 toxin and its metabolites – including the toxicity of these compounds to humans and animals (including documented human cases of T-2 toxin poisoning), the conditions under which these toxins occur, the crops and food-producing animals affected, the effect of climate change on their occurrence and distribution, the currently accepted analytical methods used and the current regulatory guidance limits and the potential changes to these – have provided the currently available data, in addition to identifying important knowledge gaps.

Analysis of oats on the island of Ireland highlighted that the mycotoxins T-2, HT-2 and ochratoxin A present an ongoing challenge to the industry. Should implementation of lower limits for the sum of T-2 and HT-2 toxins be realised due to legislative changes, the study has shown that this is likely to result in higher rates of non-conformity, potentially leading to increased food waste and greater economic losses for the industry. Processing, such as de-hulling (removal of the husk), is sufficient to reduce the concentrations of T-2 and HT-2 in oats. Observed contamination of oats with ochratoxin A (see Objective 4), including a small number of regulatory violations, has reinforced the importance of adequate drying,

storage, monitoring and control. Furthermore, analysis of metadata related to agricultural practices emphasised higher levels of contamination in conventionally produced oats when compared to organic systems. Also, in terms of mycotoxin hotspots (areas of high concentrations due to heterogeneous distribution throughout grains along the oat supply chain, the application of fungicide and the storage of oats in farm stores led to elevated mycotoxin concentrations. Thus, fungicide application before harvest, and storage conditions following oat crop harvest, represent important critical control points for oat mycotoxin management. The findings emphasise the continued need for effective surveillance and control of mycotoxins within the industry through adequate testing regimes and Hazard Analysis and Critical Control Point (HACCP procedures.

The findings of the study were disseminated to relevant stakeholders during a workshop titled ‘Measuring Mycotoxins: Applying Smart Agriculture – Smart Science (SASS’ to mitigate against the growing and unknown issues of mycotoxins in feed and food.

The market is hugely competitive for commercially available rapid diagnostics kits delivering the simultaneous measurement of T-2 and HT-2 toxins, and most of the tests available are immunochemical methods including Enzyme-Linked Immunosorbent Assays (ELISA, Lateral Flow Devices (LFDs/Dipstick Assays and Fluorescence Polarisation Immunoassays (FPIA. In terms of performance characteristics, for important information such as antibody cross-reactivity, matrices that have been validated, such as Limits of Detection (LODs and Limits of Quantification (LOQs, are very often lacking. In some instances, the LODs do not meet the preferred specifications laid down in the European Commission Recommendation on the presence of T-2 and HT-2 toxin in cereals and cereal products (2013/165/EU) of 25 µg/kg. While the use of aqueous extraction is increasing for such rapid methods, many procedures still require the use of organic solvents that are not feasible for on-site screening by producers or processors. The study performed to evaluate the ‘fitness for purpose’ of the commercial kits when compared against confirmatory Liquid Chromatography

Tandem Mass Spectrometry (LC-MS/MS revealed that under the current EU guidance limits for the sum of T-2 and HT-2, the best performing kit was the Neogen Reveal® Q+ MAX for T-2 / HT-2 Kit. This kit was validated for oats, used an aqueous extraction, and provided value for money in terms of cost per analysis. More importantly, excellent recovery when using this kit was observed for analysis of a certified reference material for T-2 and HT-2 toxins and the false negative rate was found to be 1.1%. The other kits tested had higher incidences of false negative results. In the event of new regulatory limits under discussion being implemented, none of the kits in their current form would be 'fit for purpose'. Further development and validation is required to demonstrate accurate, reliable results at the potentially lower limits of 50 µg/kg and 500 µg/kg for processed and unprocessed oats, respectively. Currently, the EU guidance limits for the sum of T-2 and HT-2 for processed and unprocessed oats are 200 µg/kg and 1000 µg/kg, respectively.

Testing regimes used within the industry range from the implementation of a two-tier system of screening and confirmatory analysis for a number of regulated mycotoxins such as T-2/HT-2 toxins, deoxynivalenol, zearalenone, alternaria mycotoxins, ergot alkaloids, aflatoxin B1, ochratoxin A, sterigmatocystin, and nivalenol., to a few mycotoxins, namely T-2/HT-2 toxins, deoxynivalenol, zearalenone, and ochratoxin A., just T-2 and HT-2 toxins to no testing and reliance on certificates of analyses from suppliers. Furthermore, from the information received, the sampling procedures used by industry must be greatly improved to ensure accurate, reliable results for the monitoring of mycotoxins in the oats and barley. Robust sampling procedures were designed as part of the project and were implemented by a number of stakeholders within the industry.

The outputs of this research have provided a better understanding of the problems associated with mycotoxins in oats, in particular T-2 and HT-2 toxins on the island of Ireland. Improved agricultural practices and increased surveillance and control will serve to improve grain quality and safety, protect products and brands, reduce

food waste, and enhance profit margins. Ultimately, consumers will be better protected in terms of mycotoxin exposure.

Key Project Recommendations

1. Further research on the ecology of *F. sporotrichioides* and *F. langsethiae,* as well as the influence of interacting environmental factors on their growth and activation of biosynthetic genes, as these are still not fully understood. Predictive models of *Fusarium* growth and subsequent mycotoxin production would be beneficial in predicting the risk of contamination and thus aid early mitigation.
2. Communicate to processors the importance of adopting a two-tier testing system for the detection and quantification of mycotoxins in grains. On-site testing by farmers/producers using rapid methods would be prudent to identify non-compliant crops. Confirmatory tests should be performed on non-compliant crops using an accredited method. As the recognised gold standard is LC-MS/MS, simultaneous measurement of a range of important mycotoxins, including T-2 and HT-2, would provide occurrence data and identify the risks posed to consumers.
3. Further research on the use of high-resolution mass spectrometry on compliant and non-compliant crops would be useful to indicate what other metabolites of T-2 and HT-2 toxins or emerging mycotoxins are prevalent in cereals such as oats and barley. This would support the prediction of what analyses should be performed in those commodities as climatic factors possibly alter the fungal and mycotoxin profiles. Moreover, as the toxicological effects and occurrence of some metabolites become clearer, this would help the industry be proactive rather than reactive.
4. Industry needs to improve/harmonise its sampling arrangements as this is crucial for the accurate determination of mycotoxins. Information and training need to be provided for this specific area.
5. In terms of the overall characteristics and performance of the rapid kits tested during this study, the Neogen Reveal® Q+ MAX for T-2 / HT-2 Kit preformed best. However, it should be noted that not all rapid test kits were

evaluated, and if guidance levels change for the sum of T-2 and HT-2, from 200 µg/kg for processed oats and 1000 µg/kg for unprocessed oats to maximum regulations of 50 µg/kg and 500 µg/kg for processed and unprocessed oats, respectively, this would need to be taken into consideration.

6. Companies need to undertake their own test kit validation, but this is a time-consuming and expensive operation. If the companies operate as a collective on one test kit, a single validation could be organised amongst them.
7. The potential changes to T-2 and HT-2 regulations, as outlined in point 5, will cause problems in terms of the performance of the test kits.

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Introduction

The island of Ireland is an important and increasingly large producer of the cereal crops oats and barley, and while some of the output is used for animal feeds, a significant proportion is used in the food and beverage industries. Globally, barley and oats are ranked fourth and sixth, respectively, in terms of tonnes produced and can be cultivated in temperate regions, making them particularly suitable to the climate on the island of Ireland. Nutritionally, oats and barley are considered functional foods, so in addition to health benefits resulting from their rich source of dietary fibre, essential amino acids and vitamins and minerals, they contain β-glucan that helps reduce cholesterol and control blood sugar. Typical oat-derived foods include breakfast cereals, breads, biscuits, infant food, muesli, granola bars and, more recently, oat dairy alternatives. While the bulk of barley produced is for the malting and brewing industries, it is also used in the production of breakfast cereals, malt vinegar, malt extract, in various cooked foods and in dairy alternative beverages. Furthermore, with increasing interest by consumers on plant-based alternatives, the development of the oat and barley sectors has already been targeted.

Natural contamination of cereal grains with fungal pathogens, both pre- and post-harvest, is a continuing and growing problem worldwide, as many of these fungal species produce mycotoxins that have serious implications for human and animal health. Of the hundreds of mycotoxins identified, only eleven have been legislated for in human food and animal feed; they include aflatoxins, fumonisins, ochratoxin A (OTA), zearalenone, deoxynivalenol, T-2 and HT-2 toxin. Production of these natural toxins is determined by specific environmental and management conditions, and climate change is expected to continue to drive contamination of these crops, necessitating greater surveillance and control to safeguard the food chain.

The aim of this research was to review (and where possible improve/refine) current rapid screening methods for the mycotoxin T-2 and its metabolites in cereals for human consumption. In addition, a survey was conducted of the relevant processing and primary production sectors in both jurisdictions on the island of Ireland to

determine what testing they have implemented for mycotoxins. This assisted in the clarification of deficits in T-2 toxin control and ultimately in the implementation of workable solutions to protect public health, address legislative obligations, and augment the resilience of the industry. The core objectives of the project were:

- Objective 1: Literature review of the most up-to-date scientific and regulatory data for the T-2 toxin and its metabolites, the specific environmental conditions required for their production, and the impact of climate change on their occurrence and distribution.
- Objective 2: Undertake a thorough analysis of the currently available commercial rapid analytical techniques for the screening of T-2 and HT-2 in cereals for direct human consumption.
- Objective 3: Undertake a survey of relevant processing and primary production sectors to determine what testing they currently conduct for mycotoxins, to fully understand the shortfalls in relation to mycotoxin monitoring and control within the industry.
- Objective 4: Conduct a survey of oats and barley (n≥100) using mass spectrometry to identify contamination levels of the mycotoxins of interest and an analysis of oat survey data and sample metadata to identify trends/major weaknesses in the supply chain.
- Objective 5: Perform a thorough evaluation of a selected number of rapid test kits to ascertain their fitness for purpose and determine how to adapt the most robust methods for better performance. If required, undertake a training workshop on the use of the best performing rapid testing kits.
- Objective 6: Engage with all stakeholders to discuss the implications of the findings of the project and produce a series of conclusions and recommendations for stakeholders on the island of Ireland.

Project aims and objectives

Objective 1

Literature review of the most up-to-date scientific and regulatory data for T-2 toxin and its metabolites, the specific environmental conditions required for their production and the impact of climate change on their occurrence and distribution.

A rigorous, systematic review of the current published literature was undertaken on T-2 and HT-2 toxin contamination in cereal crops, specifically oats and barley, and the implications for food safety. The review documented relevant toxicological data to both humans and food-producing animals, highlighting any potential risks associated with indirect exposure in humans through the consumption of animal-derived foods. Moreover, any documented human poisonings resulting from T-2 and HT-2 were reported. EU and UK regulations for the control of these mycotoxins in food intended for human consumption were detailed. Special emphasis was placed on a detailed examination of the statistical information that forms the basis of the risk assessment that leads to the establishment of indicative (regulatory) limits. These included toxicological data, the available occurrence data for these toxins in cereal crops, knowledge of the distribution of toxin concentrations within cereal lots, exposure/consumption data, and the availability of analytical methods for toxin determination. Prospective changes to the regulations for these toxins in the EU and UK and the potential impact on the cereal industry in terms of control and mitigation were discussed. Furthermore, peer-reviewed scientific publications were explored to evaluate the conditions under which the mycotoxins are formed and to ascertain how the changing climate will impact not only the production of the toxins but also their distribution on the island of Ireland. Finally, available analytical techniques, ranging from rapid screening tests to confirmatory mass spectrometry to the use of high-resolution mass spectrometry to help control and mitigate against these mycotoxins, were examined.

Objective 2

Review of the commercially available rapid diagnostics for the screening of T-2 and HT-2 in cereals.

There is a wide variety of analytical tests available for mycotoxin testing along the supply chain. These range from sophisticated confirmatory/reference methods to rapid screening assays. For growers, suppliers and processors along the supply chain, more user-friendly, inexpensive and rapid techniques are favoured. However, these methods must be accurate, reproducible and provide the required sensitivity for regulatory compliance. A review of the state-of-the-art diagnostic tools for the detection of mycotoxins was undertaken. It included a comprehensive list of commercially available screening kits to detect T-2 and HT-2 in cereals. The methods were examined in terms of their performance against existing EU and UK legislative requirements, including limits of detection (LOD), quantification ranges and antibody cross-reactivity. Details as to the number of tests that can be performed, the commodities the kits can be applied to, extraction procedures, estimated time to deliver results and their ease of use were tabulated and compared between the different manufacturers. The products were also categorised according to whether the tests were qualitative or quantitative (lateral flow devices (LFDs); enzyme-linked immunosorbent assays (ELISA)).

Objective 3

Survey of relevant processing and primary production sectors to determine what testing they currently conduct for mycotoxins, to fully understand the shortfalls in relation to mycotoxin monitoring and control within the industry.

The anticipated focus was to engage with individual growers, suppliers, the Irish Grain and Feed Association, the Northern Ireland Grain Trade Association, the Irish Farmers Association, the Ulster Farmers Union and brewing companies such as the Bushmills Distillery, Hilden Brewing Company and the Guinness Brewery. Existing collaborations with industry stakeholders were developed further. A scoping survey was undertaken with the stakeholders to gather information regarding current testing

regimes – i.e., the site where samples are analysed, whether in-house or by contract laboratories, by what means (rapid techniques or confirmatory methodologies), how many are analysed, and from which areas of the supply chain, i.e., on arrival at processing centres, throughout storage, etc. Of particular importance were the sampling regimes employed by the industry to ensure that homogenous sub-samples are provided for testing, as this is the greatest source of erroneous results for mycotoxin testing.

This knowledge, in addition to baseline data from the oats and barley survey and the evaluation of the test kits, allowed a risk assessment to be conducted for the supply chain to determine shortfalls in relation to monitoring and control. These outcomes will facilitate the implementation of improved HACCP management systems to protect public health and safeguard the resilience of the industries.

Objective 4

Survey of oats and barley.

A comprehensive survey was undertaken of oats and barley to determine the mycotoxins that frequently contaminate these cereals in Ireland and the UK, the levels of the contaminants and to provide a profile of co-contaminating toxins. Analysis of the survey data and sample metadata enabled the identification of trends and major weaknesses within the oat supply chain. This work will facilitate enhanced mitigation strategies amongst stakeholders. It was not possible to undertake this study for the barley supply chain due to a lack of samples.

Particular consideration was given to the sampling of grains for mycotoxin analysis, the importance of which cannot be overestimated. The most significant errors associated with mycotoxin testing are derived from incorrect sampling and as a direct consequence of the heterogeneous nature of grain contamination, resulting in mycotoxin hotspots.

The ASSET laboratory at Queen's University Belfast (QUB) employed state-of-the-art liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the mycotoxin profiles and contamination levels in the oat and barley survey samples.

The methodology for the regulated mycotoxins present in cereal crops has been developed and implemented on the AB SCIEX Triple Quad™ 5500+ System, a sensitive platform that enables measurement to low ppb concentrations.

Objective 5

Through stakeholder engagement and preliminary kit analyses, there is already some feedback from the industry that rapid tests for mycotoxins are not fit for purpose. The survey aimed to explore this to ascertain where the problems lie. Are end-users not suitably equipped to perform the tests? Do they require training to overcome this? Is it poor kit performance, or are sampling procedures not being performed correctly and thus accounting for huge discrepancies in results?

Following the review of the commercially available rapid diagnostic kits, several manufacturers' kits were selected (based on claimed performance) for an in-depth evaluation of their actual performance and therefore their fitness for purpose for the analysis of T-2 and HT-2 toxins in oat and barley grains. A comparative study of LC-MS/MS and rapid test kits was performed at QUB. This involved selecting a series of samples (n = 100), below, at, and above the regulatory limits for the mycotoxins of interest. In addition, representatives from the industry participated in studies using the best performing kit to allow assessment of end-user proficiency. This involved analysis of the same samples by the industrial partner, QUB and the kit manufacturer.

As a result of these evaluations – the comparative study with LC-MS/MS and end-user proficiency testing, the performance of the kits – the next steps were determined. It was agreed, should the performance of the kits be poor, QUB would select a number of kits (two) and seek to make improvements. To do this, sample preparation techniques would be examined, with the aim of providing techniques that are more robust and thus deliver more accurate and reliable results. However, in light of the results and the fact that one test kit, the Neogen Reveal® Q+ MAX for T-2 / HT-2 Kit performed well, this study was not performed. Alternatively, if the difficulties lie with industry in performing the analyses, comprehensive training for staff will be undertaken in a follow-up workshop.

Objective 6

Recommendations for stakeholders on the island of Ireland.

These will be widely disseminated through the ***safe*food** Knowledge Network and other means of reaching as wide a range of interested parties on the island as possible. In addition, workshops will facilitate feedback and recommendations to stakeholders. During these workshops, the results of the project and a range of mitigation strategies will be discussed.

Objective 1

Literature review of the most up-to-date scientific and regulatory data for T-2 toxin and its metabolites, the specific environmental conditions required for their production, and the impact of climate change on their occurrence and distribution.

Introduction

Mycotoxins are secondary metabolites of fungi produced by various genera of fungi including *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* (CAST, 2003; Luo et al., 2021). They are ubiquitous in a wide range of crops and the products derived from them (CAST, 2003; Steyn, 1995). Of the 400 or so identified mycotoxins, only a small number are considered important economically in terms of the adverse health effects they exert on humans and animals and the financial losses that may result from contamination of crops, including reduced yields and commodity values (Bennett and Klich, 2003; CAST, 2003). Accordingly, these metabolites are subject to regulatory maximum limits or guidelines in food and animal feed; they include aflatoxins, fumonisins, OTA, zearalenone, deoxynivalenol, T-2 and HT-2 (EC, 2006a; EC, 2006b; EU, 2010; EU, 2013; EC, 2003).

One of the major classes of mycotoxins posing serious hazards to humans and animals and causing severe economic impact to the cereal industry are the trichothecenes (McCormick et al., 2011). They are a large family (over 200 toxins) of structurally related compounds produced by a broad range of species of fungi such as *Fusarium*, *Cephalosporium*, *Myrothecium*, *Trichoderma*, *Stachybotrys*, *Spicellum*, *Trichothecium* and others in maize, oats, wheat, barley, rye, rice, walnut and tomato (Bennett and Klich, 2003; CAST, 2003; McCormick et al., 2011). The most important of these fungal genera are the *Fusaria* since they have adapted to a broad range of habitats, are a global problem, and produce the greatest range of trichothecenes (CAST, 2003). Although detected globally in cereals such as maize, oats, wheat, barley and rye, they are generally considered as temperate climate mycotoxins (Eskola et al., 2020). In addition to the production of mycotoxins, some species are important plant pathogens causing disease in oats, barley, wheat and maize

(McCormick et al., 2011). Although trichothecenes are the most chemically diverse of the mycotoxins, only a few are important to human and animal health. The most common metabolites identified in agricultural produce are diacetoxyscirpenol, monoacetoxyscirpenol, T-2 and HT-2 toxin, neosolaniol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, deoxynivalenol, nivalenol and fusarenon-x (EC, 1994).

These toxins are sesquiterpene alcohols or esters containing a 12, 13-epoxide group derived from a common tricyclic skeleton (Plattner et al., 1989). The tricyclic nucleus is known as trichothecene and the epoxide at C-12 and C-13 is essential for toxicity (Desjardins et al., 1993). Trichothecenes are further classified as macrocyclic or non-macrocyclic, which are determined by the presence of either an ester or ester-ester bridge between carbons 4 and 15. Macrocyclic trichothecenes include satratoxins, roridins, verrucarins and atranones and are produced primarily by *Myrothecium*, *Stachybotrys* and *Trichothecium* species. The smaller non-macrocyclic trichothecenes are subdivided into Type A, having a hydrogen or ester-type side chain at C-8, and Type B, which have a ketone group at this position. Type A trichothecenes include T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol while nivalenol, deoxynivalenol or vomitoxin and fusarenon-x comprise the Type B trichothecenes (Bennett and Klich, 2003). T-2 and HT-2 toxins are the focus of this review and Table 1 highlights the specific side chains of these toxins.

Table 1: Specific side chains of T-2 and HT-2 toxins.

Trichothecene	**R1**	**R2**	**R3**	**R4**	**R5**
T-2 Toxin	-OH	$-OCOCH_3$	$-OCOCH_3$	-H	$-OCOCH_2CH(CH_3)_2$
HT-2 toxin	-OH	-OH	$-OCOCH_3$	-H	$-OCOCH_2CH(CH_3)_2$

T-2 and HT-2 toxins are non-volatile low molecular weight compounds (MW 250-500). They are fairly insoluble in water but extremely soluble in many solvents, e.g., ethyl acetate, acetone, chloroform, methylene chloride and diethyl. The metabolites are remarkably stable; inactivation of T-2 toxin only occurs if subjected to 10 minutes

at 482°C or 30 minutes at 260°C. Hence, T-2 and HT-2 toxins are not degraded during normal food processing. They are stable at neutral and acidic pH and therefore not hydrolysed during digestion, nor are they affected by irradiation or in saline environments. An effective method used to deactivate the toxins is treatment with a 3% - 5% solution of sodium hypochlorite, the efficiency of which may be increased by addition of alkali (Rocha et al., 2005; Wannemacher and Wiener, 1997).

Exposure to these mycotoxins can happen by various means: ingestion of contaminated produce, adsorption through the skin following contact with contaminated grains, and inhalation.

This review, following the Campbell Methods Guide for information retrieval (Kugley et al., 2017) aimed to:

- Elucidate the toxicological effects of T-2 toxin and its metabolites in animals and humans.
- Report the regulatory limits for T-2 toxin and its metabolites.
- Highlight the specific environmental conditions required for their production, including information on their biosynthetic pathways.
- Ascertain the impact of climate change on the occurrence and distribution of these toxins.
- Identify the state-of-the-art methods of analysis for T-2 and HT-2 toxins.
- Expose knowledge or evidence gaps.

The methodology used for the systematic review is described in Appendix A.

Toxicity of T-2 and HT-2 toxins

The risks posed by these toxins have been assessed *in vitro*, in experimental animals, and in livestock following consumption of naturally contaminated feed. There have been a few incidences where humans exposed to these mycotoxins have exhibited toxic infections. The toxins are lipophilic and therefore easily absorbed through skin, gut and pulmonary mucosa (Wannemacher and Wiener, 1997). The pharmacokinetics revealed that, regardless of the species tested or the

route of administration, T-2 toxin was very rapidly metabolised and excreted in the urine and faeces (IARC, 1993; Wannemacher and Wiener, 1997). The International Agency for Research on Cancer (IARC) concluded that as there was no available data related to the carcinogenic effects of T-2 toxin in humans, and there was limited evidence of carcinogenicity in animals, T-2 toxin is not classified as a human carcinogen (Group 3) (IARC, 1993).

Typical clinical effects of T-2 toxin, through oral, dermal or inhalation exposure, include gastric and intestinal lesions, haematopoietic and immunosuppressive effects, anorexia, lassitude, and nausea; suppression of reproductive functions; and acute vascular effects resulting in hypotension and shock. Dermal exposure is exhibited by skin necrosis and inflammation and oral exposure by lesions in upper gastrointestinal tract. Corneal injury may be observed in those exposed to T-2 aerosols (Wannemacher and Wiener, 1997).

Toxic effects in animals

T-2 and HT-2 toxins are capable of inducing acute and chronic effects in animals depending on dosage, the route and duration of exposure, animal sensitivity, and the age, sex and health of the animal (Adhikari et al., 2017; Yiannikouris and Jouany, 2002). Clinical symptoms of T-2 and HT-2 mycotoxicoses in animals include weight loss, decreased feed conversion and feed refusal, vomiting, diarrhoea, skin problems, haemorrhage, decreased egg production, abortion and – in severe cases – death (CAST, 2003; Čonková et al., 2003; Yiannikouris and Jouany, 2002). Moreover, these toxins are potent inhibitors of protein synthesis and are immunosuppressive, although less so than type B trichothecenes (Bondy and Pestka, 2000; Rocha et al., 2005). Generally, T-2 and HT-2 are more toxic due to their dermatotoxic effect, which results in necrosis and haemorrhage of the intestinal mucosa (Diaz, 2005). Many studies have been performed on animals to help us to first understand the toxicity and metabolism of these trichothecenes, and secondly their mechanism or mechanisms of action at the cellular level. Another indication of the potency of these trichothecenes is obtained from LD_{50} values; that is, the amount of the chemical required to kill one-half of a population of organisms in a short time.

While these are useful, reports of natural infection by consumption of naturally contaminated grain are of greater value. Table 2 outlines acute toxicities of T-2 and HT-2 toxins in various animal species.

Table 2. Acute toxicity of the chosen trichothecenes in a range of animal species, LD_{50} (mg/kg).

Trichothecene	**Species**	**Administration Method**										
		IV	**IP**	**SC**	**PO**	**IM**	**IN**	**IH**	**IC**	**IG**	**IT**	**D**
T-2 Toxin	Mouse	4.2-7.3	5.2-9.1	2.1-3.3	7-10.5			0.24		9.6-10.5	0.16	6.6
	New-born mice			0.15								
	Rat	0.7–1.2	1.3-3.0	0.6-2.0	3.8-5.2	0.5-0.9	0.6	0.05	0.01	2.3-5.2	0.1	4.3->380
	Cat			<0.5								
	Pig	1.2			4.0							
	Chick				1.84-4.0							
	Rabbit					1.1						10.0

Trichothecene	Species	Administration Method										
		IV	**IP**	**SC**	**PO**	**IM**	**IN**	**IH**	**IC**	**IG**	**IT**	**D**
T-2 Toxin	Guinea pig	1.0-2.0		1.0-2.0	3.0	1.0		0.6-2.0		3.1-5.3		2.2->80
	Monkey					0.8						>8.0
	Trout				6.1							
HT-2 Toxin	Mouse		9.0									

IV = Intravenous, IP= Intraperitoneal, SC = Subcutaneous, PO = Oral, IM = Intramuscular, IN = Intranasal, IH = Inhalation, IC = Intracerebral, IG = Intragastric, IT = Intratracheal, D = Dermal (CAST, 2003; EC, 1994; Wannemacher and Wiener, 1997)

Poultry

Poultry are extremely sensitive to T-2 toxin and birds suffering from T-2 toxicosis display oral lesions, dermatitis and irritation of the intestines, thus leading to loss of appetite or feed refusal (Diaz, 2005; Hayes and Wobeser, 1983). Another toxic effect is altered feather structure or 'Helicopter disease' (Diaz, 2005; Murugesan et al., 2015; Wyatt et al., 1975). Consumption of contaminated feed containing 2-6 mg T-2 toxin/kg induced a reduction in feed conversion efficiency, weight gain and feed intake (Raju and Devegowda, 2000; Wyatt et al., 1975; Yang et al., 2016). Concentrations as low as 0.5-1 mg/kg affected the epithelial cells of the oral mucous membranes, causing oral lesions, indicating the animals are more sensitive to lesions than growth retardation (Chi et al., 1977; Diaz et al., 1994). Moreover, as an irritant, T-2 toxin has caused necrosis of proventricular mucosa and gizzard erosion. It is also known to cause tibial dyschondroplasia in broilers, a metabolic disease of young poultry that affects the growth of bone and cartilage (Diaz, 2005; Pinton et al., 2012).

Additional symptoms observed include decreased egg production, poor shell quality, (Raju and Devegowda, 2000) and regression of ovaries in laying birds (CAST, 2003; Diaz, 2005). A report of T-2 toxicosis on a farm highlighted that egg production was reduced by approximately 22%, the number of cracked eggs increased by 12%, egg breakage was in the region of 18%, incidences of blood spots increased from 0-3% and oral lesions were observed in over 85% of laying hens (Diaz, 2005). Other reported impacts on productivity included poor hatchability and a high mortality of goslings and turkey poults, reduced serum total protein and increased concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Diaz, 2005; Yang et al., 2016). Common immunosuppressive effects of T-2 toxin are leucopoenia, regression of the bursa of Fabricius and increased susceptibility to Salmonella infection (CAST, 2003; Diaz, 2005; Murugesan et al., 2015).

Pigs

Haemorrhages on the serosa of the liver, stomach and oesophagus, blood in the intestines and abdominal cavity and a cream-coloured paste on the lining of the oesophagus and the ileum have been reported in pigs as a result of exposure to T-2 toxin (Kanora and Maes, 2009; Pinton et al., 2012). In addition, various feeding trials have been completed. Animals fed T-2 toxin concentrations of 5-10 mg/kg feed exhibited reduced feed intake and reduced weight gain (Harvey et al., 1994; Harvey et al., 1990). Reduction in feed intakes have also been seen in seven-week-old piglets fed contaminated feed at a concentration of 0.5 mg T-2/kg feed, in addition to decreased plasma leucocytes, confirming the immunosuppressive nature of the toxin (Rafai et al., 1995a; Rafai et al., 1995b). In sows, T-2 toxin has been shown to induce infertility and abortion, thus highlighting its endocrine-disrupting capabilities (D'Mello et al., 1999). Dermatitis of the nose and at the corners of the mouth are typical symptoms resulting from T-2 exposure (Diaz, 2005). The influence of T-2 toxin on the enteric nervous system important in the regulatory processes in the gastrointestinal tract and in the adaptive and protective responses to toxins were summarised in a recent review (Gonkowski et al., 2020). The findings suggested that low T-2 exposure might affect digestive motility, secretion, sensory nerve conduction and the regulation of intestinal wall blood-flow (Gonkowski et al., 2020).

Ruminants

While ruminants are reported to be less sensitive to the effects of mycotoxins due to the efficiency of rumen bacteria in detoxifying these toxins (Kemboi et al., 2020; Ogunade et al., 2018), the available literature suggests that cattle are more sensitive to T-2 toxin compared with other trichothecenes (Gallo et al., 2015). The main effects described were lesions and haemorrhage in the gastrointestinal tract, enteritis, altered immunity and changes in metabolism (Gallo et al., 2015). It was postulated that T-2 toxin induced immune suppression in cattle due to a reduction in serum concentrations of IgM, IgG and IgA (Mann et al., 1983), decreasing neutrophil function and lymphocyte blastogenesis (Mann et al., 1984). In addition, necrosis of lymphoid tissues was shown to be triggered by T-2 toxin (Buening et al., 1982), and

bovine infertility and abortion resulting from consumption of T-2 toxin contaminated feed was reported (Placinta et al., 1999). In calves, consumption of this toxin at levels of 10-50 mg/kg in feed led to ulceration of the abomasum and sloughing of the papilla in the rumen (Cheeke, 1998), while dairy cows demonstrated haemorrhagic syndrome after consuming mouldy corn contaminated at 1 mg T-2 toxin/kg feed (Hsu et al., 1972). In addition, because of the cytotoxicity of T-2 toxin, severe irritation of the upper respiratory tract and haemorrhagic ruminitis have been reported in cattle following consumption of contaminated feed (Fink-Gremmels, 2008). In dairy cattle, the observed effects of T-2 toxin were feed refusal, gastrointestinal lesions, haemorrhagic gastroenteritis, depression, apathy, anorexia, hindquarter ataxia and knuckling of the rear feet. Moreover, the oestrus cycle was absent and there was a reduction in milk production (Kemboi et al., 2020). An incidence of poisoning of sheep by consumption of T-2 contaminated feed described both the acute and chronic effects observed (Ferreras et al., 2013). In the acute phase, sheep were found to be listless, displaying anorexia, ruminal atony and soft faeces, and there was a marked reduction in their water consumption. Almost 20% of the sheep died and the animals exhibited rumenitis, ulcerative abomasitis, exocrine pancreatic necrosis, a reduction in white blood cells, inflammation of the heart muscle and oedema of the brain and skin (Ferreras et al., 2013). Chronic pathology revealed the animals presented with weight reduction, reproductive inefficiency, inflammation of the gastrointestinal tract, oral lesions, myocardial fibrosis, immune suppression and altered serum enzymes (Ferreras et al., 2013).

Domestic animals

Little published information exists regarding the effects of T-2 or HT-2 toxins on domesticated animals. In a study evaluating the effect of T-2 toxin on white rabbits over a period of 32 days, two rabbits died. Serum enzymes concentrations were altered and some liver cell damage was observed. These results further supported the immunotoxicity of this trichothecene. Plasma progesterone levels were affected, thus suggesting reproductive effects (Diaz, 2005). In cats, administration of T-2 toxin resulted in symptoms similar to those causing alimentary toxic aleukia, a human disease caused by consumption of T-2 toxin contaminated grains. Clinical

observations included vomiting, blood in the faeces, dehydration, weight loss, lethargy, ataxia, shortness of breath and anorexia. Furthermore, bone marrow aplasia, lymphatic tissue alterations, bleeding diathesis, reduced haemostasis and changes in proliferative tissues were demonstrated (Diaz, 2005). In mares dosed orally with T-2 toxin for 32-40 days, oral lesions were observed in three animals, but no reproductive effect was reported (Caloni and Cortinovis, 2010).

Rodents

In rats, the immunopathology of low-dose chronic exposure to T-2 toxin was evaluated and the results indicated that both humoral and cell-mediated immune responses were suppressed (Rahman et al., 2021). In another study in Wistar rats, injury to cardiac tissue was observed on days 28 and 60 following a single injection of T-2 toxin (0.23 mg/kg SC) (Jaćević et al., 2020). To elucidate the mechanisms behind T-2 induced anorexia, changes in gut satiety hormones peptide YY3-36 (PYY3-36) and glucose-dependent insulinotropic polypeptide (GIP) in plasma were evaluated. Mice were exposed both orally and by intraperitoneal injection of 1mg/kg bw T-2 and HT-2. The results highlighted decreased food intake and elevated PYY336 and GIP concentrations, indicating that these play a role in T-2 and HT-2 induced anorexia (Zhang et al., 2017).

Aquaculture

Few studies have been reported regarding the effects of these trichothecenes on fish. Growth retardation, reduced feed intake efficiency and dose-dependent depression of haematocrit and haemoglobin concentrations were observed in rainbow trout following a 16-week experiment where they were fed >2.5mg/kg T-2 toxin. Intestinal haemorrhaging, enlarged spleens and gall bladders were observed in adult trout fed 15 mg/kg T-2 toxin (Matejova et al., 2017). Similarly, low haematocrit values, poor weight gain, reduced feed conversion rations and gastric lesions were demonstrated in catfish fed T-2 toxin (Matejova et al., 2017).

Toxic effects in humans

In humans, there have been a few reports of intoxications associated with these trichothecenes. The most notable is alimentary toxic aleukia (ATA), which affected a large proportion of the population (most of which were aged between 10 and 40) in the former U.S.S.R. from 1932 until 1947 and was thought to be due to the consumption of overwintered grain contaminated with T-2 toxin and DAS (Peraica et al., 1999; Peraica et al., 2014). The mortality rate was 60%. Initial exposure resulted in gastroenteritis, gastritis, vomiting, diarrhoea, abdominal and oesophageal pain (Bennett and Klich, 2003; Peraica et al., 1999; Wannemacher and Wiener, 1997). In addition, excessive salivation, headache, dizziness, weakness, fatigue, tachycardia, fever and sweating may also present (Wannemacher and Wiener, 1997). A longer exposure of 3-4 weeks caused vertigo, an unpleasant taste in the mouth, leukopenia, granulopenia and progressive lymphocytosis, and if further exposure occurred, the terminal phase developed. This stage was characterised by haemorrhagic diathesis of the nasal, oral, gastric and intestinal mucosa, angina, petechial rash and gangrenous laryngitis, leading to aphonia and death by asphyxia (Peraica et al., 1999; Wannemacher and Wiener, 1997). The final recovery stage lasted several weeks to two months; however, it was associated with secondary infections such as pneumonia (Peraica et al., 2014; Wannemacher and Wiener, 1997). Where the disease outbreak occurred, 5-40% of grain samples showed the presence of *Fusarium* sporotrichioides and *Fusarium* poae, whereas in regions where no disease was present only 2-8% of grain samples proved positive for these fungi (Peraica et al., 1999). It has been subsequently demonstrated that T-2 toxin was the probable cause (Wannemacher and Wiener, 1997). Since the ATA outbreak in the former U.S.S.R., no further human mortalities have been reported due to the consumption of trichothecene contaminated cereals.

T-2 toxin has been linked to Kashin–Beck disease (KBD), an endemic, chronic joint disease typically found in rural regions of eastern Siberia, northern Korea and in central China. Common symptoms include pain, stiffness and enlargement of the joints, accompanied by restriction of movement. Although the aetiology has not been defined, high concentrations of T-2 toxin in the food in endemic areas have been reported and similar pathological cartilage changes in chicks compared with KBD

patients have been observed in experimental studies. Epidemiological studies are required to prove the link between this trichothecene and KBD (Li et al., 2016).

There have also been implications that T-2 toxin has been used in biological/chemical warfare in Afghanistan, Kampuchea and Laos from 1975 to 1984 (Tucker, 2001). Although supported by intelligence reports, epidemiological data and trichothecene analysis, the claims of the “yellow rain” attacks have been discounted in the scientific literature (Wannemacher and Wiener, 1997). According to refugee accounts, sufferers experienced severe burning of the skin and began vomiting almost immediately following exposure to the yellow rain. Additional symptoms included eye pain, blurred vision, headache, dizziness, rapid heartbeat and low blood pressure, chest pain, poor coordination, severe coughing fits, breathing distress and diarrhoea. Areas of exposed skin broke out in blisters. The mortality rate was between 10% to 20% of those exposed, with death occurring within a few days to a few weeks (Tucker, 2001). Animal deaths including chickens, dogs, pigs, cattle and water buffalo were also reported, in addition to the contamination and death of crops. Mass spectrometric analysis of leaf and stem fragments marked with yellow spots that had supposedly been collected from a battlefield in Cambodia within 24 hours of a yellow rain attack were positive for three trichothecenes, as was a sample of yellow powder scraped off foliage in Laos. In total, six positive environmental samples and 20 positive human biomonitoring samples led US intelligence to conclude that trichothecenes were being used as biological/chemical agents (Tucker, 2001; Wannemacher and Wiener, 1997). However, criticism of the method of analysis, the control samples used and the absence of any such weapon being found means that there is no unequivocal proof that trichothecenes were used in biological warfare (Wannemacher and Wiener, 1997).

European Union and United Kingdom Regulations for T-2 and HT-2 toxins

In 2013, the European Union published their recommendation as regards the presence of T-2 and HT-2 in cereals and cereal products (EU, 2013). In infected cereal grains, generally, T-2 toxin will co-occur with HT-2 toxin and *in vivo* T-2 toxin

is rapidly hydrolysed to HT-2 toxin (EU, 2013; Kemboi et al., 2020). For this reason, when performing any risk assessment, it is the sum of T-2 and HT-2 toxins that is considered.

The Scientific Panel on Contaminants in the Food Chain (CONTAM panel) of the European Food Safety Authority (EFSA) established a group tolerable daily intake (TDI) of 0.1 µg/kg bw for the sum of T-2 and HT-2 (EFSA, 2011). At that time estimates of human chronic exposure based on occurrence data fell below the TDI and therefore the toxins were not deemed an immediate health risk. With respect to animal health, the risks were considered low for most animals, with the exception of cats. Limited data prevented the establishment of a No Observed Adverse Effect Level (NOAEL) or Lowest Observed Adverse Effect Level (LOAEL); therefore the recommendation does not include cat food. It was also concluded that there was no evidence of the accumulation of these toxins in the tissues of animals fed contaminated feed, which means human exposure via this route poses no public health concerns (EU, 2013). It was also recommended that more data on the occurrence of T-2 and HT-2 toxins in cereals and cereal products be collected, in addition to more information on the effects of food processing on the toxins, what factors contribute to high levels of contamination, and what mitigation strategies could be employed.

Consequently, the European Commission has set indicative limits for these toxins in cereals intended for animal and human consumption. The levels outlined by Commission Recommendation 2013/165/EU refer to the sum of T-2 and HT-2 and are outlined in Table 3. Where contamination is observed at or above these levels, further testing is required to establish if this was an isolated incident. Repetitive findings require studies to be conducted to identify the factors contributing to the high levels of contamination (EU, 2013).

A major complication and a topic of huge concern to the food industry is that the EU is currently in discussions with EU member states to set maximum levels for the sum of T-2 and HT-2 in cereals and cereal products. This has been prompted by a re-evaluation of the group TDI in 2017, when it was changed from 0.1 µg/kg bw to 0.02 µg/kg bw for the sum of T-2 and HT-2 (Arcella et al., 2017). The proposed limits are detailed in Table 3 (Agrolab, 2020; Meyer et al., 2021). What impact will this have on

the industry? How will the new levels be monitored? How will the industry mitigate against contamination? These are all pertinent questions to be answered.

Table 3. EU Indicative limits and maximum limits under discussion for the sum of T-2 and HT-2 toxins in cereals and cereals products.

