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A desk study to review current knowledge on ergot alkaloids and their potential for contamination to cereal grains

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1. Abstract

Ergot alkaloids are produced by specific fungi on cereal plants. Ergotamine, ergosine and ergocristine are the most common found in the UK but levels and occurrence vary each year. There are legislative maximum limits (ML) for ergot sclerotia, but currently no MLs are set for ergot alkaloids in Europe. It has also been suggested that ergot alkaloids may be detected in grains in the absence of sclerotia. The European Commission (EC) has stated it will review MLs for ergot alkaloids in cereal grains from July 2016, with potential new legislation to be agreed by July 2017.

The Agriculture and Horticulture Development Board (AHDB) have identified that there is a need to improve understanding of this alkaloid issue to ensure the UK can provide the most relevant evidence to the EC and supporting data to the European Food Safety Authority (EFSA) in preparation for this consultation and potential regulation.

This desk study report has been conducted to review the current literature on ergot alkaloids, including relevant information from outside of the UK. This includes whether they can be detected in grain samples in the absence of visible sclerotia, and if so, how substantiated this evidence is, with additional information on the impact of ergot alkaloids on human and animal health. The review summarises information currently available, its strengths and limitations and concludes by identifying the knowledge gaps that require further investigation and proposing how these might be addressed to inform regulators in the development of future legislation.

2. Glossary

AHDB	Agriculture and Horticulture Development Board
APCI	Atmospheric pressure chemical ionization
ARfD	Acute Reference Dose
b.w.	Body Weight
CCCF	Codex Committee on Contaminants in Foods
CE	Capillary Electrophoresis
CEN	European Committee for Standardization (Comité Européen de Normalisation)
CPS	Canada Prairie Spring wheat
CWAD	Canada Western Amber Durum wheat
CWES	Canada Western Extra Strong wheat
CWRS	Canada Western Red Spring wheat
DON	Deoxynivalenol
DDGS	Dried distillers grains with solubles
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
ESI	Electrospray ionization
EU	European Union
FLD	Fluorescence Detection
FWHM	Full Width at Half Maximum height
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
HPTLC	High-Performance Thin-Layer chromatography
HRMS	High Resolution Mass Spectrometry
KB	Karnal bunt
LB	Lower bound
LC-MS/MS	Liquid Chromatography tandem Mass Spectrometry

LC-QTOF-MS	Liquid chromatography time-of-flight mass spectrometry
LLE	Liquid-liquid extraction
LOAEL	Lowest Observed Adverse Effect Level
LOD	Limit of Detection
LOQ	Limit of Quantification
MALDI	Matrix-Assisted Laser Desorption Ionization
MS	Mass spectrometry
MIP	Molecularly Imprinted Polymer
ML	Maximum Limit
QUECHERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RSD	Relative Standard Deviation
SPE	Solid Phase Extraction
TDI	Tolerable Daily Intake
TEA	Total Ergot Alkaloids
TOF	Time-of-Flight
UB	Upper bound
UHPLC-HRMS	Ultra Performance Liquid Chromatography High Resolution Mass Spectrometry
UPLC-MS/MS Ultra	Performance Liquid Chromatography tandem Mass Spectrometry
UV	Ultra-Violet
VS	Veterinary Surgeons
WHO	World Health Organisation

3. Introduction

3.1. Introduction

There are at least twelve ergot alkaloids (and epimers) which are produced when ergot fungus infects cereal plants. Ergotamine, ergosine and ergocristine are the most common ergot alkaloids produced in the UK but their levels and frequency varies each year. Although processors reject grain when sclerotia are observed, it has been suggested that there could be physical transfer of alkaloids produced from ergot sclerotia onto grain or that these alkaloids are translocated within the cereal plants. Thus, even in the absence of physical sclerotia, alkaloids may still be detected in grain samples. Currently, analysis is dependent on lengthy 'confirmatory' laboratory testing for alkaloids, which is expensive (AHDB has stated that the UK cost of this testing could be c. £25m p.a.) and only 2-3 laboratories in the UK can undertake them. The usual time taken for this type of test is ten to fourteen days, so it cannot be used by processors on site.

Although maximum levels for ergot sclerotia are in place within Member States (and in Codex guidance) for cereals, there is currently no legislation setting maximum levels for ergot alkaloids in Europe. The EC has stated that it will be reviewing the maximum acceptable limit for ergot alkaloid levels in cereal grains from July 2016, with a potential for new legislation to be agreed by July 2017. There is, therefore, an urgent need to review understanding of this alkaloid issue to ensure the UK can provide the most relevant evidence and supporting data to EFSA in preparation for this consultation and potential regulation.

3.2. Review targets

One focus of this review is the effects of ergot alkaloids on human and animal health. Human ergotism was first recognised in the Middle Ages and even though modern grain cleaning methods have now largely eliminated human exposure to ergot alkaloids, it still remains an issue from an agricultural point of view (Bennett and Klich, 2003). Fungal alkaloids are not inactivated by rumen microflora, and thus can have significant effects on rumen function. There have been several cattle ergotism outbreaks where ingestion of feed contaminated with a cocktail of ergot alkaloids has resulted in symptoms such as diarrhoea, lameness and gangrene (EFSA, 2012). More recently, ergot toxicosis was shown to be the cause of death of eight calves fed a pelleted creep feed in the USA (Leuschen *et al.*, 2014). Ergovaline and other ergot alkaloids such as ergotamine have been reported to cause cardio vascular, pulmonary and body temperature effects on sheep (McLeay *et al.*, 2002). The ingestion of alkaloids in dairy cattle has been correlated with decreased feed intake, reduced milk

production and loss of body weight. There is evidence that ergot alkaloids can have a negative effect on dairy reproduction by decreasing the secretion of luteinising hormone, plasma prolactin and follicle-stimulating hormone. This, combined with the fact that ergot alkaloids act as dopamine receptor agonists and vaso-constrictors, explains why they interfere with ovulation, luteal function and pregnancy maintenance leading to reduced pregnancy rates and increased embryo mortality (Klotz, 2015).

The other factor considered in this review is whether alkaloids can be detected in the absence of visible sclerotia. In the past, the presence of alkaloids was presumed to be due to the presence of sclerotia, or fragments of dust due to the high concentration of alkaloids found in the sclerotia (high mg/kg concentrations). However at a recent World Mycotoxin Forum, in June 2016, Dr Shelley Tittlemier presented results that showed that alkaloids were present in the honeydew that exudes during part of the growth cycle (see Figure 1 below, AHDB, 2014). The concentrations reported were < 1 mg/kg in the honeydew, however this would be enough to contaminate grain at low µg/kg levels without visible presence of sclerotia. The review will consider evidence to confirm or rule out this possible contamination route, and will also review all other possible sources of alkaloids in grain products.

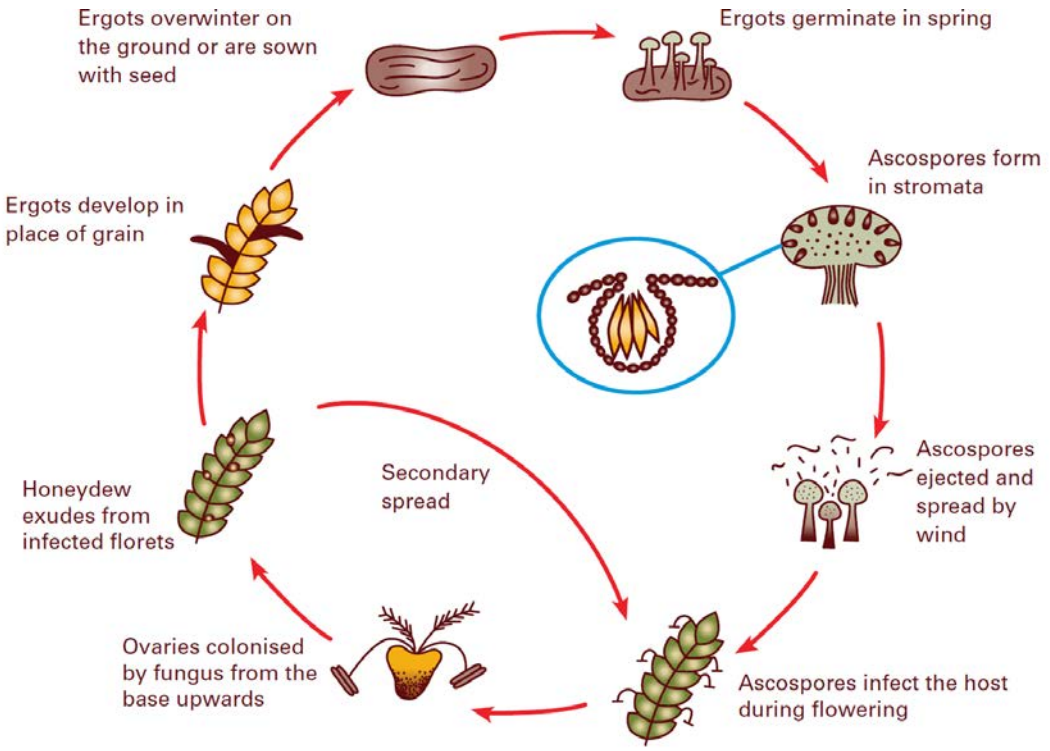


Figure 1. Ergot life cycle (AHDB, 2014)

4. Literature search methodology

4.1. Peer review literature

A literature review at Fera Science Ltd. accessed numerous secondary sources indexing a substantial body of the relevant scientific literature. Web of Science, provided access to:
Web of Science™ Core Collection (1981-present)
BIOSIS Citation Index℠ (1985-present)
CABI : CAB Abstracts® and Global Health® (1973-present)
Current Contents Connect® (1998-present)
FSTA® - the food science resource (1969-present)
KCI-Korean Journal Database (1980-present)
MEDLINE® (1950-present)
Russian Science Citation Index (2005-present)
SciELO Citation Index (1997-present)
Zoological Record® (1993-present)

In addition, Science Direct gave access to approximately 3,800 journals and more than 35,000 book titles.

Terms of reference for the literature search are given in Table 1.

Table 1. Search terms used during the literature search.

Ergocornine	Cereal
Ergometrine	Oilseeds
Ergocristine	Toxicology
Ergotamine	Analyses
Ergosine	Methodology
Ergocryptine	Exposure
Ergocorninine	Rye
Ergometrinine	Wheat
Ergocristinine	Triticale
Ergotaminine	Barley
Ergosinine	Millet
Ergocryptinine	Oats
Ergopeptine	Ergotism
Ergot alkaloids	Biomarkers
Occurrence	Prevention
Sclerotia	Good agricultural practice
Grain	Tillage
Human health	Reduction
Animal health	

Both Web of Science and Science Direct were searched using all the terms provided in Table 1. Three search strings were used, all with the timespan of 2000 – 2016, and the results were as follows (Table 2):

Table 2. Results of search strings for Web of Science and Science Direct.

Search Number	Web of Science	Science Direct
1	334	24
2	146	21
3	180	14
Total	**660**	**59**

The results were considered by experts and once irrelevant and duplicate results were removed, the final results were as follows (Table 3):

Table 3. Combined results for searches of Web of Science and Science Direct

Search Number	Combined Results
1	227
2	66
3	60
Total	**353**

4.2. Recording results – peer reviewed literature

Results were stored in EndNote software record storage and retrieval system.

4.3. Grey literature search

A grey literature review was also carried out to complement the peer reviewed literature identified in other sources. The search included online databases, including PubMed, Defra, Gov.UK, Open Grey, The National Archives, Bielefeld Academic Search Engine (BASE), COPAC, All Academic Research, Federal Science Library (Canada), Royal Society of Chemistry and Google Scholar. COPAC allows users to search the catalogues of ca. 90 libraries including the UK national libraries, University libraries, and specialist libraries.

The objective of the grey literature review was to collate non-peer reviewed reports and data on ergot alkaloids, the analyses, exposure, toxicology and incidence on an international scale. It included all material relative to the search terms given in Table 1. Again, a timespan of 2000

- 2016 was applied. The search was carried out between the 25th September and the 5th October 2016.

Grey literature results from the online databases were limited, relative to open access search engines, such as GoogleScholar. GoogleScholar's search criteria limit the results available to view to the first 1000 results. This introduces a degree of bias as the results are automatically sorted through the Google algorithm, although this is not really pertinent for this study as during this search some grey literature sources gave a greater number of results than GoogleScholar. Table 4 summarises the number of results of the grey literature searches, including where there was a nil return. There were a large number of duplicates found between the grey literature sources and the peer reviewed literature.

Table 4. Total number of relevant grey literature results from each of the sources searched

Source	**Total number of results**
BASE	43
COPAC	3
Defra	2
GoogleScholar	15
Open Grey	1
PubMed	368 (including duplicates)
Royal Society of Chemistry	10
Defra	0
Gov.uk	0
The National Archives	0
All Academic Research	0
Federal Science Library	0
Total	**442**

4.4. Recording results – grey literature

Results were recorded in Microsoft Excel and Endnote™ and included search terms, total number of references returned, number of published references returned, number of grey literature references returned that fit the search criteria and the URL for the documents.