Commodity	**Indicative limits for the sum of T-2 and HT-2 (µg/kg)**	**Maximum limits under discussion (µg/kg)**
Unprocessed Cereals:		
Barley (including malting barley) and maize	200	100
Oats (with husk)	1000	500
Wheat, rye, other cereals	100	50
Cereals for direct human consumption		
Oats	200	50
Maize	100	50
Other cereals	50	20

Commodity	**Indicative limits for the sum of T-2 and HT-2 (µg/kg)**	**Maximum limits under discussion (µg/kg)**
Cereal products for human consumption:		
Oat bran and flaked oats	200	50
Cereal bran except oat bran, oat milling products other than oat bran and flaked oats, and maize milling products	100	50
Other cereal milling products	50	20
Breakfast cereals including formed cereal flakes	75	20
Bread (including small bakery wares), pastries, biscuits, cereal snacks, pasta	25	10
Cereal-based foods for infants and young children	15	10
Cereal products for feed and compound feed:		
Oat milling products (husks)	2000	

Commodity	Indicative limits for the sum of T-2 and HT-2 (µg/kg)	Maximum limits under discussion (µg/kg)
Other cereal products	500	
Compound feed, with the exception of feed for cats	250	

T-2 and HT-2 Production

Phytopathogenic fungi in the *Fusarium* genus are known to cause economically important diseases such as *Fusarium* head blight (FHB) of wheat and barley, as well as ear rot of maize, in all climatic zones across the world, leading to substantial decreases in both the yield and quality of crops (Goswami and Kistler, 2004; Martínez et al., 2020; Munkvold, 2003). Additionally, more than 300 species of *Fusarium* have been reported to cause diseases in crops, with FHB the major economically important fungal disease of cereal crops (Goswami et al., 2004; Kazan et al., 2018). FHB is mainly caused by the hemi-biotrophic pathogenic species, *F. graminearum* (*Gibberella zeae*). Other notable *Fusarium* species associated with FHB include *F. culmorum*, *F. poae*, *F. avenaceum* and *F. nivale* (Stępień and Chełkowski, 2010). Aside from the poor crop yield and quality, *Fusarium* species also produce vast numbers of mycotoxins in infected grains, with T-2 and HT-2 toxins being amongst the most important mycotoxins from a food and feed safety perspective (Ferrigo et al., 2016; Ismaiel et al., 2015).

More than 200 trichothecenes have been identified and based on their chemical structures they are divided into four types (A, B, C and D) (Proctor et al., 2018). However, type A (such as T-2 and HT-2 toxins) and B (including deoxynivalenol (DON) and nivalenol (NIV)) are the known frequent contaminants of agricultural commodities worldwide (Mousavi Khaneghah et al., 2020; McCormick et al., 2011). So far, more than 15 genes have been reported to be involved in the T-2 and HT-2 biosynthetic pathways. Farnesyl pyrophosphate (FFP), a compound synthesised through methylerythritol 4-phosphate and mevalonate- independent pathways, has been shown to be the main precursor or substrate for trichothecene A biosynthesis (Chen et al., 2019; Suzuki et al., 2012). FFP is cyclised by trichodiene synthase enzyme encoded by TRI5 gene, to produce trichodiene (Chen et al., 2019; Kimura et al., 2007). Thereafter, enzymes encoded by TRI4, TRI101, TRI11 and TRI3 genes sequentially catalyse the conversion of trichodiene to calonectrin (Chen et al., 2019; Kimura et al., 2007).

Most *Fusarium* species that produce type A trichothecenes share similar enzymatic reaction steps, i.e., the conversion of FPP to calonectrin. However, depending on

the *Fusarium* strain, chemotype and geographical distribution, different mycotoxins can be produced by trichothecene-producing *Fusarium* species (Cardoza et al., 2011). In DON-producing *F. graminearum* strains, TRI1 gene mediates the direct conversion of calonectrin to 7,8-dihydrocalonectrin, with subsequent biosynthesis of DON (Meek et al., 2003). In NIV-producing *F. graminearum strains*, TRI7 and TRI13 are the important genes of this *F. graminearum* chemotype strain and are inactive in DON producers (Lee et al., 2001; 2002). The two alternative pathways proposed for the biosynthesis of NIV and its acetylated derivatives (4-acetyl-NIV and 15-acetyl-NIV) are TRI13-TRI7-TRI1-TRI8 pathway, using calonectrin as the substrate, and TRI13-TRI7-TRI8 pathway, using 3,15-acetyl DON as the substrate (Lee et al., 2001; 2002) (Figure 1). Regarding T-2 and HT-2 toxins production, the allelic variations of TRI1 gene is responsible for the structural differences seen in type A and type B trichothecenes (Alexander et al., 2011). In *F. sporotrichioides* and *F. langsethiae*, the major producer of T-2, TRI1 hydrolyses 3,4,15-triacetoscirpenol to yield 3-acetylneosolaniol, while TRI16, a C-8 acyltransferase, catalyses the conversion of 3-acetylneosolaniol to 3-acetyl-T-2 toxin. T-2 toxin is produced following the deacetylation of 3-acetyl-T-2 toxin by TRI8 gene (Meek et al., 2003). HT-2 is the major metabolite of T-2 toxin, and it is formed through hydrolysis of T-2's acetyloxy group at position 4S.

Worldwide occurrence of T-2 and HT-2 in oats and barley

Data on the global occurrence of T-2 and HT-2 in feed and food have shown significant temporal and geographical variations (Ramos-Dias et al., 2021). In addition to agronomic factors, climatic factors, particularly temperature and moisture, markedly affect fungal growth and the colonisation of cereals, leading to varying concentrations of mycotoxins (especially T-2 and HT-2) across different climatic zones (Van Der Fels-Klerx et al., 2010; DeColli et al., 2021; Kolawole et al., 2021a). For instance, in North America and other Asian countries, incidences of FHB are very high in wheat, maize and barley, resulting in high accumulation of key *Fusarium* mycotoxins such as deoxynivalenol, zearalenone, T-2 and HT-2, and fumonisins (Islam et al., 2021). However, in Northern and Western European countries, oats are

less susceptible to FHB. This is because of the lack of visual symptoms on the long pedicels between oat spikelets, which prevent the spread of fungal mycelia throughout the panicle (Martin et al., 2018; Imathiu et al., 2017). Nonetheless, previous mycotoxin surveys have shown oat grains from this region are frequently contaminated with high levels of T-2 and HT-2 compared to tropical regions and other cereals such as wheat and maize (Edwards, et al., 2017; DeColli et al. 2021; Kolawole et al., 2021a, Ramos-Dias et al., 2021) (Table 4).

A very high prevalence of T-2 and HT-2 was found in 458 unprocessed oat samples collected between 2002 and 2005 from various oat fields in the UK (Edwards et al., 2009a). T-2 and HT-2 was detected in 84% and 92% of samples analysed, with combined mean levels (T-2 and HT-2) of 570 µg/kg and a maximum concentration of 9990 µg/kg (Edwards et al., 2009a). Similarly, Edwards et al. (2017) reported a mean of 450 µg/kg of sum of T-2 and HT-2 in UK oat samples collected over three years (2006 to 2008). Unprocessed oat samples analysed for multi-mycotoxins, representative of Irish oat production in 2015 and 2016, also showed a very high prevalence of T-2 and HT-2 toxins (51%), with a mean concentration of 770 µg/kg (DeColli et al., 2021). A three-year monitoring (2013–2015) of *Fusarium* mycotoxins in Swiss oat grains harvested at different times showed annual variations (65–76%) in T-2 and HT-2 prevalence, with mean and maximum concentrations of 1091 µg/kg and 3789 µg/kg, respectively (Schöneberg et al., 2018). High levels of T-2 and HT-2 were also detected in oats from Norway (Hofgaard et al., 2016), Finland (Hietaniemi et al., 2016) and Sweden (Fredlund et al., 2013). Conversely, the mean of the sum of T-2 and HT-2 concentrations (87.9 µg/kg) in unprocessed Canadian oats collected between 2017 and 2018 was significantly lower compared to the levels of T-2 and HT-2 detected in European oats (Ramos-Dias et al., 2021; Xue et al., 2019). A summary of the prevalence and concentrations of sum of T-2 and HT-2 in oats and barley collected from various countries or regions is presented in Table 4.

Table 4. Worldwide occurrence of T-2 and HT-2 toxins in oats and barley.

Region	Year	Commodity	Number of samples	Positive samples (%)	Average	Range (µg/kg)	Reference
Egypt	2021	Oats	10	70	35.4	14.3 – 74.4	Tahoun et al., 2021
Canada	2016 -2018	Oats	168	81	39.0	10 – 1155.2	Islam et al., 2021
Croatia	2017-2018	Oats	30	70	87.9	9.5 – 21.8	Kiš et al., 2021
Europe	2013-2019	Oats	281	98	103.1	5.1 – 1000	Meyer et al., 2021
Lithuania	2010-2018	Oats	72	—	182	—	Kochiieru et al., 2020
Finland	2005-2006	Oats	804	100	348	25 – 17,451	Schöneberg et al., 2018

Region	Year	Commodity	Number of samples	Positive samples (%)	Average	Range (µg/kg)	Reference
Norway	2004-2009	Oats	289	76	105.2	10.2 – 658.1	Hofgaard et al., 2016
UK	2006-2008	Oats	303	85	450	10 – 8399	Edwards et al., 2017
Switzerland	2013 – 2015	Oats	325	76	225.5	10.1 - 3789	Hietaniemi et al., 2016
Croatia	2017-2018	Oats	30	100	87.9	14.3 – 21.8	Kiš et al., 2021
Ireland	2015-2016	Oats	208	51	256	53 – 3405	DeColli et al., 2021
Ireland	2020	Oats	202	62	138	5 – 3064	Kolawole et al., 2021a

Region	Year	Commodity	Number of samples	Positive samples (%)	Average	Range (µg/kg)	Reference
Hungary	2014-2015	Oats	29	10	56	50.1 – 69.2	Tima et al., 2016
Croatia	2017-2018	Barley	66	41	22.6	12.2 – 52.1	Kiš et al., 2021
Czech	2012-2017	Barley	117	20	—	11.8 – 199.0	Svoboda et al., 2019
Italy	2011-2014	Barley	691	32	127.8	26.0 – 787.0	Morcia et al., 2016
Hungary	2014-2015	Barley	29	14	58.0	52.0 – 79.0	Tima et al., 2016

—: Not stated

Impact of climate change on T-2 and HT-2 production

Fusarium growth and the production of secondary metabolites have been shown to be highly dependent on many ecophysiological or abiotic factors (Magan & Medina, 2011; Xu et al., 2014). Temperature, carbon dioxide and water activity are the main factors that have a strong influence on fungal development, transcriptional activation of mycotoxin biosynthetic genes and mycotoxin production (Verheecke-Vaessen et al., 2019). However, exposure of mycotoxigenic fungi to individual abiotic factors have less modifying effects compared to combined or interacting multiple environmental variables (Magan & Medina, 2011). Therefore, depending on the combination of abiotic factors, mycotoxin biosynthetic pathways in mycotoxigenic fungal species can either be activated or inhibited (Verheecke-Vaessen et al., 2021; Kolawole et al., 2021b).

Both T-2 and HT-2 toxins are produced by specific *Fusarium* species including *F. acuminatum*, *F. sporotrichioides*, *F. poae*, and *F. langsethiae* (Edward et al., 2012; Imathu et al., 2017). In Europe and other temperate regions, *F. sporotrichioides* and *F. langsethiae* have been reported as the major producers of T-2 and HT-2 toxins (Edwards et al., 2012). Several *in vitro* and *in vivo* studies on climatic factors influencing the growth and T-2 and HT-2 production by *F. sporotrichioides* and *F. langsethiae* showed that both species grow within the temperature range of −2°C to 35°C, and water activity above 0.98_{aw} (Magan and Medina 2016; Imathiu et al. 2017). Furthermore, optimal conditions for toxin biosynthesis were recorded at 20–30°C and $0.98–0.99_{aw}$ (Medina and Magan 2011). A decrease in fungal growth and toxin production were observed when water stress increased (Medina and Magan 2011). Previous field experiments also showed that warm and wet weather conditions before anthesis (May to August) favour the infection of oat head and the accumulation of T-2 and HT-2 by *F. langsethiae* (Xu et al., 2014; Opoku et al. 2017; Edwards et al., 2017). In addition to weather conditions, agronomic practices (organic or conventional), previous crop, tillage and oat variety can also significantly influence T-2 and HT-2 accumulation in oat grains (Kolawole et al., 2021a; Edwards 2009a).

A considerable body of knowledge regarding the climatic conditions influencing the growth and production of T-2 and HT-2 has been established. However, there is currently no information on whether infection and toxin production by these *Fusarium* species occur before harvest or during storage. Furthermore, the ecology of *F. sporotrichioides* and *F. langsethiae*, as well as the influence of interacting environmental factors on their growth and the activation of biosynthetic genes, are still not fully understood. By identifying the impact of key factors and their interactions on mycotoxin accumulation and *Fusarium* species growth, a prediction model could be developed to predict the risk of contamination pre- and post-harvest, and thus allow for earlier interventions. Furthermore, as the EU is currently considering setting new legislative limits for both toxins in cereals, there is a need for continuous monitoring, particularly in oats and barley.

Analytical Methods

Overview

Prior to the analysis of a sample, a representative homogenous food or feed sample must be provided as the toxigenic fungus and associated mycotoxins produced are not always uniformly distributed throughout a lot. A 2006 study by Whitaker et al. indicated that the true concentration of a bulk sample lot could not be determined with 100% accuracy or certainty, and that sampling must follow a strict protocol (Whitaker, 2006). In particular, if a small sample size is selected, the variation in results will increase and therefore larger sample and sub-sample quantities are preferred to ensure an even distribution of particle size, which is usually carried out by blending and/or grinding. Despite the sampling issues, detailed sampling protocols specific for mycotoxins in foodstuffs, including the *Fusarium* produced mycotoxins such as T-2 and HT-2 toxins, have been published in the EU (EC, 2006c) and by the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the U.S. Department of Agriculture (www.gipsa.usda.gov).

In addition, the EU regulation (EU, 2013) includes guidelines on the performance criteria required for the testing of T-2 and HT-2 toxins, specifying that where there is a lack of fully validated methodology, a ‘‘fitness-for-purpose’’ approach may instead

be used. This is in part due to errors in the methodology that can be introduced during the analysis, such as the homogeneity of the sample, the stability and recovery of the analytes to be measured, instrument bias, measurement conditions, reagent purity, and the skill and experience of the operator.

The analytical procedures are classified and characterised by several facets, such as being fully quantitative, semi-quantitative or qualitative (screening). The type of analysis performed dictates the simplicity of the test, the speed of analysis and the level of technical skill required to perform the assay. Screening assays such as enzyme-linked immunosorbent assays (ELISAs) and lateral flow devices (LFDs) are simpler to use and report on, as well as delivering more rapid results, as they can be applied in the field where an answer may be required as soon as is reasonably possible. By comparison, fully quantitative methodologies are usually performed on more technological platforms, such as liquid chromatography coupled to mass spectrometry (LC-MS), which serve to increase both the speed and analytical complexity, but with a more accurate end result. Furthermore, the use of fully quantitative methods is more expensive than screening methods, leading to a greater cost (Meneely et al., 2011).

Extraction methodologies

In order to extract toxins from the matrix to be analysed, several techniques can be employed, including accelerated solvent extraction (ASE), ultrasonic extraction, liquid-liquid or solid-liquid extraction, all depending on the matrix to be analysed and the type of analysis to be performed. This usually requires an admixture of organic solvent such as methanol or acetonitrile along with water in various ratios, sometimes acidified with formic acid or acetic acid. The addition of water to the sample helps the organic solvent to penetrate the solid matrix, whereas the addition of acid helps break some bonds between the mycotoxin and matrix, both of which aid the extraction efficiency (Yang et al., 2020). However, the choice of extraction solvent is dependent on several factors, such as the number of analytes in the final methodology, the range of polarity in the suite of analytes and their stability in the extraction solvent chosen. In many cases the extraction of the *Fusarium* type A

trichothecenes such as T-2 and HT-2 are extracted using an admixture of an organic solvent with water, with ratios of acetonitrile: water between (50:50, v:v) and (80:20, v:v), with the addition of 0.3 - 10% formic or acidic acid (Malachová et al., 2014; Sulyok et al., 2020; Tamura et al., 2015; Seo et al., 2021). Incidentally, use of acetonitrile can be substituted for methanol in some cases. The use of aqueous buffers is also utilised for extraction, mainly for use with rapid test kits such as ELISAs, LFDs and fluorescence polarisation immunoassays (FPIAs), and is also dependant on the clean-up step employed afterwards, where use of organic solvents may affect the antibody used. In this case, the extract must be diluted with an aqueous buffer before being applied to the test kit (Nakhjavan et al., 2020; D'Agnello et al., 2021; Arola et al., 2017).

Various physical techniques have been reported for the mixing and extraction of the analytes from the sample using the desired extraction solvent, with use of rotation, sonication or shaking commonly employed, for as little as three minutes up to a maximum of 90 minutes (D'Agnello et al., 2021; Sulyok et al., 2020).

Sample clean-up

As well as extracting the analytes of choice from the matrix, one of the main issues is the co-extraction of matrix compounds and the associated matrix effects, which can lead to issues with the analysis. These include, but are not limited to, signal suppression or enhancement (SSE), poor chromatography, false positives due to the presence of isobaric compounds, and poor or incomplete recovery of the target analytes (Lehotay et al., 2015). Therefore, after the initial extraction, a sample clean-up step is usually employed in order to remove as much matrix as possible before analysis, which also serves to decrease the limit of detection (LOD) and limit of quantification (LOQ) should this be a requirement. This clean-up step is usually a requirement for confirmatory analytical methods, but not for most screening assays such as ELISAs, LFDs and biosensor assays, due to the specificity of the antibody used and sample dilution. However, if the LOD/LOQ is not at a suitable level, some form of clean-up step may be required to aid this (Meneely et al., 2011).

In recent years, many different techniques have been employed to remove or reduce matrix effects, such as solid phase extraction (SPE), liquid–liquid extraction (LLE), QuEChERS (quick, easy, cheap, effective, rugged and safe), solid-phase micro-extraction (SPME), immunoaffinity column (IAC) and dispersive liquid-liquid micro-extraction (DLLME), to name a few (Yang et al., 2020). Although these methodologies provide good sensitivity due to the removal of unwanted matrix components, their use limits the number of analytes that can be incorporated due to the selectivity and/or specificity of the techniques employed, i.e., the stationary phase or sorbent used in SPE or SPME, or the antibody used in IACs. However, in the analysis of T-2 and HT-2, this is not typically an issue as only two structurally and physio-chemically related compounds are to be analysed, with most IACs being selective for both, and with the same being true for any SPE column chosen. One thing to note, however, is that most LC-MS methods for the analysis of T-2 and HT-2 are generally not specific for those alone but are usually incorporated alongside numerous other mycotoxins in the analysis of various matrices (Malachová et al., 2014; Nathanail et al., 2015; Sulyok et al., 2020).

Solid Phase Extraction

As one of the most universal sample clean-up techniques, Solid Phase Extraction (SPE) is readily used for the extraction and concentration of mycotoxins from various matrices. It is based on the principle of the partitioning of the analytes between the stationary and liquid phase. The sample extract is applied to a pre-conditioned SPE column, with the analyte(s) retained on the column and then washed to remove impurities, with the target analytes eluted and the resulting eluate evaporated to dryness and reconstituted for analysis (Meneely et al., 2011). This technique affords enrichment of the analytes by their adsorption to the stationary phase; therefore, it is important to choose an appropriate SPE column to improve the selectivity (Yang et al., 2020). There are numerous SPE cartridges with varying chemistries available on the market used in the analysis of T-2 and HT-2 in foodstuffs. These include: Oasis HLB cartridges, MycoSep columns, Strata-XL cartridges, Oasis HLB cartridges, Bond Elute Mycotoxin columns (Gottschalk et al., 2007; Girolamo et al., 2020; Tolgyesi and Kunsagi, 2013; Miro-Abella et al., 2018; Klötzel et al., 2006). One thing

to note from the aforementioned analyses is that none were specific to T-2 and HT-2 only; instead they are multi-methods analysing a range of mycotoxins, including the *Fusarium*-produced type-A trichothecenes.

Similar to SPE, micro-extraction techniques such as solid-phase micro-extraction (SPME), stir-bar sorptive extraction (SBSE) and micro-extraction by packed sorbent (MEPS) are used for sample clean-up prior to analysis. SPME is more commonly employed alongside GC-MS, whereas MEPS, a miniaturised version of SPE, is fully compatible with LC-MS, with the latter now more routinely employed for the analysis of mycotoxins, and in particular the type A trichothecenes such as T-2 and HT-2. Furthermore, due to their size, SPME and MEPS are more suited to small volumes of biological fluids for the analysis of mycotoxins and their metabolites rather than in foodstuffs (Abdel-Rehim et al., 2020).

Immunoaffinity Columns

An Immunoaffinity Column (IAC) is based on the premise of attaching an antibody to an inert support that binds specifically to the analyte of interest while allowing interfering components to pass through the column. As with SPE, pre-conditioning, removing unwanted impurities, and elution of the target analytes are required. However, in this instance, the extract must be aqueous and contain little or no organic solvent due to it having a detrimental effect on the antibody-antigen binding event. Depending on the selectivity of the antibody used, IACs generally afford low LOQs in comparison to other sample clean-up techniques. Several commercial companies have produced IACs for the type A trichothecenes such as T-2 and HT-2. Examples in the use of IACs before analysis include: the analysis of compound feed and foodstuffs from southern Italy using EASI-EXTRACT T-2 & HT-2 immunoaffinity column alongside LC-MS/MS, the analysis of *Fusarium* toxins including both T-2 and HT-2 in cereals and cereal-derived products using a Myco6in1+ column, the determination of T-2 and HT-2 in cereals including oats using an Easi-Extract T-2 & HT-2 immunoaffinity column, and the use of immunoaffinity columns in tandem prior to multi-mycotoxin analysis in food matrices (Di Marco Pisciottano et al., 2020; Lattanzio et al., 2014; Pascale et al., 2012; Trebstein et al., 2008; Wilcox et al.,

2015). One caveat of the latter methodology is that to retain all the mycotoxins of choice, it was necessary to use two IACs in tandem, which is not particularly cost-effective. However, if analysing T-2 and HT-2 only, the use of one would suffice.

The use of IAC as a sample clean-up technique has improved the analysis of trichothecenes enormously, even though a major drawback was the cost of the commercial columns, added to the fact they are designed for single use only. It has, however, been demonstrated that many IACs produced in-house may be re-used up to 100 times before observing any significant deterioration, which gives them an advantage over the use of SPE cartridges (Meneely et al., 2011).

QuEChERS

QuEChERS is another sample clean-up technique that rapidly expanded from use in the analysis of pesticides to its use in food for the analysis of mycotoxins. The method itself combines a liquid extraction and salt partitioning, followed by a clean-up step using dispersive SPE (dSPE). As well as being simple to use, it is relatively cheap in comparison to other techniques such as SPE and IAC and has reduced solvent consumption and therefore solvent waste. The use of the dSPE is similar in one sense to that of SPE in that each has a specific chemistry and can limit the number of analytes included in a method. However, with most methods for the analysis of the type A trichothecenes, particularly T-2 and HT-2, this is not an issue. Some studies in the use of QuEChERS for the analysis of the *Fusarium*-produced type A trichothecenes, including the target analytes T-2 and HT-2 toxins, include the investigation of 11 mycotoxin residues in compound feeds, the simultaneous determination of 11 mycotoxins including aflatoxins, fumonisins and T-2 and HT-2 in cereal-derived products, the analysis of plant-based beverages including those derived from oats, and a method for the simultaneous determination of 20 *Fusarium* toxins in cereals including barley by High-Resolution Liquid Chromatography-Orbitrap Mass Spectrometry (Seo et al., 2021; Annunziata et al., 2017; Miró-Abella et al., 2017; Tamura et al., 2015).

Furthermore, some studies have taken the QuEChERS technique and modified it to exclude the addition of the dispersive SPE such as primary secondary amine

sorbent (to remove sugars and fatty acids) after the salting out process. This simplifies the technique and can result in the inclusion of more analytes in the final method, with the trade-off of an extract that is not as clear of matrix impurities. Due to the analysis being directed toward the analysis of T-2 and HT-2 only, the former is not an issue. Some examples of this modified approach in the analysis of mycotoxins include the analysis of four major *Fusarium* mycotoxins, including T-2 and HT-2 in oats, the multi-detection of 22 mycotoxins in various animal feeds, and the analysis of 14 mycotoxins in feed, with the latter methodology including an extra lipid removal step (Nakhjavan et al., 2020; González-Jartín et al., 2021; Kolawole et al., 2021a).

Other sample clean-up techniques

Another sample clean-up technique that is now commonly employed for the analysis of mycotoxins in feed and foodstuffs is dilute-and-shoot (DnS). This technique simply involves taking an aliquot of the sample extract and diluting before filtration and analysis, typically by LC-MS. There are numerous examples of this in feed and foodstuffs, such as those carried out by Sulyok et al. (2006) for the analysis of 39 mycotoxins in wheat, the analysis of 295 bacterial and fungal metabolites including T-2 and HT-2 in four model food matrices by Malachova et al. (2014), and the expansion of this to over 500 mycotoxins and other secondary metabolites in feed, again containing both the target mycotoxins (Sulyok et al., 2006; Malachová et al., 2014; Sulyok et al., 2020). One thing to note in the use of this technique is that it is not specific to any class of mycotoxins and is more commonly used for the creation of multi-methods, with the matrix being reduced rather than removed, leading to higher LOQs for most analytes. However, it can be useful for certain labs where screening is important. In this instance, the extract can be analysed for the target toxins, such as T-2 and HT-2, but can also be analysed further by using databases for other possible contaminants or adulterants. One caveat of this technique is that it is difficult to validate multi-methods to any legislation such as EC directive 2002/657 (EC, 2002) due to the number of analytes and the resulting complexity, something that is addressed in the paper by Steiner et al. (2020) looking at LC-MS-based multiclass methods for the quantification of food contaminants (Steiner et al., 2021).

Another emerging clean-up technique is the use of immunomagnetic beads based on metal-organic framework materials (MOFs). Using MOFs conjugated to monoclonal antibodies allowed the purification of several mycotoxins, including T-2 and HT-2, from various flours; when the process is compared to IAC purification, the study indicated no difference between the clean-up methodologies (Han et al., 2020). Therefore, there is the potential for this to be extended to oats and barley for the clean-up of T-2 and HT-2 from the matrices of choice.

Analysis

There are a wide variety of analytical tests available for the analysis of the *Fusarium*-produced T-2 and HT-2 toxins, ranging from sophisticated confirmatory/reference methods which can be fully quantitative to rapid screening assays which are semi-quantitative or qualitative. The confirmatory methods generally used chromatographic separation, with gas chromatography coupled to flame ionisation detection (GC-FID) or mass spectrometry (GC-MS) being the method of choice for the major type A trichothecenes (D'Agnello et al., 2021; Pereira et al., 2015). However, due to the low volatility of these mycotoxins, a derivatisation step after the sample clean-up step is required, which adds to the complexity of the methodology and potential for human error. Of late, this has been superseded by liquid chromatography (LC), whether high-performance liquid chromatography (HPLC) or ultra-high-performance liquid chromatography (UHPLC). These are usually coupled to either high resolution mass spectrometry (HRMS) or low resolution mass spectrometry (LRMS), more commonly referred to as a triple quadrupole (MS/MS), with use of spectrofluorometric (FLR) or ultraviolet (UV) detection also employed (D'Agnello et al., 2021; Pascale et al., 2012; Tamura et al., 2015). However, similar to use of GC-MS, a derivatisation step is required for fluorescence detection, whereas this is not the case when using LC-HRMS or LC-MS/MS. These latter methods rely on considerable laboratory investment in terms of the equipment, as well as skilled personnel to operate them and to interpret the data produced (Nolan et al., 2019). Therefore, a simpler analytical approach is required further along the supply chain, which is more user-friendly, inexpensive and rapid. However, these

methods must be accurate, reproducible and provide the sensitivity required for regulatory compliance.

The European Food Safety Authority (EFSA) published a scientific opinion in 2011 of the risk to human and animal health concerning the presence of both T-2 and HT-2 toxins in food and feed due to T-2 being a carcinogen, mutagenic and causing immunosuppression (EFSA, 2011). Although no legal limits for T-2 and HT-2 toxins have been set in the EU to date, there is a recommendation of the levels in various feed and foodstuffs (Recommendation 2013/165/EU; Table 3). However, it is a requirement that analyses must detect the presence of both T-2 and HT-2 toxins in food and feed. To that end, and to improve the quality of raw materials such as oats, barley and their products in the agri-food industry, rapid tests have increasingly been utilised on-site for sample analysis and to validate food safety management systems. These rapid tests do not require much if any scientific expertise, and can be used as complimentary, high-throughput screening before being sent to a laboratory for confirmatory analysis. With climate change and the resulting increase in mycotoxin contamination on the island of Ireland and globally, and particularly T-2 and HT-2 contamination of oats and barley in this instance, there is the need for more routine analysis of these commodities to safeguard human and animal health, as well as to reduce the economic impact on growers and farmers.

In this section, the analytical techniques employed for the analysis of T-2 and HT-2 in oats, barley and cereal products are reviewed. This will include quantitative, semi-quantitative and qualitative (screening) methodologies, including those validated to EC directive 2002/657 and those considered state-of-the-art. Various analytical methods have been employed and will be covered, including GC-MS, LC-MS/MS and LC-HRMS, as well as the rapid tests which are mainly immunochemical, such as ELISA, LFDs/Dipstick Assays, Surface Plasmon Resonance (SPR) biosensors and Fluorescence Polarisation Immunoassays (FPIA) (Oplatowska-Stachowiak et al., 2017). Novel techniques, including spectroscopy and electrochemical biochip assays, are investigated.

Rapid Immunochemical Tests: An Overview

There are many rapid diagnostic tests on the market for T-2 and HT-2 toxins, with the majority being immunochemical methods including ELISAs, LFDs, SPR and FPIA, as mentioned above.

The speed of these test kits is particularly important in the agri-food industry because, when raw materials are delivered, it is important that a sample can be tested rapidly for compliance and therefore avoid disruption to the supply chain (Alldrick, 2014). Another advantage with rapid diagnostic kits is their ease-of-use and portability, which are important aspects to consider for producers monitoring the regulated mycotoxins such as T-2/HT-2. Use of such kits facilitates on-site testing, negating the requirement for laboratory equipment or the need for skilled laboratory staff, which reduces the cost per analysis and in turn encourages farmers and producers to routinely test (Zachariasova et al., 2014). Some of these test kits also use an aqueous buffer or water as the extractant, which is always advantageous when not being used in a laboratory setting so as to minimise solvent waste and damage to the environment.

However, there are some notable disadvantages associated with use of rapid immunochemical test kits. The most significant of these is the specificity of the antibody used and the associated cross-reactivity and/or matrix effects that interfere with the signal. These often lead to over-estimation and therefore increased measurement uncertainty of the measured concentration, or in some instances, false positives (Nolan et al., 2019; Meneely et al., 2011). One of the most important aspects in a rapid test kit is the specificity or cross-reactivity of the antibody utilised. For example, metabolites of T-2 toxin, T-2 Tetraol or T-2 triol may cross-react with the antibody, leading to an over-estimation of the levels of T-2, as these metabolites are not included in legislation for food. Conversely, another metabolite of T-2 toxin, HT-2 toxin, is included in the legislation as the regulatory limits refer to the sum of T-2 and HT-2. Therefore, the antibody must show good specificity for this metabolite in order that an accurate result be achieved, otherwise there could be an under-estimation reported (Meneely et al., 2011; Nolan et al., 2019). Furthermore, if a test kit is required for a different matrix, a full validation must be performed to ensure it is

'fit for purpose' and will provide the necessary accuracy, sensitivity and precision as directed.

Enzyme Linked Immunosorbent Assays (ELISA)

ELISA methods are considered the gold standard for screening assays in the food sector and are routinely employed by end users as they are cost-effective, high-throughput and relatively straightforward to use. Continual improvements are made to those already on the market due to changes in validation criteria or to extend an existing kit to a new food commodity, as most ELISA kits are specific to certain matrices. They are an excellent tool for rapid analysis to assess whether a food commodity is compliant and can enter the food chain. However, as detailed above, they can cross-react with other proteins and/or matrix components, leading to over-estimation and false positives. Therefore, any sample with a concentration higher than the EU recommended level should be sent to a laboratory for confirmatory analysis.

ELISAs are colorimetric assays performed in microtitre plates, with the assay itself being competitive due to the low molecular weight of the target compounds (< 1 kDa). Briefly, a specific antibody to the target analyte, either mono- or polyclonal, is used to coat the wells of a 96-well microtitre plate. The sample is then added to the wells, which is then followed by the addition of the target analyte (T-2 and HT-2) that is conjugated to an enzyme. Competition between the unlabelled and labelled antigen (T-2 and HT-2) for antibody binding sites occurs during the incubation period. The plate is then washed to remove unbound material and the enzyme-labelled bound antigen is measured by the addition of an enzyme substrate that reacts to produce a colour change. The absorbance is measured at a specific wavelength using a plate reader, with the absorbance being inversely proportional to the amount of toxin present (Nolan et al., 2019).

As indicated, any antibody used in an immunochemical assay must show specificity for both T-2 and HT-2, as it is the sum of both that must be reported. However, some commercial ELISA kits exhibit poor cross-reactivity with HT-2 toxin and thus an underestimation of results would follow. These include kits from Hygiena LLC (3%

cross-reactivity with HT-2 toxin), and R-Biopharm AG (RIDASCREEN® T-2 Toxin ELISA and RIDASCREEN® FAST T-2 Toxin ELISA) (7% cross-reactivity with HT-2 toxin). Furthermore, the AgraQuant® T-2 Toxin ELISA test (Romer Labs Diagnostic GmbH) and MaxSignal® T-2 ELISA Kits (PerkinElmer) do not specify the cross-reactivity profile and therefore may be unsuitable for the testing of both toxins. In fact, a 2013 study carried out by Aamot et al. indicated that ELISA kits that could simultaneously and reliably detect both T-2 and HT-2 were required, given EU legislation. Their study to estimate the sum of T-2 and HT-2 in oat samples using the RIASCREEN assays above indicated that it was necessary to re-calculate data from both ELISA kits from the known cross-reactivities of each kit to establish the actual concentrations for HT-2 toxin. The R^2 value for the correlation with the LC-MS/MS confirmatory method was determined as 0.61 and 0.83 for the 'Fast ELISA' and 'Standard ELISA', respectively (Aamot et al., 2013).

Apart from commercially available ELISA kits, some have been developed in-house that analyse for both T-2 and HT-2. These include a T-2 and HT-2 ELISA based on a T-2 monoclonal antibody and which indicated a 125% cross-reactivity with HT-2. The newly developed ELISA was validated in accordance with the recent guidelines for the validation of semi-quantitative screening methods for mycotoxins included in Commission Regulation (EU) No 519/2014, and was carried out in several matrices, including barley and oats. The accuracy of the ELISA was then confirmed through proficiency testing and reference samples (Oplatowska-Stachowiak et al., 2017). A HT-2 toxin-specific ELISA assay was developed based on an anti-immune complex (IC) scFv antibody fragment, genetically fused with alkaline phosphatase (AP). The primary antibody recognised both T-2 and HT-2; however, the anti-IC antibody made the assay specific to HT-2 only, with the assay performance tested in both barley and oats. With the ELISA specific for HT-2 only, this would not meet the criteria, as the sum of both T-2 and HT-2 must be reported. However, the authors stated that a similar assay with anti-IC antibodies could also be developed for T-2 that would allow for an accurate multiplex measurement (Arola et al., 2017). The group of Zhang et al. (2021) then developed what they referred to as a competitive Amplified Luminescent Proximity Homogeneous Assay (AlphaLISA) using a T-2 antibody, which negates the need for washing the plate and results in a low coefficient of

variation. Although the authors did not specify the cross-reactivity of the antibody with HT-2, the detection range of both was identical, with the half-maximal inhibitory concentration (IC_{50}) of 2.28 ng/ml and 2.75 ng/ml for T-2 and HT-2, respectively (Zhang et al., 2021).

Further developments with ELISAs have been made using nanomaterials, such as the use of magnetic nanoparticles (NMP), which are suspended in the reaction media and used as a solid support for the antibody. This facilitates a greater coverage of the reaction media with antibody, leading to an increased probability of antibody-antigen conjugation and therefore less incubation time (Nolan et al., 2019). As traditional ELISAs use antibody recognition and Horseradish Peroxidase-catalysed 3,3'5,5'-Tetramethylbenzidine to generate colour and therefore a signal to determine the concentration present, and which usually require a plate reader, efforts have been made to improve the colorimetric signal. This is due to the signal produced not being suitable for naked-eye detection. Therefore, acid–base indicators have been seen as ideal signal reporters for naked-eye detection, with various enzymes being utilised to change the pH through catalysis of the substrate to produce hydrogen or hydroxide ions. Another recent advancement is the use of plasmonic ELISA using gold nanoparticles as candidates for colorimetric indicators, with these advancements potentially negating the need for specific plate readers and therefore also being used in the field (Majdinasab et al., 2021). At present, the majority of these developments have been in the analysis of OTA and aflatoxin B1 (AFB1) rather than for HT-2/T-2. One study by McNamee et al. (2017) created a multiplex nanoarray-based ELISA technique via nano-spotting of mycotoxin-protein conjugates into single wells of a microtitre plate analysing three mycotoxins, including T-2, in which the assay also detected HT-2, with the antibody showing 74% cross reactivity, with the method validated in wheat (McNamee et al., 2017). A further example is the design of a highly sensitive chemiluminescent ELISA (CL-ELISA) for the detection of T-2 and its metabolite HT-2. The study by Li et al. (2017) used an anti-T-2 mAb and a SuperSignal chemiluminescence substrate solution to generate the signal which showed superior sensitivity than traditional colorimetric ELISA substrates previously used, with LODs of 8.84 and 5.62 ppb for T-2 and HT-2, respectively. The antibody used also indicated no cross-reactivity with other

structurally related mycotoxins, further improving its specificity and therefore its use for the detection of the sum of T-2 and HT-2. This study was conducted in rice but there is the potential to further develop it for use in the matrices of choice, provided the method performance limit can be met (Li et al., 2017).

Lateral Flow Immunoassay/Device (LFD)

As with ELISAs, LFDs are competitive immunoassays. The extracted sample is applied to a sample pad, which then traverses along the membrane. Once the extract reaches the conjugate release pad, the dry conjugate containing the labelled antibody is hydrated; if the analyte is present in the sample extract, it binds to the antibody and continues along the strip. If no antigen (toxin) is present, the free antibody binds to the test line. Therefore, the presence of a coloured line is inversely proportional to the amount of toxin present, with the labelled antibody used as a signal reagent. LFDs will have a cut-off level which is the point of discrimination between a positive and negative sample and this level must meet the regulatory requirements for the maximum permissible level (Nolan et al., 2019). LFDs can either be qualitative or quantitative, with the latter providing a concentration after the test, in which case some sort of reader must be used. Many LFDs have the ability to match the sensitivity of an ELISA, thus enabling on-site testing. Again, to ensure accurate results, the antibody used must cross-react with both T-2 and HT-2 toxins, as it is the sum of the two toxins that is reported for compliance.

While there are a number of commercial LFD kits available for the analysis of these trichothecenes in cereals, few specify their applicability to oats and barley. Therefore, a full validation in the matrix of choice is recommended to ensure accuracy and sensitivity.

As well as commercially available LFDs, there are also those produced in-house for research purposes and to extend their capabilities. One example is that of Foubert et al. (2017a), who developed a multiplex lateral flow immunoassay (LFIA) for the determination of four mycotoxins, including both T-2 and HT-2-toxin in barley, using different coloured luminescent quantum dots (QDs) as labels. The T-2 antibody utilised exhibited 110% cross-reactivity with HT-2, with the assay showing good

sensitivity, being rapid (15 min) and having a low false-negative rate (<5%). The same group also compared the use of colloidal gold nanoparticles (CG) to quantum dots (QDs) as labels for the LFIA, indicating that the QDs gave better sensitivity in comparison and that the results were easier to interpret (Foubert et al., 2017a; Foubert et al., 2017b).

Fluorescence Polarisation Immunoassays (FPIAs)

This methodology employs fluorescence for the detection of the analytes of choice, using both excitation and emission wavelengths. The principle is based on the fact that when a fluorophore in solution is exposed to plane polarised light at its excitation wavelength, the subsequent emission is depolarised. The format of this methodology is a competitive assay, whereby a toxin (T-2 and HT-2) is covalently linked to a fluorophore to make a fluorescent tracer molecule. The tracer molecule then competes with the toxin in the sample extract (if present) for a limited amount of toxin-specific antibody. In the absence of any toxin(s) being present, the antibody binds the tracer, resulting in high polarization. Conversely, if there is toxin(s) present in the sample extract, less of the tracer molecule will bind the antibody and a greater fraction exists unbound, resulting in lower polarization. The results are read using instruments that can determine the amount of fluorescence polarization and therefore the amount of toxin(s) present, with the degree of polarization being inversely proportional to the mycotoxin concentration. The method does not require separation between free and bound tracer; therefore no washing steps are required, negating the incubation step as no colour development is required, therefore reducing assay time and increasing throughput. One caveat of the no washing step is that it can lead to matrix effects that can cause interference in analysing the results. In order to reduce or eliminate these interferences, some sort of pre-treatment may be required in order to prevent an overestimation of the toxin level, with this adding to the assay time (Nolan et al., 2019). Numerous test kits are available based on FPIA; however, the majority to date are not for the determination of T-2 and HT-2 but for other mycotoxins such as DON, aflatoxins and fumonisins. They have, however, been validated in the matrices of choice for these analytes

(oats and barley) and therefore there is the potential for them to be tailored to these analytes of choice (Wolf and Schweigert, 2018).

An example in the use of FPIA for the mycotoxins of choice is that is employed by Lippolis et al (2019). In their study, they developed a FPIA using an HT-2-specific antibody with 100% cross-reactivity for T-2 toxin using two differing extraction protocols, one organic and one aqueous, for the analysis of both toxins in wheat. Their FPIA had an LOD of 10 ppb for both toxins and a false positive rate of <0.1%, which meets the criteria for acceptability of an analytical method for quantitative determination of T-2 and HT-2 as laid down by the EU. Both methods were validated in accordance with the guidelines for validation of screening methods included in Regulation (EU) No. 519/2014. Apart from not being applied to the matrices of choice, the main flaw in the design was that the antibody used showed high cross-reactivity (80%) for both T-2 and HT-2 glucosides, although this was part of the study. However, if any of these glucosides were present in a sample, the overall level reported would be elevated and could potentially breach the permitted level, even though the sum of HT-2/T-2 may well be below this (Lippolis et al., 2019). This may be relevant, as the glucosides could potentially be converted back to their native form on ingestion and contribute to the overall toxicity. However, at present the regulations are for the sum of HT-2/T-2 only.

Biosensors

Biosensors are composed of two elements: the molecule that reacts with the analyte of choice and the transducing element that converts the physical change into a measurable signal. The recognition element is usually an antibody-antigen, enzyme-substrate or receptor-biospecific molecule, with the transducer usually optical or electrochemical (Bueno et al., 2015). In the former, an antibody specific to the toxin(s) is mixed with the sample extract before application to the chip (sensor surface) so that any free mycotoxin(s) in the sample will bind to the antibody, which results in no free antibody binding to the probe on the sensor surface. Conversely, if a sample is negative, the antibody is free and will therefore bind to the probe on the sensor surface. Any binding changes the resonance frequency of the surface

plasmons, resulting in a change of the intensity of the reflected light that is detected by the biosensor device (Nolan et al., 2019). SPR-based biosensors are considered reliable and sensitive and have the added advantage of reusability with regeneration of the biosensor chip surface. They are also quantitative as they can be run against a calibration curve to determine the concentration.

Surface plasmon resonance (SPR) has been used for the analysis of the sum of HT-2/T-2 in various cereals and cereal-based baby foods (Meneely et al., 2010; Meneely et al., 2012), with the latter study by Meneely et al. (2012) being multiplex as it included another trichothecene, DON. Importantly, there was no cross-reactivity of DON with the HT-2 antibody used, and vice versa. In 2011, an ultrasensitive method for the detection of T-2 was developed through the combination of a molecularly imprinted polymer (MIP) with SPR, with MIPS displaying high selectivity and specificity to a particular analyte. The LOD of the assay was 0.05 pg/ml, making this an extremely sensitive method; however, there were no details on the cross-reactivity of this T-2-MIP with HT-2 (Gupta et al., 2011).

A 2014 review by Meneely & Elliott indicated the need to develop and manufacture portable and multiplex SPR instruments, although in the case of HT-2/T-2, multiplexing is not an issue. Further developments since then have included the study carried out by Joshi et al. (2016), who developed a multiplex competitive inhibition immunoassay using a portable nanostructured imaging surface plasmon resonance (iSPR) instrument for the detection of six mycotoxins in barley, including T-2, with the T-2 antibody showing cross-reactivity of 76% with HT-2. The LOD for T-2 was calculated as 0.6 ppb, with an in-house validation indicating that T-2 could be detected at the European Union regulatory limits. This study therefore highlighted the potential of this prototype for rapid on-site screening for mycotoxins. One thing to note from the study was that analysis of naturally contaminated barley samples using this assay gave a T-2 level of 46% less than the known value, most likely due to an incomplete extraction of the analyte from the matrix (Joshi et al., 2016). A further study by Hossain et al. (2018) developed an iSPR assay for the detection of T-2 and its glucoside, albeit in wheat rather than oats or barley. The antibody used showed <1% cross-reactivity with HT-2 and therefore would not be suitable for on-

site screening due to not being able to report on the sum of T-2 and HT-2 (Hossain et al., 2018).

SPR biosensors from Biacore AB, widely used in academic research, have demonstrated their applicability for mycotoxin testing. However, there are issues regarding their commercialisation for mycotoxin analysis due to the data analysis requiring technical expertise, while the miniaturisation of such instrumentation needs optimisation in order to maintain high sensitivity. Both aspects will limit the ability for on-site use.

Spectroscopy

The use of spectroscopy for the analysis of mycotoxins has been explored, with the majority of these being non-invasive/non-destructive, and therefore maintaining the integrity of the sample. These techniques include near-infrared reflectance spectroscopy (NIR), Fourier-transform infrared spectroscopy (FTIR) and surface-enhanced Raman spectroscopy (SERS). In essence, these techniques involve light of a specific wavelength being shone on the surface of the food matrix, with this generating spectral data. In order to interpret the spectra, models must be built in order to assess the data generated, this due to the interpretation of spectral data being difficult as well as many of the spectra overlapping. One emerging technique in this area is the use of surface-enhanced Raman spectroscopy (SERS), with there being an increasing interest in the use of SERS for mycotoxin analysis due to the availability of appropriate nanostructures for substrates required for this technique. When using SERS, the type of substrate used is crucial for analyte detection due to the substrate determining the signal enhancement, sensitivity, selectivity and reproducibility, with the substrate loosely divided into two groups: colloidal substrates (silver or gold) and solid surface-based substrates. The latter reduces the high variance seen with colloidal substrates, generating a highly reproducible and long-term stable substrate (Martinez and He, 2021). In order to use SERS, and unlike other spectroscopy techniques, the sample must be extracted, with liquid-liquid extraction (LLE) or solid-liquid extraction (SLE) often used depending on the matrix. This also extracts unwanted matrix components which may interfere with the

spectral data and therefore need to be removed or reduced, with this achieved through use of techniques such as SPE, IAC and QuEChERS. To date, although there are some studies using spectroscopy for the analysis of mycotoxins, there are very few focused on HT-2/T-2. One example is a multiplex SERS-based lateral flow immunosensor assay developed by Zhang et al. (2020) to detect six mycotoxins, including T-2, with an LOD of 8.6 pg/ml for T-2 toxin. The monoclonal antibody against T-2 also had a cross-reactivity of 119% for HT-2, indicating that it meets the criteria for screening due to its ability to report the sum of both T-2 and HT-2 (Zhang 2020).

Confirmatory Analysis

Although the techniques discussed above are useful, especially for on-site screening, any sample that is non-compliant after a screening test must undergo confirmatory analysis. This can be performed using GC or LC (HPLC/UHPLC) as the separation technique, with these usually coupled to a detector such as flame ionisation detection (FID), mass spectrometry (MS), a photodiode array (PDA) using ultraviolet light (UV), or fluorescence detection (FLR). All of these have been used extensively in the analysis of mycotoxins, with gas chromatography coupled to mass spectrometry (GC-MS) being the method of choice for the major type A trichothecenes such as T-2 and HT-2. However, as mentioned previously, a derivatisation step is required due to their low volatility, which adds to the complexity and potential for error in the methodology. Similarly, for analysis using LC-FLR, a derivatisation step is also required, whereas this is not the case when using LC-UV or LC-MS. HPLC coupled to UV/PDA or FLR has also been frequently used in the analysis of type A trichothecenes, with the latter requiring pre- or post-column derivatisation prior to analysis, while use of UV/PDA detection is generally not employed due to the lack of a strong chromophore in both molecules and therefore only being applicable for samples with high concentrations of these compounds, potentially leading to false-negatives. At present, there is no standardised or official method for the analysis of T-2 and HT-2 in food and/or feed, but it is an EU recommendation (since the limits applied are guidance limits rather than regulatory

limits) that member states monitor and report on the sum of both T-2 and HT-2 in food and feed. Furthermore, the LC-MS method must be validated in-house to evaluate its performance, ensuring that it is compliant with the acceptability criteria specified in Commission Regulation 401/2006/EC (EC, 2006c) to ensure it is fit for purpose. Of late, LC-MS has become the gold standard and the most extensively used technique for confirmatory analysis, with both triple quadrupole (QqQ) and high resolution (HRMS) detectors both used, LC-MS/MS and LC-HRMS respectively (Meneely et al., 2011). These systems offer high selectivity, specificity, low detection levels and can be used to analyse for multiple analytes in a short period of time.

Analysis using GC-MS, LC-UV/PDA and LC-FLR

An example in the use of GC-MS is that of Pereira et al. (2015), who simultaneously analysed 12 trichothecenes including T-2 and HT-2 in cereal-based baby foods, using QuEChERS as the clean-up step. Their method indicated LODs of 6.76 and 6.4 ppb for T-2 and HT-2, respectively, with a total run time of just over 21 minutes (Pereira et al., 2015). Another study utilising GC was that by Carballo et al. (2018), who analysed 27 mycotoxins including T-2 and HT-2 in ready-to-eat foods, including some cereal-based ones using GC-MS/MS, with some of the analytes detected using LC-MS/MS. Their method used a QuEChERS-based methodology and indicated LODs of 0.75 and 0.15 ppb for T-2 and HT-2, respectively, for the cereal-based products, with a run-time of just over 20 minutes (Carballo et al., 2018). Use of HPLC-UV/PDA and HPLC-FLR has also been utilised in the analysis of T-2 and HT-2. Examples of this include the study carried out by Soleimany et al. (2011), analysing 12 mycotoxins in cereals including T-2 and HT-2, with LODs of 9.4 and 6.2 ppb, respectively. However, the instrument setup was quite complex as it combined HPLC with both PDA and FLR detection alongside a photochemical reactor for enhanced detection and post-column derivatisation, which would therefore not be ideal for routine analysis (Soleimany et al., 2011). Other examples include the determination of T-2 and HT-2 in cereals using LC-FLR, the study of T-2 and HT-2 in cereals and cereal-based products including barley, the analysis of T-2 and HT-2 in oats using LC-PDA, and the determination of T-2 and HT-2 in oats using IAC and LC-FLR (Donnelly et al., 2006; Ok et al., 2013; Pascale et al., 2012; Trebstein et al.,

2008). All of these analyses meet the requirement of the European Commission, which states that the LOD of the analytical method should be less than or equal to 25 ppb for the sum of T-2 and HT-2 (EU 2013).

Although the above methods are suitable, there are issues with the complexity of the analysis due to derivatisation for GC and FLR analyses, or because UV/PDA is not ideal due to neither molecule containing strong chromophores. As a result, the majority of modern techniques for mycotoxin confirmation and quantification in cereals and cereal-derived foodstuffs use LC-MS, with both LC-MS/MS and LC-HRMS performed (Nathanail et al., 2015). Use of such instrumentation allows analysis of the analytes of choice but facilitates the simultaneous analysis of modified or 'masked' forms such as T-2 and HT-2 glucosides, which, although not covered by legislation, may be of interest due to potentially being converted back to their native forms whilst traversing through the gastrointestinal tract (Hossain et al., 2018). Various detectors coupled to LC systems have been employed for confirmatory analysis of T-2 and HT-2. These include triple quadrupole instruments (QqQ or MS/MS), high-resolution instruments such as orbital ion traps (Orbitrap), time-of-flight (TOF) and hybrid systems such as quadrupole-ToF (QToF), and even ambient MS such as direct analysis in real time (DART) (Kolawole et al., 2021a; Lattanzio et al., 2015; Busman, 2015).

Analysis using LC-MS techniques

The use of LC-MS has become the gold standard for confirmatory analysis of mycotoxins, with the number of analytes in methods increasing over time due to improvements in QqQ instrumentation electronics facilitating faster cycle and dwell times, permitting the inclusion of more analytes. One very recent example of this is the study conducted by Sulyok et al. (2020), which analysed more than 500 secondary microbial metabolites including T-2 and HT-2 toxins (Sulyok et al., 2020). Another reason for increased analyte number is the use of dilute-and-shoot (DnS) for sample clean-up, which allows the incorporation of numerous analytes due to the technique not being selective or specific in consequence of its having no stationary phase-based chemistry. The creation of multi-methods allows numerous analytes to

be incorporated, which in the case of analysing T-2 and HT-2 is not necessary, as it is only those two analytes which must be reported on. However, as mentioned previously, it may be important to analyse for other forms of these toxins, such as their glucosides, that may add to their overall toxicity on ingestion, as well as other metabolites such as T-2 triol and T-2 tetraol. Analysing for other *Fusarium* mycotoxins such as DON and ZEN, as well as some of the more important emerging mycotoxins according to EFSA may also be advantageous, as although they are not regulated at present, routine analysis may highlight emerging threats brought about by climate change, which may aid implementation of guideline or regulatory levels. Further to this, as indicated in the study by Kolawole et al. 2021, the type of farming, crop season and harvest date all are factors that can influence mycotoxin prevalence and concentration, and this may alter the mycotoxin profile as environmental changes occur over time (Kolawole et al., 2021a).

Over the years there have been numerous studies using LC-MS for confirmatory analysis of T-2 and HT-2, with the majority not specific for T-2 and HT-2 only but incorporating other ‘relevant’ mycotoxins dependant on the matrix analysed. Although the focus of this review is oats and barley as foodstuffs for human consumption, these commodities are also used in the formulation of complete feed for ruminants, porcine and equine (González-Jartín et al., 2021). There has also been an increase in use of oats for other food products, such as in the production of oat milk and oat yoghurt. In order to analyse oats, barley, feed or cereal-based food products based on these, some sort of sample clean-up may be applied after extraction to remove unwanted matrix components which may interfere with the performance of the analytical method. These include the use of techniques listed in the section above, such as SPE, IAC, QuEChERS or modified forms of this, and dilute-and-shoot (DnS), which all serve to lower the LOD and LOQ of the analytical method.

There are many examples of use of LC-MS in the analysis of T-2 and HT-2 for oats, barley, cereal-based foods and animal feed. These include the study by Gottschalk et al. (2007) analysing nine type-A trichothecenes in oats and oat products including T-2 and HT-2, as well as the T-2 metabolites T-2 triol and T-2 tetraol using LC-MS/MS and MycoSep SPE columns for sample clean-up. Analysis was carried out

on an API 4000 (Applied Biosystems) in electrospray positive ionisation (ESI+) mode, with a run time of 35 minutes. The authors did not stipulate the LOD of individual analytes, but it was stated to be in the range of 0.01-0.3 ppb for T-2 and HT-2. Interestingly, their study indicated that T-2 tetraol played a major role in the overall contamination due to the mean levels being higher than T-2 and concluded that it should be routinely monitored (Gottschalk et al., 2007). Another method developed by Lattanzio et al. (2011) was validated using LC-MS/MS alongside SPE for the analysis of cereal-based foods which included barley and oats. Their method used a QTrap MS (Applied Biosystems) operated in both ESI+ and ESI- modes, simultaneously analysing nine mycotoxins, including those of interest. The analysis time was >30 minutes for the nine analytes, although 13C-labelled internal standards of each mycotoxin were also incorporated, with LODs of 0.5 and 1.1 ppb for T-2 and HT-2, respectively, in barley flour, and LODs of 1.2 and 2.4 ppb for T-2 and HT-2, respectively, in oat flour (Lattanzio et al., 2011). Another study by Soleimany et al. (2012) used DnS alongside LC-MS/MS for the analysis of several (11) mycotoxins, including T-2 and HT-2, in commercial cereals, including barley and oats. Their analysis was carried out in both ESI+ and ESI- modes with a 25-minute run time, achieving an LOD and LOQ for both toxins of 5 and 10 ppb respectively (Soleimany et al., 2012). The LOD of this methodology is higher than those previously discussed, most likely due to the use of DnS as the clean-up step instead of SPE or IAC; however, the overall analytical method still meets the performance criteria as set down by the EC for analytical methods (EC, 2006c).

In 2017, a study by Annunziata et al. developed a ‘fast, easy and cheap’ method for the analysis of eight mycotoxins in cereal-derived products intended for human consumption, including barley. They used a QuEChERS-based clean-up alongside LC-MS/MS, with analysis performed on an API 3000 QqQ MS (Applied Biosystems) in ESI+ and a run time of 30 minutes. The method was validated with LODs and LOQs of 1.3 and 2.5 ppb for both toxins, respectively, with their method applicable for use in both official and research facilities (Annunziata et al., 2017). Furthermore, a survey carried out between 2013 and 2019 of mycotoxins in oats for food consumption by Meyer et al. (2021) aimed to improve the database on the occurrence of specific mycotoxins in milling oats due to ongoing discussions in the

European Commission on regulatory limits for certain mycotoxins. Their study looked at the 16 predominantly occurring trichothecenes, including T-2 and HT-2 toxins, in 281 commercial milling oats samples across 11 European provenances, including the UK, Ireland, Denmark, Sweden, Finland, Estonia, France, Germany, Lithuania, Poland and Latvia. Their analysis was carried out using an Agilent 6495 MS/MS in both ESI+ and ESI- modes using a MycoSpin SPE cartridge for sample clean-up, with an overall run time of 11 minutes, making this method more rapid than any of the aforementioned, with a LOQ of 5 ppb for both T-2 and HT-2. The mean concentration of the sum of T-2 and HT-2 toxins was 149 µg/kg, while the highest contamination found was a sample from Ireland with a level of 1290 µg/kg for the sum of T-2 and HT-2 (Meyer et al., 2021).

In 2021, two studies on oats from the island of Ireland were performed, with analysis using LC-MS/MS and a QuEChERS-based approach (De Colli et al., 2021; Kolawole et al., 2021a). The methodology used by De Colli et al. (2021) analysed 42 mycotoxins, including T-2, HT-2, T-2-triol and T-2-glucoside (T-2G) and also included 13C-labelled internal standards of T-2 and HT-2. Analysis was carried out on a Waters Quattro Premier XE QqQ MS operated in both ESI+ and ESI- modes, with the majority of analytes separated using a 15-minute gradient. One note on this method was that the supernatant was split after the centrifugation step due to the response for some non-polar analytes being very intense compared to other analytes, resulting in saturation of the MS signal and linearity issues. Therefore, these were analysed on a separate four-minute gradient without concentration of the extract but did not include the analytes of interest. Of those mycotoxins identified, several of the major type-A trichothecenes were present, including T-2 and HT-2 and T-2 triol and T-2-glucoside, with the most frequently quantified being HT-2 (51%) and T-2 (41%) toxins, with gluten-free oats containing significantly lower concentrations of HT-2 compared to conventionally produced oats. As mentioned, this work highlights the need for the routine testing of oats with multi-analyte methods to generate knowledge on the occurrence of other mycotoxins that have been to date rarely investigated. The study by Kolawole et al. (2021a) was less comprehensive in its suite of toxins but included both T-2 and HT-2 along with other regulated type-B trichothecenes, DON and ZEN. This methodology was rapid, with

separation in under seven minutes using a SCIEX 5500+ QqQ (AB SCIEX). Initially, a simple DnS approach was applied after extraction with acetonitrile:water:acetic acid (79:20:1, v/v/v); however, the extract was not suitable for injection even after filtration. Instead, a QuEChERS-based approach was used but without the addition of any dSPE. Furthermore, this method has been extended in-house by the research group at Queen's University Belfast to include other regulated mycotoxins, such as aflatoxins B, B1, G, G1, OTA and fumonisins B1 (FB1), using the same analytical conditions.

There are other methodologies that use LC-HRMS, and although the newer hybrid-systems have the quadrupole functionality for quantification, they are not routinely used for confirmatory quantitative analysis. These systems are also not ideally suited to the analysis of only a few analytes, such as in the case of the methodology required. Instead, they are more routinely used in the qualitative analysis of sample extracts for numerous analytes across different classes, with each extract usually run in both ESI+ and ESI- modes. Due to the differing polarities of mycotoxins and indeed other potential contaminants from different classes, the gradients and therefore run times used in LC-HRMS are usually longer in order to achieve better resolution of adjacent peaks. This, along with running the extracts in both polarities, serves to increase the run time of the methodology. Once the data has been generated, databases that have been generated either in-house or provided online from the various vendors are used to search for various contaminants, including mycotoxins. This type of approach is usually considered non-targeted; however, with the matrix and an idea of the potential contaminants known, the approach can be considered targeted untargeted. An advantage of HRMS over QqQ systems is the ability to retrospectively mine the data generated from a sample extract for emerging threats to see if it was present. A further advantage for use of LC-HRMS is that standards are required for an LC-MS/MS method, some of which are not available or are expensive, whereas confirmation of analytes can be confirmed through use of databases.

Some examples in the use of LC-HRMS for T-2 and HT-2 include the studies by Tamura et al. (2015) and Romera et al. (2018) (Tamura et al., 2015; Romera et al., 2018). The former analysed 20 *Fusarium* toxins in cereals, including barley, using an

LC-Orbitrap MS with a two-step clean-up which included use of QuEChERs followed by purification using a Multistep 229 Ochra multifunctional cartridge. This was done in order to lower the detection level of the method as HRMS instruments are generally not as sensitive as QqQ instruments. However, for routine analysis, this is not cost-effective. The latter study used ultra-high performance liquid chromatography mass spectrometry (UPLC-MS/MS) for the simultaneous analysis of numerous mycotoxins in compound feed for swine, sheep, poultry, cattle and equine, with these confirmed by the ultra-high performance liquid chromatography with quadrupole time-of-flight (UPLC-QToF), with many other fungal metabolites (mycotoxins) also identified through analysis using UPLC-QToF. This approach indicated that there may be many more mycotoxins present than are covered by the LC-MS/MS method, which is targeted to the analytes of interest. In this case, screening a sample extract using LC-HRMS may be beneficial as it can direct the analyst as to which quantitative method should be applied; however, again, this is not cost-effective, especially in relation to the analysis of the target analytes covered by this review. From a research standpoint though, both methods are useful for analysis of the target commodities for the analytes of choice.

Conclusion

This systematic review has highlighted that T-2 toxin and its metabolite HT-2 toxin are detrimental to the health of animals and humans alike. These toxins are potent inhibitors of protein synthesis. Moreover, they are immunosuppressive and dermatotoxic, causing necrosis and haemorrhage of the intestinal mucosa. Clinical symptoms of T-2 and HT-2 mycotoxicoses in animals include weight loss, decreased feed conversion and feed refusal, vomiting, diarrhoea, skin problems, haemorrhage, decreased egg production, abortion and – in severe cases – death. With respect to human health, these mycotoxins have been associated with several poisonings, the most significant being the alimentary toxic aleukia that caused the death of 60% of those infected in the former U.S.S.R. from 1932 until 1947. Furthermore, although the aetiology has not been confirmed, it is believed that T-2 may play a causal role in Kashin–Beck disease (KBD), an endemic, chronic joint disease typically found in

rural regions of eastern Siberia, northern Korea and in central China. In addition, it has been implied that T-2 toxin has been used in biological warfare, although, again, these claims been disputed in the scientific literature.

In terms of the current regulatory or indicative limits applied to the sum of T-2 and HT-2, discussions are ongoing as to the implementation of maximum limits that will be much lower than those currently adhered to. For example, for cereals for direct human consumption, the limits could change from 200 µg/kg to 50 µg/kg for oats, and from 50 µg/kg to 20 µg/kg for other cereals. This would have a serious economic impact on the cereal industry, not least on the island of Ireland where the main crops produced are oats and barley. Increased surveillance and mitigation strategies would add an extra burden on farmers and producers.

Further research is required on the ecology of the *Fusarium* species producing T-2 and HT-2 toxins. Furthermore, the environmental factors that influence the growth and activation of the biosynthetic genes responsible for these toxins are not fully elucidated. This information will be vital to help predict contamination and allow early interventions to reduce contamination.

Many analytical methods exist for the determination of T-2 and HT-2 toxins in foods and feeds. Screening assays are hugely important for on-site testing to give rapid results of compliance/non-compliance. As such, these tests must be easy to perform and interpret. Many rapid diagnostic kits are available commercially and farmers/producers should take advantage of these to monitor their crops. Of course, confirmatory analysis must be performed in a laboratory setting using sophisticated technology. The state-of the-art is LC-MS, which allows the simultaneous determination of multiple mycotoxins, thereby identifying the mycotoxins and concentrations that may pose a risk to humans and animals. Moreover, distribution patterns of mycotoxins due to changing climatic conditions and agronomic factors could be identified, thereby providing useful information for mitigation. Use of high-resolution mass spectrometry to characterise metabolites of T-2 and HT-2 and emerging mycotoxins would be advantageous in predicting what testing would be required in a changing climate, and thus promote a proactive approach to the continued risk of mycotoxin contamination in cereal crops.

Objective 2

Review of the commercially available rapid diagnostics for the screening of T-2 and HT-2 in cereals)

T-2 and HT-2 Testing

A wide variety of analytical tests are available for mycotoxin testing along the supply chain. These range from sophisticated confirmatory/reference methods to rapid screening assays. The confirmatory methods generally use chromatographic separation (gas chromatography or high-performance liquid chromatography) coupled to mass spectrometry (MS), ultraviolet (UV), flame-ionisation detection (FID), UV diode array (DAD), fluorescence and electron capture (Meneely et al., 2011). These methods rely on considerable laboratory investment in terms of equipment and skilled personnel and are time-consuming and expensive to administer (Nolan et al., 2019). Therefore, for growers, suppliers and processors along the supply chain, more user-friendly, inexpensive and rapid techniques are favoured. However, these methods must be accurate, reproducible and provide the required sensitivity for regulatory compliance.

Test Procedure

All methodologies applied to the analysis of T-2 and HT-2 toxins require similar step-by-step procedures prior to the final testing of the sample.

Sampling and sample preparation

Obtaining a representative sample for analysis is critical for accurate determination of the concentration of the toxins as they are heterogeneously distributed through cereal samples, leading to possible inaccuracies from “hotspots” (Whitaker, 2006). If this step is not performed correctly, it will result in undesirable consequences in terms of health outcomes and economic losses to producers (Whitaker, 2006). As

sampling is crucial, the European Commission has laid down specific methods of sampling for these toxins in food and feed, including different types of lots, numbers of incremental samples to be removed from the lots, and the weight of the aggregate sample from which the representative test sample is taken (EC, 2006c; EC, 2009).

The aggregate sample collected for cereals ranges from 1 kg to 10 kg depending on the size of the initial lot, which means further processing is required to reach the desired sample size. Grinding of the sample in a mill to reduce particle size and therefore provide a more homogenous sample is performed without increasing the variation or uncertainty between subsamples (Whitaker, 2006). From this, subsamples are removed for extraction and analysis.

Extraction

To extract the toxins from food matrices, a solvent or solvent-water mixture is commonly used. The choice depends upon the chemical characteristics of the compounds, sample type, health and safety concerns, and the analytical method employed (Meneely et al., 2011). The most commonly used tend to be 60-70% methanol, 84% acetonitrile, aqueous extraction buffers, and to a lesser extent ethyl acetate or 70% methanol containing 4% sodium chloride (see Table 5). Extraction efficiency and reproducibility is key (Meneely et al., 2011).

Clean-up

After extraction, the sample can be treated to reduce or remove impurities that may interfere with the analysis and quantification of the toxins. However, for the majority of rapid screening assays it is unnecessary, provided detection of the analytes is not compromised (Meneely et al., 2011). The two most commonly applied are solid phase extraction (SPE) and immunoaffinity columns (IACs). The former is a non-specific method whereby the sample is loaded onto a column packed with a stationary phase, (e.g., silica, C8, C18), the mycotoxins bind to this phase, and a wash removes impurities or interfering substances prior to the toxins being eluted with solvent. IACs are very specific. The column-packing has specific antibodies

incorporated so that when the sample is loaded, the toxins will bind to the column and impurities will run through. The toxins are eluted using an antibody denaturing reagent. Analysis is performed on the resulting extracts.

Review of commercially available rapid diagnostic kits

To safeguard health by improving the quality of raw materials and their products, rapid tests have been increasingly promoted to validate food safety management systems used in the agri-food industry. In a survey conducted in 17 countries (11 in the EU and six non-EU), the authors reported that 66% of respondents used rapid test kits for an array of contaminants, including mycotoxins (Lebesi et al., 2010). This is further substantiated by the fact that in 2020, the global mycotoxin testing market was estimated at US$ 946 million and projected to reach US$ 1,337 million by 2025 (Markets and Markets Research, 2021). This has been driven by a number of factors, namely, legislative demands across many countries in the world often resulting in border rejections and product recalls, increased contamination of products, climate change, and consumers' heightened awareness.

The rapid diagnostics market is hugely competitive and the vast majority of tests available for T-2 and HT-2 are immunochemical methods including Enzyme Linked Immunosorbent Assays (ELISA), Lateral Flow Devices (LFDs)/Dipstick Assays, and Fluorescence Polarisation Immunoassays (FPIA). The companies providing these kits include Aokin AG, Charm Sciences Incorporated, Elabscience Incorporated, Envirologix Incorporated, Eurofins Tecna Laboratories, Hygiena LLC, Neogen Corporation, Romer Labs Diagnostic GmbH, PerkinElmer Incorporated, R-Biopharm AG and Vicam LP, (Table 5). It should be noted that there are also multiplex immunoassay formats enabling the detection of a number of the regulated mycotoxins. These include the Biochip Array Technology, (Randox Food Diagnostics), for the measurement of nine mycotoxins (Plotan et al., 2016), the Flow Cytometry instrument from Foss for the detection of six mycotoxins and the Myco 5-in-1 PLUS test produced by Vicam LP for the determination of up to six regulated mycotoxins.

Table 5. Commercially available rapid diagnostic tests for the detection of T-2 and HT-2 in cereals.

Manufacturer	Kit	Matrix	Detection Method	Extraction Solvent	LOD* (ppb)	Range** (ppb)	Antibody Cross-reactivity Profile	Test Time***	Tests / Kit	Additional Information
Aokin AG	Aokin Mycontrol T-2/HT-2	Oats, Wheat, Corn, Other grains	Quantitative Fluorescence Polarisation Immunoassay	Methanol based SPE Clean-up	-	-	-	15 minutes	20/100	Reader required
Charm Sciences Inc.	ROSA T-2 and HT-2 Quantitative Test	Barley, Corn, Oat Groats, Wheat,	Quantitative Lateral Flow Device	70% methanol	-	25-200 100-2000	-	10 minutes	-	Reader required
Elabscience Inc.	T-2(T-2 Toxin) Lateral Flow Assay Kit	Cereals, Feed	Qualitative Lateral Flow Device	Ethyl Acetate	10 ppb	-	-	5 minutes	50	-

Manufacturer	Kit	Matrix	Detection Method	Extraction Solvent	LOD* (ppb)	Range** (ppb)	Antibody Cross-reactivity Profile	Test Time***	Tests / Kit	Additional Information
	T-2(T-2 Toxin) ELISA Kit	Corn, Oats, Peanuts, Feed	Quantitative ELISA	60% Methanol	0.05	0-4.05	-	45 minutes	96	Reader required
	T-2(T-2 Toxin) ELISA Kit	Cereals, Feed	Quantitative ELISA	70% Methanol	1	0-81	T-2 100% ZEN 59% HT-2 <1%	20 minutes	96	Reader required
Envirologix Inc.	QuickTox Kit for QuickScan T-2/HT-2 Flex	Corn	Quantitative Lateral Flow Device	Extraction Buffer	25-50	50-900 900-2500 25-600	AFB1 <1% DON <1% FB1 <1% OTA <1% ZEN <1%	5 minutes	50	Reader required

Manufacturer	Kit	Matrix	Detection Method	Extraction Solvent	LOD* (ppb)	Range** (ppb)	Antibody Cross-reactivity Profile	Test Time***	Tests / Kit	Additional Information
Eurofins Tecna Laboratories	B ZERO T-2	Cereals, Feed	Quantitative ELISA	70% methanol & 4% NaCl	25-40	0-1000	T-2 100% HT-2 72%	20 minutes	24/48	Reader required
	Celer T-2	Cereals, Feed	Quantitative ELISA	70% methanol & 4% NaCl	25-40	0-1000	T-2 100% HT-2 72% DON <1%	20 minutes	48/96	Reader required
Hygiena LLC	Helica™ T-2 Toxin ELISA	Cereals, Feed	Quantitative ELISA	70% methanol	12.5	12.5-600	T-2 100% HT-2 3%	30 minutes	96	Reader required
Neogen Corporation	Reveal® Q+ MAX for T-2 / HT-2	Wheat, Oats, Corn	Quantitative Lateral Flow Device	Aqueous Extraction	50	50-500	-	5 minutes	25	Reader required

Manufacturer	Kit	Matrix	Detection Method	Extraction Solvent	LOD* (ppb)	Range** (ppb)	Antibody Cross-reactivity Profile	Test Time***	Tests / Kit	Additional Information
	Reveal® Q+ for T-2/HT-2	Corn, Corn Products	Quantitative Lateral Flow Device	Water	50 ppb	50-600 ppb	-	6 minutes	25	Reader required
	Veratox® for T-2/HT-2	Barley, Corn, Oats, Wheat	Quantitative ELISA	70% methanol	25	25-250	T-2 100% HT-2 100%	10 minutes	48	Reader required
R-Biopharm AG	RIDA® QUICK T-2 / HT-2 RQS ECO	Oats, Wheat, Corn	Quantitative Lateral Flow Device	Aqueous Extraction Buffer	50	50-1000	-	5 minutes	20	Reader/App required
	RIDASCREEN® T-2 / HT-2 Toxin	Oats, Corn, Barley, Wheat	Quantitative ELISA	Water-based Extraction	12	10-360	T-2 100% HT-2 85%	45 minutes	96	Reader required

Manufacturer	Kit	Matrix	Detection Method	Extraction Solvent	LOD* (ppb)	Range** (ppb)	Antibody Cross-reactivity Profile	Test Time***	Tests / Kit	Additional Information
							T-2 Triol <0.5% T-2 Tetraol <0.5%			
	RIDASCREEN® T-2 Toxin	Cereals, Feed	Quantitative ELISA	84% Acetonitrile	3.5-56	3.5-560	T-2 100% HT-2 7% Acetyl T-2 <114% Iso T-2 2%	90 minutes	96	Reader required
	RIDASCREEN® FAST T-2 Toxin	Cereals, Feed	Quantitative ELISA	70% Methanol	< 20	50-400	-	15 minutes	48	Reader required

Manufacturer	Kit	Matrix	Detection Method	Extraction Solvent	LOD* (ppb)	Range** (ppb)	Antibody Cross-reactivity Profile	Test Time***	Tests / Kit	Additional Information
Romer Labs Diagnostic GmbH	AgraQuant® T-2 Toxin ELISA test	Grains, Cereals, Others	Quantitative ELISA	70% methanol	10	25-500	-	15 minutes	48/96	Reader required
PerkinElmer Inc.	AuroFlow™ AQ T-2/HT-2 Strip Test	Corn, Wheat	Quantitative Lateral Flow Device	Water-based	50	50-500	-	5 minutes	25	Reader required
	MaxSignal® T-2 ELISA Kit	Seed, Feed, Cereal	Quantitative ELISA	-	10	-	-	< 30 minutes	96	Reader required
Vicam LP	T-2/HT-2-V AQUA	Grains, Feed	Quantitative Lateral Flow Device	Water-based Extraction	10	10-800	-	5 minutes	25	Reader required

*Limit of detection (LOD); **Quantification range (ppb); ***Incubation following sample preparation; - Not detailed

Enzyme Linked Immunosorbent Assays

Commercial ELISAs are commonly employed to screen cereals for mycotoxins. They allow the qualitative or quantitative measurement of mycotoxins in food and the principle is based on the use of specific antibodies and colour changes. Most often the assay is performed in microtitre plates. As mycotoxins are low molecular weight compounds, the assays are competitive. Briefly, a pre-titrated concentration of specific antibody is coated onto the wells of a 96-Well microtitre plate. A sample or standard is added to the wells of the plate, followed by the mycotoxin conjugated to an enzyme. Competition between unlabelled and labelled antigen (mycotoxin) for antibody binding sites occurs during a specified incubation period. After washing, to remove unbound material, the labelled bound antigen is measured by the addition of a suitable enzyme substrate producing a colour change. The absorbance reading is inversely proportional to the amount of toxin present (Nolan et al., 2019).

Lateral Flow Devices

Lateral flow devices all use the bio-molecular interaction of an antibody/antigen and the signal generated by means of the antibody or antigen conjugated to colloidal metals such as silver or gold, a visible of fluorescent dye, enzymes or latex beads impregnated with visual or fluorescent dyes to enable reading of the device. As is the case for ELISA, since these toxins are small molecular weight compounds (< 2000 Daltons), the test format is an indirect (inhibition) assay. The extracted sample is applied to the sample pad and moves along the membrane by capillary action. Once it reaches the conjugate release pad, the dry conjugate (labelled antibody) is re-hydrated. If analyte is present, it binds to the antibody and continues to flow along the strip. If no toxin is present, the free antibody will bind to the test line (analyte of interest); therefore, the presence of a coloured line is inversely proportional to the amount of analyte present. The control line validates the test (composed of bound anti-species antibody) (Nolan et al., 2019). These rapid assays can either be qualitative, i.e., they detect the presence or absence of the toxin or quantitative, providing a concentration level in the product (Li et al., 2014).

Early versions of LFDs were predominantly qualitative assays. However, improvements in reagents, component materials and reader technologies, along with manufacturing processes, mean quantitative results are achievable. In addition, the developments in reader technology and advancements in raw materials, such as labels, means a lateral flow rapid test can match the sensitivity of an ELISA assay.

Fluorescence Polarisation Immunoassays

This format has not been commercialised to the same extent as ELISAs or LFDs. It is a homogeneous assay as it occurs only in solution. The principle is that when a fluorophore in solution is exposed to plane polarised light at its excitation wavelength, the subsequent emission is depolarised. Therefore, for a competitive assay, toxin is covalently linked to the fluorophore to make a fluorescent tracer. The tracer competes with toxin (from the sample) for a limited amount of toxin-specific antibody. In the absence of toxin, the antibody binds the tracer, restricting its motion and causing a high polarization. Conversely, in the presence of toxin, less of the tracer is bound to the antibody and a greater fraction exists unbound in solution, where it has a lower polarization. The results are read in fluorescence polarization reading instruments (Lippolis and Maragos, 2014; Maragos, 2009).

Advantages and disadvantages of rapid immunological diagnostic tests

Commercially available test kits provide several major advantages over confirmatory laboratory-based methods for regulatory compliance. They are rapid and easy-to-use, comparatively inexpensive, and require few procedural steps. Many are portable, often have a reader/integrated incubator; they are sensitive and can be either qualitative or quantitative. Some may be blockchain enabled and, with increasing vigilance regarding health and safety, many rely on aqueous extraction of the analyte rather than use of solvents (Aamot et al., 2013; Alldrick, 2014; Anfossi et al., 2016; Zachariasova et al., 2014). The speed of the test kit is particularly important in the agri-food industry (Zachariasova et al., 2014). Often raw materials

are delivered as and when required, therefore there is no significant stock to rely on and the lots being delivered are needed for immediate use. In this instance, it is vital that compliance-testing produces very rapid results to enable quick decision-making and avoid disruption to the business. Raw material lots can therefore be accepted or rejected within a very short timeframe (Alldrick, 2014). The ease-of-use and portability are crucial for farmers and producers monitoring the regulated mycotoxins. Use of such kits will allow on-site testing without the requirement of any special laboratory equipment or indeed the need for trained laboratory staff, thereby reducing the cost per sample significantly (Zachariasova et al., 2014).

Notwithstanding the importance of the advantages offered by rapid diagnostic kits, there are a number of notable disadvantages associated with their use. The most significant, which affect reliable detection, are the specificity of the antibody and interfering substances or matrix effects. Generally, these may lead to an over-estimation and increased measurement uncertainty of mycotoxin concentrations or false positives (Alldrick, 2014; Meneely et al., 2011; Nolan et al., 2019; Zachariasova et al., 2014). The specificity or cross-reactivity profile of the antibody employed in test kits is extremely important to ensure accurate results. For example, an antibody raised against T-2 toxin may also be specific to the metabolites T-2 Tetraol or T-2 triol, resulting in over-estimation of concentrations, as these metabolites are not legislated for in foods or feeds. By way of contrast, and given that the regulatory limits refer to the sum of T-2 and HT-2, the fact that their toxicities are similar and that T-2 is rapidly hydrolysed to the metabolite HT-2 *in vivo*, an under-estimation of results would follow if the antibody has poor specificity towards HT-2 (Meneely et al., 2011). In addition, the matrix or food commodity may increase or decrease the signal and hence lead to over-estimation or under-estimation of results. To circumvent the issue of matrix interference, manufacturers will specify the commodities for which the test kit has been fully validated (Li et al., 2014; Nolan et al., 2019). If the test kit is required for a different matrix, a full validation is required to ensure it is fit for purpose and will provide the necessary accuracy, sensitivity and precision. As outlined previously, the extraction solutions used in preparing samples for analysis have often been based on organic solvents such as methanol, acetonitrile or ethyl acetate. Obviously, the drive is to reduce these, from both a

health and safety and environmental point of view, and while many manufacturers still employ such extractants, some have introduced aqueous extractions for these toxins. Of course, these must provide comparable extraction efficiencies to guarantee reliable data; however, they offer a more attractive option for industry in terms of cost, health and safety and environmental policies. Another disadvantage with many rapid tests is that they must be performed at ambient temperature, which may prove difficult under certain circumstances. Other drawbacks include the fact that different protocols may be required for different commodities/products, that dust and dirt may affect the readers, and that if small numbers of tests are performed on a regular basis, the use of ELISAs or FPIA may prove too expensive. Therefore, careful consideration of the selection of the most applicable rapid diagnostic kit to the supplier/producer needs to be undertaken.

Performance characteristics of the commercial rapid diagnostic test kits

This review identified 20 commercially available rapid diagnostic kits for the determination of T-2 and HT-2 toxins in agricultural commodities.

As EU indicative limits (Table 3) refer to the sum of T-2 and HT-2, it is imperative that the antibody is specific for both toxins. Of the test kits identified, results have indicated that some of the products are not fit for purpose. Those that failed to meet these requirements included products where there was little specificity towards HT-2 toxin and, if the kit was marketed for T-2 toxin only, without stipulating cross-reactivity towards HT-2. The risk with using these is the possibility of under-estimation of the concentrations of the targeted mycotoxins. All tests, both LFDs and ELISAs provided by Elabscience Inc., are marketed for the testing of T-2 toxin. The cross-reactivity of the T-2 toxin antibody has not been declared for two of these products, and for the third it is <1% against HT-2 toxin, and therefore not suitable. In addition, the antibody used in the ELISA manufactured by Hygiena LLC has low specificity (3%) for HT-2 toxin, so again it could not be used to obtain accurate results. Similarly, the RIDASCREEN® T-2 Toxin ELISA and RIDASCREEN® FAST T-2 Toxin ELISA (R-Biopharm AG) would under-estimate results, as the antibody

cross-reactivity with HT-2 is only 7% or not detailed, respectively. For the AgraQuant® T-2 Toxin ELISA test (Romer Labs Diagnostic GmbH) and PerkinElmer MaxSignal® T-2 ELISA kits, the cross-reactivity profile has not been specified and are thus unlikely to be suitable for the testing of both toxins. In contrast, Eurofins Tecna Laboratories market two ELISA kits, B ZERO T-2 and Celer T-2, but both are applicable for measuring the sum of T-2 and HT-2, with 72% cross-reactivity against HT-2 toxin. Of the remaining products, and although antibody specificity has not been stated, the kits are aimed at the measurement of both toxins. These include an FPIA test (Aokin AG), quantitative LFDs by Charm Sciences Inc., Envirologix Inc., Neogen Corporation, R-Biopharm AG, PerkinElmer Inc. and Vicam LP, and quantitative ELISAs produced by Neogen Corporation and R-Biopharm AG. In total, according to the manufacturers' specifications with respect to cross-reactivity, 12 kits meet the requirements.