4.5. Summary of results

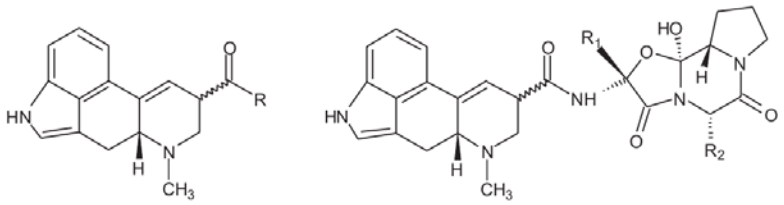
In total, 353 results were found for the peer review search, with an additional 442 from the grey literature (although this included some duplicates), giving 795 results.

Reports identified in the Grey Literature have been used in the main text of this report to supplement to findings from other sources. Press reports and suppliers' literature have been excluded due to potential reporting bias.

5. Ergot alkaloids in grain samples

5.1. Summary of current knowledge

Ergot alkaloids are mycotoxins produced by several members within the fungal orders of *Hypocreales* and *Eurotiales* with *Claviceps purpurea* being the most widespread species found within Europe. These fungal species are known to infect more than 400 plant species, including economically important cereal grains such as rye, wheat, triticale, barley, millet and oats. Ergot alkaloids are classified into four major groups; clavinet alkaloids, simple lysergic acid derivatives, ergopeptine alkaloids and ergopeptam alkaloids. The two groups of most relevance to this review are the lysergic acid derivatives that include ergometrine, and the ergopeptine group, also known as the cyclol ergot alkaloids and includes ergotamine which are found in sclerotia from *Claviceps purpurea.* The alkaloids identified in sclerotia of *Claviceps purpurea*, and reported in recent literature data, are ergometrine, ergotamine, ergosine, ergocristine, ergocryptine (a mixture of α- and β- isomers), ergocornine, and the corresponding -inine epimers that can undergo interconversion under various conditions, the ergopeptinines. The latter are (S)-diastereoisomers produced by epimerisation at the C-8 position, of which respective names end with the '-inine' suffix (Figure 2) (Bryla *et al.,* 2015). The former are derivatives of lysergic acid, whereas the latter are relatives of isolysergic acid (Müller *et al.,* 2009). The isomerisation speed and ratio of isomers depend on conditions (particularly pH and temperature) and on the alkaloid structure (Kokonnen *et al.,* 2010).



alkanolamides ergopeptides

Compound	Type	Moiety R	Moiety R_1	Moiety R_2
Lysergic acid diethyl amide (LSD)	Alkanolamide	$NH(C_2H_5)_2$	–	–
Ergometrine/-inine	Alkanolamide	$NHCH(CH_3)CH_2OH$	–	–
Ergocornine/-inine	Ergopeptide	–	$CH(CH_3)_2$	$CH(CH_3)_2$
Ergocristine/-inine	Ergopeptide	–	$CH(CH_3)_2$	$CH_2C_6H_5$
Ergokryptine/-inine	Ergopeptide	–	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
Ergosine/-inine	Ergopeptide	–	CH_3	$CH_2CH(CH_3)_2$
Ergotamine/-inine	Ergopeptide	–	CH_3	$CH_2C_6H_5$

Figure 2. Chemical structures of the major ergot alkaloids.

In nature, ergot alkaloids are organic bases. Acid dissociation constant values, pKa, for ergopeptines range from 5.5 (ergocristine) to 6.0 (ergometrine), while those for ergopeptinines range from 4.8 (ergocorninine) to 6.2 (ergometrinine). Natural ergopeptines are always accompanied by ergopeptinines. Ergopeptines may transform into ergopeptinines during long-term storage (particularly if storage conditions are improper) or during the extraction of ergot alkaloids from cereals during analysis. Different epimers may have different biological and physicochemical properties. Ergopeptines are reportedly more active biologically than ergopeptinines (Krska, *et al.*, 2008).

High levels of ergot alkaloids in cereal based food and feed products can cause ergotism (gangrene and/or hallucinations and convulsions) in both humans and in animals and at lower levels of contamination they can cause vasoconstriction and reproductive effects (Schiff, 2006). An EFSA opinion in 2012 (EFSA, 2012) concluded that vasoconstrictive effects provided a suitable reference point for establishment of a group acute reference dose of 1 µg/kg body weight (b.w.) and a group tolerable daily intake of 0.6 µg/kg b.w. per day. The EFSA panel concluded that the available data did not indicate concern for any population subgroup but indicated that dietary exposure estimates were related to a limited number of food groups and that a possible unknown contribution from other foods which could not be discounted. It was also recommended that 'Collection of analytical data on occurrence of ergot alkaloids in relevant food and feed commodities should continue'. Currently, ergot alkaloids in food are not regulated under EU Regulations though the Commission adopted a

Recommendation on the monitoring of the presence of ergot alkaloids in feed and food (EU Recommendation; 2012/154/EU). Following this, the setting of Maximum Levels (MLs) is seen as a means of managing the risks from ergot alkaloids in food, where some Member States are already enforcing their own legislative levels, e.g. Germany (BfR, Stellungnahme Nr. 024/2013, 2013). Recommendation 2012/154/EU stated the intention that MLs would be established by July 2017, however negotiations are ongoing within the EU.

However, it will not be possible to enforce emerging Regulations unless suitable testing methods are available. Currently ergot alkaloids pose an analytical challenge, with only a few laboratories in the UK able to perform the analysis. There are currently no validated, rapid methods of analysis that can be used at grain storage facilities or at milling houses prior to the use of the raw product in preparing food and feedstuffs. Instead, methods which use state of the art detection technologies tend to be focussed on the analysis of the ergot alkaloids in processed grains, food and feedstuffs. Stakeholders in the industry as well as enforcement bodies have expressed concern that due to the lack of validated, rapid methods of analysis it is not be possible for them to ensure that compliant grain enters the food chain. Current analytical methods are laboratory based and need specialised laboratory equipment and analytical calibration standards. At present, the main methods employed by laboratories use high performance liquid chromatography with fluorescence detection (HPLC-FLD) and high performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) to allow the determination of individual ergot alkaloids in food and feed commodities at relevant levels with sufficient confidence. Turn-around times are 10 to 14 days, meaning these methods are not suitable for either enforcement authorities or industry for rapidly analysing for ergot alkaloids. A current project at Fera, funded by European Committee for Standardization (CEN), aims to validate an LC-MS/MS method for ergot alkaloids to support future legislation. The ergot alkaloids pose additional difficulties for laboratories undertaking their analysis as some of the key compounds are classified as Category 1 drug precursors and in the UK, a UK Home Office Licence is required for their use, storage and distribution. Similar arrangements exist in other countries to comply with European wide drug pre-cursor legislation, which restricts the buying, selling or storing of the relevant compounds (EC, 2004).

There has been an increase in interest in ergot alkaloids in recent years following reports from EFSA (EFSA, 2012) and an EU Recommendation (EU Recommendation; 2012/154/EU). In addition, there is evidence that the incidence of grain rejections in Canada due to the presence of ergot alkaloids has grown year on year since 1999 (Menzies, 2016). There have been two significant and comprehensive collections of articles published recently about ergot alkaloids. A special issue of Toxins Journal published in 2015 included papers reviewing many aspects

of ergot alkaloids from their use as therapeutic drugs, to effects on animals, the genetics of the infecting fungi, biosynthesis of the toxins, transfer to edible tissues, analytical methods and biology and management of ergots in rye and other cereals (Schardl, 2015). The other major recent publication is an on-line book published by Frontiers in Chemistry – "Recent investigations of ergot alkaloids incorporated into plant and/or animal systems", edited by Smith and Klotz (2015). This book covers a range of topics although mainly focusses on exposure and effects on different animals. It should be noted however, that many of the articles relate to exposure via ingestion of ergovaline and tall fescue grass and pasture, and not via infection from grain materials.

5.2. Current legislation

A maximum level of 0.05% ergot was specified in Regulation 2000/824/EC (EC, 2000) for durum wheat, wheat and rye (bread grain or cereals) taken over by intervention agencies. According to Regulations 2003/1784/EC (EC, 2003) and 2005/1068/EC (EC, 2005) intervention on rye has been suspended but a maximum value of 0.05% remains for ergot in durum wheat and wheat according to Regulation 2009/1272/EU (EU, 2009). In Regulation 2015/1940/EU (EU, 2015) a maximum level of 0.5 g/kg ergot sclerotia is laid down for certain unprocessed cereals with the exception of corn and rice. This amends Regulation 2006/1881/EC (EC, 2006) that controls various contaminants in food, and makes provision for limits for ergot alkaloids to be set for unprocessed cereals (except corn and rice), cereal milling products, bread, pastries, cakes, biscuits, cereal snacks, breakfast cereals and pasta, and baby food by July 2017. However, currently, individual ergot alkaloids are not regulated in grain and grain-based food at the European level but some national controls are in place. Monitoring ergot alkaloids in feed and food is strongly recommended by Recommendation 2012/154/EU (EU, 2012) and Regulation 2015/1940/EU (EU, 2015), with a maximum level for the sum of the major ergot alkaloids in relevant food categories currently being considered (EU, 2015). In the United States, there are limits of 0.3% ergot bodies (sclerotia) in wheat and rye for both human consumption and animal feed, and 0.1% for barley, oats and triticale. However, there are no maximum levels established for ergot alkaloids in any grains. In Canada, guideline levels for *S. cornutum* (*Claviceps purpurea)* have been determined by the Canadian Grain Commission for the grading of cereal grains ranging from 0.01% for the highest quality grades, up to 0.1% for the lowest quality grades, values are laid out in primary grade determinant tables by variety (Canadian Grain Commission; https://www.grainscanada.gc.ca/oggg-gocg/04/oggg-gocg-4e-eng.htm). In Germany, as a comparison, the toxicological assessment by the Federal Institute for Risk Assessment (BfR, 2013) indicates 1000 µg/kg of total ergot alkaloids (TEA) as the "guidance level" for cereals,

flour and cereal based foods. This assessment refers to Article 14 of Regulation 2002/178/EC (EC, 2002) stating that food may not be placed on the market if it is harmful. This total ergot alkaloid value is calculated taking account of an ergot sclerotia maximum level of 0.05% for durum wheat and wheat and an average content of 0.26% ergot alkaloids in pure *C. purpurea* in Central Europe, as stated by EFSA (EFSA, 2005). However in the EFSA opinion of 2012, a more recent publication was cited that reported the range for the ergot alkaloid content in pure *C. purpurea* for Europe, to vary from 0.01-0.24 % with a mean value 0.076 % ergot alkaloids in sclerotia (EFSA, 2012).

5.3. Occurrence of ergot alkaloids

Much ergot alkaloid occurrence data comes from literature on mixed mycotoxins. The table shown in Appendix 1 summarises information in papers from the last 5 years for ergot alkaloids occurrence and includes a brief description of the information found in each paper. Appendix 1 also contains a detailed summary of the concentrations of ergot alkaloids found in the different matrices analysed. In addition, some occurrence data is summarised in the EFSA opinion of 2012 (EFSA, 2012). Data summarised here are more recent.

Boestfleisch *et al.* (2015) have illustrated the geographical incidence of the invasive ergot *Claviceps purpurea* var. *spartinae* which has become recently established in the European Wadden Sea on common cordgrass, is genetically homogeneous and the sclerotia contain high amounts of ergot alkaloids. Ergocristine, α-ergocryptine and their epimers were identified in samples in high amounts which were comparable to average values in sclerotia found on rye. Comparisons with reference strains indicated that the isolates along the Wadden Sea belonged to the clade also found in Ireland and Wales. The sclerotia found in the drift lines still contain equally high amounts of ergot alkaloids as those collected directly from the plants.

5.4. Occurrence in cereals – feed

Ergotamine was the most abundant ergot alkaloid found in wheat feed from Australia (Blaney *et al.,* 2009) with ergocornine and ergosine being predominant in wheat sclerotia from England (Bayles *et al*., 2009). It is likely that different strains of the *Claviceps purpurea*, epimerisation and other transformations occur under field, storage, and processing conditions.

Malysheva *et al.,* (2014) reported on the ‘Pattern and distribution of ergot alkaloids in cereals and cereal products from European countries”. This study is an update of a survey that the group conducted for EFSA to produce data on the six ‘ine’ and ‘inine’ epimers in foodstuffs

and animal feeds. The report incorporates the largest of the more recent surveys undertaken to estimate both human and animal exposure to these ergot alkaloids (Diana Di Mavungu *et al.,* 2011), as well as some additional data. Results for a total of 1065 samples of cereals and cereal products intended for human and animal consumption were reported and 59% of all samples analysed were contaminated with ergot alkaloids to some degree. Of significance is that the study considered the determination of the specific alkaloids in the foodstuffs rather than sclerotia. The pattern of ergocristine/ inine as the most predominant ergot alkaloid was not found in the European samples analysed in this survey. Malysheva *et al.,* (2014) rather reported ergocryptine as the predominant ergot alkaloid in rye and wheat-based food and rye feed whereas ergosine was the most abundant compound in wheat feed. The co-occurrence of all six ergot alkaloids was reported for 35% of the positive samples, and where a single ergot alkaloid was found this was most commonly ergometrine. The data obtained in this study is summarised in Table 5.