As these analytical tools are off-the-shelf products, it is crucial that they are applicable to the product (cereal) to be monitored for T-2 and HT-2, and therefore validated accordingly, as producers would not have the means, knowledge or time to conduct performance testing. Hence, the identified kits were examined in terms of the matrices in which they had been developed and validated. Oats and barley are the cereals of interest for this project. Fourteen diagnostic kits met this requirement: Aokin Mycontrol T-2/HT-2 (Aokin AG), ROSA T-2 and HT-2 Quantitative Test (Charm Sciences Inc.), T-2(T-2 Toxin) Lateral Flow Assay Kit and T-2(T-2 Toxin) ELISA Kit (Elabscience Inc.), B ZERO T-2 and Celer T-2 (Eurofins Tecna Laboratories), Helica™ T-2 Toxin ELISA (Hygiena LLC), Veratox® for T-2/HT-2 (Neogen Corporation), RIDASCREEN® T-2/HT-2 Toxin, RIDASCREEN® T-2 Toxin and RIDASCREEN®FAST T-2 Toxin (R-Biopharm AG), AgraQuant® T-2 Toxin ELISA test (Romer Labs Diagnostic GmbH), MaxSignal® T-2 ELISA Kit (PerkinElmer Inc.) and T-2/HT-2-V AQUA, (Vicam LP) were found to be applicable. That said, not all tests were validated in both oats and barley: Reveal® Q+ MAX for T-2/HT-2 (Neogen Corporation) and RIDA®QUICK T-2/HT-2 RQS ECO (R-Biopharm AG) have only been validated in oats.

The European Commission has stipulated that the Limit of Quantification should not exceed 10 ppb for T-2 and HT-2 individually, and that the LOD of an analytical

method should be less than or equal to 25 ppb for the sum of T-2 and HT-2 (EU, 2013). For the most part, from the information available, the test kits identified have indicated the LOD of the methods, although the Limits of Quantification (LOQs) have not been stated. The vast majority of the rapid methods meet the requirements laid down by the European Commission. However, this information has not been indicated by Aokin AG for the FPIA. In the case of the ROSA T-2 and HT-2 Quantitative Test produced by Charm Sciences Inc., the quantification range has been specified as 25 – 200 ppb, rather than the LOD. LFDs produced by Neogen Corporation, R-Biopharm AG and PerkinElmer incorporated state LODs of 50 ppb. While the kits still meet the performance characteristics required to accurately determine the sum of T-2 and HT-2 in unprocessed cereals, they are not suitable for cereal products or cereal-based foods for infants and children where the indicative limits fall at 25 ppb and 15 ppb, respectively. The ELISA kits B ZERO T-2 and Celer T-2 produced by Eurofins Tecna Laboratories have specified LODs of 40 ppb for oats, and therefore also fall into this category, i.e., are not applicable for the testing of cereal products or cereal-based food for infants and children.

LFDs can be used on-site to test raw materials, whereas ELISA kits need to be performed in a laboratory setting with access to a spectrophotometer or microplate reader. Furthermore, there are many steps involved with this type of immunoassay and the technician performing the test must be proficient in the use of micropipettes. As a result, LFDs are the preferred option for inclusion in the project.

Conclusions

The aim of the review was to evaluate commercially available rapid diagnostic test kits for the determination of T-2 and HT-2 toxins in oats and barley with respect to their claimed performance characteristics. For on-site testing, kits that met the following criteria were selected for possible inclusion in the project, i.e., to evaluate their performance for the measurement of the sum of T-2 and HT-2 toxins in oat and barley samples from the food supply chain:

- Ability to measure T-2 and HT-2 toxins in commodities (✓).
- Validated for oats and barley (✓).
- LODs of ≤ 25 ppb (✓).
- Laboratory facilities (X).
- Speed of test

The potential kits to be selected have been included in Table 6. They include LFDs ELISA kits. Engagement with the producers/suppliers to identify the kits they employ was sought and these products were included for evaluation, where possible. Furthermore, as some LFDs are only applicable to oats, validation may be performed in barley to ascertain if the tests provide accurate results. In addition, one ELISA kit was assessed, as these are considered the gold standard in immunoassays.

All the test kits highlighted are quantitative and employ aqueous extractions. The LFDs have few procedural steps, they are easy to use on-site, and they are rapid. Importantly, only those kits that could be delivered within the timespan of the project where the readers required to measure the toxin concentrations were available to rent were considered.

Table 6. Appraisal/selection of rapid diagnostic kits.

Manufacturer	Kit	Antibody specificity (T-2 and HT-2 toxins)	Validated for oats and barley	LOD (≤ 25 ppb)	Laboratory facilities	Speed of test (minutes)
Envirologix Inc.	QuickTox Kit for QuickScan T-2/HT-2 Flex (LFD)	✓	X	✓	X	5
Neogen Corporation	Reveal® Q+ MAX for T-2/HT-2 (LFD)	✓	X	X	X	5
	Reveal® Q+ for T-2/HT-2 (LFD)	✓	X	X	X	6
R-Biopharm AG	RIDA®QUICK T-2/HT-2 RQS ECO (LFD)	✓	X	X	X	5
	RIDASCREEN® T-2/HT-2 Toxin (ELISA)	✓	✓	✓	✓	45

Manufacturer	Kit	Antibody specificity (T-2 and HT-2 toxins)	Validated for oats and barley	LOD (≤ 25 ppb)	Laboratory facilities	Speed of test (minutes)
PerkinElmer Inc.	AuroFlow™ AQ T-2/HT-2 Strip Test (LFD)	✓	X	X	X	5
Vicam LP	T-2/HT-2-V AQUA (LFD)	✓	✓	✓	X	5

Objective 3

Report on the current mycotoxin testing regimes used within the industry.

Introduction

To help improve the resilience of the cereal primary production and processing sector in terms of mycotoxin contamination, it is essential to understand the supply chain, the critical control points within the chain, the sampling practices used, and the testing regimes employed to monitor these contaminants. To that end, engagement with stakeholders is critical. A Project Advisory Board was established, composed of primary oat producers/processors and barley processors. The aim of this objective was to evaluate the testing regimes and sampling strategies used by the stakeholder members of the Project Advisory Board to ascertain the current shortfalls in mycotoxin control within the supply chains. The information gathered aimed to facilitate improved practical mitigation approaches to protect public health, address regulatory requirements, and ultimately increase the resilience of the indigenous cereal industry.

Survey

The questions detailed below were circulated by email to the Project Advisory Board for responses to help identify any potential shortfalls in relation to mycotoxin monitoring within the industry.

Mycotoxin testing regimes:

- What mycotoxins do you currently test for?
- Where are the tests performed? On-site, or by contract laboratories?
- What methods are employed? Rapid diagnostic tests and/or confirmatory methods such as mass spectrometry?

- How many samples are typically analysed per annum and from which areas of the supply chain, i.e., incoming raw materials, throughout storage, after processing?
- Please describe the sampling procedure employed prior to testing.

Results and Discussion

Survey results

Testing Regimes: Industry feedback

From the industry feedback (Table 7), it is evident that the monitoring of mycotoxins in oats and barley is somewhat varied. While two out of the four oat primary producers/processors tested for the important *Fusarium* mycotoxins, T-2 and HT-2, deoxynivalenol and zearalenone in oats, the third only tested for T-2 and HT-2 and the fourth had no testing regime established at all. Only one commercial company involved in processing barley joined the Project Advisory Board. They rely on the certificate analysis as proof of compliance and perform no testing.

In terms of the testing applied, having a two-tier testing regime is definitely the best approach. In-house testing with screening tests, such as lateral flow devices or ELISA, will provide rapid results indicating compliance or non-compliance with current EU regulations. These results can then be confirmed using the reference or confirmatory methods at an external contract laboratory. It is essential that the rapid tests are validated for the particular matrix to ensure accurate, reliable results and only those samples that fall within the measurement uncertainty for the test (at the regulatory limits) be sent for confirmation, thus providing cost-effective monitoring for the industry. It is not clear from those who only use rapid tests what happens to samples that are non-compliant. Are these re-tested using confirmatory analytical methods? Moreover, for those with a two-tier testing system, what happens to raw materials/products that fail the indicative limits? From previous collaborative projects, the industry indicated that the performance of some rapid tests may be poor. This underpins the importance of the comparative study between the screening kits and confirmatory LC-MS/MS to ascertain whether this is the case, or if sampling, sample preparation and extraction techniques are increasing imprecision.

Table 7. Industry survey results (mycotoxin testing regimes).

Primary producer/processor	Mycotoxins tested	Methods employed	Analyses performed per annum	Types of samples tested
Company 1	T-2 and HT-2 toxins. Deoxynivalenol. Zearalenone. OTA.	LC-MS/MS and GC (Reference methods) – Contract labs. Rapid methods - (Neogen Raptor) – In-house testing.	20 per season.	Incoming raw material. Raw oats. Dried oats. Finished flaked oats.
Company 2	T-2 and HT-2 toxins. Deoxynivalenol. Zearalenone. Alternaria. Ergot Alkaloids. AFB1	Iso accredited LC-MS/MS – external lab Rapid methods - (Neogen Raptor) – In-house testing.	12 per season to the external lab for all mycotoxins listed. 200 samples on Raptor for T-2 and HT-2 and deoxynivalenol.	Standard oats. Organic oats. Finished flaked oats.

Primary producer/processor	Mycotoxins tested	Methods employed	Analyses performed per annum	Types of samples tested
	OTA. Sterigmatocystin. Nivalenol.			
Company 3	T-2 and HT-2 toxins.	Rapid method - Charm test - external lab.	20 per season.	Incoming raw material. Dried oats. Stored oats. Finished flaked oats.
Company 4	None	N/A	N/A	N/A
Company 5	None	N/A	N/A	N/A

Mycotoxin test procedure

Regardless of the method of analysis chosen for mycotoxins in grains, there are four important factors that will determine the reliability of the results obtained: sampling, sample preparation, sample extraction and the analysis. At each stage there will be a certain amount of variability or error and thus the total error of the test procedure is the sum of these.

Sampling is crucially important as mycotoxins are heterogeneously distributed throughout grains. They can be concentrated in areas known as “hotspots” while the rest of the batch may contain very low concentrations or no mycotoxins at all. If sampling procedures are not adequate (representative), this may lead to an over-estimation of the concentration, resulting in rejections, economic loss and food waste or, alternatively, be under-estimated, resulting in contaminated materials entering the food chain and posing a health risk to consumers. In light of the importance of this, the European Union issued recommendations for sampling for the official control of mycotoxins in foodstuffs (EC, 2006c). Depending on the lot size, a number of incremental samples must be collected from different places within the lot, which are combined to provide an aggregate sample. The EU sampling recommendations for cereals and cereal products are detailed in Tables 8 and 9.

Table 8. EU sampling methods for lots ≥ 50 tonnes.

Lot weight (tonnes)	**Weight or number of sub-lots**	**No. of incremental samples**	**Aggregate sample weight (kg)**
≥1,500	500 tonnes	100	10
> 300 - < 1 500	3 sub-lots	100	10
≥ 50 - ≤ 300	100 tonnes	100	10

Table 9. EU sampling methods for lots ≤ 50 tonnes.

Lot weight (kg)	Number of incremental samples*	Aggregate sample weight (kg)
≤ 50	3	1
≥ 50 - ≤ 500	5	1
≥ 500 - ≤ 1,000	10	1
≥ 1,000 - ≤ 3,000	20	2
≥ 3,000 - ≤ 10,000	40	4
≥ 10,000 - ≤ 20,000	60	6
≥ 20,000 - ≤ 50,000	100	10

*Incremental sample ≥ 100 g

The aggregate sample is divided to subsequently form the test samples. The methods of taking these incremental samples to ensure a random representative sample will depend on whether the lots are static or dynamic. To obtain a representative sample for testing, if the aggregate sample is very large, i.e., ≥ 1-2 kg, it must be thoroughly mixed in a large rotating drum or by hand mixing on a clean area. Subsampling to obtain a 1-2 kg subsample may be achieved through coning and quartering or the use of a sample divider. The representative sample must then be homogenised by grinding to reduce the particle size: the smaller the particle size, the smaller the subsample size can be without increasing error or uncertainty. Although variability will be seen between subsamples, it will be much less in the comminuted sample. Reference samples and test samples must be derived from this sample. Even the most precise analytical methods will not produce accurate, reliable results if sampling is not representative and the sample not homogeneous.

Sampling: Industry feedback

Company 1 highlighted their sampling protocol. The batch size of 60 tonnes requires that at least 100 incremental samples be removed from the lot to prepare an aggregate sample of no less than 10 kg. However, only a 2 kg aggregate sample was taken, therefore it is not representative of the original batch of cereal. Furthermore, no information was given as to how these incremental samples were taken, or how many. Therefore, the results of any testing performed will not accurately reflect the true level of contamination of the grains; the results may be under-estimated or over-estimated.

Company 2: Unprocessed grains (farm samples) are lifted at harvest and sent for analysis. Moisture, bulk density and mycotoxins etc. are among the tests performed on a sample before the newly harvested oats are approved for delivery. For the majority of these samples, the company's farm liaisons will be on-site to lift a representative sample – spearing the sample three to five times in different locations and mixing this together to form the aggregate sample. For samples not lifted by farm liaisons, the farmer is walked through the procedure before lifting the sample. Processed grain (flake) samples are lifted directly from the conveyor that is feeding the packing machines in production.

From the information supplied, it is evident that there are shortfalls in the procedures followed. There is no indication of the size of the bulk samples from which the aggregate samples are produced or the number of incremental samples taken. Furthermore, for processed samples, there is no mention of milling the samples to ensure homogeneity prior to sample analysis. Adoption of a suitable sampling plan, (although delivered to the participants), may not have been followed in all cases; therefore, the importance of this must be emphasised during the workshop (see Objective 6).

In the case of Company 3, raw oat samples were collected from farms; conventionally and organically dried oats (in silos) were sampled in addition to processed oats (hulls and groats) and the finished product. 500g samples were received for testing. No information was supplied on the sampling procedures,

highlighting the need for further discussions regarding this to emphasise the importance of obtaining a representative sample.

Sample preparation trial

To highlight the importance of ensuring homogeneity by milling the sample to as small a particle size as possible, a small trial was performed to demonstrate the difference in analytical results obtained. Fifty processed oat samples were analysed by the LC-MS/MS detailed under Objective 4, with no milling. The remainder of the same samples were subsequently milled and re-analysed using the same method. Results are shown for the concentrations determined for the sum of T-2 and HT-2 and deoxynivalenol in Figures 1 and 2 and in Table 1 in Appendix B.

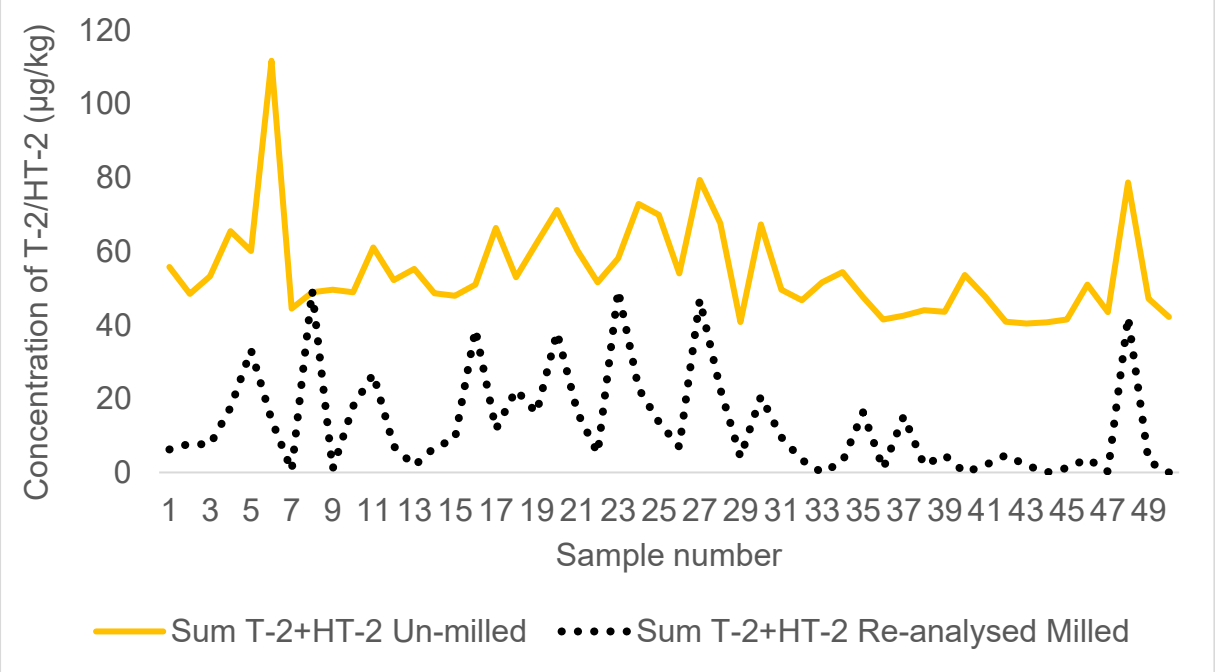


Figure 1. Comparison of LC-MS/MS results for the sum of T-2 and HT-2 (no milling and milling) in processed oats.

The solid yellow line depicts the concentrations of the sum of T-2 and HT-2 toxins in processed oats that were not milled to <0.5 mm prior to analysis, and the black

dotted line represents analysis of the same samples following milling to <0.5mm prior to analysis. The x-axis represents the number of samples tested and the y-axis represents the concentration of the toxins (µg/kg).

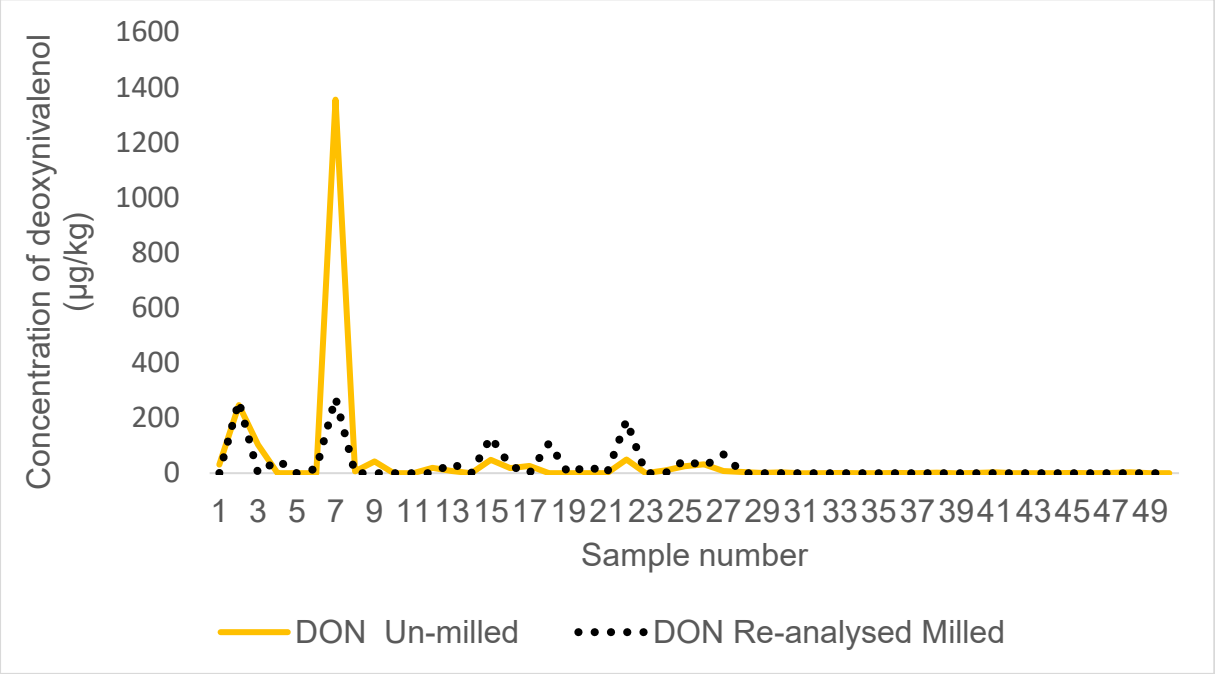


Figure 2. Comparison of LC-MS/MS results for deoxynivalenol (no milling and milling) in processed oats.

The solid yellow line depicts the concentrations of the deoxynivalenol in processed oats that were not milled to <0.5 mm prior to analysis, and the black dotted line represents analysis of the same samples following milling to <0.5mm prior to analysis. The x-axis represents the number of samples tested and the y-axis represents the concentration of the toxin (µg/kg).

The results illustrate that for the analysis of T-2 and HT-2, the concentrations measured were for the most part lower following milling than the levels detected in the un-milled samples. For deoxynivalenol, the results were more variable, with higher concentrations detected in milled samples compared with un-milled samples, and vice-versa. It should be noted that the samples tested in this instance all met current indicative limits for the sum of T-2 and HT-2 of 200 µg/kg, whether milled or

un-milled. If these were to change to the lower limit of 50 µg/kg under discussion, and the samples were not milled prior to analysis, 27 out of the 50 samples tested (54%) would be classed as non-compliant (false positives), causing economic loss and food waste. Similarly, for deoxynivalenol, one sample (no milling) was non-compliant with respect to the current regulatory limit of 750 µg/kg with a concentration of 1355 µg/kg; however, once milled and re-analysed, only 273 µg/kg of deoxynivalenol was detected, which is well below the legal limit. These observations stress the importance of correct sample preparation, i.e., comminution of the sample to ensure accurate results for mycotoxin testing.

Conclusions

The aim of this Objective 3 was to gain a comprehensive understanding of the testing regimes the industry (Project Advisory Board) undertake in order to control and mitigate against mycotoxins entering the food chain.

The results emphasise that testing is varied throughout the industry. It ranges from the use of both screening and confirmatory analyses for a number of regulated and unregulated mycotoxins, to screening tests for a small number of mycotoxins, to no testing and a reliance of certificates of analysis from suppliers. Furthermore, from the information received, the sampling and sample preparation procedures used by industry can be greatly improved, providing accurate reliable results for the monitoring of mycotoxins in the oats and barley sectors.

The impact of better testing of mycotoxins will ensure the industry moves from a reactive approach in dealing with mycotoxins to a proactive, industry-led approach. Further impacts will include improved grain quality and safety, product and brand protection, the adoption of new technologies, and reduced waste and therefore enhanced margins. With the drive to increase plant-based foods on the island of Ireland, having such guidelines in place will give confidence to the farming community to diversify into these crops. Finally, consumers of oat- and barley-based foods will be better protected in terms of mycotoxin exposure.

To ensure the resilience of the industry, these shortcomings must be addressed through engagement with the industry stakeholders.

Objective 4

Survey of oats and barley to identify contamination levels of the mycotoxins of interest using mass spectrometry and analysis of oat survey data and sample metadata to identify trends/major weaknesses in the supply chain.

Introduction

The island of Ireland is an important and increasingly large producer of the cereal crops of oats and barley and while some of the output is used for animal feeds, a significant proportion is used in the food and beverage industries. In 2021, crop yields for barley and oats were estimated at 1556.4 and 238.3 (x 1000) tonnes, respectively, in Ireland, while in Northern Ireland the reported yields were 137 and 11 (x 1000) tonnes, respectively, for barley and oats. In economic terms, the crops were worth an estimated total of €326M in Ireland and £32M in Northern Ireland (Statista, 2022).

Globally, barley and oats are ranked fourth and sixth, respectively, in terms of tonnes produced and can be cultivated in temperate regions, making them particularly suitable to the climate on the island of Ireland. Nutritionally, oats and barley are considered functional foods, so in addition to health benefits resulting from their rich source of dietary fibre, essential amino acids and vitamins and minerals, they contain β-glucan, which helps reduce cholesterol and control blood sugar. Typical oat-derived foods include breakfast cereals, breads, biscuits, infant food, muesli, granola bars and, more recently, oat dairy alternatives (De Colli et al., 2021). While the bulk of barley produced is for the malting and brewing industries (Teagasc, 2022), it is also used in the production of breakfast cereals, malt vinegar, malt extract, in various cooked foods and in dairy alternative beverages (Badea and Wijekoon, 2021).

Natural contamination of cereal grains with fungal pathogens, both pre- and post-harvest, is a continuing and growing problem worldwide, as many of these fungal species produce mycotoxins that have serious implications for human and animal

health. Of the hundreds of mycotoxins identified, only eleven have been legislated for in human food and animal feed, and include aflatoxins, fumonisins, OTA, zearalenone, deoxynivalenol, T-2 and HT-2 toxin. Production of these natural toxins is determined by specific environmental and management conditions, and climate change is expected to continue to drive contamination of these crops, necessitating greater surveillance and control in order to safeguard the food chain (Van Der Fels-Klerx et al., 2010; DeColli et al., 2021; Kolawole et al., 2021a; Ramos-Dias et al., 2021). Current risks to cereal production on the island of Ireland include the damp weather conditions that can impact adversely on disease levels, harvest moisture and quality, in addition to the fact that often there is continuous cereal cropping and the absence crop rotation.

Of these pathogens, the *Fusarium* fungi are probably the most damaging toxigenic fungi affecting small cereal grains and maize globally, and typically one or more species may infect a plant at one particular time (Imathiu et al., 2013). *Fusarium* head blight (FHB), one of the most important diseases associated with the fungal genus, causes yield loss and a reduction in grain and seed quality, in addition to the production of mycotoxins that pose health risks. Wheat, barley and maize are often affected by these fungal pathogens, in addition to other cereal crops such as oats and rye (Miller, 2008). *Fusarium* species have been found in all European cereal-growing regions: the most commonly found species include *F. graminearum*, *F. culmorum*, *F. poae*, *F. nivale*, and *F. avenaceum* (Stępień and Chełkowski, 2010). The most frequent worldwide contaminants in agricultural commodities associated with these fungal pathogens include the type A trichothecenes (T-2 and HT-2 toxins) and type B trichothecenes (Deoxynivalenol and Nivalenol) (Khaneghah et al., 2020; McCormick et al., 2011), all of which are important from a food safety perspective (Ferrigo et al., 2016; Ismaiel et al., 2015).

Several studies have not only highlighted the contamination of oats and barley with T-2 and HT-2 toxins in Ireland and the United Kingdom, but also that different levels of contamination were observed in conventional and organic crops. Analysis of 458 harvested oat samples in the United Kingdom from 2002 until 2005 revealed that the *Fusarium* mycotoxins T-2 and HT-2 were the most frequently detected in 92% and 84% of samples, respectively. The mean concentration for the sum of T-2 and HT-2

was reported as 570 µg/kg, with a maximum of 9990 µg/kg. Highly significant differences were found in the occurrence of these toxins in conventional and organic farming practices, with predicted mean concentration of T-2 and HT-2 five times lower in organic oat crops (Edwards, 2009a). Similarly, Edwards (2017) reported a mean of 450 µg/kg for the sum of T-2 and HT-2 in oats harvested in the United Kingdom over the three years from 2006 to 2008. In a parallel study performed in barley from 2002 to 2005, the prevalence and concentrations of T-2 and HT-2 were much lower when compared with unprocessed oats, with detection in 36% and 12% of samples, respectively, and a maximum concentration of 138 µg/kg (sum of HT-2 and T-2 toxins). In this study of 446 samples, no differences were observed in prevalence rates in conventional and organic barley (Edwards, 2009b). DeColli et al. (2021) surveyed mycotoxin contamination in 208 unprocessed oat samples harvested in 2015-2016 from conventional, organic and gluten-free farming systems in Ireland. T-2 and HT-2 were detected in 41% and 51% of samples, respectively, with concentrations ranging from 55–1102 µg/kg for T-2 toxin and 53– 3405 µg/kg for HT-2 toxin. In agreement with the study performed by Edwards (2009a), this survey also found that T-2 and HT-2 were more prevalent in conventionally produced oats (82% and 70%, respectively) than in organic or gluten-free oats (DeColli et al., 2021).

To understand the current challenges of mycotoxin contamination, and specifically the *Fusarium* toxins T-2 and HT-2, on the island of Ireland and the UK, monitoring of oats and barley in these regions is essential.

This study details findings of T-2 and HT-2 contamination in unprocessed and processed oats from the 2021 harvest, in addition to unprocessed barley samples, again from the 2021 harvest. Furthermore, the survey included all regulated mycotoxins such as aflatoxins, fumonisins, OTA, zearalenone and deoxynivalenol.

Materials and Methods

Chemicals and materials

Analytical grade ammonium acetate, acetic acid, Ultra high performance liquid chromatography (UPLC) grade methanol and acetonitrile were purchased from Sigma-Aldrich (Gillingham, UK). Ultra-pure water (18.2 MΩ-cm) was produced in-house using a Millipore water purification system (Millipore, Cork, Ireland). Mycotoxin standards for the aflatoxins AFB1, AFB2, AFG1, AFG2, fumonisin B1 (FB1), fumonisin B2 (FB2), OTA, zearalenone (ZEN), deoxynivalenol (DON), T-2 and HT-2 were obtained from Romer Labs (Runcorn, UK). Individual stock standards (1 mg/ml) were prepared for each mycotoxin and stored in amber vials at a temperature of -20°C until use. Multi-mycotoxin working standards were prepared weekly from the stocks and stored as per stock standards.

Sample collection and preparation

Samples were provided by members of the Project Advisory Board. Where possible, a 1kg aggregate sample was supplied as detailed under current European Union legislation (Commission Regulation (EC) No 401/2006). If this was not feasible, a representative sample following the EU guidelines was provided for analysis. A comprehensive sampling plan was provided, details of which are included in Appendix C. Prior to analysis, all samples were milled to a fine powder (particle size <50 µm) using a CGoldenwall multifunction grinder and stored at -20°C to preserve integrity. The total number of samples provided and analysed was 281; 229 oat samples and 52 barley samples.

Sample extraction

A dilute-and-shoot sample extraction procedure was employed. Briefly, 1g of finely milled material was weighed into a 15ml centrifuge tube. Extraction solvent: acetonitrile:water:acetic acid (79:20:1, v/v/v) was added (4ml for barley samples or 5ml for oat samples) and the sample vortexed at 2500 rpm for 90 minutes using a

multi-vortexer. Following centrifugation at 5000 rpm for 15 minutes, a 1ml aliquot was removed and mixed 1:1 with acetonitrile:water:acetic acid (20:79:1, v/v/v) in an Eppendorf tube. The mixture was vortexed for 30 seconds and filtered through a 0.2 µm PTFE syringe filter into an amber LC-MS/MS vial for analysis.

LC-MS/MS parameters

Chromatographic separation was performed on an SCIEX ExionLC™ AD system with detection via SCIEX triple Quad 5500+ QTrap Ready LC-MS/MS system equipped with Turbo V™ ionisation source SCIEX, MA, USA). The Mass Spectrometer was operated in both positive and negative electrospray ionisation mode. Detection and quantification were accomplished using targeted analysis via a scheduled Multiple Reaction Monitoring (sMRM). For each analyte, two MRM transitions were monitored, a precursor ion and two product ions. Details of these transitions and the operating conditions are outlined in Table 10.

Table 10. Optimised MS/MS parameters for the analytes quantified.

Analyte	**Precursor ion (m/z)**	**Product ion (m/z)**	**Declustering Potential (DV)**	**Collision Energy (eV)**	**Collision Cell Exit Potential**
Aflatoxin B_1	313.061	285.1	121	33	14
	313.061	241.1	121	53	14
Aflatoxin B_2	315.074	287.2	141	37	14
	315.074	259.1	141	41	14
Aflatoxin G_1	329.055	243.2	131	37	18
	329.055	311.1	131	31	16
Aflatoxin G_2	331.057	313	106	35	16

Analyte	Precursor ion (m/z)	Product ion (m/z)	Declustering Potential (DV)	Collision Energy (eV)	Collision Cell Exit Potential
	331.057	245.2	106	41	14
Deoxynivalenol	297.097	249.1	91	21	20
	297.097	203.2	91	21	20
Fumonisin B_1	722.316	704.3	1	41	38
	722.316	334.4	1	53	10
Fumonisin B_2	706.309	336.1	126	49	20
	706.309	354.3	126	47	18
OTA	404.092	239	81	33	12
	404.092	358.1	81	21	18
T-2 Toxin	484.3	215.2	76	29	18
	484.3	185.1	76	31	11
HT-2 Toxin	442.257	263.102	71	19	14
	442.257	215.102	71	19	22
Zearalenone	317.1	175	-100	-34	-13
	317.1	131.1	-100	-42	-8

Separation was achieved using a Gemini C18, 100 x 4.6 mm, 5µm, 110 Å with the column maintained at 30°C. Gradient elution using mobile phases of methanol/water/acetic acid 10:89:1 (v/v/v) (Mobile Phase A: MPA) and methanol/water/acetic acid 97:2:1 (v/v/v) (Mobile Phase B: MPB), both containing 5 mM ammonium acetate buffer, were used. The binary gradient elution was as follows in Table 11.

Table 11. Chromatography Gradient Elution Conditions.

Time (min)	MPA (%)	MPB (%)
0	99	1
1.0	99	1
3.0	50	50
9.0	1	99
11.5	1	99
12.0	99	1
14.0	99	1

A flow rate of 1.0 ml/minute was maintained with a sample injection volume of 5 µl. Total run time was 14.0 minutes.

Mass spectrometer parameters were as follows: Curtain Gas (CUR) = 35; Collision Gas (CAD) = 9; ion spray voltage: 4.5 kV (ESI+) and 4.5 kV (ESI-); temperature = 600°C; ion source gas 1 (GS1) = 60 and ion source gas 2 (GS2) = 50.

Results and Discussion

In total, 229 oat samples (unprocessed and processed) and 52 barley samples from Ireland and the United Kingdom were analysed for the regulated mycotoxins. One sample of oats was from Britain with the remainder from Ireland and Northern Ireland. All processed oats were from Northern Ireland. Five samples of barley came from Britain and the remainder from Northern Ireland. Results tables for all samples can be found in Appendix 4 (Tables 1-5).

In oats, the *Fusarium* mycotoxins T-2 and HT-2 were observed in 216 samples (94.3%). Deoxynivalenol and zearalenone were detected in 65 (28.4%) and 14 (6.1%) samples, respectively. Additionally, OTA was detected in 18 samples (7.9%) of oats tested.

In barley, T-2 and HT-2, deoxynivalenol and zearalenone were the only mycotoxins detected, all of which fell below current regulatory limits. Occurrence rates were 78.8%, 3.8% and 50% for T-2 and HT-2, deoxynivalenol and zearalenone, respectively.

Figure 3 highlights the prevalence of the regulated mycotoxins in unprocessed and processed oats produced conventionally and organically, and those detected in barley.

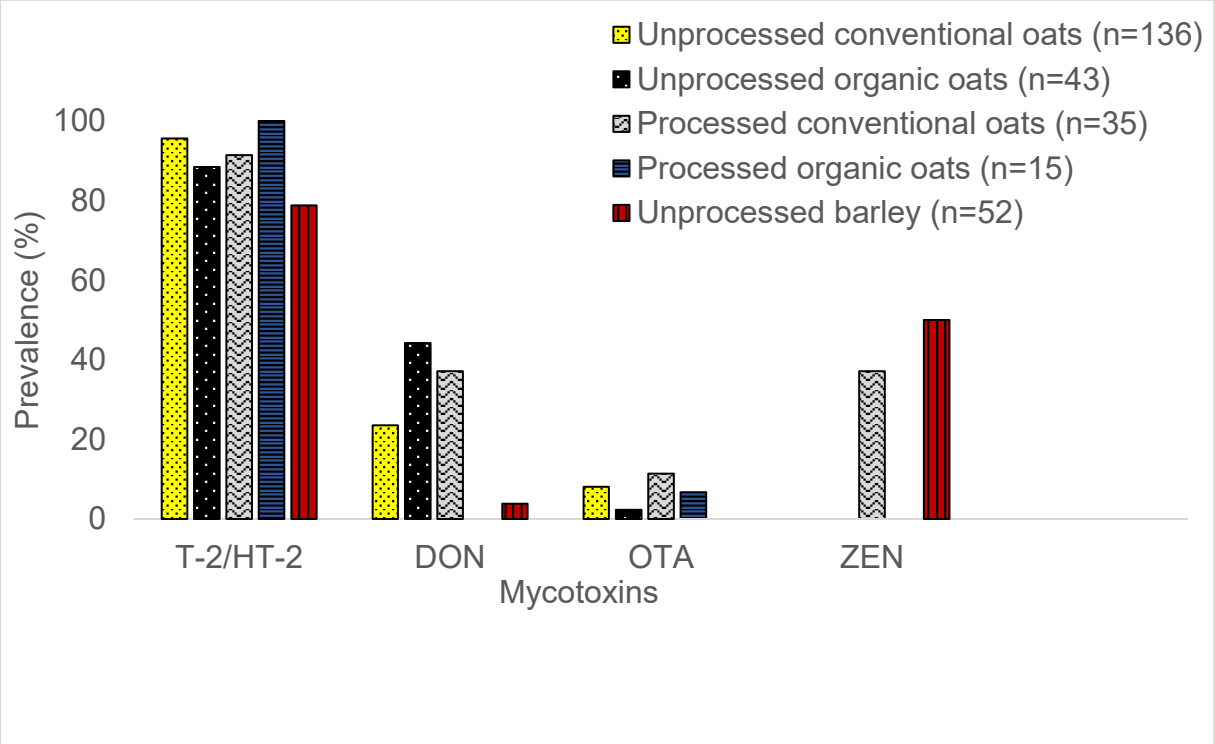


Figure 3. Prevalence of regulated mycotoxins detected in oats and barley survey samples.

The graph demonstrates that T-2 and HT-2 toxins were present in all oat and barley samples, ranging from 79% to 100% prevalence. Conventionally produced unprocessed oats were contaminated with multiple mycotoxins, T-2 and HT-2, deoxynivalenol and OTA, the prevalence of which were 95.6%, 23.5% and 8.1%, respectively. For organically produced unprocessed oats, a similar profile was exhibited, with T-2 and HT-2 occurring in 88.4% of samples, and deoxynivalenol and

OTA contaminating 44.2% and 2.3% of samples, respectively. In conventionally produced processed oats, four mycotoxins were present: T-2 and HT-2 (91.4%), deoxynivalenol (37.1%), OTA (11.4%) and zearalenone (37.1%). All (100%) processed organic oats were contaminated with T-2 and HT-2 toxins and 6.7% contained levels of OTA. No deoxynivalenol or zearalenone were detected in these samples. In unprocessed barley, T-2 and HT-2 toxins were present in (78.8%) of samples, zearalenone in 50% of samples and deoxynivalenol was detected in 3.8% of samples.

Unprocessed oats

Results of unprocessed oats (Table 12) highlighted that, of the 179 samples tested, contamination with T-2 and HT-2 was observed in 169 (94.4%) of samples; of these, 29 (16.2%) contained T-2 and HT-2 at concentrations above the current EU indicative limits of 1000 µg/kg. It's worth considering the ongoing discussions regarding the potential change of the regulatory limits for the sum of T-2 and HT-2 in unprocessed oats being reduced from 1000 µg/kg to 500 µg/kg; if this were to happen, then 61 of the samples tested (34.1%) would exceed these limits. Although deoxynivalenol and zearalenone were detected in 52 (29.1%) and 1 (0.6%) samples respectively, contamination levels did not exceed the regulatory limits, and in fact fell well below these limits. Low prevalence of OTA was observed, with only 13 (7.3%) samples contaminated; however, 5 (2.8%) of these samples exceeded the regulatory limits of 5 µg/kg. Co-occurrence of T-2 and HT-2, and deoxynivalenol, occurred in 50 samples (28.6%), T-2 and HT-2, and OTA, in 10 samples (5.7%), and T-2 and HT-2, and zearalenone, in 1 (0.6%) sample. Only one sample (0.6%) contained all five mycotoxins.

Table 12. Results of unprocessed oat analysis highlighting samples exceeding the indicative (regulatory) limits of 1000 µg/kg for T-2 and HT-2, 1750 µg/kg for deoxynivalenol, 5 µg/kg for OTA and 100 µg/kg for zearalenone.

Samples (n = 179)	**T-2 and HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
No. of samples	169	52	13	1
Prevalence (%)	94.4%	29.1%	7.3%	0.6%
No. of samples	29	0	5	0
Exceed regulations (%)	16.2%	0%	2.8%	0%
Median (µg/kg)	314.6	4.7	3.1	/
Range (µg/kg)	0.4 - 3993.3	0.4 - 504.1	1.4 - 125.9	4.7

The results for unprocessed oats were further analysed according to the farming system used, i.e., conventional or organic production, to ascertain prevalence of these *Fusarium* toxins, in particular T-2 and HT-2. Figure 4 illustrates the results. Of the 136 conventionally produced oat samples analysed, T-2 and HT-2 were detected in 130 samples (96%) with 23 (16.9%) exceeding the EU indicative limits. Of the 43 organically produced oats tested, 39 samples (90.7%) were contaminated with T-2 and HT-2, with 6 (14%) exceeding the limits. If the regulatory limits were to be changed, 50 samples (36.8%) and 11 samples (25.6%) would be non-compliant for unprocessed conventional and organic oats, respectively (Figure 4).

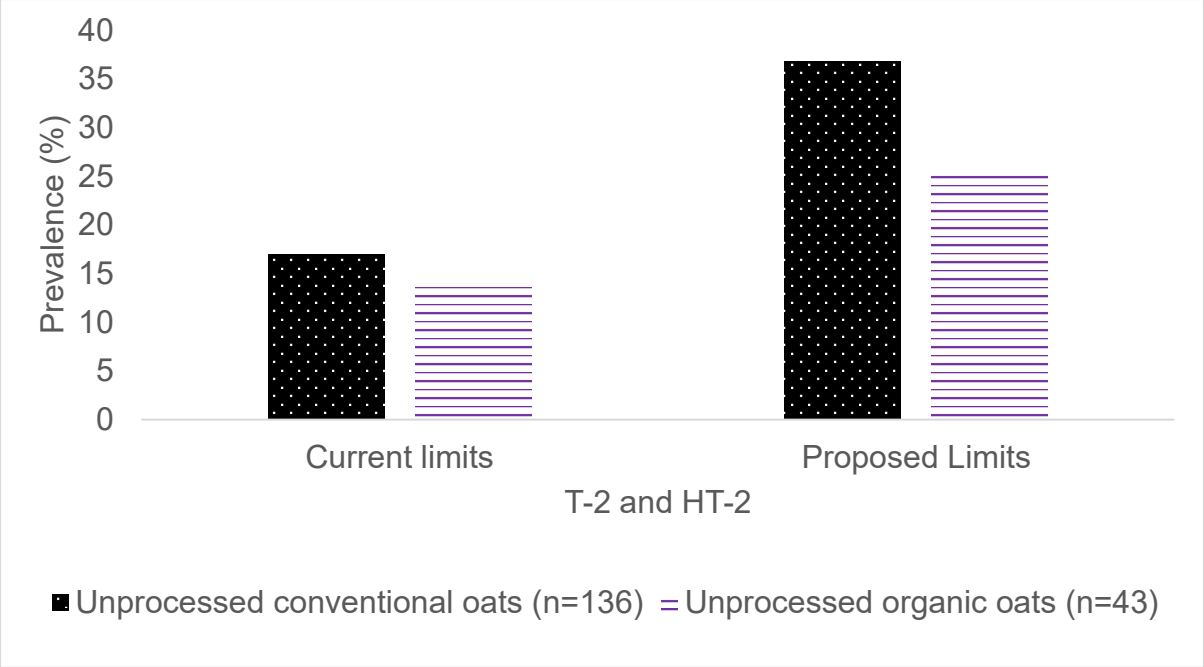


Figure 4. Prevalence of non-compliances in terms of current and proposed regulatory limits.

The black columns with the white dots represent the prevalence of conventionally produced unprocessed oat samples and the purple-lined columns represent the prevalence of organically produced unprocessed oat samples.

This chart clearly shows the increased percentage of non-compliances/violations should a reduction in the limits permitted be enforced.

Figures 5 and 6 show the concentration distribution of T-2 and HT-2 toxins in unprocessed conventionally produced and organic oats. Limit lines highlight those that exceed the current and proposed regulatory limits for the sum of T-2 and HT-2 toxins.

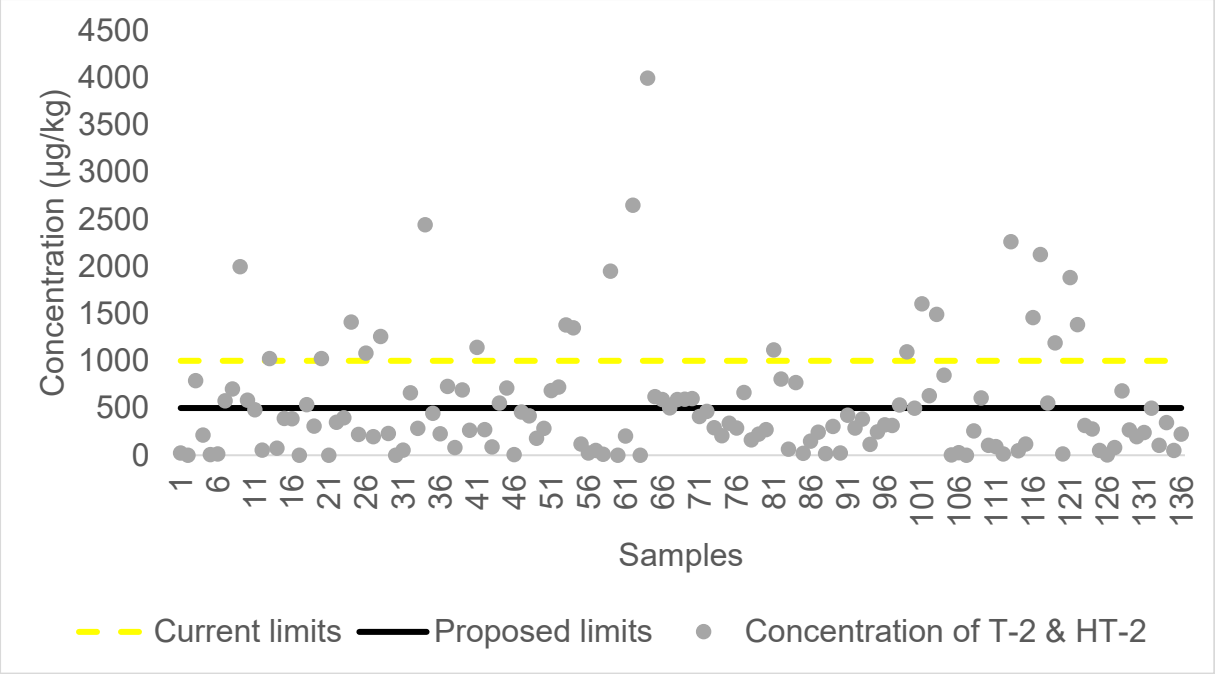


Figure 5. Conventionally produced unprocessed oats survey highlighting those exceeding current indicative limits and proposed new regulatory limits.

The solid black line represents the proposed regulatory limits and the dashed yellow line signifies the current EU indicative limits. The grey dots denote the concentrations of the sum of T-2 and HT-2 detected in individual oat samples (n = 136).

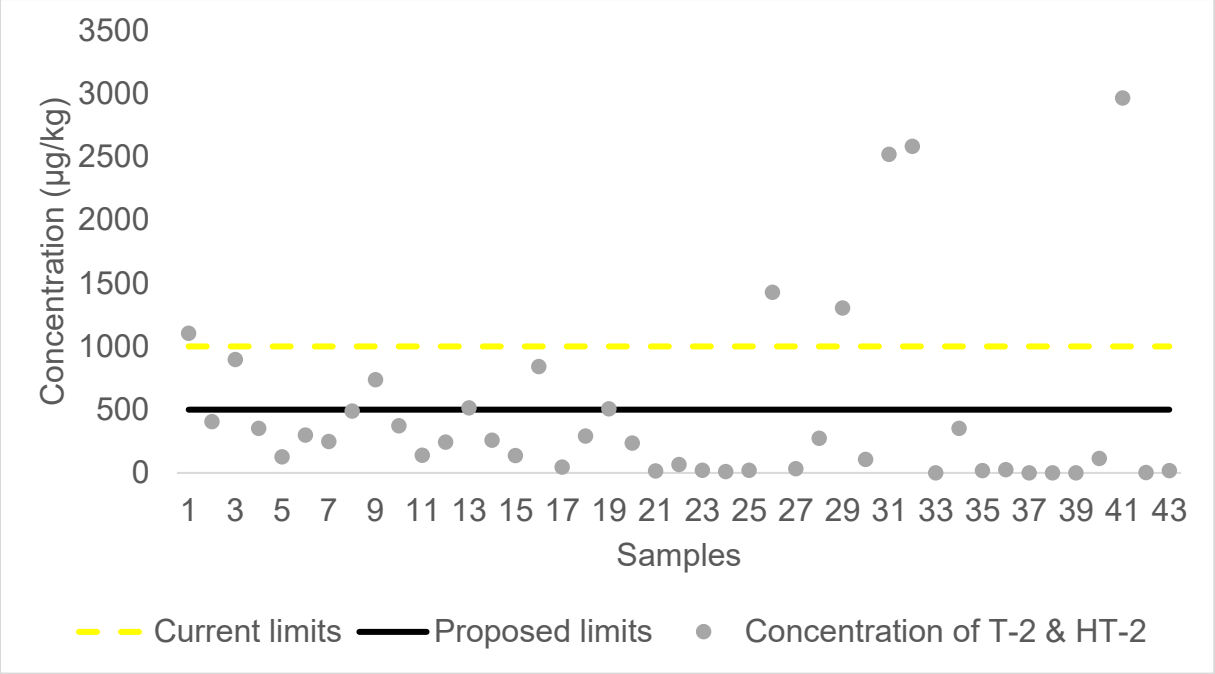


Figure 6. Organically produced unprocessed oats survey highlighting those exceeding current indicative limits and proposed new regulatory limits.

The solid black line represents the proposed regulatory limits and the dashed yellow line signifies the current EU indicative limits. The grey dots denote the concentrations of the sum of T-2 and HT-2 detected in individual oat samples (n=43).

Deoxynivalenol and OTA were also present in conventionally produced unprocessed oats in 32 samples (23.5%) and 11 samples (8.1%), respectively. Regulatory limits for OTA (5 µg/kg) were exceeded in five samples (3.7%). For organically produced oats, the presence of deoxynivalenol, OTA and zearalenone were detected in 20 samples (44%), two samples (4.7%) and one sample (2.3%), respectively. Regulatory limits for these mycotoxins were not exceeded.

Processed cereals

Results of processed oats (Table 13) highlights that, of the 50 samples tested, T-2 and HT-2, deoxynivalenol, OTA and zearalenone were detected in 94%, 26%, 10%, 26% and 98% of samples, respectively. All mycotoxin concentrations fell well below

regulatory limits except for three samples (6%) that contained OTA above the regulatory limits (3 µg/kg) for processed oats.

Table 13. Results of processed oats analysis highlighting samples exceeding the indicative (regulatory) limits of 1000 µg/kg for T-2 and HT-2, 1750 µg/kg for deoxynivalenol, 5 µg/kg for OTA and 100 µg/kg for zearalenone.

Samples (n = 50)	**T-2 and HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
No. of samples	47	13	5	13
Prevalence (%)	94%	26%	10%	26%
No. of samples	0	0	3	0
Exceed regulations (%)	0%	0%	6%	0%
Median (µg/kg)	7.8	47.4	4.4	0.9
Range (µg/kg)	0.2-49.4	15.4-273.2	0.6-52.4	0.5-3.3

As with the unprocessed samples, the data were examined in terms of the farming systems used. Of the 35 conventionally produced samples, 32 (91.4%), 13 (37.1%), four (11.4%), 13 (37.1%) and 34 (97.1%) were contaminated with T-2 and HT-2, deoxynivalenol, OTA, zearalenone and fumonisins, respectively. Three samples (8.6%) were found to exceed the regulatory limits of 3 µg/kg for OTA. No other regulatory limits were violated. In the organic processed oats, 100% of samples (15) were contaminated at low levels with T-2 and HT-2 and fumonisin mycotoxins. Additionally, OTA was detected in one sample (6.7%); again, this fell below the legislative limit. There was no prevalence of deoxynivalenol or zearalenone in these processed oat samples. Figures 7 and 8 illustrate the concentration distribution of T-2 and HT-2 in processed conventionally produced and organic oats. Limit lines

highlight that no samples exceed the current and proposed regulatory limits for the sum of T-2 and HT-2 toxins.

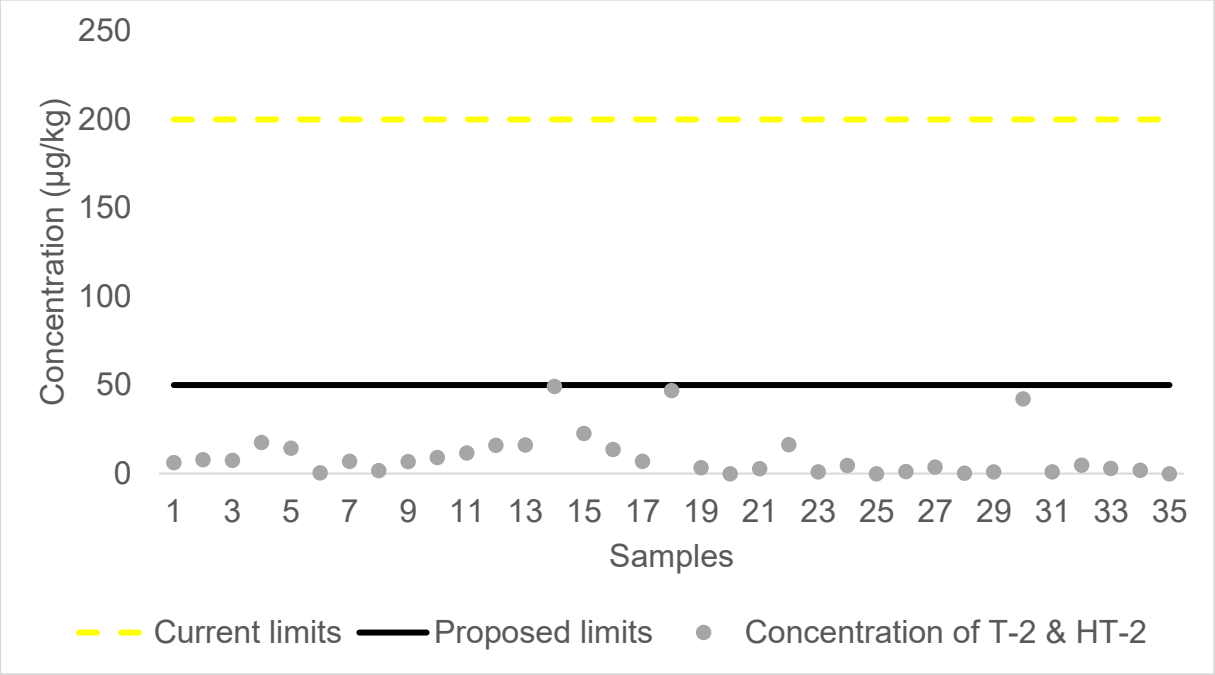


Figure 7. Conventionally produced processed oats survey highlighting no violations of current indicative limits or proposed new regulatory limits.

The solid black line represents the proposed regulatory limits, and the dashed yellow line signifies the current EU indicative limits. The grey dots denote the concentrations of the sum of T-2 and HT-2 detected in the oats (n = 35).

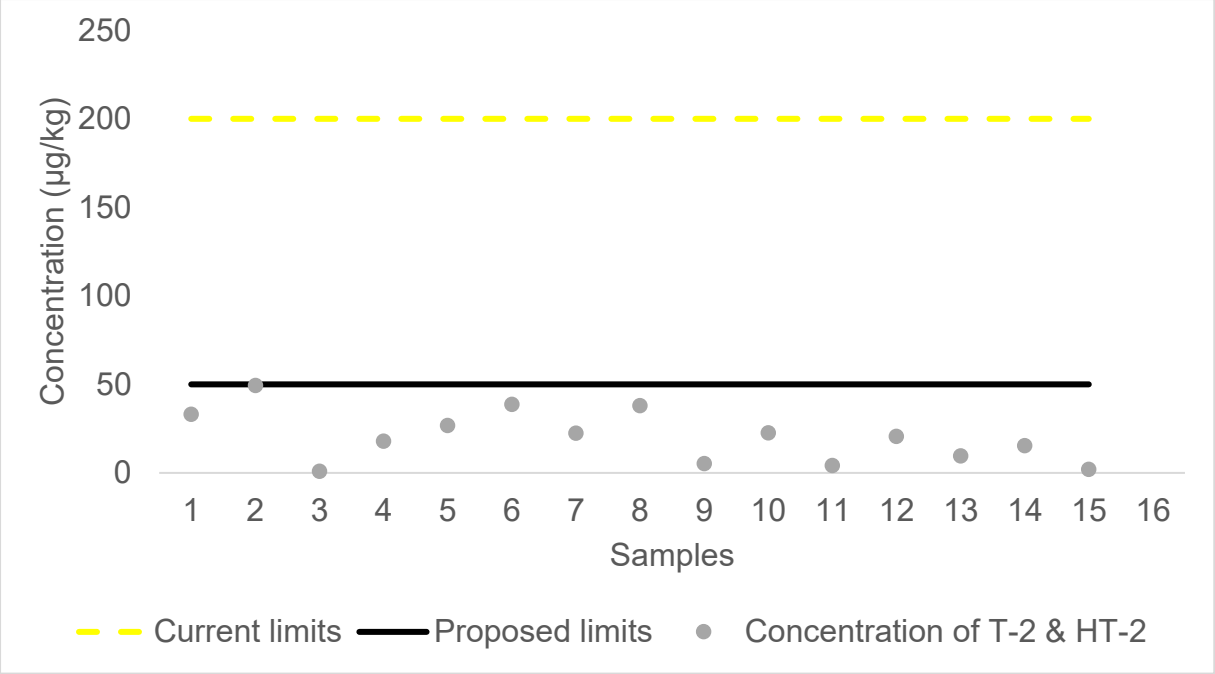


Figure 8. Organically produced processed oats survey highlighting no violations of current indicative limits or proposed new regulatory limits.

The solid black line represents the proposed regulatory limits, and the dashed yellow line signifies the current EU indicative limits. The grey dots denote the concentrations of the sum of T-2 and HT-2 detected in the oats (n = 15).

Conclusions

The aim of this study was to conduct a survey of oats and barley sourced from Ireland and the UK to identify contamination levels of the regulated mycotoxins using mass spectrometry. The observed results show that contamination with *Fusarium* mycotoxins, in particular T-2 and HT-2, are an ongoing issue for oat producers.

In terms of unprocessed oats, T-2 and HT-2 were detected in 94.4% of samples tested, with 16.6% of these violating current EU indicative limits set for the sum of these compounds. In addition, the unprocessed samples were also analysed for other regulated mycotoxins. While deoxynivalenol and zearalenone concentrations all fell well below the legislative limits, OTA exceeded these in 2.8% of samples. Co-occurrence of T-2 and HT-2 and deoxynivalenol, T-2 and HT-2 and OTA, and T-2

and HT-2 and zearalenone were observed, and one sample contained all four mycotoxins. In organic unprocessed oats, the prevalence of the sum of T-2 and HT-2 was 90.7% compared with 96% for conventional unprocessed oats. This agrees with other surveys performed. Should the limits be reduced following the ongoing discussions with policy makers to 500 µg/kg (from 1000 µg/kg), the prevalence of non-compliance would increase, creating challenges for the sector.

In relation to processed oats, while the prevalence of T-2 and HT-2 was high (94%), no regulatory violations were observed, thus supporting the fact that industrial processes, for example de-hulling, are effective in reducing the contamination levels of these toxins in oats. As with the unprocessed oats, other regulated mycotoxins such as deoxynivalenol, zearalenone and fumonisins were detected, albeit at concentrations well below the legislative limits. Again, non-compliance was found with respect to OTA, where 6% of samples exceeded the limit of 3 µg/kg.

The fact that OTA has been observed in a small number of samples and in some cases has exceeded the regulatory limits for both unprocessed and processed oats, suggests that there has been a problem with the storage of these, i.e., the oats may not have been dried to the specified moisture content of < 15% as outlined in the Commission Recommendation 2006/583/EC (EU,2006d). This was not investigated as part of this research and needs to be monitored and investigated by the industry itself.

No violations were observed in barley for any of the mycotoxins tested, although T-2 and HT-2, deoxynivalenol and zearalenone were detected. It is difficult to draw any conclusions from these analyses, however, due to the small sample size (52).

The results emphasise the continued need for effective surveillance and control of such mycotoxins within the industry through testing and robust HACCP, the principles of which are outlined in Commission Recommendation 2006/583/EC (EU, 2006d).

Analysis of oat survey data and sample metadata to identify trends and major weaknesses in the supply chain

Introduction

Oat crops sown in spring and harvested in August are called spring oats, while crops sown in October and harvested in summer are called winter oats. Oat cultivation starts with land ploughing, the selection of varieties and other agronomic practices, including liming and fertilisation. At full maturity stage, oats are harvested, cleaned, dried, sorted (based on size) and stored in silos, on-farm or at central stores, before being transported for milling. Once the crop reaches the mill, each batch of oats is tested to ensure they satisfy the required specification in terms of quality and safety. The crops go through a further cleaning process to remove foreign particles before the de-hulling and milling processes. In terms of the milling process, oats are passed through a complex arrangement of rollers, grinders and sieves to produce different end products, including flakes and flour. Oat flour is used by bakeries to make bread, biscuits and cakes. Oat flakes can be used for porridge, granola and cereal bars (Clemens & Klinken, 2014).

Oat crops are highly susceptible to *Fusarium* infection both before and after harvest. This drastically reduces oat crop yield and quality (Martinelli et al., 2014; Hautsalo et al., 2020). The most important *Fusarium* species in oats include *F. graminearum*, *F. poae*, *F. langsethiae*, *F. avenaceum*, *F. sporotrichioides*, and *F. culmorum* (Hautsalo et al., 2020). Aside from losses in yield and quality due to *Fusarium* infection, the accumulation of toxic *Fusarium* secondary metabolites, including deoxynivalenol, zearalenone, T-2 and HT-2 toxins, is also a major concern to farmers (Kolawole et al., 2021a). These toxic secondary metabolites (known as mycotoxins) are hazardous to human and animal health (Ostry et al., 2016; Kolawole et al., 2020). A wide range of adverse health effects, including anorexia, immunotoxicity, reduced body weight, hepatotoxicity and infertility, have been linked to the consumption of food and feed contaminated with zearalenone, deoxynivalenol, T-2 and HT-2 toxins (Kolawole et al., 2020; Sobrova et al., 2010; Knutsen et al., 2017).

To safeguard human and animal health, the European Commission (EC) established maximum limits for deoxynivalenol and zearalenone in cereals and cereal products,

while an indicative limit was set for the sum of the T-2 and HT-2 toxins in cereals intended for human consumption (Commission Regulation (EC) No. 1881/2006) (Tables 3 & 14) (EC 2006b; EU (2013); EFSA, 2014). Moreover, following an evaluation of the occurrence and toxicity associated with chronic dietary exposure to deoxynivalenol, zearalenone and the sum of T-2 and HT-2, the European Food Safety Authority (EFSA) established tolerable daily intakes (TDI) of 1, 0.25, and 0.02 µg/kg body weight (bw) per day, respectively (Knutsen et al., 2017; EFSA 2014; EFSA, 2016).

Table 14. Maximum EU limits for deoxynivalenol and zearalenone in unprocessed cereals and finished products intended for human consumption.

Mycotoxin	**Food**	**Regulatory Limit (µg/kg)**
Deoxynivalenol	Unprocessed oats	1750
	Oat flour, meal, bran, or germ	750
	Bread, pastries, biscuits, cereal snacks and breakfast cereals	500
Zearalenone	Unprocessed oats	100
	Oat flour, meal, bran, or germ	75
	Bread, pastries, biscuits, cereal snacks and breakfast cereals	50

This report presents the results of a survey of mycotoxin contamination of oats produced on the island of Ireland from 2020 to 2021. Important and specific stages in the supply chain of oats, with the potential to promote mycotoxin production or critical to the management of oat mycotoxin contamination, are also highlighted.

Materials and Methods

Chemicals and materials

These have been previously described in this chapter.

Sample collection

In total, 310 oat samples were collected from farmers and oat processors across the island of Ireland between July 2021 and September 2022. These samples were analysed for the occurrence of deoxynivalenol, zearalenone, T-2 and HT-2 using state-of-the-art mass spectrometric instruments and techniques. Furthermore, agronomic data including crop varieties, fungicide application, storage conditions and duration, farming practice, processing techniques and mycotoxin testing method were collected. Prior to analysis, all samples were milled using a CGoldenwall multifunction grinder to a fine powder (particle size < 50 µm) and stored at -20°C to prevent further contamination.

Sample extraction and LC-MS/MS parameters

These have been described previously in this chapter.

Statistical analyses

The software R 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria), together with the ggpubr (v0.4.0), faraway (v1.0.7), chisq. posthoc.test (v0.1.2), and MASS packages were used for statistical analyses. All continuous variables were tested for normality of distribution by the Shapiro-Wilk test, and the null hypotheses were all rejected ($p < 0.05$).

Frequency distributions (i.e., prevalence) were compared between groups by Pearson's Chi-squared test. Logistic regression was used to evaluate the effect of a continuous variable on a dependent categorical variable, Spearman's rank correlation was used to compare pairs of continuous variables, and the Kruskal–

Wallis rank-sum test was used to compare categories of a continuous variable. Where significant differences were identified in a group of categorical variables, post-hoc pairwise comparisons were performed by Dunn's rank-sum test using the Benjamini–Hochberg procedure for correcting family-wise error rate. Null hypotheses were rejected, and association considered significant at (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$. Finally, where pairwise significant differences were identified, post-hoc power calculations were then also performed to ensure the reliability of the observation. Multiple logistic regression was used to model the occurrence of each mycotoxin, and multiple linear regression was used to model mycotoxin levels amongst positive detections. Multivariate models were evaluated by Akaike Information Criterion to optimize selection of independent variables.

Results and Discussion

The developed and validated LC-MS/MS method met the criteria defined in the SANTE/12089/2016 and EC/456/2002 documents. A total of 64 (32%) samples did not contain detectable levels of deoxynivalenol, zearalenone, T-2 and HT-2 toxins. Amongst the positive detections, type A trichothecenes (~ 90%) were the most frequent contaminants, with T-2 and HT-2 each observed in 301 (88%) and 297 (87%) samples, respectively, and co-occurring in 109 (54%) samples. Zearalenone and deoxynivalenol were detected in 20 (10%) and 13 (6%) samples, respectively, at a mean concentration of 358 µg/kg and 39.5 µg/kg, respectively.

T-2 and HT-2 were found to be co-occurring with deoxynivalenol and zearalenone in 69% and 55% of samples, respectively. All the four mycotoxins (deoxynivalenol, zearalenone, T-2 and HT-2) were found to co-occur in only two (1%) of the samples analysed. Only one mycotoxin was detectable in 26 (13%) samples, with half of those being HT-2. The most common pattern of occurrence was contamination with both type A trichothecenes only, which accounted for 67% of detected positive samples and 46% of all analysed.

The concentration of T-2 and HT-2 ranged between 5.1 and 3064.2 µg/kg, with up to 20 samples (0.06%) contaminated with levels of T-2 and HT-2 above the EU guidance limits for these toxins in oats (Table 15). Furthermore, a strong positive

correlation (R^2 = 0.78) was observed between the levels of T-2 and HT-2 detected in the samples. This positive relationship is not surprising as both mycotoxins are type A trichothecenes produced by the same fungal species through similar metabolic pathways (Kolawole et al., 2021b). No significant positive relationships were observed between the concentrations of T-2 and HT-2 and deoxynivalenol or zearalenone. The relationships appear to be mutually exclusive, i.e., when T-2 and HT-2 was present at high concentration, the concentration of deoxynivalenol or zearalenone was low. This could be because deoxynivalenol and zearalenone are produced by different *Fusarium* species with different environmental requirements, or because these fungal species actively compete against one another within the same environmental niche. A summary of *Fusarium* mycotoxins and concentrations found in 310 oat samples analysed using LC-MS/MS is shown in Table 15.

Table 15. Basic descriptive statistics for detections of mycotoxins in oat samples, including samples above the European Commission's regulatory limits in unprocessed oats: 1750 µg/kg deoxynivalenol; 100 µg/kg zearalenone; 1000 µg/kg for sum of T-2 & HT-2 toxins, applied here to T-2 & HT-2 individually as well.

	Deoxynivalenol	**Zearalenone**	**T-2**	**HT-2**	**T-2 + HT-2**
No. of positive samples (%)	38 (11%)	73 (21.2%)	297 (86.6%)	301 (87.8%)	-
Average (µg/kg)	359.8	39.5	133.2	209.2	318.3
Min – Max (µg/kg)	10.1 – 1985.3	10.3 – 287.4	5.1 – 1165.6	5.0 – 2190.3	5.1 – 3064.2
No of samples above Reg.	2	5	2	9	20
Correlation (T-2 and HT-2)	-	-		**0.7834**	

Mycotoxin hotspots along the oat supply chain

The farm metadata were used to identify specific mycotoxin hotspots along the oat supply chain. The fungicide application stage was found to be important in determining the scale of oat mycotoxin contamination pre-harvest, while the type and condition of storage post-harvest is a vital pinch point.

Oat crops sprayed with fungicide had significantly higher concentrations of T-2 and HT-2 toxins when compared to oats without fungicide application ($p < 0.001$) (Figure 9). Generally, fungicides in the class of triazole, strobilurin and azole are commonly used in the EU and UK to reduce or control *Fusarium* head blight in wheat and panicle blight in oats (Kolawole et al, 2021). Whilst these fungicides are not specifically registered for reducing mycotoxins, they are effective in controlling fungal disease outbreak, which may indirectly reduce mycotoxin production. Although the mechanism behind elevated mycotoxins following fungicide application is not fully understood, some *in vitro* studies have shown that the application of fungicides at certain climatic conditions and sub-lethal doses can cause mycotoxigenic fungal species to increase mycotoxin biosynthesis (Kolawole et al, 2021b).

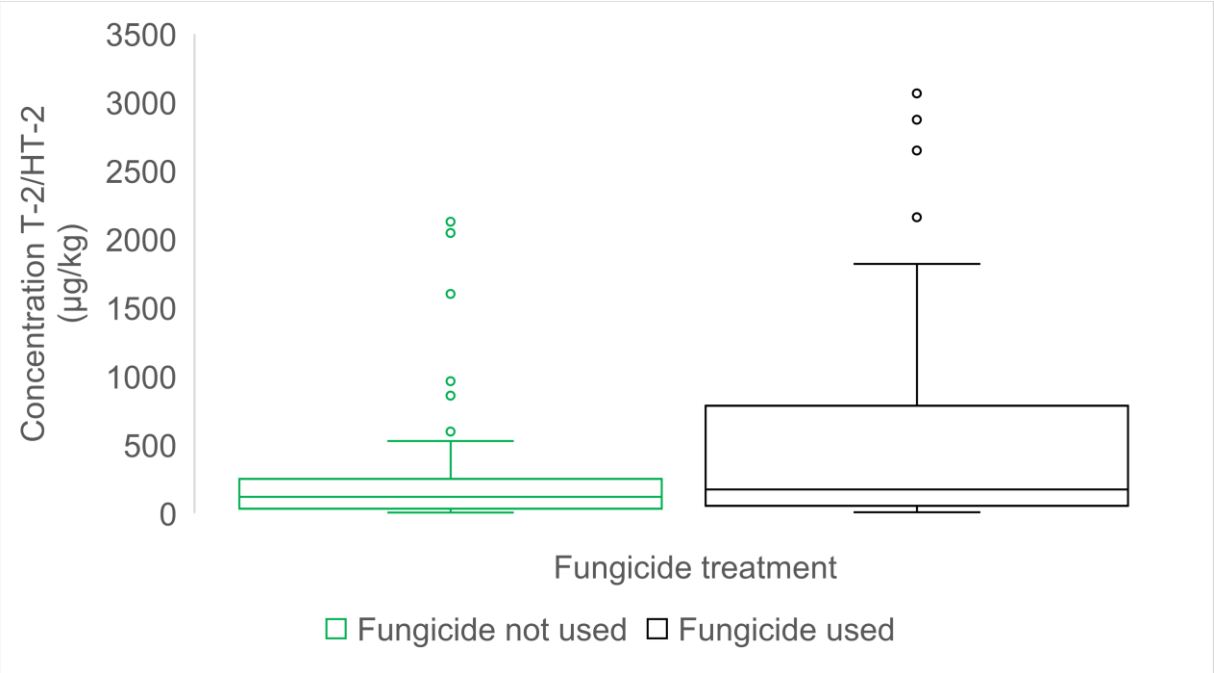


Figure 9. Box plot of T-2 and HT-2 levels in oat crops sprayed with or without fungicide.

The box plot describes the distribution of the data comparing oat sprayed with fungicide and those that were not. On the left (in green), the results depict oats with no fungicide application, and on the right (black) the results highlight oats sprayed with fungicide. As is shown, oats treated with fungicide contained higher concentrations of T-2 and HT-2 toxins. The bottom and top of each box represent the 25th and 75th percentile (the lower and upper quartiles, respectively), while the line inside each box represents the 50th percentile (the median). The ends of the whiskers represent 5th and 95th percentile.

Oat samples stored in silos had a significantly lower T-2 and HT-2 content compared to oats stored on-farm, as shown in Figure 10. The prevalence of type A trichothecenes was significantly lower ($p = 0.024$) by approximately 15% in oat crops stored in silos compared to crops stored in farm stores.

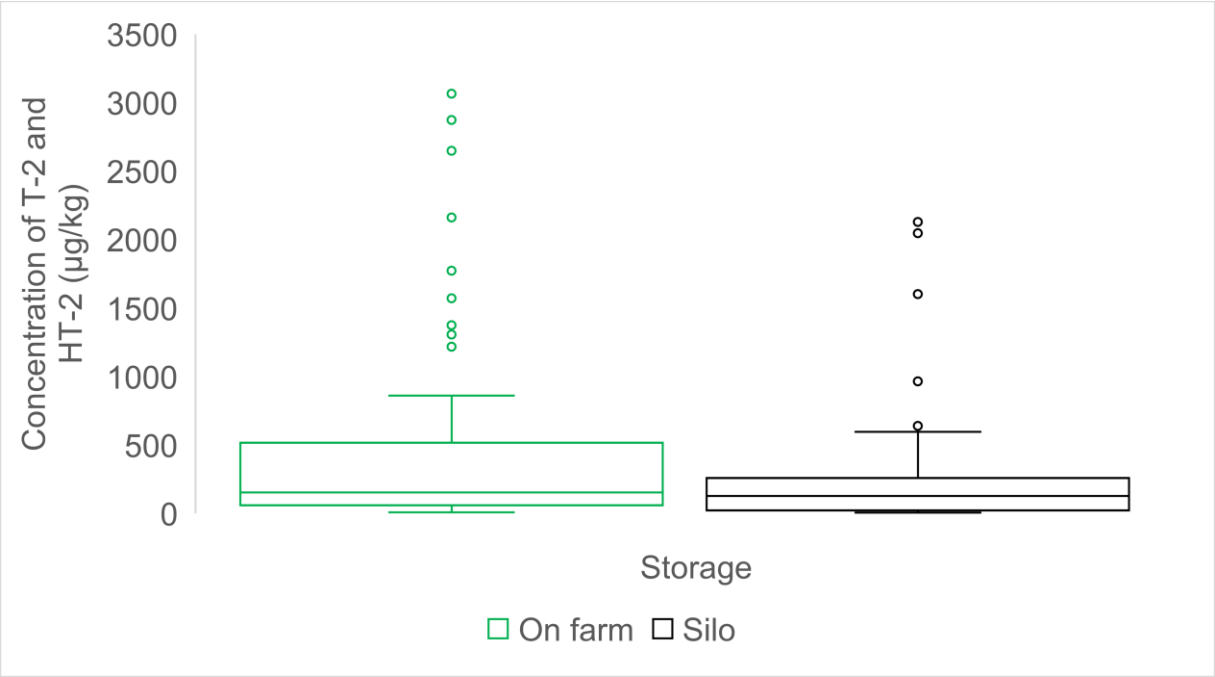


Figure 10. Box plot of T-2 and HT-2 levels in oat crops stored in silos or farm store.

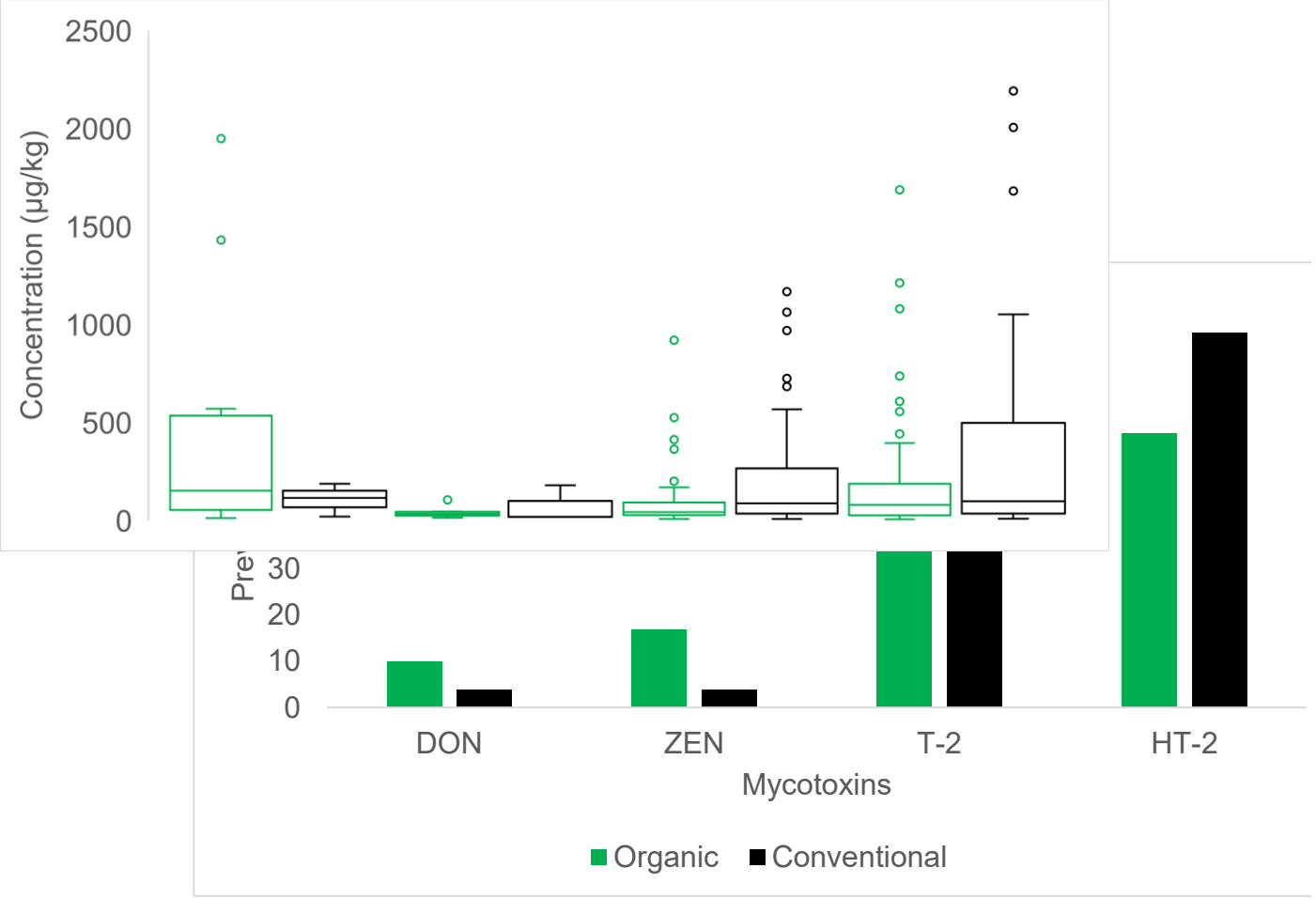
The box plot describes the distribution of the data comparing oats stored in farm stores and silos. On the left (in green) the results depict oats stores in farm stores and on the right (in black) the results highlight oats stored in silos. As is shown, oats

stored in silos were contaminated with lower concentrations of T-2 and HT-2 toxins. The bottom and top of each box represent the 25th and 75th percentile (the lower and upper quartiles, respectively), while the line inside each box represents the 50th percentile (the median). The ends of the whiskers represent 5th and 95th percentile.

Farming systems

Contamination with type A trichothecenes was significantly more prevalent ($p < 0.001$) in conventional crops (76%) compared to organic (53%). T-2 and HT-2 were each detected in 77% and 80% of conventional crops, respectively, but only 50% and 59% of organic crops, respectively. Deoxynivalenol and zearalenone were found in a contrasting pattern, contaminating 10% and 17% of organic crops, respectively, but only 4% of conventional crops contained detectable levels of either mycotoxin (Figure 11, Top). The black columns illustrate mycotoxin contamination in conventionally produced oats and the green columns depict mycotoxin contamination in organically produced oats.

The levels at which these mycotoxins were detected were not found to be significantly different between farming systems, except for T-2 only. Both the mean and median concentrations of T-2 in conventionally grown crops were more than double those found in organic crops, and the overall distributions were found to be significantly different ($p = 0.028$). Though the mean HT-2 concentration was also 75% higher in conventional crops, the difference in distributions was not statistically significant, nor was the difference between farming systems for combined type A trichothecenes (Figure 11, Bottom).



DON ZEN T-2 HT-2

Figure 11. Prevalence of each mycotoxin (top) and the distribution of detected levels (bottom), by type of farming system (organic is shown in green and conventional in black).

Conclusions

Mycotoxin contamination of cereals, exacerbated by the changing climate and agronomic practices, is a cause of increasing concern. This report presents the results of the survey of mycotoxin contamination of oats grains produced on the island of Ireland from 2021 to 2022. The results show that contamination with *Fusarium* mycotoxins, particularly T-2 and HT-2 toxins, is a concern for oat producers. T-2 and HT-2 toxins were detected in more than 90% of the 310 oat samples analysed. Zearalenone and deoxynivalenol were detected in 20 (10%) and 13 (6%) samples, respectively, at a mean concentration of 358 µg/kg and 39.5 µg/kg, respectively. The concentration of T-2 and HT-2 ranged between 5.1 and 3064.2 µg/kg, with up to 20 samples contaminated with levels of T-2 and HT-2 above the EU guidance limits for these toxins in oats. Furthermore, a strong positive correlation ($R^2 = 0.78$) was observed between the levels of T-2 and HT-2 detected in the samples. Contamination with type A trichothecenes was significantly more prevalent ($p < .001$) in conventional crops (76%) compared to organic (53%). T-2 and HT-2 were each detected in 77% and 80% of conventional crops, but only 50% and 59% of organic crops, respectively. Deoxynivalenol and zearalenone were found in a contrasting pattern, contaminating 10% and 17% of organic crops, respectively, but only 4% of conventional crops contained detectable levels of either mycotoxin.