Table 5 Descriptive statistics for the total ergot alkaloid content in the different matrix groups. (Reproduced from; Malysheva *et al.,* 2014)

	Rye food	Wheat food	Multigrain food	Rye feed	Wheat feed	Triticale feed
Total number of samples (n)	226	332	186	157	137	27
Incidence of positive samples (%)	84	67	48	52	27	44
Mean (µg/kg)	87	62	7	311	19	62
Median (µg/kg)	28	7	<LOQ	1	<LOQ	<LOQ
Maximum (µg/kg)	1,121	591	123	12,340	701	1,103
Minimum (µg/kg)	<LOQ[1]	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
75th percentile (µg/kg)	87	54	8	54	1	5
90th percentile (µg/kg)	233	246	23	615	12	154
95th percentile (µg/kg)	393	375	36	1,456	52	787

[1] LOQ = limit of quantification: 1 µg/kg.

It is of note that the highest levels and frequencies of contamination were reported for rye feed collected in Switzerland. Total ergot alkaloid content ranged from 833 to 12,340 µg/kg in 6 out of 7 samples, with the total alkaloid content in the other sample being 61 µg/kg. This was not representative generally of levels in samples analysed from Europe, with wide variances of generally low level medians and averages to occasional much higher levels of over 1,000 µg/kg being detected. An example was given of rye feed samples analysed from Germany where the majority of samples contained an alkaloid content of < 15 µg/kg from ten samples with the remaining two samples giving values of 1260 and 1,145 µg/kg. No Dutch rye feed was found to be contaminated at levels above 500 µg/kg and were the 'cleanest' in general in the European samples analysed.

Wheat feed samples were generally low relative compared to rye feed. Extremes of low level medians and averages with occasional higher total ergot alkaloid levels were found e.g., 313 µg/kg in one Dutch sample, 379 µg/kg in one sample from the Czech Republic and one of 701 µg/kg from Denmark.

Generally triticale samples were not as highly contaminated with ergot alkaloids as rye and wheat feed, as noted earlier, although one sample from the Czech Republic contained 1,103 µg/kg.

Rye feed was the only sample where the ergot alkaloid content exceeded 2,000 µg/kg (4.5% of samples) and showed the largest variation in concentration. In this case, a mean of 311 µg/kg and a median of 1 µg/kg were observed. Wheat feed was characterised by the highest number of negative (< LOQ) samples (73%) and a median at < LOQ level. The triticale feed samples showed higher levels than the wheat. The total ergot alkaloid content was above 154 µg/kg in 10% of the triticale samples with a maximum of 1,103 µg/kg, whilst the concentration at the 90th percentile for wheat feed samples was 12 µg/kg and the maximum was 701 µg/kg. Representative data showing the relative distributions of the ergot alkaloids in the food and feed samples is shown as the authors published it in Figure 3.

5.5. Occurrence in cereals – food

The most extensive summary of ergot alkaloid occurrence in cereals and cereal products for human consumption was given by Malysheva *et al.* (2014) described above. They found 59% of samples to be contaminated with ergot alkaloids to some extent and 55% contained levels of 'ine' isomers above the LOQ. Contamination with the 'inine' isomer was found in 51% of these samples. The frequency for contamination was higher in food than in feed with the number of positive samples highest in rye food (84%), followed by wheat food (67%), rye feed (52%), multigrain food (48%) and triticale feed (44%), while only 27% of wheat feed contained ergot alkaloids. The range of ergot alkaloids determined ranged from < LOQ to 12,340 µg/kg with an average content of 89 µg/kg. The median results for individual 'ine' and 'inine' ergot alkaloids were 1 and 2 µg/kg respectively. Samples with an alkaloid content of < 1 µg/kg were considered negative regardless of the LOQ.

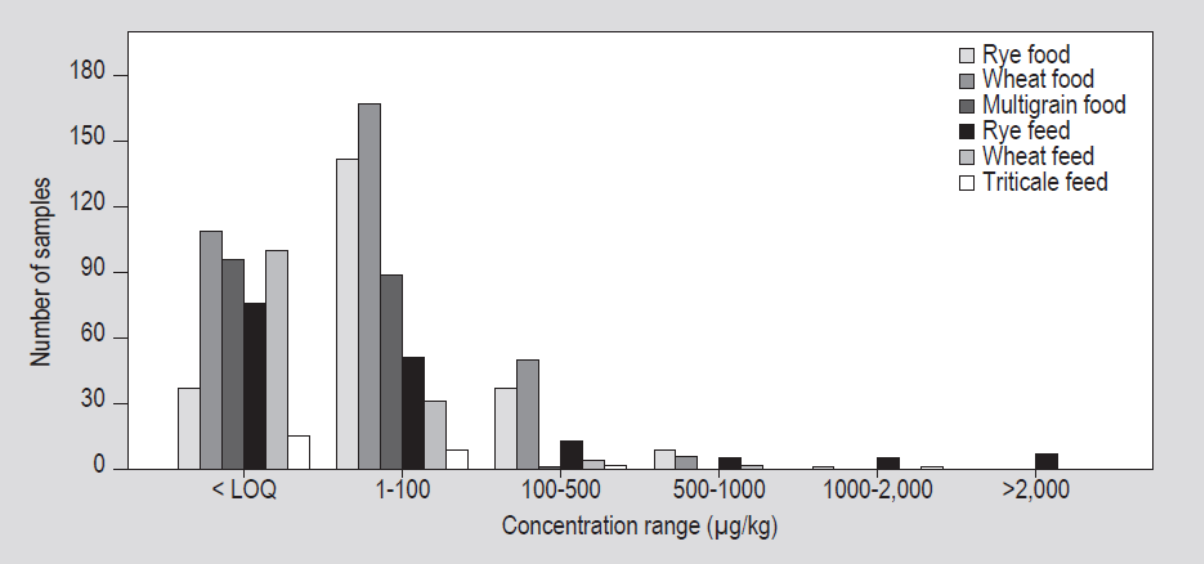


Figure 3. Distribution of the total ergot alkaloid content in different matrix groups based on the total content (Reproduced from Malysheva *et al.,* 2014)

From Figure 3 the highest incidence of contamination in the samples analysed lay in the 1 - 100 µg/kg concentration range. Interestingly, multigrain food samples contaminated at levels of 100 - 500 µg/kg were clearly the lowest, and were not determined at > 500 µg/kg for this matrix group. The mean content in multigrain food was 7 µg/kg and the maximum was 123 µg/kg (Table 5 above).

For cereals grown conventionally, a maximum level of 591 µg/kg with an average of 89 µg/kg was determined, while the same statistics for organically grown produce were 72 µg/kg and 8 µg/kg respectively.

Further breakdown of the data of Malysheva *et al.* (2014) reflects potential exposure to ergot alkaloids determined in food matrices from the different types of flours, breads, crispbreads, etc. made up of rye, wheat or multigrain. The incidence of ergot alkaloids in the flour samples was high; 62% in wheat flour, 84% in rye flour and 94% in multigrain flour. Rye flour gave the highest maximum value at 1,121 µg/kg but with a low median of 34 µg/kg. The highest median was noted for rye grain intended for human consumption at 731 µg/kg, although the data were not shown. With respect to bread samples, rye bread and multigrain bread had the highest maxima of 94 and 67 µg/kg respectively compared with wheat bread at 17 µg/kg. Rye bread and rye based crispbread had a similar profile of 18 and 12 µg/kg averages and medians of 4 and 10 µg/kg respectively. The maximum in rye bread was higher than in rye-based

crispbread (29 µg/kg). In wheat food, ergot alkaloids were detected in 84% of the wheat bran samples (115 out of 137). Among the negative wheat bran, 15 samples were of organically grown origin.

The main ergot alkaloids occurred with the corresponding epimers. The total content varied from < LOQ to 590 µg/kg with a mean value of 133 µg/kg and a median of 64 µg/kg. Wheat grain had the lowest concentration range with a maximum of 2 µg/kg. The total content in multigrain food was somewhat similar among the different product types. The largest observation was detected in multigrain crispbread at 123 µg/kg whilst the highest median was at 10 µg/kg in multigrain flour. Only 6% of multigrain flakes were positive for ergot alkaloids; these samples also characterised by the lowest alkaloid concentrations with a maximum value of 1 µg/kg.

In wheat bran, lot to lot variability was extensive in 66 samples produced in Belgium comprising 15 lots from the same manufacturer (Malysheva *et al.* (2014)). Levels from 159 to 590 µg/kg were seen in some lots from 2011, while other lots from the same year were as low as < LOQ to 114 µg/kg with the median lying between 18 and 63 µg/kg. Most of the lots sampled in 2012 from the same manufacturer contained low concentrations e.g. < LOQ to 76 µg/kg although two lots were found to contain high concentrations with maximum levels of 523 and 396 µg/kg.

Unlike rye feed samples, ergot alkaloids were found in the majority of rye food samples. Some examples of the available data are that French, Estonian and Polish samples tested positive in 93, 97 and 100% cases respectively. Examples of the spread of these values are the French the median value of 135 µg/kg, and the minimum and maximum of < LOQ to 928 µg/kg, whereas Polish and Estonian samples showed approximately the same concentrations at the 75th percentile of 121 and 124 µg/kg respectively. In one sample from the UK the level was found to be 1,121 µg/kg total ergot alkaloid content.

Wheat food and multigrain foods generally had low levels of contamination. The most contaminated wheat food samples contained maxima of 590 and 544 µg/kg in samples from Belgium and France respectively. The highest maximum alkaloid concentration was found in a multigrain sample from Belgium which contained 123 µg/kg. The only two samples from the UK and Germany were contaminated at relatively low levels; with 8 and 30 µg/kg found for the UK and German samples, respectively. For Dutch samples, the median was <LOQ indicating that about half of the samples were not contaminated with ergot alkaloids. 14 out of 20 multigrain food samples (70%) from France were contaminated at levels not higher than

52 µg/kg. However it was noted that many of the multigrain products were processed foods, and that heat treatment can result in a reduction of ergot alkaloids.

Whilst the data have been further broken down, probably the most relevant representation of the data lies in the mean and maximum concentrations (µg/kg) of the individual ergot alkaloids in different matrix groups (Appendix 1).

The authors observed that a high correlation was found between individual and total alkaloid content suggesting that any ergot alkaloid could be used to indicate the total alkaloid content. However, this should be taken with caution as co-occurrence of six ergot alkaloids was only noted in 35% of the positive samples, and more data would be needed to support this statement. Overall the highest frequencies of contamination were noted in food samples when compared with feed. However, the highest levels of total ergot alkaloids found in individual samples were found in feed (up to 12,340 µg/kg in Swiss rye feed). Multigrain food had generally lower ergot alkaloid contamination compared with other investigated foods, but these were on the whole processed products that had undergone some form of heat treatment. Interestingly samples produced via conventional cultivation of the raw material were found to contain higher levels than samples from organic production. This is possibly due to guidelines for organic farming advising against the use of hybrid species which are more susceptible to *Claviceps sp.* infection.

Bürk *et al.* (2006) reported results of 66 commercial rye products collected from the Bavarian marketplace. None of the samples exceeded a total alkaloid content of 1000µg/kg, a guidance level used for ergot alkaloids in cereal products in Germany (BfR, 2013). The highest levels were found in rye breads, of 23 samples tested 14 contained ergot alkaloids, the highest level found was 258 µg/kg. Other products, including rye crispbread, pumpernickels and rye-bread rolls contained lower levels of alkaloids in the range of <LOQ (between 0.1 and 1 µg/kg) and 91 µg/kg in a sample of rye-bread rolls (Bürk *et al.*, 2006).

The presence of ergot alkaloids in rye flour from Saxony were reported with approximately 50% of samples positive and the highest level found was 1063 µg/kg (Reinhold and Reinhardt, 2011). A small survey of UK rye-based cereal products was reported in 2009 by Crews *et al*. in which a small number (28) of samples were analysed and ergot alkaloids were detected in 25 out of 28 products. All eleven crispbreads contained ergot alkaloids at concentrations up to 340 µg/kg, and rye breads contained up to 121 µg/kg. The other products tested, loaf breads, bread-mix flours and crackers contained only low levels (Crews *et al.*, 2009). Lopez *et al.,* (2016) reported ergot alkaloids in all cereal composites in a total diet study in the

Netherlands. Only low levels (<12 µg/kg for each epimer pair) were found, ergocristine (-inine) was the most commonly found alkaloid in all products, and rye products did not contain higher levels than other cereal products.

Bryla *et al.* (2015) reported measurable levels of alkaloids in most of the analysed samples (particularly rye flour samples). A single sample of flour contained ergot alkaloids at a combined level of 1,216 µg/kg. The authors stated that their results, although they were obtained from a relatively small number of samples, suggested that ergot alkaloid levels in rye-based food products (and fodder) should be monitored on a wider scale.

Additionally, alkaloids were determined in ergot sclerotia that were isolated from rye grains and the total content was nearly 0.01% (97.9 mg/kg). However, the alkaloid profile was dominated by ergocristine at 44.7 mg/kg (45.6% share in total), which is not among the most commonly found alkaloids in tested food products. Ergocorninine at the mass fraction of 0.2 mg/kg (0.2% share in total) was the least abundant alkaloid. The absolute levels and contribution of individual compounds are detailed in the publication. As noted in the 'Exposure' section below, as well as here, these profiles change so it cannot be estimated from these analyses for the sclerotia alone what the contribution to the food or grain might be.