Fungicide application and storage of oats in farm stores led to elevated mycotoxin contamination. Thus, the application of fungicide before harvest and storage conditions post-harvest were found to be important critical control points for oat mycotoxin management.

Objective 5

Assessment of the performance characteristics of selected rapid diagnostic kits.

Introduction

There are many commercial diagnostic kits available for the regulated mycotoxins, the majority of which are immunoassays. In a preliminary comparative study between liquid chromatography tandem mass spectrometry (LC-MS/MS) and a diagnostic kit for the detection of the sum of T-2 and HT-2 in oats, a poor correlation was observed between the two analytical approaches. The kit results highlighted consistent underestimation of the contamination levels when compared with confirmatory LC-MS/MS.

The aim of this study was to select a number of commercially available rapid diagnostic kits (based on claimed manufacturers' performance) for an in-depth evaluation of their actual performance, and therefore their fitness for purpose for analysing the sum of T-2 and HT-2 in oat grains. Following preliminary assessment (Objective 2), three lateral flow devices (LFDs) were selected for on-site testing, in addition to an Enzyme-Linked Immunosorbent Assays (ELISA), a laboratory-based method for evaluation and comparison against the state-of-the-art technology, LC-MS/MS. The commercially available kits for the measurement of the toxins have been detailed in Table 5. Only those that could measure both T-2 and HT-2 were selected in the first instance. Secondly, the kits that used water-based extractions were prioritised over those using solvent-based extraction methods. The LFD kits meeting these criteria included:

- Envirologix Incorporated; QuickTox Kit for QuickScan T-2/HT-2 Flex
- Neogen Corporation; Reveal® Q+ MAX for T-2/HT-2
- R-Biopharm AG; RIDA®QUICK T-2/HT-2 RQS ECO
- PerkinElmer Incorporated; AuroFlow™ AQ T-2/HT-2 Strip Test
- Vicam LP; T-2/HT-2-V AQUA

Only one ELISA kit met the criteria:

- R-Biopharm AG; RIDASCREEN® T-2 and HT-2 Toxin

Other important considerations included whether the kits were validated for oats, the ability to rent a reader to perform the analyses, and the availability and delivery of kits/readers within the time-span of the project. Subsequently, three LFD kits were selected in addition to an ELISA kit. Two LFDs were not validated for oats; however, they were included in the comparative study since they met the selection criteria outlined previously. Moreover, all test kits used aqueous extraction methods, a reader (if required) was offered (rental) to perform the analyses, and the delivery of kits/readers were available for delivery within the timespan of the project. From the survey of oats and barley to identify contamination levels of T-2 and HT-2 using mass spectrometry, 100 oat samples below, at and above the EU guidance limits were selected for testing using these commercial rapid immunological test kits. The outcome of the evaluation was to identify where the problems lie (if any). Are end-users not adequately trained to perform the tests or do they require training? Is it poor kit performance? Or are sampling procedures not being performed correctly and accounting for huge discrepancies in results? The assessment will provide invaluable insight for stakeholders.

Materials and Methods

Chemicals and materials

Analytical grade methanol was purchased from Sigma-Aldrich (Gillingham, UK). Ultra-pure water (18.2 MΩ-cm) was produced in-house using a Millipore water purification system (Millipore, Cork, Ireland). Test kits were purchased from Neogen Europe Limited (Ayr, UK), SGR Scientific Limited (Swords, Ireland), Bio-Check UK (St. Asaph, UK), and Fannin Ltd (Dublin, Ireland).

Sample collection and preparation

Industry stakeholders (the Project Advisory Group) provided samples for analysis. Where possible, a 1kg aggregate sample was supplied, as detailed under current European Union legislation (Commission Regulation (EC) No 401/2006). If this was

not feasible, a representative sample following the EU guidelines was provided for analysis. Prior to analysis, all samples were milled to a fine powder (particle size <50 µm) using a CGoldenwall multifunction grinder and stored at -20°C to preserve integrity. The number of samples selected and analysed was 100. Only oat samples were tested, as no T-2 or HT-2 toxins were detected in barley.

Test procedures

Sample extractions and analyses were performed according to the kit manufacturers' instructions, as outlined below.

Test Kit 1

Sample Extraction

1. Weigh out 10g ± 0.1g of sample into extraction cup.
2. Add contents of one extraction packet to the extraction cup.
3. Add 50 ml distilled or deionized water to the extraction cup.
4. Vigorously shake, using hand or mechanical means, for three minutes, or blend for one minute.
5. Allow the sample to settle, filter with a filter syringe or Whatman #4 filter paper to collect a minimum of 3 ml filtrate into a sample collection tube. Alternatively, pipette 1 ml of sample into a 2.0 ml micro-centrifuge tube, and centrifuge for 30 seconds using a microcentrifuge (approx. 2,000 x g).

Test Procedure

1. Place the appropriate number of sample dilution cups into a sample cup rack. Label cups.
2. Add 100 µl of sample extract to each sample dilution cup.
3. Add 1,500 µl of sample diluent to the sample dilution cup. Mix by pipetting up and down five times.
4. Fully insert a test strip into a cartridge.

5. Insert the cartridge containing the test strip into any of the three ports within the reader.
6. The bar code on the test strip will be read. The system identifies the type of test strip and the lot number. If the lot number is not found in the system, the bar code reader in the front of the reader will turn on automatically.
7. Scan the QR code found on the tube containing the test strips. The information will be stored on the reader.
8. Enter Sample ID if desired.
9. Add 400 µl of sample extract from the sample dilution cup to the cartridge.
10. The system will start automatically.
11. Additional samples can be started in the other ports while the first sample is processing.
12. Results will be displayed on the reader screen after the five-minute testing period is complete.

Dilution Procedure

Samples greater than 500 ppb will need to be diluted and re-tested.

1. Add 100 µl of sample filtrate to a sample collection tube.
2. Add 400 µl distilled or deionized water to the sample collection tube. Mix well by pipetting up and down five times or cap the tube and shake.
3. Add 100 µl of diluted sample extract (from step 2) to each sample dilution cup.
4. Add 1,500 µl of sample diluent to the dilution cup with sample extract. Mix by pipetting up and down five times.
5. Insert the cartridge containing the test strip into any of the three ports within the reader.
6. The bar code on the test strip will be read. The system identifies the type of test strip and the lot number. If the lot number is not found in the system, the bar code reader in the front of the reader will turn on automatically.
7. Scan the QR code found on the tube containing the test strips. The information will be stored on the reader.
8. Enter Sample ID if desired.

9. Add 400 µl of sample extract from the sample dilution cup to the reader cartridge.
10. The system will start automatically.
11. Additional samples can be started in the other ports while the first sample is processing.
12. Results will be displayed on the reader screen after the five-minute testing period is complete.

NOTE: The reader will not calculate your dilution. Final result displayed will need to be multiplied by five.

Test Kit 2

Test Procedure

1. Calibrate the reader by scanning in the barcode for the specific commodity and the lot of strips being used.
2. Weigh 5g ± 0.1g ground sample into extraction tube. Add 25 ml extraction buffer to extraction tube.
3. Vortex at maximum speed for two minutes. Filter the extract into a clean tube for no more than five minutes.
4. Transfer 100 µl of filtered extract to the LFD by dropping (~1 drop/second) vertically into the sample well. Allow the strip to develop for five minutes on a flat surface.
5. Insert the LFD into the reader. Press the centre key to take a reading. To print, use the arrow keys to move the cursor to “P” and press the centre button.
6. To run the next sample, use the arrow keys to move the cursor to “NT” and press the centre button on the keypad.

Test Kit 3

Test Procedure

1. Power on and calibrate the reader with supplied Lot ID calibration codes.
2. Combine 10g of ground sample and the contents of one powder pack into an extraction bag.

3. Add 37 ml distilled or deionized water into the bag.
4. Seal the bag, then shake for 1 min 30 sec.
5. Transfer 10 ml of liquid extract into the assembled filter funnel.
6. Allow the sample to filter until approximately 2-3 ml of filtrate has been collected.
7. Add running buffer to a clean capped tube.
8. Add sample extract to the capped tube and shake to prepare the sample mix.
9. Transfer 150 µl of sample mix to the appropriate capless tube and add a new test strip.
10. Insert strip into the cassette and place in reader.
11. The reader will automatically record a result and display the results on the screen.

Test Kit 4

Sample Extraction

The samples should be stored in a cool place, protected from light. A representative sample (according to accepted sampling techniques) should be ground and thoroughly mixed prior to proceeding with the extraction procedure.

For preparation of oat samples, a special extraction buffer is needed. Please use the extraction buffer oats concentrate contained in the kit and dilute it 1:10 (e.g., 10 ml + 90 ml distilled water) to obtain ready-to-use extraction buffer oats. Ready-to-use extraction buffer oats should be stored at 2-8°C and expires after approx. 8-10 weeks.

1. Weigh 5g of ground sample and add 25 ml of ready-to-use extraction buffer oats*.
2. Shake the sample for 10 min (overhead).
3. Centrifuge: 10 min / 3000g / at room temperature (20-25 °C).
4. Dilute the supernatant 1:2 (1+1) with methanol/distilled water (70/30; v/v), e.g., 1 ml supernatant + 1 ml of methanol/distilled water (70/30; v/v).
5. Use 50 µl per well in the assay.

*Sample size may be increased if required, but the volume of ready-to use extraction buffer oats must be adapted accordingly, e.g., 25g in 125 ml ready-to-use extraction

buffer oats or 50g in 250 ml ready-to-use extraction buffer oats. Buffer contained in the kit will then be sufficient for a lower number of oat samples, respectively.

NOTE: At high T-2 and HT-2 toxin concentrations (> 360 ppb) the 1:2 diluted extract solution must be further diluted (e.g., 1:10 (1+9) with methanol (35 %) means 50 µl of the diluted extract solution + 450 µl methanol (35 %)). This results in an additional dilution factor of 10.

Test Procedure

Bring all reagents to room temperature (20-25°C) before use. A Phosphate Buffered Saline-Tween buffer is needed as wash buffer; please use the wash buffer salt contained in the kit. Dissolve the total content of the pouch in one litre of distilled water. The ready-to-use washing buffer expires after approx. 4-6 weeks, stored at 2-8°C.

Alternatively, dissolve the contents of the pouch in 100 ml of distilled water to obtain a 10-fold concentrated wash buffer. This 10-fold concentrate expires after approx. 8-12 weeks, stored at room temperature (20-25 °C). Use one part of this concentrate and dissolve with nine parts of distilled water to obtain the ready-to-use wash buffer.

Carefully follow the recommended washing procedure. Do not allow microwells to dry between working steps.

1. Insert a sufficient number of wells into the microwell holder for all standards and samples to be run in duplicate. Record standard and sample positions.
2. Add 50 µl of standard or prepared sample to separate duplicate wells; use a new pipette tip for each standard or sample.
3. Add 50 µl of conjugate to the bottom of each well.
4. Add 50 µl of antibody to each well. Mix gently by shaking the plate manually and incubate for 30 min at room temperature (20-25 °C).
5. Pour the liquid out of the wells and tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all the wells with 250 µl of wash buffer and pour out the liquid again. Repeat two more times.

6. Add 100 µl of substrate/chromogen to each well. Mix gently by shaking the plate manually and incubate for 15 min at room temperature (20-25 °C) in the dark.
7. Add 100 µl of the stop solution to each well. Mix gently by shaking the plate manually and measure the absorbance at 450 nm. Read within 30 minutes after addition of stop solution.

Results

Calculate the results as follows:

Absorbance standard (or sample)/absorbance zero standard x 100 = % absorbance.

The zero standard is thus made equal to 100 % and the absorbance values are quoted in percentages. The values calculated for the standards are entered in a system of coordinates on semilogarithmic graph paper against the T-2 and HT-2 concentration [µg/l].

In order to obtain the T-2 and HT-2 concentration in µg/kg actually contained in a sample, the concentration read from the calibration curve must be further multiplied by the corresponding dilution factor. When working in accordance with the regulation stated, the dilution factors are as follows:

Oats, corn/maize, barley, wheat x 10, or greater if the samples have been diluted further.

Therefore, the measurement range of the test is 10 to 360 µg/kg (ppb) T-2 and HT-2 for oats and grain (or in the range of 100 to 3600 µg/kg (ppb) following a further sample dilution of 1:10).

Results and Discussion

In total, 100 oat samples (55 unprocessed, 44 processed and one ERM®, European certified reference material, T-2 and HT-2 in oat flakes) were analysed for the sum of T-2 and HT-2 using the four rapid test kits described, in addition to confirmatory analysis by mass spectrometry. Only oats were included in the study as no T-2 or HT-2 toxins were observed in barley following the survey conducted using LC-

MS/MS (Objective 4). Results for all samples have been detailed in Table 1 in Appendix E. The comparative study was performed on 92 samples in total, since some rapid tests results were invalid, i.e., the control line of the lateral flow device could not be seen, or if the resulting concentrations fell outside the kit range, when no concentration value was obtained and were therefore removed from the sample group. Invalid results (i.e., the control line did not develop) were observed in Test Kit 1 and Test Kit 2 (sample numbers 201 and 205, respectively). The number of samples showing results greater than the range was five: one measured using Test Kit 2 (sample 100) and four measured using Test Kit 3 (sample numbers 71, 100, 134 and 157). Using Test Kit 4, two samples were determined to be less than the range (sample numbers 167 and 198 were selected from the 229 samples analysed in the survey. The sample numbers were not changed to enable easy comparison with LC-MS/MS). Figures 12 – 15 outline the observed results for both processed and unprocessed oat samples from Test Kits 1-4, respectively, compared with the results from mass spectrometry. Generally, the rapid tests tended to underestimate the results when compared with the confirmatory method.

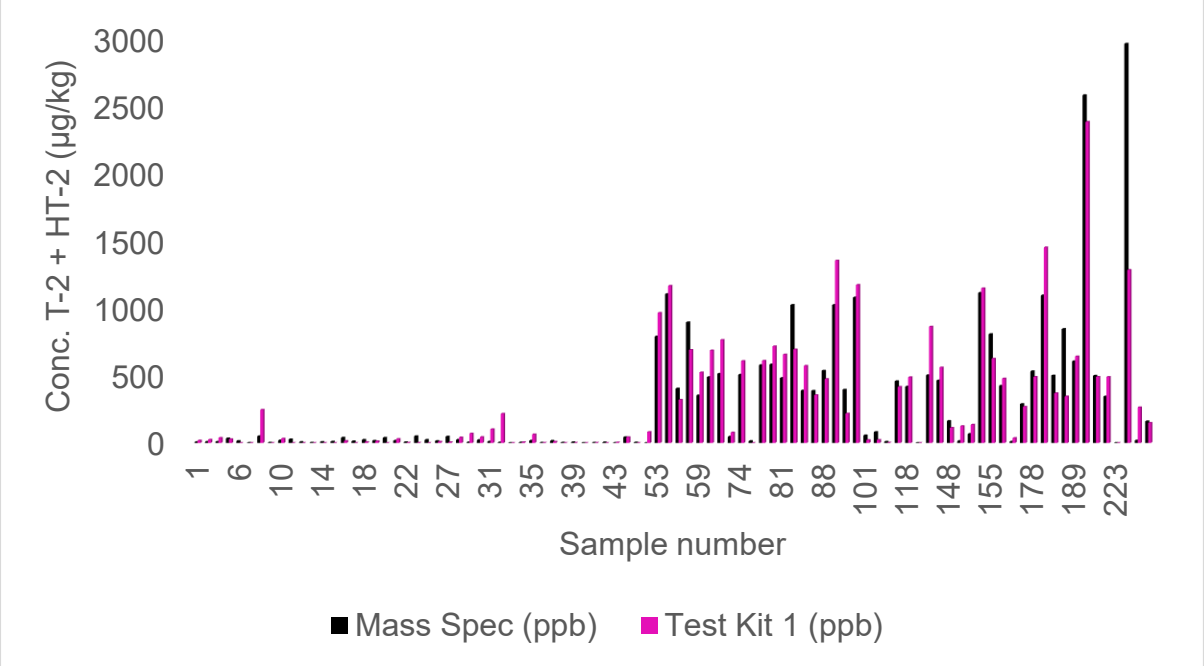


Figure 12. Comparison of the sum of T-2 and HT-2 by mass spectrometry and Test Kit 1.

Mass spectrometry concentrations of the sum of T-2 and HT-2 are shown as the black bars and the concentrations derived using Test Kit 1 are shown as magenta bars. Sample numbers up to and including 50 refer to processed oat samples, sample numbers thereafter are processed oat samples.

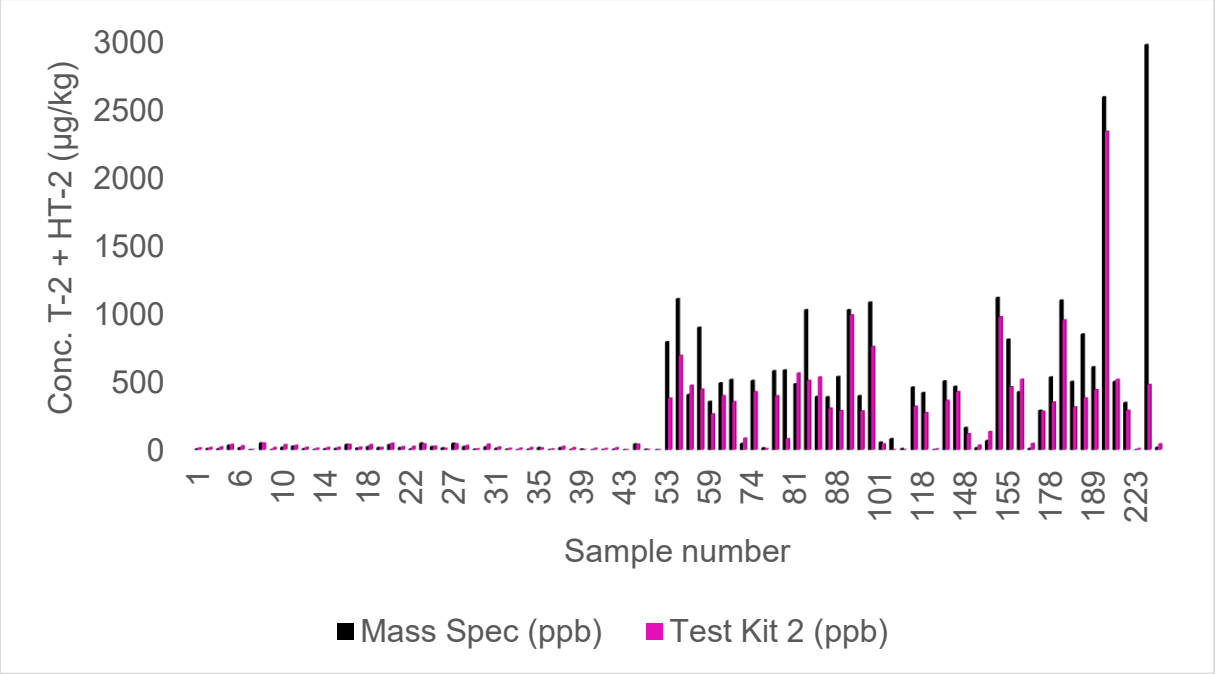


Figure 13. Comparison of the sum of T-2 and HT-2 by mass spectrometry and Test Kit 2.

Mass spectrometry concentrations of the sum of T-2 and HT-2 are shown as the black bars and the concentrations derived using Test Kit 2 are shown as magenta bars. Sample numbers up to and including 50 refer to processed oat samples, sample numbers thereafter are processed oat samples.

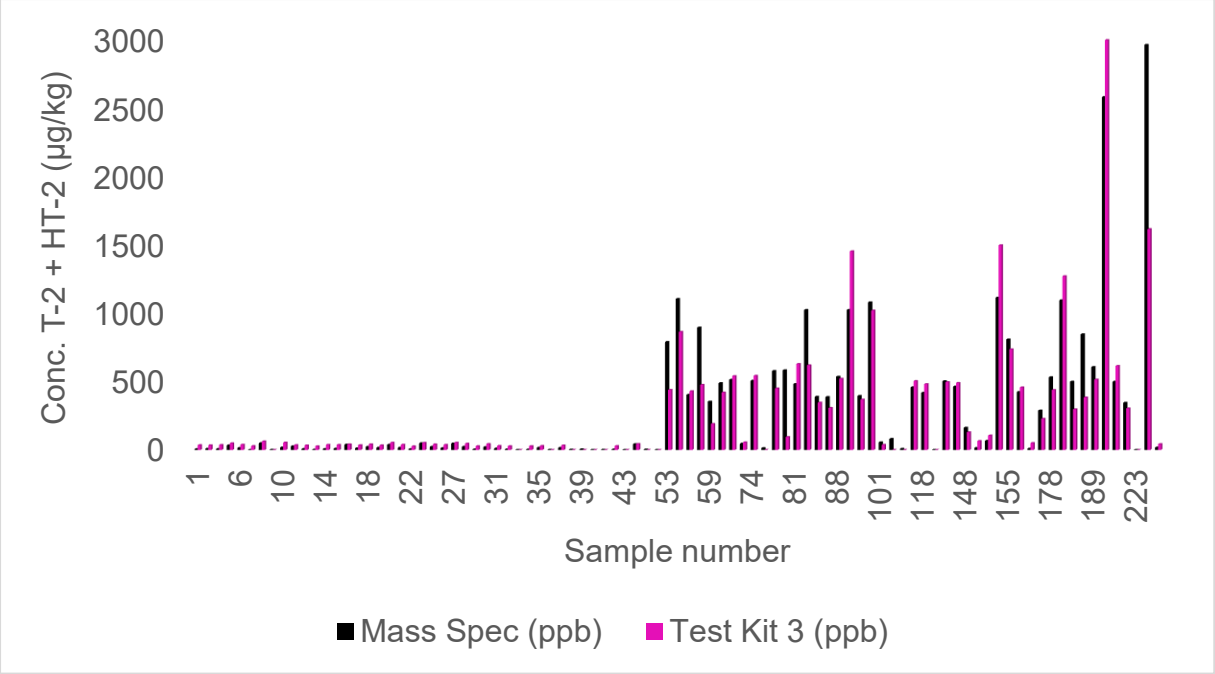


Figure 15. Comparison of the sum of T-2 and HT-2 by mass spectrometry and Test Kit 3.

Mass spectrometry concentrations of the sum of T-2 and HT-2 are shown as the black bars and the concentrations derived using Test Kit 3 are shown as magenta bars. Sample numbers up to and including 50 refer to processed oat samples, sample numbers thereafter are processed oat samples.

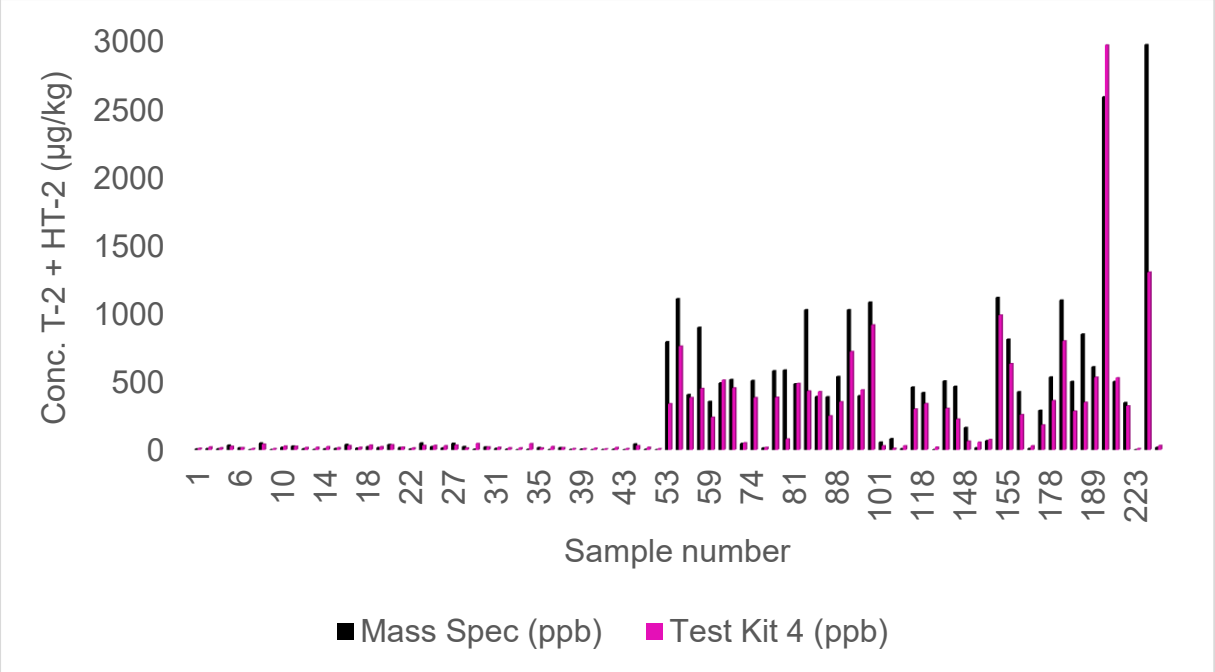


Figure 15. Comparison of the sum of T-2 and HT-2 by mass spectrometry and Test Kit 4.

Mass spectrometry concentrations of the sum of T-2 and HT-2 are shown as the black bars and the concentrations derived using Test Kit 4 are shown as magenta bars. Sample numbers up to and including 50 refer to processed oat samples, sample numbers thereafter are processed oat samples.

A European Certified Reference Material for the sum of T-2 and HT-2 in oat flakes was tested by each method. The reference concentration values were 80 µg/kg for T-2 toxin and 80 µg/kg for HT-2 toxin, therefore 160 µg/kg (ppb) for the two. Figure 16 outlines the performance of the rapid methods compared with mass spectrometry.

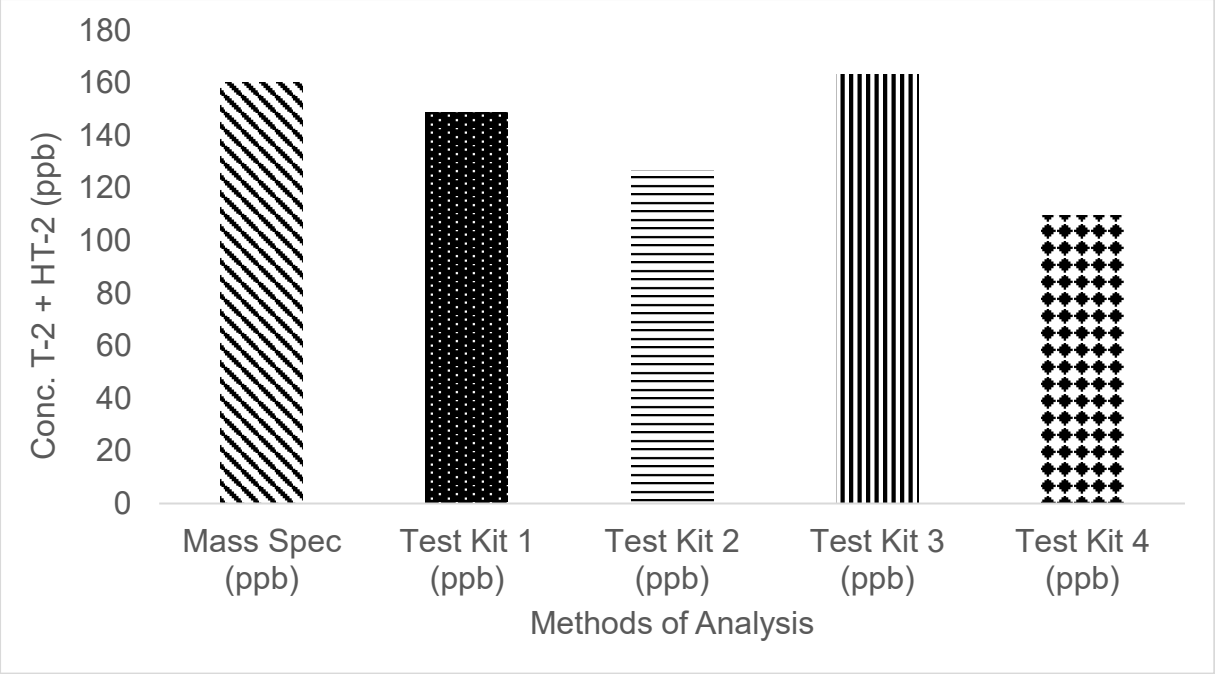


Figure 16. Comparison of rapid methods and mass spectrometry for the determination of the sum of T-2 and HT-2 in a European Certified Reference Material for oat flakes.

The observed results of the European Certified Reference Material indicated that two rapid test kits, Test kit 1 and Test Kit 3, performed very well, with concentrations of 149 µg/kg and 163 µg/kg obtained, respectively. Test Kits 2 and 4 displayed under-estimation of the sum of T-2 and HT-2 toxins (127 µg/kg and 110 µg/kg, respectively); however, this may be attributed to the cross-reactivity profile of the antibody included in the kit. Despite requesting this information for Test Kit 2, cross-reactivity is still unknown; therefore, it cannot be assumed that this is the reason for the underestimation of results (observed concentration of 126.6 µg/kg). In contrast, for Test Kit 4, the antibody was raised against HT-2 toxin, therefore the cross-reactivity with HT-2 toxin is 100% and that for T-2 toxin 85%. This would account, in part, for the underestimation of the observed results of 109.7 µg/kg. Matrix effects may also contribute. It should also be noted that Test Kit 2 has not been validated in oats; therefore, some optimisation would be required to improve the accuracy.

One-Way ANOVA was used to determine if the mean differences between the methods tested were significantly different. A p value of 0.45 indicated that the

results obtained from each method were not significantly different, and therefore provided no definitive answer as to which test(s) performed better.

Compliance/non-compliance in relation to the EU guidance limits

The current indicative limits set by the European Union for processed and unprocessed oats are 200 µg/kg and 1000 µg/kg, respectively. To ascertain if the performance of the kits met the current regulatory requirements, the results were evaluated in terms of being negative or positive against the set standards, a qualitative approach. The results are summarised in Table 16 (a full table of results is included in Appendix E, Table 2).

Table 16. False positive/false negative rates for the rapid test kits (existing guidance limits)

Rapid Test Kit	Number of False Positives	False Positive Rate (%)	Number of False Negatives	False Negative Rate (%)
Test Kit 1	2	2.2	1	1.1
Test Kit 2	0	0	7	7.6
Test Kit 3	0	0	2	2.2
Test Kit 4	0	0	6	6.5

The results highlight that, generally, all kits tend to underestimate the concentration of the sum of T-2 and HT-2 in processed and unprocessed samples, with the exception of two results for Test Kit 1. For Test Kit 1 and Test Kit 3, the rates of false negatives/positives fall within the accepted tolerance of <5% (EU, 2014). However, for Test Kits 2 and 4, although the false positive rate is 0%, the false negative rate was determined as 7.6% and 6.5%, respectively. This increases the risk of contaminated cereals and cereal products entering the human food chain. However, it should be noted that for Test Kit 2, three of the seven false negative sample results fell at ≥ 950 µg/kg, which is very close to the guidance limit. Depending on the cut-off limits determined, these may not be considered as negative samples and

therefore the false negative rate of the kit would then be 4.3%, thus meeting the accepted criteria. For Test Kit 4, one sample out of the six false negatives was found to contain ≥ 950 µg/kg. If the same rationale were applied as for Test Kit 2, five false negatives would result; however, the 5.4% false negative rate would still fail the accepted tolerance. Given that Test Kits 2 and 3 have not been validated for the determination of T-2 and HT-2 in oats, the kits have performed very well. While Test Kit 3 met the false negative/false positive criteria, Test Kit 2 would require some optimisation to deliver increased accuracy.

The same qualitative evaluation was applied to the results in terms of the proposed new regulatory limits under discussion (i.e., 50 µg/kg for processed oats and 500 µg/kg for unprocessed oats). As observed from the summarised results (Table 17), the performance of the kits, in their current form, would fail to meet the criteria as laid down by the European Commission (EU, 2014).

Table 17. False positive/false negative rates for the rapid test kits (proposed regulatory limits)

Rapid Test Kit	Number of False Positives	False Positive Rate (%)	Number of False Negatives	False Negative Rate (%)
Test Kit 1	11	12	3	3.3
Test Kit 2	4	4.3	13	14.1
Test Kit 3	7	7.6	7	7.6
Test Kit 4	2	2.2	11	12

The only kit to meet the tolerance of ≤ 5% of false negatives was Test Kit 1; however, it exceeded the limit for false positive results. In relation to false positives, Test Kits 2 and 4 fell within the set criterion but did not meet the specifications for the rate of false negatives. Test Kit 2 failed to meet both criteria. Therefore, optimisation and full validation would be required for all kits to meet the proposed regulatory limits. The full results are tabulated in Appendix E (Table 3). Furthermore, should the legislation change, the sensitivity of Test Kits 1 and 3 would need to be improved as the LOD for each is currently reported as 50 µg/kg.

Comparative study of Test Kit 1

A small study was undertaken to compare the results of the same samples when tested in different laboratories using Test Kit 1. Three laboratories were involved in the study. The samples were selected from those provided by the stakeholders.

Results of 61 oat samples (both processed and unprocessed) tested by two laboratories using Test Kit 1 were compared. The results highlighted that there were substantial differences in eight out of the 61 samples tested (13.1%) (Appendix E, Table 4). This would mean the difference between compliant and non-compliant results. These results are illustrated in Figure 17.

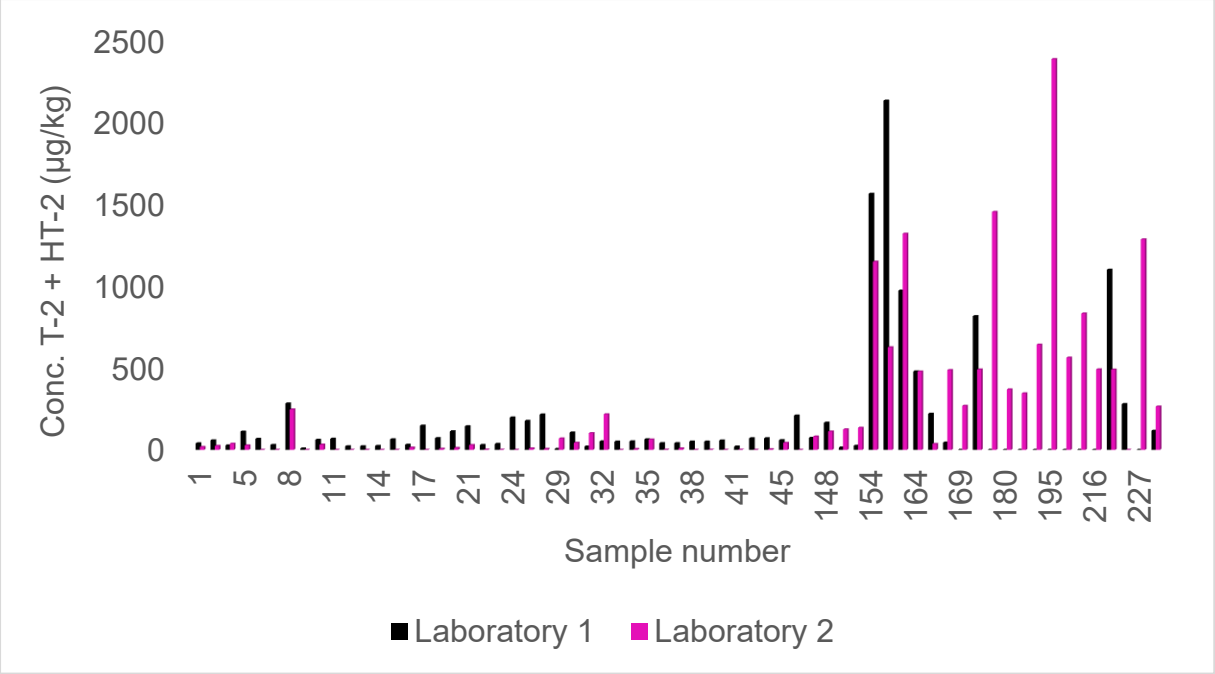


Figure 17. Comparison of Test Kit 1 between two laboratories.

The black bars depict the concentrations of the sum of T-2 and HT-2 for laboratory 1 while the magenta bars represent those concentrations found by laboratory 2. Sample numbers up to and including 50 refer to processed oat samples, sample numbers thereafter are processed oat samples.

Nineteen processed samples were analysed by three laboratories (Figure 18) and the observed results showed that one sample was found to be non-compliant by one laboratory but compliant by the other two laboratories (Appendix E, Table 5). In most instances, the results determined by laboratory 1 were higher than those found by laboratories 2 and 3.

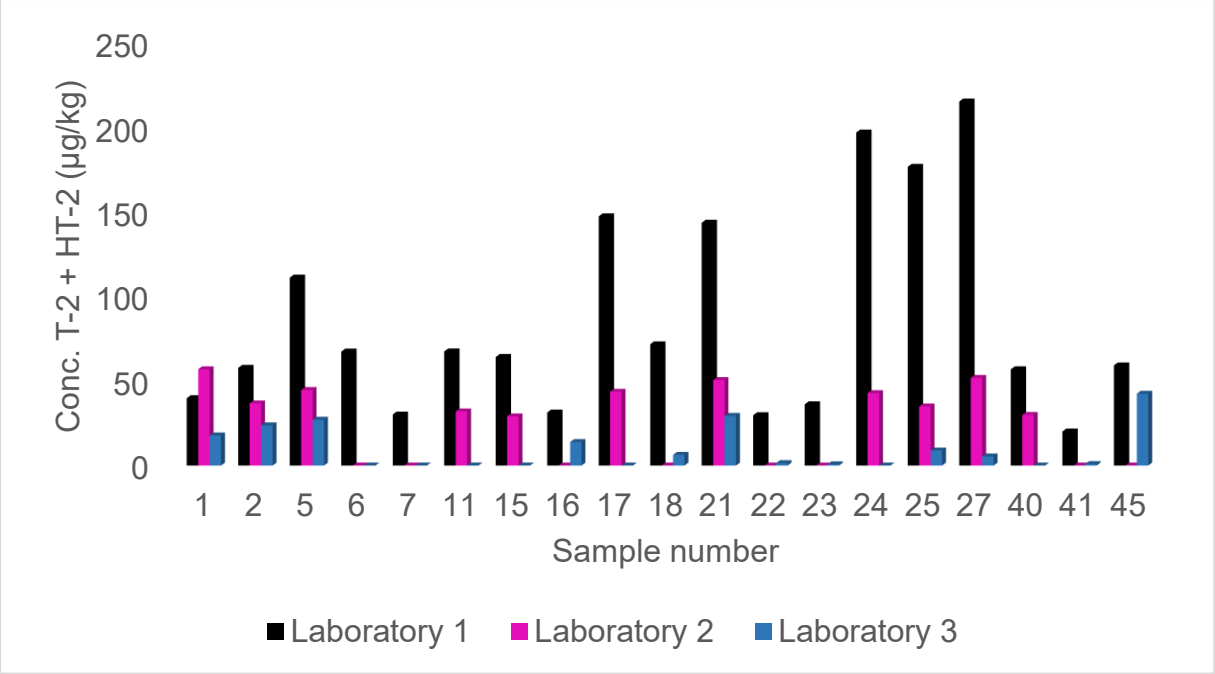


Figure 18. Comparison of Test Kit 1 between three laboratories.

The black bars depict the concentrations of the sum of T-2 and HT-2 for laboratory 1, the magenta bars represent those concentrations found by laboratory 2 and the blue bars represent concentrations determined by laboratory 3. All samples tested for this trial were processed oat samples.

Although it is not clear why the results differ so much, it may be down to sampling and sample size or possibly sample preparation. The samples tested at each laboratory came from the same aggregate sample; however, it is unknown how many incremental samples (including the weight) were used to prepare the aggregate sample or how this sample was split to form the sub-samples for testing. This is a crucial step in mycotoxin analysis. Furthermore, sample preparation protocols must be adhered to, and the sample milled to a fine powder prior to extraction. Further discussions with the industry are necessary to understand the entire process.

Conclusions

The aim of this study was to select a number of commercially available rapid diagnostic kits (based on claimed manufacturers' performance) for an in-depth evaluation of their fitness for purpose for analysing the sum of T-2 and HT-2 toxins in oat and barley grains. Following the survey (Objective 4), only oat samples were selected, as barley samples were not contaminated with T-2 and HT-2 toxins. Four rapid test kits were assessed against state-of-the-art LC-MS/MS. Although Test Kits 2 and 3 are not validated for oats, they were included as they met the remaining set eligibility criteria.

One hundred oat samples were prepared according to the manufacturers' protocols and tested using the rapid test kits. Ninety-two samples were used for the comparative study owing to invalid results, i.e., the control lines failing to develop or due to no numerical values being observed (> range or < range). Generally, under-estimation of results was observed using the rapid test kits; however, statistical analysis using One-Way ANOVA revealed the methods were not significantly different from each other, and thus provided no definitive answer as to which test(s) performed better. Evaluation of a T-2 and HT-2 European Certified Reference Material highlighted that Test Kits 1 and 3 performed very well. Test Kits 2 and 4 displayed under-estimation of the sum of T-2 and HT-2 toxins. As Test Kit 2 has not been validated for this matrix, the results indicate that further optimisation of sample preparation may be required to eliminate possible matrix effects. In addition, the cross-reactivity profile of this antibody is unknown and therefore may account, in part, for lower concentrations. In the case of Test Kit 4, the cross-reactivity of the antibody will contribute to the lower concentrations observed, but matrix interference may also play a role.

Assessing the kits in terms of false positives and false negatives, both Test Kits 1 and 3 were within the accepted EU criteria (≤ 5%). Observed results for Test Kits 2 and 4 did not meet the criterion for false negative rates, with both exceeding 5%; however, false positive rates were 0%. Several concentrations were close to the guidance limit of 1000 µg/kg for unprocessed oats. If a cut-off or threshold value were to be calculated and implemented, the false negative rate for Test Kit 2 may

subsequently fall within the permitted tolerance. However, applying this to Test Kit 4 may not alter the outcome. This emphasises the need for the industry to perform their own validation of the test kits and establish a cut-off/threshold value in terms of compliant/suspect samples. All kits were easy to use and provided results within the specified time. All reagents were provided in the kits except for the extraction buffer required for Test Kit 2.

In terms of the regulatory limits under discussion, and assessing the false negative and false positive rates, the results highlighted that none of the kits in their current form would be fit for purpose. Further development and validation would be required to demonstrate accurate, reliable results at the lower limits. Moreover, the LOD for Test Kits 1 and 3 is 50 µg/kg. If the limits were changed, this would not meet the control legislation as the maximum concentration permitted for processed oats under discussion is 50 µg/kg.

Under the current legislative standards for the control of T-2 and HT-2 toxins in oats, two kits demonstrated reliable results in terms of comparison with LC-MS/MS and false negative/false positive results, i.e., Test Kits 1 and 3. The other kits did not perform as well, one of which was not validated for the tested matrix.

The performance of the test kits has been summarised in Table 18. Considering these characteristics, the best performing kit is Test Kit 1, the Neogen Reveal® Q+ MAX for T-2/HT-2 Kit, with cost per analysis and the percentage rate of false negatives being the most important features considered. Test Kit 3 would be the second choice.

Table 18. Kit Performance

	Test Kit 1	**Test Kit 2**	**Test Kit 3**	**Test Kit 4**
Matrix – oats	Yes	No	No	Yes
Limit of detection	50 ppb	10 ppb	50 ppb	12 ppb
Test time (incubation following sample preparation)	5 minutes	5 minutes	5 minutes	45 minutes
Ease of use	Easy	Easy	Easy	Technical skills required
Kit reagents supplied	All supplied	Extraction buffer purchased separately	All supplied	All supplied
Cost per analysis	£7.20	£16.80	£7.40	£7.44
False negative rate (%)	1.1	7.6	2.2	6.5
False positive rate (%)	2.2	0	0	0
Recovery (ERM sample) (%)	93	79	102	69

Objective 6

Engage with all stakeholders to discuss the implications of the findings of the project and produce a series of conclusions and recommendations for stakeholders on the island of Ireland.

Summary

Two workshops were organised by QUB to disseminate the findings of the project to the stakeholders. The first was an industry-wide seminar designed to disseminate not only the findings of the research but also how the application of Smart Agriculture - Smart Science (SASS) can be used to mitigate against the ever-increasing challenges posed by the presence of mycotoxins (regulated and emerging) in food and feed.

The second workshop held was company specific and designed to help stakeholders establish a robust and efficient sampling regime to ensure accurate measurement of mycotoxins. QUB described the EU recommendations for the sampling of cereal grains for mycotoxins analysis and provided advice to those involved in the process from on-farm sampling to obtaining a representative sample for analysis. Subsequently, a sampling standard operating procedure was created and discussed. A final sampling method has been approved and initiated within the company.

Workshop 1

Title: Measuring Mycotoxins: Applying Smart Agriculture – Smart Science (SASS) to mitigate against the growing and unknown issues of mycotoxins in feed and food.

Date/Time: Thursday, 2nd of February 2023: 10:00 - 14:30.

Venue: Deramore Suite, Malone Boutique Hotel, 60 Eglantine Avenue, Belfast

Organiser(s): The Institute for Global Food Security in partnership with Food Fortress, ***safe*food** and Agritox.

Agenda: The Food Fortress annual general meeting was held prior to the workshop. During the seminar, four presentations were included. 1. How to improve the resilience and sustainability of the food and feed sectors by effective control and mitigation of mycotoxins. 2. The impact of mycotoxins on livestock performance and environmental sustainability. 3. Recent survey data on the presence of the regulated mycotoxins in oats and barley produced on the island of Ireland and an evaluation of rapid test kits to measure T-2 and HT-2 toxins in oats. 4. The development and implementation of an LC-MS/MS method as a 'Toxicity Alert System' in the animal feed sector. The workshop programme is detailed in Appendix F.

Attendees: Invitations were sent to all companies in Food Fortress, in addition to other animal nutrition companies, food producers and testing industries. Approximately 50 people attended the meeting. A full list of the companies that attended are included in Appendix F.

Workshop 2

Title: Sampling Strategies

Date/Time: Thursday, 16th of February 2023 at 11:00

Venue: Online – Microsoft Teams Meeting

Organiser(s): The Institute for Global Food Security.

Agenda: This was a meeting tailored to meet the specific demands of stakeholders to design, establish and implement a robust sampling strategy for the monitoring of mycotoxins in processed and unprocessed grains.

Current sampling strategies were evaluated. Discussions followed on how to improve upon these to ensure accurate testing results for mycotoxins are obtained. Considerations included the batch sizes of grains, how they are stored, the number of incremental samples required, the size of the aggregate sample, mixing the aggregate sample and finally sub-sampling to obtain a representative sample for analytical testing. The stakeholders involved have written Standard Operating

Procedures based on Commission Regulation (EC) No 401/2006 (EC 2006c) that have now been implemented.

Project Discussion and Key Findings

The primary aims of this study were to review the most up-to-date scientific and regulatory data on T-2 toxin and its metabolites, in addition to assessing (and where possible improve/refine) current analytical methods for the rapid screening for the presence of T-2 and HT-2 toxins in cereals for direct human consumption. To do this, the project engaged from the outset with the relevant sectors of the cereal primary production and processing industries in both jurisdictions on the island of Ireland to determine shortfalls in relation to monitoring and control for T-2 toxin and its metabolites, and by extension, other mycotoxin contaminants within the supply chain.

The scientific review highlighted the known toxicological effects of T-2 and HT-2 toxins on both animals and humans, one of the key aspects in the establishment of regulatory limits to safeguard health. Using this information and occurrence data for these toxins in food, the European Safety Authority (EFSA) published a Scientific Opinion in 2011 on the risks for animal and public health related to the presence of T-2 and HT-2 in food (EFSA, 2011). A group tolerable daily intake (TDI) of 0.1 µg/kg body weight was established for the sum of T-2 and HT-2 toxin; however, they concluded that toxicological and occurrence data was lacking for these toxins. Nevertheless, indicative limits for the sum of T-2 and HT-2 were introduced in the European Union in 2013 (EU, 2013). In 2017, following the availability of more data, a new group TDI for T-2 and HT-2 of 0.02 µg/kg body weight was established (Arcella et al., 2017). This has prompted new discussions on setting maximum limits for the sum of these toxins in foods. The proposed new limits are lower than those already in existence, and are therefore expected to have a huge impact on the cereal industry in terms of the monitoring of, and mitigating against, these contaminants.

While the biosynthetic pathways for T-2 and HT-2 toxins are well understood and a considerable body of knowledge regarding the climatic conditions influencing the growth and production of these toxins is available, gaps in the research have been identified. The ecology of *F. sporotrichioides* and *F. langsethiae*, as well as the

influence of interacting environmental factors on their growth and activation of biosynthetic genes, are still not fully understood. Predictive models of *Fusarium* growth and subsequent mycotoxin production would be beneficial in predicting the risk of contamination and thus aid early mitigation.

As indicated, with discussions ongoing in relation to establishing new maximum limits for the sum of T-2 and HT-2 toxins, more surveillance of these toxins in cereals is required. Therefore, on-site testing by farmers/producers using rapid methods would be prudent in order to identify non-compliant crops. In the event of samples being positive, i.e., above the current EU indicative levels, for these toxins, confirmatory tests should be performed. As the gold standard is LC-MS, simultaneous measurement of a range of mycotoxins, including T-2 and HT-2, would provide occurrence data and the risks posed to consumers. Use of high-resolution mass spectrometry on compliant and non-compliant crops would be useful to indicate what other T-2 and HT-2 metabolites or emerging mycotoxins are prevalent in cereals such as oats and barley. This would support the prediction of what analyses should be performed in those commodities as climatic factors possibly alter the fungal and mycotoxin profiles. Moreover, as the toxicological effects and occurrence of some metabolites become clearer, this would help the industry to be proactive rather than reactive.

As regards the monitoring and control of T-2 and HT-2, commercially available rapid diagnostic kits were reviewed in relation to the existing guidance limits set by the European Union and also the proposed maximum limits under discussion. Initial evaluation was based on their performance characteristics (manufacturers' specifications) against current regulatory requirements. Of the 20 kits identified, 12 kits were applicable for the measurement of the sum of T-2 and HT-2 with respect to antibody cross-reactivity. As oats were the cereals of choice for the project, validation of the test kits in these matrices was prioritised for this exercise. Of the kits selected according to specificity, six were validated in the matrices of choice, while the remaining kits were applicable to other cereals. The European Commission has stipulated that the Limit of Quantification (LOQ) should not exceed 10 µg/kg for T-2 and HT-2 individually, and that the LOD of an analytical method should be less than or equal to 25 µg/kg for the sum of T-2 and HT-2 (EU, 2013). For the most part,

from the information available, the test kits identified have indicated the LOD of the methods, although the Limits of Quantification (LOQs) have not been stated. The vast majority of the rapid methods meet the requirements laid down by the European Commission; however some, while being applicable to the determination of these toxins in unprocessed cereals, would not be suitable for cereal products or cereal-based foods for infants and children where the indicative limits lower to 25 µg/kg and 15 µg/kg, respectively. Another important element considered when evaluating the rapid tests is the extraction solution used for sample preparation. Often the extraction solutions used in preparing samples for analysis have been based on organic solvents such as methanol, acetonitrile or ethyl acetate. The drive is to reduce these from both a health and safety and environmental point of view, and while many manufacturers still employ such extractants, some have introduced aqueous extractions for these toxins. This is particularly important for on-site testing, where the storage, use and disposal of organic solvents is not feasible.

LFDs are the preferred choice for on-site testing rather than ELISAs as their use requires less technical expertise, and no access to a spectrophotometer or microplate reader is required. That said, as ELISA are considered the ‘gold standard’ for rapid tests, both platforms were selected for the comparative study.

Using state-of-the-art liquid chromatography coupled to mass spectrometry, a survey of oats and barley sourced from Ireland and the UK was undertaken to determine the prevalence of not only the *Fusarium* mycotoxins T-2 and HT-2 but also all other regulated mycotoxins, including aflatoxins, deoxynivalenol, zearalenone, fumonisins and OTA. In total, 229 oat samples (unprocessed and processed) and 52 unprocessed barley samples from Ireland and the United Kingdom were analysed for the regulated mycotoxins.

The results indicated that contamination of oats with the mycotoxins T-2, HT-2 and OTA represent an ongoing challenge to the industry. Should the proposed lower limits for the sum of T-2 and HT-2 be implemented, the study has shown that this may result in higher rates of non-conformity, potentially leading to greater economic losses for the industry and increased food waste. That said, in examining the results of the processed oats (although a small sample number), no violations were

observed, and even if the permitted limits are lowered, it seems that processing, such as de-hulling, is sufficient to reduce the concentrations of T-2 and HT-2 in oats.

In barley, T-2 and HT-2, deoxynivalenol and zearalenone were the only mycotoxins detected, all of which fell below current regulatory limits.

OTA was detected in a small number of samples and in some cases exceeded the regulatory limits for both unprocessed and processed oats. This suggests that there has been a problem with the storage of these, i.e., the oats have not been dried to the specified moisture content or have been damaged during harvesting, or by insects or rodents, for example. This needs to be monitored and investigated by the industry to ensure that all possible controls are adopted to mitigate contamination with mycotoxins, particularly during storage.

The findings of this survey emphasise the continued need for effective surveillance and control of mycotoxins within the industry through adequate testing regimes and HACCP.

Analysis of oat survey data and sample metadata to identify trends/major weaknesses in the supply chain was conducted on a different sample set of oats (310 samples) collected between July 2021 and September 2022. The samples were analysed for the occurrence of deoxynivalenol, zearalenone, T-2 and HT-2 using LC-MS/MS. Furthermore, agronomic data including crop varieties, fungicide application, storage conditions and duration, farming practice, processing techniques and mycotoxin testing method were collected. As with the survey described previously, this study showed that contamination of oats with *Fusarium* mycotoxins, particularly T-2 and HT-2 toxins, represent a huge challenge for oat producers. These toxins were also found to be more prevalent in conventionally produced oats compared with organically produced oats. In addition, the application of fungicide and the storage of oats in farm stores led to elevated mycotoxin concentrations. Thus, fungicide application before harvest, and storage conditions following oat crop harvest, represent important critical control points for oat mycotoxin management.

Following the initial survey described, only oat samples were selected for comparison between LC-MS/MS and rapid test kits, as the barley samples were not contaminated at high levels with T-2 and HT-2 toxins. Four rapid test kits were

assessed against state-of-the-art LC-MS/MS: three LFDs and one ELISA kit. All test kits were selected based on their ability to measure both T-2 and HT-2 toxins, their use of aqueous extraction methods, matrices they have been validated for, the ability to rent a reader to perform the analyses, and the availability and delivery of kits and readers within the timespan of the project. Of the kits highlighted for possible inclusion in the study, only two LFDs and one ELISA were validated in oats; however, one of these LFD kits was not available for delivery within the timespan of the project, therefore other kits were chosen. In terms of the remaining two LFD kits selected, their performance characteristics met the remaining criteria for the study, even though they were not validated for oats.

One hundred oat samples supplied by the project stakeholders were prepared according to the kit manufacturer's protocols and tested using the LFDs or ELISA. Ninety-two samples were used for the comparative study owing to invalid results, i.e., the control lines failing to develop or due to no numerical values being observed (> range or < range). Invalid results (i.e., the control line did not develop) were observed in Test Kits 1 and 2 (one sample for each kit). The number of samples showing results greater than the range was five, one measured using Test Kit 2 and four measured with Test Kit 3. Using Test Kit 4, two samples were determined to be less than the range. Statistical analysis using One-Way ANOVA revealed that the results generated from the different kits were not significantly different from each other, and thus provided no indication of the best-performing kit. Evaluation of a certified reference material containing 160 µg/kg T-2 and HT-2 toxins highlighted that Test Kits 1 and 3 performed best, with concentrations of 149 µg/kg and 163 µg/kg obtained, respectively. Test Kits 2 and 4 displayed under-estimation of the sum of T-2 and HT-2 toxins (127 µg/kg and 110 µg/kg, respectively). As Test Kit 2 was not validated for this matrix, the results indicate that further optimisation of sample preparation may be required to eliminate potential matrix effects. Furthermore, the cross-reactivity profile of the antibody is unknown. In the case of Test Kit 4, the cross-reactivity of the antibody was likely to be a contributing factor to the lower concentrations observed, but matrix interference may also have played a role.

Assessing the kits in terms of false positives and false negatives (according to the current EU guidance limits for the sum of T-2 and HT-2 toxins), results for Test Kits 1 and 3 fell within the accepted EU criteria (≤ 5%). Results observed for Test Kits 2 and 4 did not meet the criterion for false negative rates, with both exceeding 5%; however, false positive rates were 0%. Removing results ≥ 950 µg/kg (close to the guidance limit of 1000 µg/kg for unprocessed oats), the false negative rate for Test Kit 2 fell within the permitted tolerance; however, in applying this to Test Kit 4, the result was still above 5%. This emphasises the need for the industry to perform their own validation of the test kits and to establish a cut-off/threshold value in terms of compliant/suspect samples. All kits were easy to use and provided results within the specified time. All reagents were provided in the kits, except for the extraction buffer required for Test Kit 2.

Looking at the regulatory limits under discussion (i.e., 50 µg/kg for processed oats and 500 µg/kg for unprocessed oats) and assessing the kits in terms of false negatives/positives highlighted that none of the kits in their current form would be fit for purpose. Further development and validation would be required to demonstrate accurate, reliable results at the lower limits. Moreover, the LOD for Test Kits 1 and 3 is 50 µg/kg. If the limits were to be changed, this would not meet the control legislation, as the maximum concentration permitted for processed oats in the legislation under discussion is 50 µg/kg.

Under the current legislative standards for the control of T-2 and HT-2 toxins in oats, two LFD kits – Test Kits 1 and 3 – demonstrated reliable results in terms of comparison with LC-MS/MS and false negative/false positive results. The other kits did not perform as well, albeit one was not validated for the tested matrix (oats). Test Kit 1, the Neogen Reveal® Q+ MAX for T-2 / HT-2 Kit, performed best for the characteristics outlined in Table 18.

Using the best performing kit, inter-laboratory studies were performed (between two and three laboratories) on a small number of samples to ascertain the performance of the kits and the analysts. The first study involved analysis of 61 samples (both processed and unprocessed) tested by two laboratories. The results highlighted that there were substantial differences in eight out of the 61 samples tested (13.1%) that would mean the difference between compliant and non-compliant results. Following

this, 19 processed samples were analysed by three laboratories and the observed results showed that one sample was found to be non-compliant by one laboratory but compliant by the other two laboratories. In most instances, the results determined by one of the laboratories were higher than those found by the other two. Although it is not clear why the results differed so much, it may be down to sampling and sample size or potentially sample preparation. The samples tested at each laboratory were derived from the same aggregate sample; however, it is unknown how many incremental samples (including the weight) were used to prepare the aggregate sample or how this sample was split to form the sub-samples for testing. This is a crucial step in mycotoxin analysis and accounts for the greatest variability in results. Furthermore, it is imperative that sample preparation protocols are followed, and that the sample is milled to a fine powder prior to extraction. To highlight the importance of ensuring homogeneity by milling the sample to as small a particle size as possible, a small trial was performed in processed oat samples. Samples that had not been milled were analysed and then subsequently milled and re-analysed by LC-MS/MS. Following milling, the concentrations measured for T-2 and HT-2 toxins were for the most part lower than the levels detected in the un-milled samples. For deoxynivalenol, the results were more variable, with higher concentrations detected in milled samples compared with un-milled samples, and vice-versa. However, one sample (no milling) was non-compliant with respect to the current regulatory limit of 750 µg/kg with a concentration of 1355 µg/kg, yet once the sample was milled and re-analysed, the concentration determined was 273 µg/kg, which is well below the legal limit. While these samples met the regulatory guidelines of 200 µg/kg for the sum of T-2 and HT-2, were the limits to change to 50 µg/kg, and the samples not milled prior to analysis, then 27 of the 50 samples tested (54%) would have been classed as non-compliant (false positives), causing economic loss and food waste.

To safeguard human health, monitoring of mycotoxins in foodstuffs is paramount in order to ensure human exposure is limited. To achieve this, effective testing regimes must be established within the cereal industry, consisting not only of reliable and accurate analytical methods, but also, given the difficulties associated with accurate analysis of mycotoxins, efficient sampling strategies. Therefore, testing regimes and

sampling strategies used by the stakeholder members of the Project Advisory Board were assessed to determine the current shortfalls in mycotoxin control within the supply chains. From the industry feedback, it is evident that monitoring of mycotoxins in oats and barley varies a great deal. Of the four companies surveyed, two of the primary oat producers/processors tested for the important *Fusarium* mycotoxins T-2 and HT-2, deoxynivalenol and zearalenone in oats, while the third company only tested for T-2 and HT-2. The fourth company had no testing regime established. Only one company processed barley, and it performed no testing, relying instead on a certificate analysis as proof of compliance. Two of the companies have established a two-tier testing approach for the presence of mycotoxins in oats, i.e., in-house testing with screening tests such as lateral flow devices or ELISA, with subsequent confirmatory analysis for suspect positives at external laboratories using reference/accredited methods. This is the most effective approach and not only provides cost-effective monitoring for the industry but also protects company brands and, ultimately, public health. For others, while some testing is undertaken, they fall short in terms of the mycotoxins they analyse for and the type of testing that is used i.e., screening tests with no confirmatory analyses.

In relation to sampling procedures in place, shortfalls were again evident. Initial batch sizes, aggregate sample weights, the number (and weights) of incremental samples and how the samples were split to sub-samples for testing, were not consistent across the industry.

This information enabled further discussions with the industry, to assess their sampling procedures in terms of how to harmonise and improve them, ensuring that the sampling procedures are robust, the recommended aggregate sample sizes are taken from the correct number of incremental samples, and careful consideration is taken to ensure the samples are split into homogeneous sub-samples for testing. Furthermore, the observed results stress the importance of correct sample preparation to ensure accurate results for mycotoxin testing. As a result, one company has already designed, established and implemented a robust sampling strategy for the monitoring of mycotoxins in processed and unprocessed grains.

The knowledge gained through this project will facilitate improved practical mitigation approaches to protect public health, address regulatory requirements and ultimately increase the resilience of the indigenous cereal industry.

Project Conclusions

This project provides an in-depth review of the most up-to-date scientific and regulatory data on T-2 and HT-2 toxins; small grain surveys to assess the extent of contamination with these toxins in oats and barley and how agronomic practices may influence the production of them; engagement with industry to gain a better understanding of their established testing/monitoring regimes, including sampling; and an evaluation of a range of commercial rapid test kits.

The systematic review highlighted that these mycotoxins inhibit protein synthesis and animal exposure, which may result in feed refusal, reduced feed conversion, vomiting, decreased egg production, abortion, immunological disorders, skin dermatitis, haemorrhagic lesions and, in some cases, death. In terms of human health, T-2 toxin has been linked to the alimentary toxic aleukia (ATA) and Kashin–Beck disease (KBD), and has been implicated in biological warfare in the 1970s and 1980s. As a result, guidance limits were introduced for the presence of the sum of T-2 and HT-2 toxins in cereals and the products derived from them. With the availability of more data, the tolerable daily intake was changed from 0.1 µg/kg body weight to 0.02 µg/kg body weight, prompting further discussions in the European Union to introduce maximum limits much lower than those currently adhered to. This will result in serious economic impact on the cereal industry, not least on the island of Ireland, where the main crops produced are oats and barley. Increased surveillance and mitigation strategies would add an extra burden on farmers and producers.

While there is great understanding as to the ecological and environmental factors that influence the activation of the biosynthetic pathways and thus the production of these toxins, further research is required to fully elucidate these mechanisms to help in the prediction of contamination and to allow early interventions, and therefore reduce the mycotoxin burden on these crops.

In terms of the monitoring and surveillance of these toxins, there are a huge number of available technologies for their detection and measurement, ranging from sophisticated confirmatory/reference methods to rapid screening assays. All

methodologies are important in the challenge against mycotoxins. Rapid screening assays are invaluable for farmers and producers to monitor their crops for contamination, while confirmatory analysis provides indisputable evidence of compliance or non-compliance against the regulations. Using the sophisticated LC-MS/MS technology enables the simultaneous measurement of multiple mycotoxins in grains, thereby providing a comprehensive assessment of the mycotoxins present and their concentrations. Additionally, changes in the distribution of mycotoxins due to the changing climate or agronomic factors may be identified and mitigation strategies tailored. Combining the information will promote a proactive approach to the continued risk of mycotoxin contamination in cereal crops.

The survey indicated that the contamination of oats with T-2 and HT-2 toxins is an ongoing challenge for the industry, requiring routine monitoring and surveillance of raw grains and final products. Additionally, the results demonstrated that in a small number of samples, OTA was detected. This is a storage mycotoxin which occurs as a result of insufficient drying of the grains and/or poor storage. Therefore careful consideration should be given to the storage of grains through the entire supply chain to avoid contamination with OTA.

In relation to agricultural practices that may influence the extent of contamination, it was observed that the farming system (conventional or organic), fungicide application and storage facilities all impacted the prevalence of T-2 and HT-2 toxins. In terms of the farming system, concentrations of T-2 toxin in conventionally produced oats were more than double those found in organically produced oats. Oat crops sprayed with fungicide had significantly higher concentrations of T-2 and HT-2 toxins when compared to oats without fungicide application, and oat samples stored in silos had a significantly lower T-2 and HT-2 toxins content compared to oats stored on-farm.

The report on the current mycotoxin testing and sampling regimes used within the industry, the survey of oats and barley to identify contamination levels of the mycotoxins of interest using mass spectrometry, and the comparative study with commercially available rapid diagnostic tests have led to the following conclusions:

- Industry needs to improve and harmonise its sampling arrangements.

- In terms of the overall characteristics and performance of the rapid kits tested during this study, the Neogen Reveal® Q+ MAX for T-2/HT-2 Kit performed best according to defined characteristics and is fit-for-purpose for the screening of these mycotoxins in unprocessed and processed oats. However, it should be noted that not all rapid test kits were evaluated.
- Companies need to undertake their own validation, but this is a time-consuming and expensive operation. Were the companies to operate as a collective using one test kit, a single validation could be supported.
- The potential changes to T-2 and HT-2 regulations would cause problems in terms of the performance of the test kits.

Added Value and Anticipated Benefits of Research

Due to the underpinning research that has been conducted on mycotoxins in oats and the establishment of a collaborative network with key industry stakeholders, this project has led to added value and anticipated benefits in several areas.

In terms of scientific knowledge, the review provides an overview of the data currently available on T-2 and HT-2 toxins, in addition to identifying important knowledge gaps. The research also presents a better understanding of the problems associated with mycotoxins on the island of Ireland, including the occurrence of multiple mycotoxins, levels of contamination, and the challenges of analysis of samples along the supply chain. Furthermore, through analysis of the samples and farm metadata provided by stakeholders, the influence of specific farming practices and production techniques were revealed and the resultant vulnerabilities in the supply chain exposed.

Through stakeholder engagement, the need for improvements and the harmonisation of sampling and testing regimes have been highlighted. With the knowledge that T-2 and HT-2 toxins and other mycotoxins remain a threat to the cereal industry on the island of Ireland, the industry will move from a very reactive approach to dealing with mycotoxins to a proactive, industry-led approach. This will lead to improved grain quality and safety, product and brand protection, the adoption of new technologies, reduced waste and therefore enhanced margins. There is a drive on the island of Ireland to increase plant-based food and the development of the oats and barley sectors have already been targeted. Having industry guidelines in place as to the monitoring and mitigation of these toxins will help drive innovation in the sector in terms of more farms switching to crops they have not previously produced.

Ultimately, consumers of oats and barley-based foods and beverages produced from grains grown on the island of Ireland will be better protected in terms of mycotoxin exposure.

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Appendices

Appendix A

Systematic Review Methodology

The Campbell Methods Guide (Kugley et al., 2017) was followed for the retrieval of information for the systematic review. The PRISMA Extension for Reviews checklist (Tricco et al., 2018) was applied to elucidate the most up-to-date scientific and regulatory data for T-2 toxin and its metabolites, the specific environmental conditions required for their production, and the impact of climate change on their occurrence and distribution, with a focus on the island of Ireland. Relevant articles were initially identified using title and abstract screening and secondly by full text. The records were double screened by two researchers and any discrepancies settled by a third researcher. All citations were exported to EndNote and duplicates removed prior to full text screening. The screening results have been presented in a modified PRISMA chart.

Research Aims

This review aimed to:

a. Elucidate the toxicological effects of T-2 toxin and its metabolites in animals and humans.
b. Report the regulatory limits for T-2 toxin and its metabolites.
c. Highlight the specific environmental conditions required for their production, including information on their biosynthetic pathways.
d. Ascertain the impact of climate change on the occurrence and distribution of these toxins.
e. Identify the state-of-the-art methods of analysis for T-2 and HT-2 toxins.
f. Expose knowledge or evidence gaps.

Search Strategy

A systematic review of peer-reviewed and grey literature was conducted to understand the toxicology of T-2 toxin and its metabolites, the regulatory limits set for these toxins, the environmental conditions required for growth, including their biosynthetic pathways, the impact climate change may have on their occurrence and distribution, and the state-of-the-art methods of analysis employed. Electronic searches of the following databases were conducted: Scopus, Web of Science and PubMed. The following keywords and search strings were applied to the electronic literature databases outlined previously. These key search terms were also used to search for grey literature. This was piloted in Scopus. ((TITLE-ABS-KEY (Trichothecenes OR T-2 OR HT-2 OR fusarium) AND (TITLE-ABS-KEY) (oats AND barley) AND (TITLE-ABS-KEY) (food AND regulations OR feed AND regulations) AND (TITLE-ABS-KEY) (Mycotoxicoses OR immunity effects OR haematopoietic effects OR hepatotoxicity OR nephrotoxicity OR reproductive effects OR teratogenic effects OR dermal toxicity OR carcinogenesis) AND (TITLE-ABS-KEY) (analytical OR screening OR confirmatory OR LC-MS OR ELISA OR lateral flow OR immunoassay OR spectroscopy) AND (TITLE-ABS-KEY) (climate change OR in vitro OR filed study OR occurrence OR biosynthesis OR biosynthetic genes OR survey)). In addition, the Google search engine was interrogated to identify relevant international and government agencies associated with food safety regulations for T-2 and HT-2 toxins and to identify additional grey literature relating to the topic.

Inclusion Criteria

Studies were identified by searching literature published in English only between the inception of the databases and 2021. For regulations, analysis, occurrence, distribution, biosynthetic pathways and the impact of climate change, only publications between 2000 and 2021 were selected. For the toxicological information, publications were selected from the start of the database, as much of this research was conducted decades ago. No restrictions were placed on geographical regions and no particular study design was specified. The search

strategy was piloted to test the suitability of the electronic databases and the selected keywords.

Exclusion Criteria

Publications relevant to other mycotoxins such as aflatoxins, deoxynivalenol, fumonisins, zearalenone, ochratoxin, masked mycotoxins and emerging mycotoxins were excluded. Conference proceedings and *in vitro* studies for toxicity were excluded. All duplicates were removed.

Critical Appraisal

Critical appraisal of the publications ensured that only relevant high-quality studies were included in the review, and low-quality studies excluded. This step was based on the Critical Appraisal Skills Programme (CASP) Tools Checklists (CASP, 2018). To be included in the review, papers had to adequately answer the following citation, title and abstract screening questions:

1. Does the citation indication publication within the period specified?
2. Is the title and abstract in English?
3. Aim of study?
4. What were the main findings of the paper?
5. Strengths?
6. Limitations?
7. Did the author acknowledge any limitations?
8. Were the findings consistent with other literature?

The studies were classified using keywords signifying their relevance to the five research aims described previously and further questions considered. In terms of the toxicological effects of T-2 toxin and its metabolites in animals and humans, the following were examined. Evidence regarding *in vivo* studies on animals, or poisonings in livestock, in addition to evidence of human poisonings linked to T-2 or HT-2. The regulatory limits assigned to the toxins were reported from EU and UK legislation. Any studies reporting the biosynthetic pathways and the genes

associated with these were included. Studies highlighting the scale of contamination, i.e., occurrence and distribution, in particular for oats and barley, were selected, as were publications describing the environmental conditions required for the production of T-2 and HT-2. Moreover, research that has explored the impact of climate change on the production of these mycotoxins was also included. In terms of analysis, publications investigating rapid screening methods and confirmatory methods were considered.

Synthesis of Information

A narrative synthesis of the extracted data was conducted, in order to address the aims of the review and to facilitate the inclusion of both qualitative and quantitative data. Scientific publications and grey literature were collated to provide an overview of the most up-to-date scientific and regulatory data for T-2 toxin and its metabolites, the specific environmental conditions required for their production, and the impact of climate change on their occurrence and distribution, with a focus on the island of Ireland.

Results

Using the described search strategy, 1,205 references were retrieved and subjected to screening and critical appraisal. Both qualitative and quantitative studies were included in the review. Overall, 151 papers passed the critical appraisal process and were included. A summary of the screening and critical appraisal approach has been detailed in Figure 1.

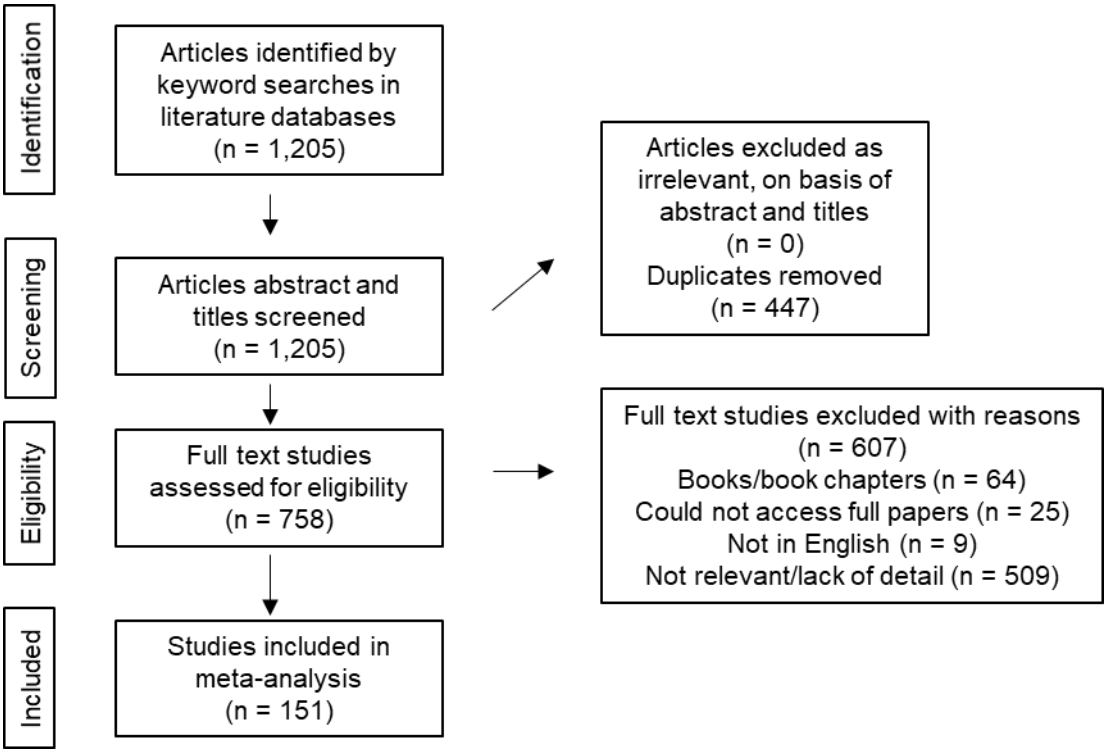

Figure 1. Summary of screening and critical appraisal processes

Appendix B

Table 1. Analysis of T-2 and HT-2 toxins and deoxynivalenol in oats with and without milling

Sample number	Sum T-2 and HT-2 Un-milled (µg/kg)	Sum T-2 and HT-2 Milled (µg/kg)	Deoxynivalenol Un-milled (µg/kg)	Deoxynivalenol Milled (µg/kg)
1	55.7	6.2	31.5	Nd
2	48.5	7.8	246.5	261.1
3	53.2	7.6	103.6	Nd
4	65.5	17.6	nd	47.4
5	60.1	33.2	nd	Nd
6	111.7	14.3	nd	15.4
7	44.4	0.5	1355.4	273.2
8	48.9	49.4	6.2	Nd
9	49.6	1.0	43.2	Nd
10	48.9	18.0	Nd	Nd
11	61.0	26.7	Nd	Nd
12	52.2	7.1	19.3	Nd
13	55.2	6.9	8.3	35.5
14	48.7	1.8	Nd	Nd
15	47.9	6.8	48.0	130.8

Sample number	Sum T-2 and HT-2 Un-milled (µg/kg)	Sum T-2 and HT-2 Milled (µg/kg)	Deoxynivalenol Un-milled (µg/kg)	Deoxynivalenol Milled (µg/kg)
16	50.9	9.1	18.2	26.0
17	66.3	38.8	25.8	Nd
18	53.0	11.7	Nd	108.7
19	62.2	22.4	Nd	nd
20	71.2	16.1	Nd	25.6
21	60.2	38.0	Nd	nd
22	51.5	16.2	49.5	191.3
23	58.1	5.2	Nd	nd
24	72.8	49.4	10.5	nd
25	69.9	22.7	24.9	46.8
26	54.0	13.7	31.6	24.7
27	79.3	46.9	7.4	68.0
28	67.6	22.7	3.9	nd
29	40.8	4.2	0.4	nd
30	67.3	20.7	3.0	nd
31	49.6	9.5	0.1	nd
32	46.7	3.3	Nd	nd
33	51.6	nd	1.3	nd

Sample number	Sum T-2 and HT-2 Un-milled (µg/kg)	Sum T-2 and HT-2 Milled (µg/kg)	Deoxynivalenol Un-milled (µg/kg)	Deoxynivalenol Milled (µg/kg)
34	54.3	1.0	Nd	nd
35	47.5	2.9	Nd	nd
36	41.5	16.3	0.4	nd
37	42.6	1.1	Nd	nd
38	44.0	1.0	2.3	nd
39	43.6	4.8	0.2	nd
40	53.6	15.3	0.3	nd
41	47.7	2.0	3.1	nd
42	40.9	4.7	Nd	nd
43	40.4	1.9	Nd	nd
44	40.7	nd	Nd	nd
45	41.5	1.2	0.3	nd
46	50.9	3.8	Nd	nd
47	43.5	0.2	1.0	nd
48	78.7	42.2	2.6	nd
49	47.1	3.0	Nd	nd
50	42.2	nd	Nd	nd

Appendix C

Sampling Protocol

Sampling for mycotoxins requires a demanding regime because the distribution is not likely to be uniform within bulk storage. Fungal growth and mycotoxins can be very localised and concentrated in certain spots, or 'hotspots', while the remainder of the lot is uncontaminated. The vast majority of error in testing occurs because of poor sampling. Therefore, it is critical that a representative sample is obtained for analysis.

The EU has laid down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs: Commission Regulation (EC) No 401/2006.

Multiple samples (incremental samples) are taken from each batch/lot and mixed to form an aggregate sample. Incremental samples should be at least 100g. The number of incremental samples and subsequent weight of the aggregate sample depend on the weight of the original lot. For the purposes of this project, work was carried out on lots of 1 tonne of grain or less in size. Should the lots be larger, the aggregate sample should be prepared according to the regulation detailed.

On-site

1. For each lot/batch, take 10 samples of 100g. The sampling must be random and removed at intervals across a moving stream (dynamic lot) or, in the case of static lots, removed from different areas, containers and bags of the same batch.
2. Combine these samples together in a clean, dry container and shake thoroughly for at least five minutes.
3. Transfer 1 kg of the aggregated sample into a clean, dry and leak-proof container (preferably, a food-quality plastic jar or container that can be securely sealed).
4. Carefully label the samples and record as much information as possible about the lots from which samples have been taken to provide as much traceability and metadata as possible (Excel file if possible).
5. Store samples in a cool, dry, and dark place.

6. Dispatch samples to the IGFS laboratory as soon as possible after collection (within 24 hours).
7. Please notify IGFS laboratory about transportation by E-mail.

Email Tobi: Oluwatobi.Kolawole@qub.ac.uk or Julie: j.p.meneely@qub.ac.uk.

Samples should be sent to Tobi Kolawole/Julie Meneely at:

14a Lennoxvale
Belfast
BT9 5BY
Northern Ireland
UK

To reduce variability of results, this 1 kg aggregate sample **must be used for all analysis**, on-site at the producers and at QUB. Therefore, if it is feasible, the aggregate sample should be sent to QUB where it will be milled and homogenised and a test portion returned to the producer for testing via their preferred rapid diagnostic kit. QUB will also test using rapid diagnostic kits (screening test) followed by LC-MS/MS (confirmatory method).

QUB can arrange the collection of samples by courier or by car, if local.

Appendix D

Table 1: Mass spectrometry results for the regulated mycotoxins in conventionally produced unprocessed oats.