Uhlig *et al.* (2013) applied a semi-quantitative LC-MS method to carry out a pilot study of mycotoxin occurrence in Norwegian grains after unusually wet growing seasons. The total number of samples tested was not explicitly stated, but the authors reported up to 25% of barley samples were positive for ergocristine/inine (max level 68 µg/kg), while 15% were positive for ergocryptine/inine (max level 59 µg/kg). Only 4% of oats were positive for any ergot alkaloid, with only trace levels (sub µg/kg) detected, while wheat was found to contain a range of alkaloids up to 79 µg/kg ergotcryptine/inine. It is hard to understand the total concentrations found in individual samples due to the way the data is reported, but the study highlighted that about a quarter of barley and wheat contained measurable ergot alkaloid compounds (Uhlig *et al.*, 2013).

Slaiding and Byrd (2013) reported the results of analysis of UK grown cereals for ergot alkaloids in crops from harvests from 2009, 2010 and 2011. Analysis was carried out on grain that had been rejected following checks for the presence of sclerotia. The highest level found was 339 µg/kg in a wheat sample. An organic wheat was analysed before and after sieving and contrary to expectation, a higher level was found after sieving to remove sclerotia, although this this could be explained by sample heterogeneity. A similar result was observed for barley, while highly contaminated rye contained lower levels of ergot alkaloids after sieving.

It is clear from these observations that the mechanism of distribution of alkaloids is still not understood and more work should be done to determine the impact that sieving may have on the distribution of ergot alkaloids.

Despite grain cleaning at mills being able to remove up to 82% of ergot bodies (sclerotia; Posner and Hibbs, 1997) these surveys continue to highlight that the alkaloids continue to enter the human food chain.

The UK Food Standards Agency has funded a project to monitor the presence of ergot alkaloids in cereals. In addition, a possible relationship between the sclerotia content and the levels of ergot alkaloids was investigated (Byrd *et al.,* 2014). In total, 209 samples of cereals (wheat, barley, oats and rye) were analysed for ergot alkaloids. Rye was found to be most frequently contaminated and at the highest concentration, with up to 370 µg/kg total ergot alkaloids being found. The results reported indicated a significant reduction in ergot alkaloid levels in samples that underwent cleaning using an industrial ergot sclerotia cleaning process although the number of samples tested was not statistically significant (Byrd *et al.,* 2014).

The only other recent survey of UK foodstuffs for ergot alkaloids was published by the Food Standards Agency in 2011 (FSA, 2011). This reported the results of a survey of 100 UK purchased cereal products. Overall low levels were found, with 12% of the samples tested found to be positive for ergot alkaloids, the range of concentrations found was 1 – 169 µg/kg. The data was submitted to EFSA.

6. Effects of Ergot Alkaloids on Animal and Human Health

6.1. Exposure of Animals

The contamination and exposure route for animals falls into two distinct sources, fescue grass and cereals and cereal derived products. Different profiles of alkaloids are found in these matrices with ergovaline and related compounds being found in fescue grass and related materials, and the ergosine / ergotamine compounds found in cereals. *Claviceps pupurea* is responsible for producing the ergopeptides principally discussed in this report and named by EFSA (2012) as being the twelve compounds of main interest with respect to discussion of legislation in 2016/2017 due to their occurrence in cereals. However, in some cases, where feedstuffs have been mixed (Craig *et al.,* 2014), as well as finding ergovaline representative of tall fescue in the straw, ergotamine has also been found in the pellets produced. Indicators

and impacts of exposure could be exacerbated and compounded by a cocktail of toxins in this case, an aspect that has so far had little investigation.

Causative levels for toxicological effects of ergotism in some animals are very low, e.g., effects on livestock being generally observed when total dietary concentrations of these compounds exceed 100 to 200 µg/kg (Evans, 2001 and Miskimins *et al.*, 2015). Consequently, the quality of these grains used in feed might need to be re-addressed to reduce exposure occurrences amongst animals. Miskimins *et al.* (2015) reported that calves weighing between 181 and 300 kg, eating approximately 5 to 7 kg of creep feed containing total ergot alkaloid concentrations of 200 µg/kg (no ergovaline from fescue etc.) showed signs of lameness and tail loss. Zachariasova *et al.* (2014) have assessed the content of mycotoxins in European feedstuffs to estimate dietary intake of farm animals. All the samples analysed were from the Czech Republic (256 samples) and the UK (87 samples) between 2008 and 2012. The UK samples were collected as part of the Quality and Safety of Feeds and Food for Europe (QSAFFE) project headed by Queens University, Belfast (http://www.qsaffe.eu/). In this work, a highly selective U-HPLC-Qtrap MS/MS analysis was used to determine the ergot alkaloid content at very low levels (µg/kg) in feedstuffs. As opposed to the general incidence of mycotoxins reported in the paper, ergot alkaloid analyses accounted for relatively few positives (Appendix 1) and generally were found at low levels. Arithmetic mean concentrations were calculated using half of the limit of quantification (LOQ) value (µg/kg). Although all the ergot alkaloids of interest for future legislation were sought in these samples, only four positives were reported, ergosine, ergocryptine, ergocornine and ergocristine. Making dry weight corrections and using average animal intakes of the various feeds, dietary intake data could be calculated. There were relatively few feedstuffs where µg/kg b.w. intakes could be calculated from test results. Ergocornine, ergocryptine and ergocristine were calculated as < 0.001; < 0.001 and 0.01 µg/kg b.w. respectively in maize-based DDGS (Dried distillers' grains with solubles), and as 0.01, 0.02 and 0.06 µg/kg b.w. for wheat-based DDGS respectively for dietary intake of dairy cattle. Therefore, the maize silage, complex compound feed for cows, malt sprouts and brewers' grains held no exposure implications for cows in this study.

For pigs, there were no calculable levels of dietary intake for ergosine in malt sprouts, maize-based DDGS or wheat-based DDGS. However, there was a calculable level of 0.17 µg/kg b.w. dietary intake of ergosine in complex compound feed for pigs. Ergocryptine was not present in malt sprouts but dietary intake values of 0.09, < 0.01 and 0.02 µg/kg b.w. were calculated in the complex compound feed for pigs, maize-based DDGS and wheat-based DDGS respectively. Likewise, ergocristine was not present in malt sprouts but dietary intake

values of 0.26, 0.01 and 0.06 µg/kg b.w. were calculated in the complex compound feed for pigs, maize-based DDGS and wheat-based DDGS respectively.

Similarly, for laying hens, dietary intake of ergot alkaloids in feedingstuffs and feedingstuff supplements (as µg/kg b.w.) was: <0.01 for ergocornine; ergocryptine, ergocristine (for maize-based DDGS) and 0.05 for ergocornine, <0.01 for ergocryptine and 0.02 for ergocristine (for wheat-based DDGS). However, the complex compound feed for chickens and laying hens and malt sprouts had no levels determined and therefore had no exposure implications.

The study noted the most contaminated feedstuff for all mycotoxins was DDGS, which was true also for the ergot alkaloids. Likewise, the highest contamination level was in the 'basic' feeding stuff of the complex compound feed for pigs.

In conclusion concentrations were generally not alarming from the legislative point of view. However, the cumulative effects of a cocktail of a high frequency of mycotoxins / ergot alkaloids analysed and for which there is very little literature published may be of concern.

Zachariosa *et al.* (2014) discussed storage and processing conditions for feedstuffs, with respect to the EU countries, and concluded that these are not comparable on a broad basis with those found in some developing countries where differences in climate make these quality criteria more difficult to maintain.

In the European survey conducted on behalf of EFSA, rye feed was the only product where the ergot alkaloid content exceeded 2,000 µg/kg (4.5% of samples) and showed the largest variation in concentration (Malysheva *et al.,* (2014)). In this case, a mean of 311 µg/kg and a median of 1 µg/kg were observed, which are significant compared to Miskimins *et al.,* 2015 values of 200 to 300 µg/kg in feed being a trigger value for effects in cattle.

6.2. Effects on animals

There are several well documented effects of ergot alkaloids on livestock and it is important to note which type of feedstuff and fungi is responsible for different types of ergot exposure. Canty *et al.,* (2014) expressed these combinations in a simple table reproduced here (Table 6).

Table 6. Mycotoxicoses in cattle and horses associated with fungal infections of grasses or cereals by members of the *Clavicipitaceae* family, (based on data from Radostits *et al.* (2007), and Mostrom *et al.* (2011), table reproduced from Canty *et al.* (2014)).

Fungus [Grass & cereal]	Mycotoxin	Disease	Clinical signs and pathogenesis
Neotyphodium lolii [Perennial ryegrass (*Lolium perennae*)]	Lolitrems (Lolitrem B), an indole-diterpene toxin	Ryegrass staggers	When disturbed gross incoordination, falling hypersensitivity. Functional derangement of nervous tissue function. No histological lesions
Neotyphodium coenophialum [Tall fescue (*Festuca arundinaceae*)]	Ergovaline, an ergot alkaloid	Fescue toxicosis	Low milk yield or weight gain, hypersalivation, seek shade. Depression of blood prolactin concentrations
		Fescue foot	Loss of tail switch, distal limbs, tail tip gangrene. Local vasoconstriction restricts blood supply
[Perennial ryegrass (*Lolium perennae*)]		Prolonged gestation	Long gestation, dystocia, abortion, stillbirth, agalactia. Vasoconstriction cause placental edema, reducing circulating prolactin
Claviceps purpurea [Cereals, rye, triticale, grains, grasses]	A range of ergot alkaloids, principally ergotamine, but also ergocristine, ergosine, ergocorine and ergocryptine	Ergotism	Lameness, gangrene of lower limbs, ear tips, loss of tail switch. Arteriolar spasm causes deficient blood supply body parts
		Hyperthermia	Hyperthermia, salivation, dyspnea. Reduced blood supply to skin reduces heat loss

Dänicke (2015) reported a duck feeding study using ergot contaminated rye, comprising of 45.2% ergot and 54.8% rye (w/w) in which the total ergot alkaloid content amounted to 436 mg/kg. This was used to prepare four different diets containing 1, 10, 15, and 20 g ergot/kg diet respectively, which corresponded to total ergot alkaloid contents of 0.6, 7.0, 11.4, and 16.4 mg/kg diet. The total included the alkaloids ergonovine, ergotamine, ergocornine, ergocristine, ergotcryptine and ergosine and their -inine isomers, all 12 'EFSA' ergot alkaloids. Dänicke discussed the total of 'key alkaloids', at a lower concentration than the total but there seems little value in viewing the data in this way as all alkaloids present contribute to the observed effects.

The ducks were allowed free feeding, one group was also allowed to choose between a control feed and the highest concentration feed. This group appeared to learn to differentiate between the control and contaminated diets from the second week and the voluntary intake of the contaminated diet reduced to less than 2%. In the main study, feed intake decreased continuously during the first week for all ergot containing diets compared to the control, with 91%, 72%, 59 and 53% of feed consumed compared to the control. This decrease was more pronounced in the second week. Although no duck died during the study, feeding from the 2 highest ergot alkaloid containing diets (Ergot 3 and 4) was stopped after 2 weeks as feed intake had decreased by more than 50% for these two groups. The author noted that several things may have led to the feed reduction, including the fact that ducks are known to be sensitive to bitter taste, and the ergot alkaloids present would presumably have caused this

type of taste. The presence of other toxic substances in ergot, such as ricinoleic acid, was also noted and needs to be considered for further investigation. Nonetheless, there were effects observed at the lowest concentration of ergot alkaloids (0.6 mg/kg TEA). The author claimed this suggests that existing ergot alkaloid limits for poultry (1 g ergot/kg unground cereal grains in EFSA regulations) may not offer sufficient protection for ducks.

Furthermore, Dänicke (2005) analysed liver, breast meat and serum from these ducks and found alkaloid residues were lower than 5 ng/g. Ergonovine was the only alkaloid detected in bile (40 ng/g) from the ducks fed Ergot 2 diet (the second lowest level diet). In contrast, Mainka *et al.* (2005) identified no adverse effects on weight gain/live performance of chickens when fed diets containing up to 11.1 mg/kg ergot alkaloids, although some clinical effects were observed for diets containing 2.8 mg and 11.1 mg total ergot alkaloids/kg. These authors stated that further studies are necessary to define the critical level of ergot alkaloids dependent on alkaloid pattern. In contrast, the negative performance of ducks when exposed to 0.6 mg/kg of ergot alkaloids, indicates that not all species of poultry are equally tolerant of dietary ergot.

Miskimins *et al.* (2015) reported twelve of 100 beef calves in central South Dakota had lost tail switches and tail tips in the summer of 2014. All twelve affected animals had tail lesions and three were suffering from early lameness. Chemical analysis demonstrated multiple ergopeptine alkaloids in the creep feed (which usually has a base of cracked corn, rolled oats, alfalfa, brewer's grain or any combination of these four) which suggested ergotism caused by *Claviceps spp*, specifically *Claviceps purpurea*. Rapid identification of the cause of unusual distal extremity lesions is important to reduce suffering of affected animals and financial losses to owners. Calves were consuming 10 to 15 lb (4.5 to 6.8 kg) of the creep feed per day at the time of the outbreak and the creep feed had been fed for more than one month. Ergot bodies are difficult or impossible to recognize in processed or pelleted feeds. Analysis of one of the feed samples detected 25 µg/kg ergosine, 30 µg/kg ergotamine, 20 µg/kg ergocornine, 35 µg/kg ergocryptine, and 95 µg/kg ergocristine for a total of 205 µg/kg in the feed. Adverse effects on livestock performance are generally observed when total dietary concentration of these compounds exceeds 100 to 200 µg/kg.

In a perspectives article, Craig *et al.* (2015) reported investigations of cases of ergotism in livestock and the associated ergot alkaloid concentrations in feed. In this article the authors considered eight examples of clinical cases that had been submitted to the Endophyte Service Laboratory (ESL). They reported a marked increase in the number of ergot cases reported to ESL in the past five to ten years from approximately once per month; to several cases per week and sometimes multiple cases each day in recent years, despite maintaining

approximately the same number of total samples tested. The worst toxicosis cases were in cattle and horses. They suggested that the increase in cases seen in the Pacific Northwest and throughout the USA is likely to be due to climate change or the lack of field burning that has been phased out throughout the years. The stoppage of burning has greatly reduced the ability to destroy *Claviceps purpurea* by fire.

The samples of feed and plant material were analysed using an HPLC method with fluorescence detection. However, one critical point to note was that the method did not measure ergonovine and the epimer forms of the other ergot alkaloids (-inines), so any concentrations discussed may be underestimated by a significant amount.

This is worth noting as although the 'inine' epimers are reported to be relatively inactive in laboratory animals (Blaney *et al.,* 2009), it has also been proposed that this form might be converted into the active 'ine' form under physiological conditions (Mulac and Humpf, 2011). Ruminants may be affected as the isomers could be inter-converted with active epimers in the rumen (Blaney *et al.,* 2009). Therefore, the 'inine' epimers should not be ignored in studies on toxicity or environmental dissemination. In the EFSA opinion of 2012, the 'inine' forms were included in the risk assessment as EFSA concluded that interconversion can occur in both acid and alkaline conditions and therefore they had to be considered in the assessment (EFSA, 2012).

Craig *et al.* (2015) have constructed a table of the relative vascular potency and efficacy of a number of ergot alkaloids, using data published from a number of other sources. They considered that ergovaline and ergotamine have equal sensitivity for vasoconstriction whereas ergonovine, ergocristine, and ergocornine are about one-tenth as powerful. The samples tested for the comparison were found to contain from ~500 µg/kg ergot alkaloids to ~62,000 µg/kg ergot alkaloids (measuring only 'ine' forms). The symptoms observed in cattle ranged from tail loss at the lowest concentrations (ca 500 µg/kg) to hooves sloughing completely off at 62,000 µg/kg, with early term abortions, low milk yield and reduced feed intake observed at the intermediate levels. In all cases, except one, the cattle had consumed pellets as nearly 100% of their feed source. The only exception was a case which used a grass seed as the feed. In this case, the total alkaloid content was ~1,500 µg/kg, however the level expressed as ergotamine equivalence was lower than any of the other samples due to the profile of the ergot alkaloids present. In this case the observed clinical symptoms were limited to moderate lameness.

Pharmacologically, the half-maximal concentration (EC_{50}) and onset concentration are measures of a compound's potency (the lower the concentration the more potent the

compound); ergotamine and ergovaline are identical in this aspect. The onset of vasoconstriction is reported as concentration (moles/L), and is viewed as the best indicator of toxicosis when evaluating levels in feedstuffs (Craig *et al.,* 2015). Using the highest and the lowest equivalent ergotamine concentrations calculated from their study, the authors used a series of assumptions around bioavailability (that it was 100%). They then postulated that the lowest concentration feed would result in an ergotamine (equivalence as the most potent form of alkaloids) blood concentration of 0.215 µg ergotamine / ml blood. This is significantly higher than the onset concentration of 0.005 µg ergotamine / ml blood. The highest concentration sample resulted in a much higher blood concentration estimate and therefore more severe clinical effects. Of course, this is based on many assumptions, does not take account of the presence of 'inine' forms, and presumes 100% bioavailability, which has not been verified. The authors stated that the clinical effects observed were due to vasoconstriction and could occur regardless of seasonal conditions as well as noting that no threshold levels have been established for different animal species.

Klotz *et al.* (2013) demonstrated that the EC_{50} for ergovaline, ergotamine, and ergocornine can be reduced by an animal's prior exposure to ergot alkaloids although it was found in one treatment regime (KY31 pasture treatment) only. These results indicated there was an increase in sensitivity to ergovaline, ergotamine, and ergocornine in the lateral saphenous veins isolated from steers that grazed this pasture (KY31), and that the chronic exposure to ergot alkaloids that occurs through grazing toxic endophyte-infected tall fescue pastures, alters vasculature at the receptor level. This may have an influence on the animal's perceived sensitivity (as reported by the clinician/researcher) and may affect the EC_{50} threshold of an individual animal. Other experiments reported in the same study found ergovaline-, ergotamine-, and ergocornine-induced contractile responses were suppressed by antagonism of the 5HT2A receptor with ketanserin. They suggested that receptor antagonism as a means of mitigating the effects of ergot alkaloids could be a solution to fescue toxicosis syndrome. However, they also noted that because the alkaloids in their study had previously been shown to bind receptors irreversibly, any potential antagonist (such as ketanserin) would have to be administered to the animals before any ergot alkaloid exposure, which could limit its potential. These findings however, introduce the possibility of mitigating the effects of ergot alkaloids by the development of a vaccine, or other veterinary intervention. Filipov *et al.* (1998) reported an initial positive response in rabbits inoculated with fescue grass alkaloids, although it seems no further work has been reported.

Current data form the basis for risk assessments and predictions about further spreading of *Claviceps purpurea var. spartinae* on *S. anglica* (common cord grass). Intoxication with

Claviceps purpurea infected *S. anglica* is less likely in humans since this plant is not part of normal human diet, however, poisoning may occur in animals grazing on infected *S. anglica*. Strickland *et al.*, (2011) reviewed the state of ergot alkaloid research concerning grazing livestock and noted that owners of horses and small ruminants often have not recognized this problem although the losses are great. A significant number of the articles about animal ergot poisoning relate to intoxication from ingestion of grass and tall fescue. Ergot poisoning is not only a problem in animal husbandry but has recently also been observed in wild animals (Norwegian cervids) probably consuming wild grasses infected with *Claviceps purpurea*. Ergocristine was the dominant alkaloid in most sclerotia extracts examined during these poisoning incidents (Uhlig *et al.,* 2007). Uhlig *et al.* (2013) in their article "Faces of a Changing Climate: Semi-Quantitative Multi-Mycotoxin Analysis of Grain Grown in Exceptional Climatic Conditions in Norway." state “as fungi may produce a variety of different metabolites, animals and humans that live on a grain-based diet are exposed to a complex chemical cocktail. Therefore, the composition of this cocktail, rather than certain groups of toxins/metabolites alone, needs to be considered when assessing the safety of grain or grain-based products. Knowledge regarding the commonly co-occurring metabolites in grain can further provide the basis for future toxicological studies on combined effects.” This is a good summary of a complex situation.

Literature data illustrate the nature of exposure to animals feeding on wild grasses and note the increasing spread of *Claviceps purpurea* in geographical locations around Europe. Of course if these areas were used for haylage then the concomitant likelihood of exposure would be increased.

Craig *et al.* (2014) established threshold levels for Fescue Toxicosis (ergovaline) and lolitrem B (another ryegrass fungal toxin) for camels, cattle, horses and sheep and they stated that concentrations in the range 300-500 µg/kg for ergovaline for cattle and horses and 500-800 µg/kg for sheep would lead to symptoms in the animals. The authors did note that in addition to cases of ergotism caused by fescue, in the Pacific Northwest, cases of ergotism due to *Claviceps purpurea* contamination in feed have been increasing. They recommended feeding trials should needed to be conducted to establish threshold of toxicity levels in livestock species for the collection of ergot alkaloids that cause this disease (mainly the EFSA 12 compounds). The cases reported included:

- Consumption of affected fescue grass and ergovaline resulting in a total of 600 cows dying, being humanely euthanized, or aborted.
- When 1500 steers in Lethbridge, Alberta, Canada, were supplemented in January with silage and wheat and barley grain, clinical signs of dry gangrene of the ears and feet became

evident, with 45 steers being euthanized or died. The grain was infected with *Claviceps purpurea* at the level of 3473 µg/kg total ergot alkaloids. This total included 262 µg/kg of ergosine, 545 µg/kg of ergotamine, 753 µg/kg of ergocornine, 613 µg/kg of α-ergocryptine, and 1299 µg/kg of ergocristine.

- A third case found 330 steers, bulls, and pregnant beef cows, on frozen pastureland in Coburg, USA, being supplemented with tall fescue straw plus pellets made from perennial ryegrass screenings combined with steamed corn. Ergovaline representative of tall fescue in the straw was found along with ergotamine in the pellets at 820 µg/kg. Within days, multiple cows were affected with vasoconstrictive dry gangrene and eventually, 44 animals lost hooves, ears, and tails, resulting in their deaths.
- These levels were not the highest reported; e.g. when 40 cattle in a cow/calf operation in a pen in rural Oregon in early January were fed on pellets, seed screenings and haylage, thirteen developed lameness and dry gangrene due to ergot toxicosis caused by *Claviceps purpurea*. Pellets were contaminated with 54,916 µg/kg total ergot alkaloids which included ergotamine (36,858 µg/kg), ergocristine (8,016 µg/kg), α-ergocryptine (3,494 µg/kg) and ergosine (4,684 µg/kg).
- In another example, tall fescue supplements spread on a field were found to contain 2,096 µg/kg ergovaline. Of 30 pregnant cows in their second trimester, five were euthanized, four lost their tails, two became lame, and two aborted.
- Finally feeding cattle with perennial ryegrass straw and a grain ration containing the toxin Lolitrem B at 3,711 µg/kg concentration affected seventeen Hereford × Red Angus male and female calves weighing between 136 and 181 kg with eight of 17 of the calves developing concurrent acute pulmonary emphysema.

The paper recommends further study into ergot alkaloid levels causing effects in animal stock subjected to such feedstuffs which would give further evidence for establishing safe limits in these feedstuffs, for different animals.

Canty *et al.* (2014) considered ergot alkaloid intoxication in perennial ryegrass (*Lolium perenne)* in Ireland. The authors stated that ”although there are differences in the primary ergopeptide produced by *Neotyphodium spp.* and *Claviceps purpurea*, the mode of action of these ergopeptides is similar and the clinical presentation of toxicity in animals can be indistinguishable. Any clinical differences between fescue toxicosis and ergotism, if observed, is likely to be due to the higher ergopeptine alkaloid concentrations in ergot (*Claviceps purpurea*) compared with fescue endophytes, and the tendency for a longer duration of exposure to fescue endophyte”.

The paper focusses on pasture and grasses, however symptoms and effects from alkaloids were described, with some threshold concentrations of ergot alkaloids cited. Adverse effects

on livestock performance have been observed when total dietary ergopeptine alkaloid levels exceed 100-200 µg/kg (levels commonly measured). Whereas for fescue toxicosis the effects of sub-acute to chronic exposures are observed at levels of 200 - 600 µg/kg of ergovaline. The authors did note that ergovaline concentrations can vary between fields and that the increasing levels of nitrogen fertiliser on pasture can lead to increased ergovaline concentrations (Canty *et al.,* 2014).

A paper by Kononenko and Burkin (2014), "Mycotoxin contaminations in commercially used haylage and silage" reported how 30 commercial haylage and silage were analysed for total ergot alkaloid levels using an enzyme linked immunosorbent assay (ELISA) approach. Of the 30 samples analysed, 5 contained <10 µg/kg, 6 contained >10 µg/kg and 2 samples contained > 100 µg/kg total ergot alkaloid levels. The authors speculated that a decreased total ergot alkaloid level in haylage when compared to silage is probably due to a low pre-disposition to ergot development and endophyte colonisation in the former. However, the impact of mechanical removal of ergot sclerotia during pre-storage treatment could also affect the levels observed.

Forgeat *et al.* (2014) reported that several herds of cattle were poisoned with ergot alkaloids in France, and presented two cases of poisoning in Charolais herds, describing the wide range of symptoms from the poisoning. In another article Scottish Agricultural College Consulting Veterinary Service (SAC C VS) summarised how they and Animal Health and Veterinary Laboratories Agency (AHVLA) both investigated outbreaks of ergot poisoning in December 2011 (SAC, 2012). The St Boswells team, from SAC C VS considered ryegrass seeds to be the source of ergot that caused lameness in 15 of a group of 30 suckler cows. Affected animals first presented with moderate lameness and unilateral or bilateral swelling of the hock joints. Approximately four weeks after the initial signs developed, six cows developed a characteristic ring of demarcation between vital and devitalized tissues around the skin covering the metatarsal bones.

Further investigation found that, because there were too many molehills, hay rather than silage was made in one field which had been baled and stacked in the field. Although all this hay had been eaten by the time of investigation, ryegrass seeds contaminated with ergot were found where the bales had been stacked, confirming the presumptive diagnosis. Unfortunately in this study, no data was given regarding levels found or if the alkaloids were of the 'ine' and 'inine' forms.

These studies emphasise the potential complexity of identifying and managing ergotism outbreaks.

6.3. Exposure of humans

One of the most recent papers by Bauer *et al.* (2016) refers to analysis of beer for ergot alkaloids, expressed in terms of ergometrine equivalents. In this study 93% of samples from the German market tested were positive for ergot alkaloids. The authors concluded that, when compared to the EFSA Tolerable Daily Intake (TDI) of 0.06 µg/kg b.w., beer is not a major contributor to human exposure. However, they did note that the frequency of relatively high values noted in this paper warrants further study of the raw materials used in the production of beer.