	Regulated Mycotoxins Concentrations (µg/kg)			
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
51	25.1	nd	nd	nd
52	nd	nd	nd	nd
53	790.4	nd	nd	nd
54	213.8	nd	nd	nd
55	7.7	nd	nd	nd
76	13.7	nd	nd	nd
77	578.0	nd	8.2	nd
78	700.8	nd	nd	nd
79	1996.0	nd	nd	nd
80	582.9	nd	nd	nd
81	482.3	nd	nd	nd
82	55.6	nd	nd	nd
83	1024.8	nd	nd	nd
84	73.0	nd	nd	nd
85	388.8	nd	nd	nd
86	387.3	nd	nd	nd

	Regulated Mycotoxins Concentrations (µg/kg)			
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
87	nd	nd	2.8	nd
88	536.2	nd	nd	nd
89	309.6	nd	nd	nd
90	1025.0	nd	nd	nd
91	nd	nd	nd	nd
92	347.9	nd	nd	nd
93	395.0	nd	nd	nd
94	1410.0	nd	nd	nd
95	219.6	nd	nd	nd
96	1080.0	nd	nd	nd
97	198.0	nd	nd	nd
98	1256.0	nd	nd	nd
99	229.3	nd	nd	nd
100	nd	nd	nd	nd
101	55.4	nd	7.1	nd
102	661.7	nd	nd	nd
103	283.1	nd	nd	nd
104	2441.7	nd	nd	nd

	Regulated Mycotoxins Concentrations (µg/kg)			
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
105	444.9	nd	1.4	nd
106	228.3	nd	nd	nd
107	727.8	nd	nd	nd
108	80.9	nd	5.5	nd
109	692.7	nd	nd	nd
110	263.6	nd	nd	nd
111	1142.3	nd	2.1	nd
112	272.0	nd	nd	nd
113	88.3	nd	nd	nd
114	553.9	nd	nd	nd
115	710.9	nd	nd	nd
116	8.2	nd	nd	nd
117	458.2	nd	nd	nd
118	417.8	nd	nd	nd
119	180.0	nd	125.9	nd
120	286.0	nd	nd	nd
121	684.3	nd	nd	nd
122	721.6	nd	nd	nd

Regulated Mycotoxins Concentrations (µg/kg)				
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
123	1379.9	nd	nd	nd
124	1348.6	nd	nd	nd
125	119.8	nd	nd	nd
126	23.3	nd	nd	nd
127	52.5	nd	nd	nd
128	9.6	nd	nd	nd
129	1947.9	nd	nd	nd
130	nd	nd	3.3	nd
131	203.0	nd	nd	nd
132	2647.4	nd	nd	nd
133	nd	nd	39.9	nd
134	3993.3	nd	2	nd
135	620.9	nd	nd	nd
136	590.1	nd	nd	nd
137	503.1	nd	nd	nd
138	591.5	nd	nd	nd
139	594.3	nd	nd	nd
140	600.6	nd	nd	nd

Regulated Mycotoxins Concentrations (µg/kg)				
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
141	410.5	nd	nd	nd
142	463.6	nd	nd	nd
143	290.7	nd	nd	nd
144	205.9	nd	nd	nd
145	337.9	nd	nd	nd
146	286.8	nd	nd	nd
147	664.0	nd	2.9	nd
148	162.7	1.1	nd	nd
149	224.7	0.8	nd	nd
153	272.1	5.6	nd	nd
154	1114.6	1.8	nd	nd
155	808.5	nd	nd	nd
156	64.4	2.7	nd	nd
157	768.0	nd	nd	nd
158	21.2	1	nd	nd
159	150.6	1.8	nd	nd
160	243.0	3.2	nd	nd
161	18.5	5	nd	nd

Regulated Mycotoxins Concentrations (µg/kg)				
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
162	304.1	3.1	nd	nd
163	23.8	6.7	nd	nd
164	424.7	1.9	nd	nd
169	288.4	205.3	nd	nd
170	381.7	20.3	nd	nd
171	116.1	5.8	nd	nd
172	247.0	3.8	nd	nd
173	322.5	0.4	nd	nd
177	314.6	1.1	nd	nd
178	532.2	6.3	nd	nd
179	1095.1	1	nd	nd
180	499.8	2.2	nd	nd
181	1602.6	nd	nd	nd
182	629.3	nd	nd	nd
183	1491.3	2.2	nd	nd
184	846.3	nd	nd	nd
185	3.7	nd	nd	nd
186	26.2	27.9	nd	nd

	Regulated Mycotoxins Concentrations (µg/kg)			
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
187	0.4	nd	nd	nd
188	258.5	nd	nd	nd
189	606.6	nd	nd	nd
190	103.7	nd	nd	nd
191	91.5	nd	nd	nd
192	13.4	nd	nd	nd
193	2260.1	nd	nd	nd
196	48.4	12.8	nd	nd
197	118.2	7.5	nd	nd
198	1458.6	nd	nd	nd
199	2127.1	nd	nd	nd
201	553.2	nd	nd	nd
202	1190.4	9.8	nd	nd
203	12.0	25.2	nd	nd
204	1881.1	8.1	nd	nd
205	1383.5	nd	nd	nd
206	316.8	nd	nd	nd
207	279.4	nd	nd	nd

	Regulated Mycotoxins Concentrations (µg/kg)			
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
208	50.7	nd	nd	nd
209	2.7	2.3	nd	nd
210	82.2	4.7	nd	nd
212	680.0	nd	nd	nd
213	266.6	nd	nd	nd
214	198.1	0.8	nd	nd
215	241.7	nd	nd	nd
216	499.3	nd	nd	nd
217	105.4	nd	nd	nd
218	345.0	nd	nd	nd
219	50.1	nd	nd	nd
220	224.4	4.7	nd	nd

Table 2. Mass spectrometry results for the regulated mycotoxins in organically produced unprocessed oats.

Regulated Mycotoxins Concentrations (µg/kg)				
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
56	1105.1	247.7	3.1	4.7
57	404.2	nd	nd	nd
58	895.3	nd	nd	nd
59	353.2	nd	nd	nd
60	127.9	nd	nd	nd
61	298.8	nd	nd	nd
62	248.8	nd	nd	nd
63	488.8	nd	2.7	nd
64	736.9	nd	nd	nd
65	372.6	nd	nd	nd
66	139.3	nd	nd	nd
67	244.2	nd	nd	nd
68	513.7	nd	nd	nd
69	257.4	nd	nd	nd
70	135.9	nd	nd	nd
71	839.6	nd	nd	nd
72	45.8	nd	nd	nd

Regulated Mycotoxins Concentrations (µg/kg)				
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
73	290.7	nd	nd	nd
74	506.7	nd	nd	nd
75	236.8	nd	nd	nd
150	14.3	5.6	nd	nd
151	66.8	9.8	nd	nd
152	19.4	4.7	nd	nd
165	9.4	3.4	nd	nd
166	21.1	4.7	nd	nd
167	1427.7	14.5	nd	nd
168	32.7	3.2	nd	nd
174	273.4	5.0	nd	nd
175	1304.3	12.5	nd	nd
176	107.8	2.6	nd	nd
194	2518.1	4.1	nd	nd
195	2583.0	nd	nd	nd
200	nd	nd	nd	nd
211	351.8	4.0	nd	nd
221	17.1	3.2	nd	nd

Regulated Mycotoxins Concentrations (µg/kg)				
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
222	25.1	248.4	nd	nd
223	nd	nd	nd	nd
224	nd	7.5	nd	nd
225	nd	5.6	nd	nd
226	115.1	nd	nd	nd
227	2964.6	24.1	nd	nd
228	4.1	504.1	nd	nd
229	17.5	7.3	nd	nd

Table 3. Mass spectrometry results for the regulated mycotoxins in conventionally produced processed oats.

Regulated Mycotoxins Concentrations (µg/kg)				
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
1	6.2	nd	nd	nd
2	7.8	261.1	4.6	1.6
3	7.6	nd	nd	0.9
4	17.6	47.4	nd	0.9
6	14.3	15.4	nd	nd
7	0.5	273.2	nd	3.1
12	7.1	nd	nd	0.8
13	1.8	nd	nd	nd
14	6.8	130.8	nd	0.6
15	9.1	26.0	nd	0.7
17	11.7	108.7	1.3	0.7
19	16.1	25.6	nd	1.1
21	16.2	191.3	nd	3.3
23	49.4	nd	4.4	nd
24	22.7	46.8	nd	1.4
25	13.7	24.7	nd	0.6
26	6.9	35.5	nd	0.5

Regulated Mycotoxins Concentrations (µg/kg)				
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
27	46.9	68.0	nd	nd
32	3.3	nd	nd	nd
33	nd	nd	nd	nd
34	2.9	nd	nd	nd
35	16.3	nd	nd	nd
36	1.0	nd	nd	nd
39	4.7	nd	nd	nd
40	nd	nd	nd	nd
41	1.2	nd	nd	nd
42	3.8	nd	nd	nd
43	0.2	nd	nd	nd
44	1.1	nd	52.4	nd
45	42.2	nd	nd	nd
46	1.0	nd	nd	nd
47	4.8	nd	nd	nd
48	3.0	nd	nd	nd
49	1.9	nd	nd	nd
50	nd	nd	nd	nd

Table 4. Mass spectrometry results for the regulated mycotoxins in organically produced processed oats.

	Regulated Mycotoxins Concentrations (µg/kg)			
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
5	33.2	nd	nd	nd
8	49.4	nd	nd	nd
9	1.0	nd	nd	nd
10	18.0	nd	0.6	nd
11	26.7	nd	nd	nd
16	38.8	nd	nd	nd
18	22.4	nd	nd	nd
20	38.0	nd	nd	nd
22	5.2	nd	nd	nd
28	22.7	nd	nd	nd
29	4.2	nd	nd	nd
30	20.7	nd	nd	nd
31	9.5	nd	nd	nd
37	15.3	nd	nd	nd
38	2.0	nd	nd	nd

Table 5. Mass spectrometry results for the regulated mycotoxins in unprocessed barley

	Regulated Mycotoxins Concentrations (µg/kg)			
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
230	15.0	nd	nd	nd
231	nd	nd	nd	nd
232	26.4	nd	nd	0.2
233	2.3	nd	nd	1.7
234	0.9	nd	nd	nd
235	2.4	nd	nd	nd
236	2.5	nd	nd	1.7
237	4.4	nd	nd	1.9
238	5.0	nd	nd	nd
239	3.3	nd	nd	1.1
240	4.1	nd	nd	nd
241	4.6	nd	nd	1.1
242	2.6	nd	nd	nd
243	4.6	nd	nd	nd
244	nd	nd	nd	nd
245	0.3	35.5	nd	10.2
246	3.5	nd	nd	nd

	Regulated Mycotoxins Concentrations (µg/kg)			
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
247	3.5	nd	nd	nd
248	4.7	nd	nd	nd
249	4.9	nd	nd	1.2
250	nd	nd	nd	nd
251	4.7	nd	nd	4.0
252	3.1	nd	nd	nd
253	3.7	nd	nd	10.0
254	4.3	nd	nd	3.8
255	1.1	nd	nd	nd
256	3.8	nd	nd	nd
257	2.7	nd	nd	nd
258	5.2	nd	nd	nd
259	0.3	nd	nd	1.3
260	5.4	nd	nd	nd
261	5.0	nd	nd	2.1
262	5.0	nd	nd	nd
263	5.2	nd	nd	nd
264	4.8	nd	nd	nd

	Regulated Mycotoxins Concentrations (µg/kg)			
Sample ID	**T-2 & HT-2**	**Deoxynivalenol**	**Ochratoxin A**	**Zearalenone**
265	5.1	nd	nd	1.2
266	nd	nd	nd	nd
267	nd	nd	nd	13.7
268	nd	nd	nd	nd
269	6.0	nd	nd	1.5
270	nd	nd	nd	2.1
271	nd	nd	nd	2.2
272	nd	nd	nd	8.0
273	nd	nd	nd	nd
274	nd	nd	nd	1.7
275	4.1	nd	nd	13.4
276	5.4	nd	nd	1.6
277	6.0	35.7	nd	2.8
278	4.6	nd	nd	3.5
279	3.2	nd	nd	2.2
280	5.2	nd	nd	nd
281	6.2	nd	nd	1.9

Appendix E

Table 1. Results of processed and unprocessed oat samples using mass spectrometry and four rapid test kits.

Sample ID	LC-MS/MS (µg/kg)	Test Kit 1 (µg/kg)	Test Kit 2 (µg/kg)	Test Kit 3 (µg/kg)	Test Kit 4 (µg/kg)
1	6.2	17.8	13.5	35.2	10.5
2	7.8	23.8	16.4	33.4	20.7
3	7.6	37.4	20.6	36.5	14.1
5	33.2	27.1	38.9	48.2	24.5
6	14.3	0	28.2	38.2	15.1
7	0.5	0	0	27.9	8.6
8	49.4	246.2	48.5	62.2	41.0
9	1.0	0	15.8	0	8.2
10	18.0	31.8	36.9	53.5	26.6
11	26.7	0	31.8	35.4	24.7
12	7.1	0	16.5	32.3	16.5
13	1.8	0	10.6	26.5	17.8
14	6.8	0	15.8	37.5	22.2
15	9.1	0	18	36.8	15.3
16	38.8	13.9	38.1	40.6	29.6
17	11.7	0	18.4	34.1	17.6

Sample ID	LC-MS/MS (µg/kg)	Test Kit 1 (µg/kg)	Test Kit 2 (µg/kg)	Test Kit 3 (µg/kg)	Test Kit 4 (µg/kg)
18	22.4	6.3	37.3	40.9	34.0
19	16.1	11.8	15.8	33.4	22.0
20	38.0	0	47.9	53	36.1
21	16.2	29.4	22.3	38.2	18.9
22	5.2	1.4	23.5	26.2	13.0
23	49.4	0.7	42.2	51.8	31.2
24	22.7	0	25.5	40.6	31.7
25	13.7	8.9	8.6	37.3	29.0
27	46.9	5.4	43	53	33.4
28	22.7	40.3	31.2	45.7	12.9
29	4.2	69.3	6.7	27	45.4
30	20.7	43	40.8	43.6	20.1
31	9.5	101.5	19.2	29.2	18.0
32	3.3	216.7	9.7	27.4	14.5
33	0.0	0	10.9	0	13.5
34	2.9	4.7	15.3	27.8	44.0
35	16.3	62.9	12.7	29.2	12.6
36	1.0	0.92	5.3	0	23.4

Sample ID	LC-MS/MS (µg/kg)	Test Kit 1 (µg/kg)	Test Kit 2 (µg/kg)	Test Kit 3 (µg/kg)	Test Kit 4 (µg/kg)
37	15.3	8.5	24.1	33	15.2
38	2.0	0	15.3	0	8.0
39	4.7	0.47	0	0	8.4
40	0.0	0	9.8	0	10.6
41	1.2	0.89	8.5	0	6.8
42	3.8	0	14.4	28.6	16.4
43	0.2	3.5	0	0	8.5
45	42.2	42.5	40.2	43.2	29.5
48	3.0	0	1.2	0	18.0
50	0.0	80.9	0	0	6.9
53	790.4	966	377.2	439.4	336.4
56	1105.1	1166.5	690	864	758.2
57	404.2	319.7	471.8	429.6	380.9
58	895.3	690.5	442.4	476.2	448.0
59	353.2	523	261.4	190	237.1
63	488.8	686.5	395.2	420.2	509.4
68	513.7	764.5	351.4	540.2	452.1
71	839.6	1122.5	966.1	> range	641.8

Sample ID	LC-MS/MS (µg/kg)	Test Kit 1 (µg/kg)	Test Kit 2 (µg/kg)	Test Kit 3 (µg/kg)	Test Kit 4 (µg/kg)
72	45.8	76.8	84.6	55.4	49.9
74	506.7	608	424.3	542.6	381.1
76	13.7	0	7.2	0	16.9
77	578.0	610	394.3	449.8	383.4
80	582.9	717	80.5	94	78.4
81	482.3	656.5	561.5	628.6	484.7
83	1024.8	694	506.8	619.5	429.7
85	388.8	572.5	532.6	346.1	425.4
86	387.3	355	303.8	307.1	247.8
88	536.2	474.5	286.2	521	350.9
90	1025.0	1354.5	986	1454.4	718.7
93	395.0	217.5	283.5	368	437.7
96	1080.0	1173.5	755.1	1020	913.0
100	0.0	1129.3	> range	> range	5092.4
101	55.4	21.1	42.1	37.1	28.4
108	80.9	21.8	0	0	10.2
116	8.2	1.2	0	0	28.6
117	458.2	416	317.9	503.8	297.5

Sample ID	LC-MS/MS (µg/kg)	Test Kit 1 (µg/kg)	Test Kit 2 (µg/kg)	Test Kit 3 (µg/kg)	Test Kit 4 (µg/kg)
118	417.8	487.5	270.3	481.4	337.7
130	0.0	0	6.1	0	19.0
134	3993.3	1477	1461.5	> range	2919.4
137	503.1	863.5	360.5	496.4	302.2
142	463.6	560.1	425.1	490	223.9
148	162.7	111.7	116.6	129	62.4
150	14.3	124.9	33.6	65.2	54.5
151	66.8	134.8	133.1	105.8	75.0
154	1114.6	1148	972.7	1497.9	985.5
155	808.5	625.5	461.3	736.2	629.5
157	768.0	1319	676.8	> range	694.8
164	424.7	478	515.9	457.6	256.1
165	9.4	36.1	46.3	50.2	28.5
167	1427.7	487	62.1	172.8	< range
169	288.4	268.6	281.4	227.3	181.3
178	532.2	490	348.9	438.6	359.0
179	1095.1	1452	949.9	1271.4	796.6
180	499.8	369	313	296.6	282.0

Sample ID	LC-MS/MS (µg/kg)	Test Kit 1 (µg/kg)	Test Kit 2 (µg/kg)	Test Kit 3 (µg/kg)	Test Kit 4 (µg/kg)
184	846.3	344.5	378.6	383.8	346.6
189	606.6	642	440	514.8	530.9
195	2583.0	2384.5	2331.1	3218	2962.2
198	1458.6	562.5	12.8	132	< range
201	553.2	Invalid	396.9	517	317.0
205	1383.5	832	Invalid	1011.2	905.9
216	499.3	490.7	515	614.8	525.8
218	345.0	489.6	288.4	303.9	321.0
223	0.0	0	8	0	8.1
227	2964.6	1284.5	477.2	1616	1299.4
229	17.5	264.1	42.1	42.5	30.6
ERM	160	148.8	126.6	163.1	109.7

Table 2. Qualitative (positive/negative) results for samples against the EU guidance limits for the sum of T-2 and HT-2 toxins (where a test kit result contradicts the LC-MS/MS result, this is highlighted in yellow(positive) or red (negative).

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
1	6.2	Negative	17.8	Negative	13.5	Negative	35.2	Negative	10.5	Negative
2	7.8	Negative	23.8	Negative	16.4	Negative	33.4	Negative	20.7	Negative
3	7.6	Negative	37.4	Negative	20.6	Negative	36.5	Negative	14.1	Negative
5	33.2	Negative	27.1	Negative	38.9	Negative	48.2	Negative	24.5	Negative
6	14.3	Negative	0	Negative	28.2	Negative	38.2	Negative	15.1	Negative
7	0.5	Negative	0	Negative	0	Negative	27.9	Negative	8.6	Negative
8	49.4	Negative	246.2	Positive	48.5	Negative	62.2	Negative	41.0	Negative
9	1.0	Negative	0	Negative	15.8	Negative	0	Negative	8.2	Negative
10	18.0	Negative	31.8	Negative	36.9	Negative	53.5	Negative	26.6	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
11	26.7	Negative	0	Negative	31.8	Negative	35.4	Negative	24.7	Negative
12	7.1	Negative	0	Negative	16.5	Negative	32.3	Negative	16.5	Negative
13	1.8	Negative	0	Negative	10.6	Negative	26.5	Negative	17.8	Negative
14	6.8	Negative	0	Negative	15.8	Negative	37.5	Negative	22.2	Negative
15	9.1	Negative	0	Negative	18	Negative	36.8	Negative	15.3	Negative
16	38.8	Negative	13.9	Negative	38.1	Negative	40.6	Negative	29.6	Negative
17	11.7	Negative	0	Negative	18.4	Negative	34.1	Negative	17.6	Negative
18	22.4	Negative	6.3	Negative	37.3	Negative	40.9	Negative	34.0	Negative
19	16.1	Negative	11.8	Negative	15.8	Negative	33.4	Negative	22.0	Negative
21	16.2	Negative	29.4	Negative	22.3	Negative	38.2	Negative	18.9	Negative
20	38.0	Negative	0	Negative	47.9	Negative	53.0	Negative	36.15	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
22	5.2	Negative	1.4	Negative	23.5	Negative	26.2	Negative	13.0	Negative
23	49.4	Negative	0.7	Negative	42.2	Negative	51.8	Negative	31.2	Negative
24	22.7	Negative	0	Negative	25.5	Negative	40.6	Negative	31.7	Negative
25	13.7	Negative	8.9	Negative	8.6	Negative	37.3	Negative	29.0	Negative
27	46.9	Negative	5.4	Negative	43	Negative	53	Negative	33.4	Negative
28	22.7	Negative	40.3	Negative	31.2	Negative	45.7	Negative	12.9	Negative
29	4.2	Negative	69.3	Negative	6.7	Negative	27	Negative	45.4	Negative
30	20.7	Negative	43	Negative	40.8	Negative	43.6	Negative	20.1	Negative
31	9.5	Negative	101.5	Negative	19.2	Negative	29.2	Negative	18.0	Negative
32	3.3	Negative	216.7	Positive	9.7	Negative	27.4	Negative	14.5	Negative
33	0.0	Negative	0	Negative	10.9	Negative	0	Negative	13.5	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
34	2.9	Negative	4.7	Negative	15.3	Negative	27.8	Negative	44.0	Negative
35	16.3	Negative	62.9	Negative	12.7	Negative	29.2	Negative	12.6	Negative
36	1.0	Negative	0.92	Negative	5.3	Negative	0	Negative	23.4	Negative
37	15.3	Negative	8.5	Negative	24.1	Negative	33	Negative	15.2	Negative
38	2.0	Negative	0	Negative	15.3	Negative	0	Negative	8.0	Negative
39	4.7	Negative	0.47	Negative	0	Negative	0	Negative	8.4	Negative
40	0.0	Negative	0	Negative	9.8	Negative	0	Negative	10.6	Negative
41	1.2	Negative	0.89	Negative	8.5	Negative	0	Negative	6.8	Negative
42	3.8	Negative	0	Negative	14.4	Negative	28.6	Negative	16.4	Negative
43	0.2	Negative	3.5	Negative	0	Negative	0	Negative	8.5	Negative
45	42.2	Negative	42.5	Negative	40.2	Negative	43.2	Negative	29.5	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
48	3.0	Negative	0	Negative	1.2	Negative	0	Negative	18.0	Negative
50	0.0	Negative	80.9	Negative	0	Negative	0	Negative	6.9	Negative
53	790.4	Negative	966	Negative	377.2	Negative	439.4	Negative	336.4	Negative
56	1105.1	Positive	1166.5	Positive	690	Negative	864	Negative	758.2	Negative
57	404.2	Negative	319.7	Negative	471.8	Negative	429.6	Negative	380.9	Negative
58	895.3	Negative	690.5	Negative	442.4	Negative	476.2	Negative	448.0	Negative
59	353.2	Negative	523	Negative	261.4	Negative	190	Negative	237.1	Negative
63	488.8	Negative	686.5	Negative	395.2	Negative	420.2	Negative	509.4	Negative
68	513.7	Negative	764.5	Negative	351.4	Negative	540.2	Negative	452.1	Negative
72	45.8	Negative	76.8	Negative	84.6	Negative	55.4	Negative	49.9	Negative
74	506.7	Negative	608	Negative	424.3	Negative	542.6	Negative	381.1	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
76	13.7	Negative	0	Negative	7.2	Negative	0	Negative	16.9	Negative
77	578.0	Negative	610	Negative	394.3	Negative	449.8	Negative	383.4	Negative
80	582.9	Negative	717	Negative	80.5	Negative	94	Negative	78.4	Negative
81	482.3	Negative	656.5	Negative	561.5	Negative	628.6	Negative	484.7	Negative
83	1024.8	Positive	694	Negative	506.8	Negative	619.5	Negative	429.7	Negative
85	388.8	Negative	572.5	Negative	532.6	Negative	346.1	Negative	425.4	Negative
86	387.3	Negative	355	Negative	303.8	Negative	307.1	Negative	247.8	Negative
88	536.2	Negative	474.5	Negative	286.2	Negative	521	Negative	350.9	Negative
90	1025.0	Positive	1354.5	Positive	986	Negative	1454.4	Positive	718.7	Negative
93	395.0	Negative	217.5	Negative	283.5	Negative	368	Negative	437.7	Negative
96	1080.0	Positive	1173.5	Positive	755.1	Negative	1020	Positive	913.0	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
101	55.4	Negative	21.1	Negative	42.1	Negative	37.1	Negative	28.4	Negative
108	80.9	Negative	21.8	Negative	0	Negative	0	Negative	10.2	Negative
116	8.2	Negative	1.2	Negative	0	Negative	0	Negative	28.6	Negative
117	458.2	Negative	416	Negative	317.9	Negative	503.8	Negative	297.5	Negative
118	417.8	Negative	487.5	Negative	270.3	Negative	481.4	Negative	337.7	Negative
130	0.0	Negative	0	Negative	6.1	Negative	0	Negative	19.0	Negative
137	503.1	Negative	863.5	Negative	360.5	Negative	496.4	Negative	302.2	Negative
142	463.6	Negative	560.1	Negative	425.1	Negative	490	Negative	223.9	Negative
148	162.7	Negative	111.7	Negative	116.6	Negative	129	Negative	62.4	Negative
150	14.3	Negative	124.9	Negative	33.6	Negative	65.2	Negative	54.5	Negative
151	66.8	Negative	134.8	Negative	133.1	Negative	105.8	Negative	75.0	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
154	1114.6	Positive	1148	Positive	972.7	Negative	1497.9	Positive	985.5	Negative
155	808.5	Negative	625.5	Negative	461.3	Negative	736.2	Negative	629.5	Negative
164	424.7	Negative	478	Negative	515.9	Negative	457.6	Negative	256.1	Negative
165	9.4	Negative	36.1	Negative	46.3	Negative	50.2	Negative	28.5	Negative
169	288.4	Negative	268.6	Negative	281.4	Negative	227.3	Negative	181.3	Negative
178	532.2	Negative	490	Negative	348.9	Negative	438.6	Negative	359.0	Negative
179	1095.1	Positive	1452	Positive	949.9	Negative	1271.4	Positive	796.6	Negative
180	499.8	Negative	369	Negative	313	Negative	296.6	Negative	282.0	Negative
184	846.3	Negative	344.5	Negative	378.6	Negative	383.8	Negative	346.6	Negative
189	606.6	Negative	642	Negative	440	Negative	514.8	Negative	530.9	Negative
195	2583.0	Positive	2384.5	Positive	2331.1	Positive	3218	Positive	2962.2	Positive

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
216	499.3	Negative	490.7	Negative	515	Negative	614.8	Negative	525.8	Negative
218	345.0	Negative	489.6	Negative	288.4	Negative	303.9	Negative	321.0	Negative
223	0.0	Negative	0	Negative	8	Negative	0	Negative	8.1	Negative
227	2964.6	Positive	1284.5	Positive	477.2	Negative	1616	Positive	1299.4	Positive
229	17.5	Negative	264.1	Negative	42.1	Negative	42.5	Negative	30.6	Negative
ERM	160	Negative	148.8	Negative	126.6	Negative	163.1	Negative	109.7	Negative

Table 3. Qualitative (positive/negative) results for samples against the proposed regulatory limits for the sum of T-2 and HT-2 toxins under discussion (where a test kit result contradicts the LC-MS/MS result, this is highlighted in yellow(positive) or red (negative).

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
1	6.2	Negative	17.8	Negative	13.5	Negative	35.2	Negative	10.5	Negative
2	7.8	Negative	23.8	Negative	16.4	Negative	33.4	Negative	20.7	Negative
3	7.6	Negative	37.4	Negative	20.6	Negative	36.5	Negative	14.1	Negative
5	33.2	Negative	27.1	Negative	38.9	Negative	48.2	Negative	24.5	Negative
6	14.3	Negative	0	Negative	28.2	Negative	38.2	Negative	15.1	Negative
7	0.5	Negative	0	Negative	0	Negative	27.9	Negative	8.6	Negative
8	49.4	Negative	246.2	Positive	48.5	Negative	62.2	Positive	41.0	Negative
9	1.0	Negative	0	Negative	15.8	Negative	0	Negative	8.2	Negative
10	18.0	Negative	31.8	Negative	36.9	Negative	53.5	Positive	26.6	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
11	26.7	Negative	0	Negative	31.8	Negative	35.4	Negative	24.7	Negative
12	7.1	Negative	0	Negative	16.5	Negative	32.3	Negative	16.5	Negative
13	1.8	Negative	0	Negative	10.6	Negative	26.5	Negative	17.8	Negative
14	6.8	Negative	0	Negative	15.8	Negative	37.5	Negative	22.2	Negative
15	9.1	Negative	0	Negative	18	Negative	36.8	Negative	15.3	Negative
16	38.8	Negative	13.9	Negative	38.1	Negative	40.6	Negative	29.6	Negative
17	11.7	Negative	0	Negative	18.4	Negative	34.1	Negative	17.6	Negative
18	22.4	Negative	6.3	Negative	37.3	Negative	40.9	Negative	34.0	Negative
19	16.1	Negative	11.8	Negative	15.8	Negative	33.4	Negative	22.0	Negative
20	38.0	Negative	0	Negative	47.9	Negative	53.0	Negative	36.15	Negative
21	16.2	Negative	29.4	Negative	22.3	Negative	38.2	Negative	18.9	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
22	5.2	Negative	1.4	Negative	23.5	Negative	26.2	Negative	13.0	Negative
23	49.4	Negative	0.7	Negative	42.2	Negative	51.8	Positive	31.2	Negative
24	22.7	Negative	0	Negative	25.5	Negative	40.6	Negative	31.7	Negative
25	13.7	Negative	8.9	Negative	8.6	Negative	37.3	Negative	29.0	Negative
27	46.9	Negative	5.4	Negative	43	Negative	53	Positive	33.4	Negative
28	22.7	Negative	40.3	Negative	31.2	Negative	45.7	Negative	12.9	Negative
29	4.2	Negative	69.3	Positive	6.7	Negative	27	Negative	45.4	Negative
30	20.7	Negative	43	Negative	40.8	Negative	43.6	Negative	20.1	Negative
31	9.5	Negative	101.5	Positive	19.2	Negative	29.2	Negative	18.0	Negative
32	3.3	Negative	216.7	Positive	9.7	Negative	27.4	Negative	14.5	Negative
33	0.0	Negative	0	Negative	10.9	Negative	0	Negative	13.5	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
34	2.9	Negative	4.7	Negative	15.3	Negative	27.8	Negative	44.0	Negative
35	16.3	Negative	62.9	Positive	12.7	Negative	29.2	Negative	12.6	Negative
36	1.0	Negative	0.92	Negative	5.3	Negative	0	Negative	23.4	Negative
37	15.3	Negative	8.5	Negative	24.1	Negative	33	Negative	15.2	Negative
38	2.0	Negative	0	Negative	15.3	Negative	0	Negative	8.0	Negative
39	4.7	Negative	0.47	Negative	0	Negative	0	Negative	8.4	Negative
40	0.0	Negative	0	Negative	9.8	Negative	0	Negative	10.6	Negative
41	1.2	Negative	0.89	Negative	8.5	Negative	0	Negative	6.8	Negative
42	3.8	Negative	0	Negative	14.4	Negative	28.6	Negative	16.4	Negative
43	0.2	Negative	3.5	Negative	0	Negative	0	Negative	8.5	Negative
45	42.2	Negative	42.5	Negative	40.2	Negative	43.2	Negative	29.5	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
48	3.0	Negative	0	Negative	1.2	Negative	0	Negative	18.0	Negative
50	0.0	Negative	80.9	Positive	0	Negative	0	Negative	6.9	Negative
53	790.4	Positive	966	Positive	377.2	Negative	439.4	Negative	336.4	Negative
56	1105.1	Positive	1166.5	Positive	690	Positive	864	Positive	758.2	Positive
57	404.2	Negative	319.7	Negative	471.8	Negative	429.6	Negative	380.9	Negative
58	895.3	Positive	690.5	Positive	442.4	Negative	476.2	Negative	448.0	Negative
59	353.2	Negative	523	Positive	261.4	Negative	190	Negative	237.1	Negative
63	488.8	Negative	686.5	Positive	395.2	Negative	420.2	Negative	509.4	Positive
68	513.7	Positive	764.5	Positive	351.4	Negative	540.2	Positive	452.1	Negative
72	45.8	Negative	76.8	Negative	84.6	Negative	55.4	Negative	49.9	Negative
74	506.7	Positive	608	Positive	424.3	Negative	542.6	Positive	381.1	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
76	13.7	Negative	0	Negative	7.2	Negative	0	Negative	16.9	Negative
77	578.0	Positive	610	Positive	394.3	Negative	449.8	Negative	383.4	Negative
80	582.9	Positive	717	Positive	80.5	Negative	94	Negative	78.4	Negative
81	482.3	Negative	656.5	Positive	561.5	Positive	628.6	Positive	484.7	Negative
83	1024.8	Positive	694	Positive	506.8	Positive	619.5	Positive	429.7	Negative
85	388.8	Negative	572.5	Positive	532.6	Positive	346.1	Negative	425.4	Negative
86	387.3	Negative	355	Negative	303.8	Negative	307.1	Negative	247.8	Negative
88	536.2	Positive	474.5	Negative	286.2	Negative	521	Positive	350.9	Negative
90	1025.0	Positive	1354.5	Positive	986	Positive	1454.4	Positive	718.7	Positive
93	395.0	Negative	217.5	Negative	283.5	Negative	368	Negative	437.7	Negative
96	1080.0	Positive	1173.5	Positive	755.1	Positive	1020	Positive	913.0	Positive

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
101	55.4	Negative	21.1	Negative	42.1	Negative	37.1	Negative	28.4	Negative
108	80.9	Negative	21.8	Negative	0	Negative	0	Negative	10.2	Negative
116	8.2	Negative	1.2	Negative	0	Negative	0	Negative	28.6	Negative
117	458.2	Negative	416	Negative	317.9	Negative	503.8	Positive	297.5	Negative
118	417.8	Negative	487.5	Negative	270.3	Negative	481.4	Negative	337.7	Negative
130	0.0	Negative	0	Negative	6.1	Negative	0	Negative	19.0	Negative
137	503.1	Positive	863.5	Positive	360.5	Negative	496.4	Negative	302.2	Negative
142	463.6	Negative	560.1	Positive	425.1	Negative	490	Negative	223.9	Negative
148	162.7	Negative	111.7	Negative	116.6	Negative	129	Negative	62.4	Negative
150	14.3	Negative	124.9	Negative	33.6	Negative	65.2	Negative	54.5	Negative
151	66.8	Negative	134.8	Negative	133.1	Negative	105.8	Negative	75.0	Negative

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
154	1114.6	Positive	1148	Positive	972.7	Positive	1497.9	Positive	985.5	Positive
155	808.5	Positive	625.5	Positive	461.3	Negative	736.2	Positive	629.5	Positive
164	424.7	Negative	478	Negative	515.9	Positive	457.6	Negative	256.1	Negative
165	9.4	Negative	36.1	Negative	46.3	Negative	50.2	Negative	28.5	Negative
169	288.4	Negative	268.6	Negative	281.4	Negative	227.3	Negative	181.3	Negative
178	532.2	Positive	490	Negative	348.9	Negative	438.6	Negative	359.0	Negative
179	1095.1	Positive	1452	Positive	949.9	Positive	1271.4	Positive	796.6	Positive
180	499.8	Negative	369	Negative	313	Negative	296.6	Negative	282.0	Negative
184	846.3	Positive	344.5	Negative	378.6	Negative	383.8	Negative	346.6	Negative
189	606.6	Positive	642	Positive	440	Negative	514.8	Positive	530.9	Positive
195	2583.0	Positive	2384.5	Positive	2331.1	Positive	3218	Positive	2962.2	Positive

Sample ID	LC-MS/MS (µg/kg)	LC-MS/MS (Pos/Neg)	Test Kit 1 (µg/kg)	Test Kit 1 (Pos/Neg)	Test Kit 2 (µg/kg)	Test Kit 2 (Pos/Neg)	Test Kit 3 (µg/kg)	Test Kit 3 (Pos/Neg)	Test Kit 4 (µg/kg)	Test Kit 4 (Pos/Neg)
216	499.3	Negative	490.7	Negative	515	Positive	614.8	Positive	525.8	Positive
218	345.0	Negative	489.6	Negative	288.4	Negative	303.9	Negative	321.0	Negative
223	0.0	Negative	0	Negative	8	Negative	0	Negative	8.1	Negative
227	2964.6	Positive	1284.5	Positive	477.2	Negative	1616	Positive	1299.4	Positive
229	17.5	Negative	264.1	Negative	42.1	Negative	42.5	Negative	30.6	Negative
ERM	160	Negative	148.8	Negative	126.6	Negative	163.1	Negative	109.7	Negative

Table 4. Results of the comparative study for Test Kit 1 (two laboratories)

Sample ID	**Test Kit 1 – Lab 1 (µg/kg)**	**Test Kit 1 – Lab 2 (µg/kg)**
1	39.9	17.8
2	57.9	23.8
3	27.0	37.4
5	111.3	27.1
6	67.5	0.0
7	30.2	0.0
8	283.5	246.2
9	8.4	0.0
10	61.6	31.8
11	67.6	0.0
12	21.9	0.0
13	21.9	0.0
14	24.2	0.0
15	64.3	0.0
16	31.4	13.9
17	147.6	0.0
18	71.8	6.3
19	113.1	11.8

Sample ID	Test Kit 1 – Lab 1 (µg/kg)	Test Kit 1 – Lab 2 (µg/kg)
21	143.8	29.4
22	29.8	1.4
23	36.3	0.7
24	197.2	0.0
25	176.9	8.9
27	215.6	5.4
29	5.7	69.3
30	105.9	43.0
31	20.4	101.5
32	51.6	216.7
33	50.1	0.0
34	51.6	4.7
35	64.6	62.9
36	40.9	0.9
37	40.9	8.5
38	50.5	0.0
39	50.5	0.5
40	57.0	0.0

Sample ID	Test Kit 1 – Lab 1 (µg/kg)	Test Kit 1 – Lab 2 (µg/kg)
41	20.2	0.9
42	70.7	0.0
43	70.7	3.5
45	59.3	42.5
48	209.1	0.0
50	73.0	80.9
148	165.9	111.7
150	14.2	124.9
151	24.7	134.8
154	1562.6	1148.0
155	2131.2	625.5
164	478.6	478.0
165	220.2	36.1
169	0.0	268.6
178	815.9	490.0
179	0.0	1452.0
180	0.0	369.0
184	0.0	344.5

Sample ID	Test Kit 1 – Lab 1 (µg/kg)	Test Kit 1 – Lab 2 (µg/kg)
189	0.0	642.0
195	0.0	2384.5
216	0.0	490.7
218	1099.3	489.6
223	279.3	0.0
227	0.0	1284.5
229	116.6	264.1

Table 5. Results of the comparative study for Test Kit 1 (three laboratories)

Sample ID	Test Kit 1 – Lab 1 (µg/kg)	Test Kit 1 – Lab 2 (µg/kg)	Test Kit 1 – Lab 3 (µg/kg)
1	39.9	57.0	17.8
2	57.9	36.8	23.8
5	111.3	44.7	27.1
6	67.5	0.0	0.0
7	30.2	0.0	0.0
11	67.6	32.0	0.0
15	64.3	29.1	0.0
16	31.4	0.0	13.9
17	147.6	43.7	0.0
18	71.8	0.0	6.3
21	143.8	50.6	29.4
22	29.8	0.0	1.4
23	36.3	0.0	0.7
24	197.2	42.9	0.0
25	176.9	35.0	8.9
27	215.6	51.9	5.4
40	57.0	30.0	0.0
41	20.2	0.0	0.9

Sample ID	Test Kit 1 – Lab 1 (µg/kg)	Test Kit 1 – Lab 2 (µg/kg)	Test Kit 1 – Lab 3 (µg/kg)
45	59.3	0.0	42.5

Appendix F

The **Institute for Global Food Security** in partnership with **Food Fortress**, ***safe*food** and **Agritox** will host a half-day workshop on the challenges of mycotoxins in food and feed.

Confirmed Programme

10:00-11:00	Food Fortress Annual General Meeting	**Mr Robin Irvine**, Food Fortress/Northern Ireland Grain Trade Association
11:00-11:10	Welcome and introductions	**Professor Chris Elliott,** Institute for Global Food Security, Queen's University Belfast
11:10-11:40	Towards a resilient and sustainable food system through efficient control and mitigation of mycotoxins	**Professor Rudolf Krska**, University of Natural Resources and Life Sciences, Vienna

		(BOKU), Austria, and Queen's University Belfast
11:40-12:10	Evaluating the impact of mycotoxins on livestock performance and environmental sustainability	**Dr Tobi Kolawole/Professor Chris Elliott**, Institute for Global Food Security, Queen's University Belfast
12:10-12:40	Assessment of rapid diagnostics for T-2 and HT-2 toxins in oats, *safe*food project Mycotoxin control in cereals: Safeguarding human food	**Dr Julie Meneely**, Institute for Global Food Security, Queen's University Belfast
12:40-13:10	The design, development, and implementation of an LC-MS method as a 'Toxicity Alert System' in the animal feed sector	**Dr Brett Greer**, Institute for Global Food Security, Queen's University Belfast
13:10-13:30	Concluding remarks	**Mr Robin Irvine**, Food Fortress/Northern Ireland Grain Trade Association **Professor Chris Elliott**, Institute for Global Food Security, Queen's University Belfast
13:30-14:30	Lunch & close	

Attendees: Measuring Mycotoxins: Applying Smart Agriculture – Smart Science (SASS) to mitigate against the growing and unknown issues of mycotoxins in feed and food.

Company/Institution Name	**Industry**
Alltech	Animal nutrition, health and feed supplements
Ballinaskeagh Grains Ltd	Animal feed raw materials
Cefetra Ltd	Food and feed raw materials
Corby Rock Mill	Animal feed
Cranswick Plc	Food company
Devenish Nutrition Ltd	Animal nutrition
Fane Valley Feeds Ltd	Animal feed
Flahavan & Sons Limited	Food company
Food Fortress Ltd	Animal feed industry co-operative
Glanbia Plc	Global nutrition company
Hyde Farm Feeds	Animal feed
John Thompson & Sons Ltd	Animal feed
Joseph Walls Ltd	Animal feed
Mason's Animal Feeds Ltd	Animal feed
Moore Animal Feeds Ltd	Animal feed
Moy Park	Poultry meat producer

Neogen Corporation	Food and animal safety
Phileo UK/Ireland Ltd	Animal feed additives
Precision Analysis Ltd	Animal feed analysis
Queen's University Belfast	Education
R & J Lyness Ltd	Animal feed
***safe*food**	Public body – food safety and nutrition
The Agri-Food and Biosciences Institute (AFBI)	The Department of Agriculture, Environment and Rural Affairs
The Northern Ireland Grain Trade Association	Represents those involved in the Northern Ireland agricultural supply trade
United Feeds Ltd	Animal feed
United Molasses Ireland Ltd	Animal feed raw materials
University of Natural Resources and Life Sciences, Vienna (BOKU)	Education
Volac International Ltd	Dairy nutrition
Whites Oats Ltd	Food company