Straumfors *et al.* (2015) investigated ergosine, ergocryptine, ergocryptinine, ergocristine and ergocristinine in grain dust from Norwegian grain elevators and compound feed mills. Levels were found to be in the low µg/kg range in line with levels regularly found in food and feed grains. Whilst this is of concern with respect to operator exposure there is little evidence of adverse health effects due to mycotoxins via inhalation of the contaminated dust.

Mulder *et al.* (2015) investigated ergot alkaloids (and tropane alkaloids) in grain based products for young children and infants in the Netherlands between 2011 and 2014. Cereals are a major part of the diet for many young children who consume relatively large amounts of food per kg body weight when compared to adults (EFSA, 2015). In total, 133 samples were analysed. Overall 47% of samples contained one or more ergot alkaloid. All 12 major EAs contributed to the sum of EAs, with ergotamine and ergocristine found at slightly higher concentrations than the other EAs. Mean ergot alkaloid levels in the three sampling years (2011, 2012, 2014) were 10.6, 6.2 and 8.6 µg/kg, respectively with a maximum concentration of 115.4 µg/kg, indicating that exposure to ergot alkaloids would not have exceeded the health-based guidance values set by EFSA in 2012 (EFSA; 2012).

The EFSA survey conducted by Diana Di Mavungu *et al.* (2011) included 182 cereal based foods purchased in Belgium but none of them were specifically for children. Therefore the survey by Mulder *et al.* (2015) gives the first good snapshot of food products intended for children and allowed an estimate of exposure to be calculated for this critical group of the population.

Mycotoxicoses in children were also discussed by Peraica *et al.* (2014) who considered, briefly, worldwide outbreaks of ergotism and illustrated that these cases are few and far between. They did note a historical case caused by alkaloids from *Claviceps fusiformis* that causes the convulsive type of ergotism that ultimately leads to mania, psychosis and delirium and is believed to be the cause of the symptoms of the teenage girls accused of witchcraft in Salem in 1692.

The EFSA opinion of 2012 collates occurrence data in foods from number of European countries, predominantly Germany, but also Switzerland, the UK and Belgium (reported by Diana Di Mavungu *et al.* (2011)). The results were then used in combination with information about European diets to calculate dietary exposure values for four age groups of the population, these being:

- Infants (<12 months)
- Toddlers, other children and adolescents (≥ 1 to < 18 years old)
- Adults (≥ 18 to < 65 years old)
- Elderly and very elderly (≥ 65 years old)

Mean chronic dietary exposure values, across the different dietary surveys and age classes, ranged from 0.007 µg/kg b.w. per day (minimum lower bound LB) to 0.173 µg/kg b.w. per day (maximum upper bound UB). The 95th percentile dietary exposure ranged from 0.014 µg/kg b.w. per day (minimum LB) to 0.335 µg/kg b.w. per day (maximum UB). The number of data sets (dietary surveys) for infants was limited (only 2) therefore the estimated exposure for this group cannot be representative of the European population. Toddlers were found to have the highest values for chronic exposure due to a combination of the consumption of foods containing EAs, and the fact that they have a higher intake of food per kg body weight than other groups. Toddlers also showed the highest acute dietary exposure to EAs. For both chronic and acute exposure, the countries with the highest consumption of rye bread and rolls had the highest exposure levels.

However, the opinion concluded that the mean and high level estimated chronic exposure of the sum of the EAs for all age groups across European dietary surveys were below the group TDI of 0.6 µg/kg b.w. per day as established by the EFSA Panel on Contaminants in the Food Chain (CONTAM) panel. They also concluded that the acute dietary exposure was below the group Acute Reference Dose (ARfD) of 1 µg/kg b.w. and do not indicate a health concern (EFSA, 2012).

6.4. Effects on humans

da Rocha *et al.* (2014) in their article “Mycotoxins and their effects on human and animal health” reviewed several mycotoxins although the review of each type of mycotoxin was fairly superficial and did not add anything new to the body of knowledge. This was quite typical of several review papers on mycotoxins and effects of health (human or animal). There are numerous articles that describe the effects of ergotism on humans.

The effects of ergots have been known since Biblical times, da Rocha *et al.* (2014) summarised various cases of ergot intoxication and described two relatively recent cases from the 1970s. One in Ethiopia between 1977 and 1978, when 140 people were affected, where the mortality rate reached 34% and also one in India, in 1975, where an outbreak of convulsive ergotism was verified which affected 78 people but no deaths were confirmed. They did note that cases of ergotism are currently rare since the majority of scleroids are eliminated during the processing in mills and only very low levels of ergot alkaloids can still be detected. However they did also state that, these alkaloids are relatively thermolabile and are almost always destroyed in the bread making process, which is not in complete agreement with other authors who have reported a reduction, but not complete loss, of alkaloids during this process.

6.5. Monitoring and mode of action

To better understand the occurrence and frequency of ergotism outbreaks, Canty *et al.* (2014) suggested ‘Developing a coordinated national sampling, testing and reporting system for ergot alkaloid in forages and feeds; and consider the need for inclusion of ergot alkaloid testing within the EU seed certification regulatory framework.’ This paper also covered ‘mode of action’. The authors reported that the biological activity of the ergot alkaloids in animal systems is largely due to the similarity of the ergoline ring structure to biogenic amines such as serotonin, dopamine, noradrenaline, and adrenaline, which allows many of the ergot alkaloids to bind to biogenic amine receptors and to elicit such effects as decreased serum prolactin and vasoconstriction. The primary pharmaceutical action of ergot alkaloids, whether of endophytic or ergot origin, involve vasoconstriction and/or hypoprolactinaemia (a decrease in serum prolactin levels). Ergot alkaloids affect many physiological systems resulting in changes in growth, reproduction, and cardiovascular effects. Ingestion of ergot alkaloids can cause a range of effects in livestock, these include poor weight gain, reduced fertility, hyperthermia, convulsions, gangrene of the extremities, and death. Susceptibility of livestock seems to vary according to species, breed, age, gender and physiological state. Ruminants are less affected than non-ruminants and hindgut fibre digesting animals. In ruminants, vasoconstrictive effects predominate and cause gangrenous and hyperthermic forms of

ergotism. The gangrenous syndrome generally occurs in low environmental temperatures and results in lameness and eventually dry gangrene. Horses are more susceptible to reproductive effects than ruminants.

A thorough review of activities and effects of ergot alkaloids on livestock has been carried out by Klotz, (2015). He describes in detail, species specific effects and the modes of action. There are also specific gender effects of ergot alkaloid exposure such as reduction in lactation caused by lowered prolactin levels as well as reduced progesterone which has been observed in heifers, cows, ewes and mares (Klotz, 2015). This has a major impact on establishment and maintenance of pregnancy and so is extremely important to livestock producers. It is known that the alkaloids interact with various receptors which can cause localised vasoconstriction, impacting on pregnancy. The consumption of EAs can also result in lower birth weights for some species because of the multiple effects the EAs can have on the placenta. Effects in male livestock are less well documented but reduced sperm motility has been noted (Coufal-Majewski *et al.* 2016).

Evans (2011) reported "ergot alkaloid intoxication results in disruption of several physiological systems, relating to reproduction, growth, cardiovascular function, and the signs of these disruptions vary with severity. There is some evidence that ergopeptines, including ergovaline, bio-accumulate in body fat/lipid stores. However, very little is known about the distribution of ergot alkaloids in the tissue of grazing livestock. This lack of knowledge may, in part, be due to technical constraints in the sensitivity and selectivity of the analytical methods available for the detection of these alkaloids generally."

7. ANALYTICAL METHODOLOGIES

Appendix 2 contains a summary of recent relevant papers identified in the literature search describing analytical methods for the determination of ergot alkaloids and summarises methodology and method performance where it is described.

Previously, HPLC with fluorescence detection (HPLC-FLD) was used for ergot alkaloid analysis, e.g. Lauber et al. (2005). At that time, analytical standard materials were not readily available for all 12 ergot alkaloid compounds but this situation has improved since then with more interest in the topic and all 12 compounds are now commercially available.

Each year, the World Mycotoxin Journal publishes an update of recent developments for analytical methods for mycotoxins. The most recent was published early in 2017 (Berthiller *et*

al., 2017). There is an evident trend within these articles for a move away from established methods using HPLC and GC to increased use of LC-MS, including multi-mycotoxin methods. The most recent article describes methods for ergot alkaloids using LC-MS, HPTLC and LC-HRMS (Berthiller *et al.,* 2017).

7.1. LC-MS Methods

Historically, commonly used methods for ergot alkaloid analysis required either chemical derivatization followed by GC-MS analysis, or LC separation with typical run times of around 45 minutes per sample. The method used currently at Fera Science Ltd., in support of CEN mandate M520 for the analyses of ergot alkaloids in food, is significantly shorter taking less than 20 minutes using UPLC followed by ESI-MS and MS/MS analysis. It is based on the method published by Krska *et al.* (2008) and is capable of determining levels of less than 1 µg/kg for each ergot alkaloid. Formal in-house validation of the method for a range of test materials, including rye flour, bread, infant food and breakfast cereal has found acceptable performance characteristics in terms of repeatability and reproducibility, with recovery values for all analytes in the range 90-110%. The method is currently undergoing formal validation via an international method validation study, and results will be published in due course.

Useful analytical observations were reported in the review by Malysheva *et al.* (2013) who investigated ionisation techniques, chromatography and sample preparation for the minimisation of matrix effects in chromatography-mass spectrometry systems. Matrix effects are well known to lead to inaccurate quantification. It was observed that signal suppression or enhancement for ergot alkaloids varied not only between grain types, with the most pronounced effect being in oat, but also to a lesser extent within one grain type. The ionization technique employed was also found to influence the occurrence of matrix effects. Generally, suppression effects were characteristic for ESI, however APCI showed high signal enhancement for most ergot alkaloids leading to overestimation of the alkaloid concentration. In general, ergometrine was most susceptible to matrix effects, as it elutes early in the HPLC system and co-elutes with sample interferences. Use of UPLC did not solve the problem of signal suppression for ergometrine, whereas for later eluting compounds, it gave better results. Matrix effects were affected by the sample preparation procedure, a liquid-liquid extraction (LLE) procedure using ethyl acetate was compared to SPE on different sorbents. For all the procedures tested, no clear signal enhancement was noted. MycoSep® and MIP columns developed especially for ergot alkaloids did not demonstrate improvements for ergometrine, but for the later eluting ergot alkaloids, these cartridges were useful in minimization of signal suppression.

Bryla *et al*. (2015) describe a method that uses Liquid Chromatography/Ion Trap Mass Spectrometry to determine ergot alkaloids in grain products. The method was developed and validated (in-house) by them for the six major ergot alkaloids produced by *Claviceps purpurea,* the ergopeptines (ergometrine, ergocornine, ergocristine, ergocryptine, ergosine, and ergotamine) and also the ergopeptinines, i.e. (S)-diastereoisomers, of which respective names end with the '-inine'. Samples were extracted with 84:16 acetonitrile/ammonium carbonate solution and after centrifugation, supernatant was transferred into a separating funnel for liquid-liquid extraction with n-hexane to eliminate fats. An aliquot of the acetonitrile/ammonium carbonate fraction was then evaporated and residue redissolved in dichloromethane/ethyl acetate/methanol/25 % aqueous ammonia mixture. This extract was purified on neutral alumina (preheated overnight at 600 °C) in a glass column. Solvent switching and solid phase clean-up gave very clean extracts as the alkaloids were separated from polar impurities in the sample matrix (Bryla *et al.,* 2015). Method performance is summarised in Appendix 2, but recoveries were in the range 63.0 to 104.6%, relative standard deviations were below 18 %, and the linear range was from 1 to 325 µg/kg, depending on the analyte, the spiking level and matrix.

Kokkonen and Jestoi (2010) published a short communication where they described a UPLC-MS/MS method for determination of ergot alkaloids. The extraction was similar to other methods, using a mixture of acetonitrile and ammonium carbonate solution. Commercially available, push through, SPE columns were used to clean up sample extracts. Preliminary in house validation was carried out with LOQs of 0.01 to 1.0 µg/kg for each alkaloid in wheat and 0.01 to 10 µg/kg in rye. Recoveries ranged from 80 to 120 %, and within day repeatability values were between 1.3 and 13.9% (n=6). However, despite the use of a clean-up step, matrix effects, both suppression and enhancement, were observed, with the response for ergormetrine being suppressed the most, in agreement with Malysheva *et al.* (2013).

7.2. High Resolution MS method (HRMS)

In the paper "Target analysis and retrospective screening of veterinary drugs, ergot alkaloids, plant toxins and other undesirable substances in feed using liquid chromatography–high resolution mass spectrometry" (Leon *et al.* (2016)), a multi-analyte method is described. This involved analysis for the ergot alkaloids: ergosine, ergosinine, ergocornine, ergocorninine, ergocryptine, ergocryptinine, ergocristine and ergocristinine as part of a multi toxin approach to detect contaminants in feed. A QuEChERS-based extraction followed by an UHPLC-HRMS approach was described. The quantitative method was validated on an optimized procedure

and gave recoveries ranging in general from 80 to 120%, with a precision expressed as relative standard deviation (RSD) of lower than 20%. Full-scan accurate mass data were acquired with a resolving power of 50000 FWHM and a mass accuracy of lower than 5 ppm. The method LOQ was less than 20 µg/kg for each individual ergot alkaloid which, when compared to other current LC-MS/MS methods (e.g. the method being validated by Fera), seems high where 1 µg/kg values are routinely achieved. However, the validation of the methodology is thorough and this would be suitable for most analytical purposes in regulating feed levels at probable legislative levels.

For post-target screening a customised theoretical database including the exact mass, the polarity of acquisition and the expected adducts formed in the instrument can be built. UHPLC-HRMS equipment is very expensive and currently generates large quantitative files which can cause data management and computer processing power issues.

Liao *et al.* (2015) describe another multi-mycotoxin method, also using high resolution mass spectrometry on a UHPLC/Q-orbital trap MS system. The described method used simple solvent extraction and reported low level, even sub µg/kg, LOQs for ergocornine, ergocristine, ergocryptine, ergometrine, ergosine, ergotamine, albeit with no reported 'inine' levels, in corn, rice, wheat, almond, peanut, and pistachio. The validation criteria reported are thorough and detailed. It is possible that the approach could be utilised for in depth investigations into ergot alkaloids in grains and feedstuffs and could be applied to meat samples for toxicological studies relating to adverse reaction 'onset' values and distribution in animals.

7.3. Other Instrumental methods with MS

Sivagnanam *et al.* (2016), describe "Rapid Screening of Ergot Alkaloids in Sclerotia by MALDI-TOF Mass Spectrometry". This most recent analytical method publication describes a novel approach using matrix-assisted laser desorption ionization (MALDI)–time-of-flight (TOF) MS to identify four ergot alkaloids. Of interest here, the method considers analysis of ergosine, ergocornine, ergocryptine, and ergocristine which were readily detected in individual sclerotia of *Claviceps purpurea*. The accuracy of identification of the identified ergot alkaloids was further confirmed by tandem MS analysis. MALDI-TOF MS has potential for high-throughput screening of ergot alkaloids because it permits rapid and accurate identification, uses simple sample preparation, and requires no derivatization or chromatographic separation using only small amounts of sample for the analysis.

Samples of individual sclerotia in this study were extracted using an approach based on the method described by Tittlemier *et al.* (2015), with minor modifications. The developed method not only benefitted from the superior features of MALDI-TOF MS analysis, such as speed, simplicity, and ease of automation for high-throughput analysis, but also gave the enhanced selectivity obtained with high-accuracy m/z measurements. Whilst this method is quick and highly selective, it requires very expensive instrumentation and in this case, it determined the specific ergot alkaloids in the sclerotia rather than in the grain or the food product which could also be important as described in the earlier sections of this report.
Also, the method has only been developed for four of the 12 compounds of interest, including none of the 'inine' forms. Furthermore, there has been little validation reported, even lacking comparison of results to a reference material and levels of detection, repeatability or robustness of the methodology are not given. Challenges may also be expected where spot contamination exists in the product as only very small areas of each sample is examined.

Another method is described by Oellig and Melde (2016) in the paper "Screening for total ergot alkaloids in rye flour by planar solid phase extraction-fluorescence detection and mass spectrometry." This is an alternative approach and, as the authors consider that only the sum of ergot alkaloids is relevant, they report a fast and easy screening method for the determination of total alkaloid content using planar solid phase extraction (pSPE), thus removing the use of HPLC to separate the individual ergot alkaloid compounds. Whilst this method is not specific to the 'ines' and 'inines' of interest here and will encompass other ergot alkaloids e.g., ergovaline, it does offer an alternative approach. An initial ammonium acetate buffered extraction step was followed by fast liquid–liquid partitioning pre-cleaning before conducting planar SPE on high-performance thin-layer chromatography (HPTLC) amino plates. A single methanol elution step was used to separate the ergot alkaloids from any remaining matrix and to collect them in a single zone. For quantitation, native fluorescence was used after dipping the plate in n-hexane/paraffin solution for fluorescence enhancement. Method validation was detailed and thorough, with LOD and LOQ values of 70 and 240 µg/kg rye, expressed as ergocristine which is well below the currently applied quality limit for rye. The fast planar SPE–FLD is a rapid, efficient and reliable method to screen for the total ergot alkaloid content in rye. HPTLC-MS additionally enabled the identification of the ergot alkaloid composition by a single mass spectrum taken from the concentrated zone and, when utilized as a "fingerprint", offered easy differentiation of *Secale cornutum* from different locations. The speed and ease of the method are offset by decreased sensitivity (higher detection limits), and these may not be low enough for the analysis of foodstuffs, although this will be dependent on the maximum levels that are set in the future.

An analytical variation of the MS theme was reported by Nielen and van Beek (2014) in: "Macroscopic and microscopic spatially-resolved analysis of food contaminants and constituents using laser-ablation electrospray ionization mass spectrometry imaging (LAESI-MSI)". As part of this multi residues method, the ergot alkaloids analysed were: ergine, erginine, ergometrine, ergometrinine, ergotamine, ergotaminine, ergocristine and ergocristinine in ergot sclerotia. Furthermore, the method included a novel form of sample submission to the instrument drawing a laser desorbed sample into the MS ion source thus eliminating a lot of extraneous 'dirt' build up that so easily affects mass spectra and causes matrix effects in the chromatography, sometimes affecting quantitation. Once again this is expensive technology and focuses on the ergot bodies or sclerotia rather than the foodstuffs. Major development work would be required to further investigate the potential of this technique with respect to foodstuffs.

7.4. Other instrumental methods

"Simultaneous separation of ergot alkaloids by capillary electrophoresis after cloud point extraction from cereal samples" was discussed by Felici *et al.,* (2015). Capillary electrophoresis (CE) has low solvent dependency and is more environmentally friendly than alternative solvent extraction systems that use larger volumes of organic solvents. The equipment is relatively cheap compared to MS but is still specialised. Ergovaline and ergotamine were analysed in cereal samples of which flour samples comprised: rye, wheat, oat, corn rice and soy, with grains of wheat and oat, cereal product for infant feeding, and composites of rice, corn, wheat, barley, sugar and additives. The results were applicable to TEA content expressed as ergotamine although the method does not enjoy the selectivity of MS technology as it relies on diode array ultra-violet (UV) detection. Of the results reported, rye flour was expectedly found to contain the highest levels of ergot alkaloids, 231 µg/kg, comparable with other EU country study publications and as reported in Appendix 1. Validation criteria were reported with limits of detection at about 2 µg/kg and recovery at a minimum of 92%.

7.5. Known problems for instrumental methods

Experience suggests that electrophoresis systems are prone to blockages with significant down time and systems can be complicated to use. Mass spectrometry based systems are also prone to dirty sample matrix effects, including UHPLC lines blocking, sample inlet probes and cones clogging up or 'dirtying' affecting response and generally have high maintenance costs associated with engineer services and maintenance.

Sample clean-up becomes an issue with respect to maintenance of 'healthy' mass spectrometry systems and should not be underestimated. Costs saved with respect to solid phase extractions systems being eliminated or filters not being used can have knock-on effects to the maintenance of the MS aspect of the analyses.

Malysheva *et al.* (2013) highlighted that even with different clean-up strategies, there is no perfect solution to reducing matrix effects for LC-MS analysis.

In the paper by Bryla *et al.* (2015), the authors report on an ion-trap mass spectrometry system the "detector is also vulnerable to matrix effects: too many trapped ions degrade the trap performance because of their interactions (the so-called space charge effect). As a result, instrument sensitivity and/or repeatability may significantly decrease. Thus, the to-be-analysed samples must be cleaned up very carefully or surplus ions from the matrix residues will 'clog' the instrument". This reinforces the need for use of a good clean-up protocol during analysis, even when a selective detector, such as a mass spectrometer is used.

7.6. Novel methods

Moench *et al.* (2016) reported on "The different conformations and crystal structures of dihydroergocristine". Dihydroergocristine (DHEC) was crystallised from different solvents and the crystals characterised. The analysis of the different crystal structures confirmed that DHEC is a suitable template for mimicking ergot alkaloids for production of molecularly imprinted polymers (MIPs). MIPs have potential to be used for extraction/clean-up as part of an analytical method and the future use of these MIPs for ergot alkaloid analysis could develop into a more cost-effective approach.

MIPs were successfully developed toward the EFSA 12 ergot alkaloids by Lenain *et al.* (2012). Metergoline was used as a template molecule for the first time. Spherical beads with a narrow size distribution were produced as a sorbent in an SPE column and used for the cleanup of barley samples prior to LC-MS/MS analysis. The MIP performed well compared to traditional cleanup giving good recoveries, reduced matrix effects for most compounds and achieved low limits of quantification (LOQ) of 10 µg/kg for ergometrine and ergometrinine. All other compounds could be quantified at 1 µg/kg except for ergosine and ergocristinine which had respective LOQs of 2 and 3 µg/kg. The MIP has the advantage of being easy and cheap to produce, and the time for the MIP-SPE procedure was short. In addition the SPE columns were reusable. The authors also reported that using different amounts of polymer had an

effect on the binding efficiency; this also means there is a potential use of these MIPs in other formats, for example, as a sensitive layer in sensor applications.

Rouah-Martin *et al.* (2012) produced several aptamers, one was found to have a high sensitivity for lysergamine. A colorimetric reaction could be achieved when the aptamer was linked to gold nanoparticles and a specific colour change was observed in the presence of lysergamine and small ergot alkaloids in wheat flour. This has the potential to be developed further into a rapid method, e.g. a dipstick test using lateral flow diffusion. Other techniques (electrochemical, piezoelectric, optical, etc.) could be used with this aptamer in the development of different sensing formats. The authors believe this aptamer also has potential for future applications for the detection of small ergot alkaloids.

Martin *et al.* (2014) published "Aptamer-based extraction of ergot alkaloids from ergot contaminated rye feed". Although aptamers were mainly developed for sensing applications, this study showed that it is also possible to use aptamers for the specific extraction of target compounds. This type of system can be easily applied for food clean-up, extraction of toxins or contaminants from various environmental or food samples, as well as the specific extraction of natural compounds from turbid matrices. In this study, DNA aptamer ligands specially selected for ergot alkaloids, were grafted onto silica gel in order to construct a solid phase extraction system with high specificity. The aptamer-functionalised silica gels were then used to extract ergot alkaloids from a contaminated rye feed sample and the resulting cleaned-up extracts analysed using LC-QTOF-MS. It was possible to specifically extract ergosine, ergocryptine and ergocornine from an ergot contaminated rye feed sample. Another detection approach could similarly be used, e.g., LC-MS/MS which is moderately less expensive. However, because of the high degree of specificity of the method, other ergot alkaloids were not retained or were present at levels that were not detectable. Aptamers have advantages over MIPs including their chemical robustness and greater specificity but therefore a more restricted range of recognition.

Malysheva *et al.* (2013) considered different clean-up methods associated with ergot alkaloid analysis, including the use of MIPs, and evaluated the effectiveness of different methods for minimising or eliminating matrix effects.

Vermeulen *et al.* (2013) reported a complete procedure for detecting ergot bodies in cereals based on near-infrared hyperspectral imaging. Using this method it was possible to detect ergot body content as low as 0.02% in grain, less than the EC intervention limit of 0.05%, with good reported repeatability and robustness. The laboratory and industrial level trials showed

that NIR hyperspectral imaging combined with chemometric tools could be used as a control method to assess and quantify the presence of ergot bodies in cereals. However this concept needed the use of specific ownership software for spectral acquisition and data treatment. Models were built to discriminate ergot bodies from cereal grains, no false negatives were found for whole ergot bodies. However, depending on the size limits set for the size of ergot bodies, some pieces of broken ergot bodies could not be correctly classified using the identification tool as they could be confused with small weed seeds. The screening size parameters could be modified depending on the size of the grain and contamination present. Nonetheless, the authors were confident that this method could be useful as an analytical screening tool in an automatic cereal control scheme, where the positive samples could be further analysed using official confirmatory methods (chemical or physical).

Less well studied or novel ergot alkaloids in cereals were considered by Arroyo-Manzanares, *et al.* (2014) who discussed a "Holistic approach based on high resolution and multiple stage mass spectrometry to investigate ergot alkaloids in cereals". Their identification strategy consisted of a few steps which made it quick to draw preliminary conclusions regarding the presence of ergot alkaloids in a sample or to directly identify an ergot alkaloid derivative. Subsequent application of the strategy in the screening of grain samples was successful and allowed identification of eleven metabolites for which commercial standards were not available.

8. Reduction of Sclerotia infestation of crops using integrated processes.

8.1. Integrated agronomic practices that could reduce ergot infection

Menzies and Turkington (2015) have published an excellent review article that covers all the relevant topics for consideration in reducing infection such as; 'Reduction of primary inoculum', 'Role of alternative hosts', 'Fungicides', 'Cultural management', 'Host genotype', 'Avoidance of infection', 'Physiological resistance', and 'Pathogen variability'. Evidence relating to mitigation strategies for crop infestation with sclerotia is detailed, this paper was reported at the Edmonton symposium, entitled, "Ergot and strip rust: continuing challenges for Canadian cereal producers" in June 2013. The authors noted that; "there are few active plant pathologists that have worked extensively with the *Claviceps purpurea* pathogen and could be considered experts on their agricultural (non-alkaloid chemistry) aspects". However, since this publication, there has been a dramatic increase in the number of papers published in this

area. The review also gives a good overview of ergot alkaloids effects, the mechanisms of infection and development and lifecycle of *Claviceps purpurea*.

Menzies (2004) conducted a study to determine if registered Canadian wheat cultivars and experimental wheat lines differed in their reactions to *C. purpurea* and to determine if different classes of wheat differed in their levels of resistance. The classes of wheat assessed were: Canadian Western Red Spring (CWRS), Canadian Prairie Spring (CPS), Canadian Western Extra Strong (CWES), Canadian Western Soft White Spring (CWSWS), and Canadian Western Amber Durum (CWAD). Of particular interest was that the durum line 'Pelissier' had significantly lower honeydew production than most lines tested. The breeding line 9260B-173A was found to be significantly more resistant than all other lines tested in terms of percentage of florets with sclerotia, size of sclerotia and honeydew production.

For hexaploid spring wheats, the line 'Kenya Farmer' has been reported to have increased resistance to *Claviceps purpurea* in several studies and has been known to have significantly lower values for percentage of florets with sclerotia, size of sclerotia and honeydew production (Menzies (2004) and Menzies and Turkington (2015)). This confirmed observations by Menzies (2004) who reported 'Kenya Farmer' to be more resistant to ergot infection than the other lines tested, but only in terms of honeydew production. Genotypes from the CWAD and CPS wheat classes generally showed more resistance to *C. purpurea* than the genotypes within the CWES and CWRS wheat classes (Menzies and Turkington 2015). The author suggested that in areas where ergot can be a problem, the growing of commercially available CWAD or CPS cultivars could help reduce the negative effects of *C. purpurea*. However the author noted the genotypes that showed more resistance tended to be the experimental genotypes and were not available on a commercial scale. One durum line in particular, genotype 9260B-173A, exhibited very good resistance to the mixture of isolates of *C. purpurea* used in this study, the author suggested it might be worth exploring if this resistance could be transferred to hexaploid spring wheats (Menzies and Turkington 2015).

Menzies and Turkington 2015 report on the increasing incidence of ergot sclerotia infected crops in Western Canada since 2002. Before 2002 there was less than 5% ergot infestation of Canadian Western Red Spring wheat reported in either Alberta, Saskatchewan, or Manitoba provinces, however the values in all three had increased to between 12 and 30% by 2011.

A summary of work on physiological resistance in Canadian wheat was given. The authors noted that assessment of ergot resistance can be time consuming and labour intensive, and therefore will be expensive. They noted that studies are underway with some lines to

characterize the resistance and its nature of inheritance and that if molecular markers for different types of resistance could be developed, it would greatly enhance efforts to develop ergot resistance lines. Another consideration they raised was that geographical issues are also important, namely, the difference in pathogen variability. They reported some initial work Menzies (unpublished) carried out to study strain variability among 42 strains of *C. purpurea* from western Canada, and five strains from the UK. Preliminary results included in this paper showed wheat line 9260B-173A, previously found to be highly resistant to different *C. purpurea* strains (Menzies, 2004) was resistant to 4 western Canadian strains of *C. purpurea*, but not to four UK strains that were tested. This has implications for breeding programmes to make sure that the strains used experimentally for testing resistance are appropriate to ensure that effective field resistance is achieved (Menzies and Turkington, 2015).

Summing up, the authors highlighted a series of harvest and post harvest practices that can be used to reduce the amount of ergot in harvested grain. These were:

1. Delaying of swathing or harvest - this can reduce the ergot content in harvested grain because the ergot bodies can be shaken off by the wind.
2. Selective harvesting of the field - to separate out heavily infested ergot grain from clean grain. Also to avoid the areas of the field around grassy ditches or headlands that are often the most severely affected. Harvesting the areas of the field close to wild grass areas separately from the rest of the field will separate out the grain which is more heavily infected.
3. Commercial cleaning of grain - gravity sorters or colour sorters can remove sclerotia from infested grain. However, this does have limitations, and heavily infested grain may not be cleaned sufficiently enough for the market. Heavily infested grain which cannot be adequately cleaned should be disposed of properly and should not be used for animal feed.
4. Crop rotation – Shorter crop rotations may contribute to the problem as a 1-year break away from cereals such as wheat or barley may not be sufficient to allow for the natural destruction of the ergot sclerotia in or on the soil. Also shorter rotations may lead to increased levels of ergot bodies in grassy areas adjacent to cereal fields, thus resulting in the build-up and spread of *C. purpurea* inoculum.
5. Limit plant stress – ensure adequate soil fertility and avoid herbicide applications close to crop flowering, and reducing late tiller development in cereals through increased seeding rates, can reduce ergot risk.
6. Preventing flower development in grassy hosts in field border areas - can reduce sclerotia production and thus a future inoculum source and therefore reduce the potential for disease development.
7. Host resistance –resistant varieties, especially physiological resistance will play a key role in providing improved ergot management.

8. Integrated process to tackle the disease triangle –a combination of the above practices is required as it is difficult for cereal producers to completely eliminate the risk of ergot. None of the recommended control measures have been found to be entirely successful in controlling ergot on their own.

Finally,

9. Investment in research is required - the sporadic nature of ergot infections from year to year has presumably been one reason there has not been a large amount of research. However, interest is gathering as it becomes more apparent that there has been an increase in incidence of this disease over the last few years. This emphasises the need for reliable analytical approaches to support biological studies.

The Government of Saskatchewan have also published a similar list of ergot management prevention strategy practices on their website (Government of Saskatchewan, 2017: https://www.saskatchewan.ca/business/agriculture-natural-resources-and-industry/agribusiness-farmers-and-ranchers/crops-and-irrigation/crop-protection/disease/ergot-of-cereals-and-grasses). The headings are similar to those listed above, and they mention; harvesting techniques, tillage, seed cleaning, planting clean seed, crop rotation, crop choice, ensuring uniform stands (sim. reduce plant stress) and sanitation. Therefore it seems there is consensus developing on the most appropriate preventative measures to take.

A review of "Biology, genetics and management of ergot (*Claviceps* spp.) in rye, sorghum, and pearl millet" was published by Miedaner and Geiger in 2015. They identified at least three mechanisms that affect ergot susceptibility in plants, these were:

- Characteristics of flowers affecting stigma receptivity, e.g., time the florets stay open, stigma size, start of stigma drying after pollination,
- The ability of a plant to pollinate and fertilize before infection occurs
- Resistance that reduces fungal infection or fungal spread in the gynoecium.

They reviewed each of these mechanisms in turn but they also noted that the situation is more complex because all of these are strongly affected by weather shortly before and at flowering and they stated that interactions between weather, fungal infection and spread, and pollen availability must be considered.

Miedaner and Geiger (2015) also reviewed breeding strategies and other control measures pre- (agronomic measures and fungicides) and post-harvest (cleaning) as methods to control

ergot contamination. In conclusion they suggested genomic selection procedures to improve selection of complex traits such as ergot resistance.

Tittlemier *et al.,* (2015) reported another LC-MS/MS method for the analysis of ergot alkaloids in cereal grains, but also reported the occurrence of ergot in two western Canadian grain samples. Data presented suggested the severity of ergot infection increased over the time period from 2002 to 2013, the authors also referring to the incidence of ergot increasing in the UK during the mid-2000s (Bayles *et al.,* 2009). Tittlemier *et al.,* (2015) once again highlighted agronomical practices, such as minimum tillage and use of grass field margins, that would need to be changed in order for an integrated process to be used effectively. The authors noted these practices would affect the presence of ergot in addition to environmental factors such as precipitation, temperature, and soil conditions, which have also been implicated as affecting ergot in cereals.

The paper also then compared the total ergot alkaloid concentrations found in the cereal grains of the 'high ergot' year in Canada of 2011 against the ergot sclerotia found by mass and proved a correlation.

The sum total of ergot alkaloids increased in proportion to the percentage of ergot sclerotia in the sample with the higher the mass of ergot in the sample giving the higher the ergot alkaloid content. Ergot alkaloid concentrations among wheat and durum samples harvested and prepared for shipment was also discussed and it was noted that the TEA in raw grain samples was higher than that found in cereal shipments. Also, the lowest mean concentration was found in barley shipments which could be a reflection of lower infection in the barley or due to cleaning prior to export as malt. Furthermore, there was a stark contrast between ergot alkaloid content in wheat samples designed for human consumption and that for animal feed. The four grades of wheat for human food were in the region of 0.05 to 0.3 mg/kg total ergot concentration whereas animal feed was in the region of 0.4 to 0.65 mg/kg.

Patterns of ergot alkaloids determined in shipped and harvested wheat and durum grains were illustrated in this paper and showed similar relative proportions where, e.g., for shipments, ergocristinine was the predominant alkaloid at 39% of the total followed by ergocristine (28%) and ergotamine (10%), with the other alkaloids accounting for the remainder. The patterns were similar for barley and rye. The most striking pattern observed in harvest samples compared to the shipped samples, was a slightly higher abundance of the biologically active 'ine' form, or R enantiomer, compared to the 'inine' form or S enantiomer. One reason

tentatively put forward is that long storage and some storage conditions can increase conversion of the S enantiomer to the R enantiomer.

Monitoring of grains may not necessarily be an indicator of eventual hazards in food and feed so monitoring should also include the finished product.

A further publication by Chen *et al*., (2016) confirmed that agronomic practices for harvesting can help manage the risk of fungal contamination and reduce the microbial load in malting barley early in the value chain, however this is generally for fungi and not specifically for *Claviceps purpurea*.

Coufal-Majewski *et al.* (2016) concluded that the most effective routes to minimise ergot establishment seem to be in the field and following good general agricultural practice including:

1. Limiting damage to kernels from birds or insects
2. Harvesting as soon as possible, especially where ergot is detected (contrary to Menzies and Turkington, 2015, above)
3. Maintaining grass verges, ensuring they are kept mown to reduce flowering and the minimise the reservoir for ergot infection.
4. Correctly storing and drying grain.
5. Rotating crops to avoid carry over of moulds, due to long survival of sclerotia in soil. 6. Increase seedling vigour and use seed treated with fungicides.

A Discussion Paper to add an annex on ergot and ergot alkaloids to the Codex Code of Practice to reduce mycotoxins in grain was tabled at the Joint FAO/WHO Food Standards Programme Codex Committee on Contaminants in Foods (CCCF) in April 2016 (Codex, 2016). This discussed many of the aspects already highlighted above, but also addressed the issue of dust and surface contamination and cleaning of grains. It was stated that ergot bodies have a softer, greasier and less dense structure than the grain kernels. This means there is a high probability that very fine ergot dust can be released by the kernels and the sclerotia rubbing against each other when moving a cereal lot containing ergot bodies. The rubbed-off material has highly adhesive properties so that it sticks to the grain surface. It was also noted that sclerotia can break very easily, triggering very fine ergot dust. This can deposit on the grain surface, in the stomach furrow and in the beard of the grain and will in effect be invisible. The paper therefore recommended avoidance strategies should be used to combat the resulting ergot alkaloid contamination of the cereal. They also recommended cleaning should be used to remove ergot bodies and dust from the consignment of cereal to the highest possible degree. The paper cited practical experience where this can be achieved by use of

a combination of different cleaning principles and systems, e.g. mechanical light-fraction separators, sieve separators, table separators, spiral separators, optic-electronic separators or colour-based optical sorting systems.

Where there is a high level of ergot, care should be taken to control the throughput capacity of the cleaning process, or a second cleaning process for the pre-cleaned grain should be used to ensure effectiveness. Dust adhering to the grain surface can be removed by cleaning the surface of the kernels e.g. via scrubbing, brushing or peeling. The grain furrow part of the kernel usually carries a lot of dirt and unwanted substances and this part of the kernel can be cleaned with a crusher. It was also recommended that in general, the dust and fine filter flours, which may contain higher content of ergot alkaloid, should be removed via a suction ventilator and an effective filter system used.

In summary it was concluded that there are some particular issues specific to ergot and therefore some controls that are different to those required for other mycotoxins and it was recommended that CCCF agree on the need for an Annex on ergot and ergot alkaloids in the Code of Practice for the Prevention and Reduction of Mycotoxin Contamination in Cereals (CAC/RCP 51-2003). This will be discussed at future Codex meetings.

8.2. Other possible mitigation measures

Hahn *et al.* (2015) succeeded in the identification of metabolites formed by microbial and enzymatic degradation of ergot alkaloids, a crucial step in the future development of feed additives for gastro intestinal detoxification of ergopeptines in farm animals.

A rapid and recently developing area for mitigating the effects of ergot alkaloids is the breakdown of these alkaloids in the feed, particularly because of higher concentrations in feed. Strategies to support development in this area were suggested by Thamhesl, *et al.,* (2015). An ergopeptine degrading bacterial strain, MTHt3, has been isolated and classified based on its 16S rDNA sequence, as a strain of *Rhodococcus erythropolis* (*Nocardiaceae, Actinobacteria*). No other tested *R. erythropolis* strain degraded ergotamine. *R. erythropolis* MTHt3 degraded all ergopeptines found in an ergot extract containing ergotamine, ergovaline, ergocristine, ergocryptine, ergocornine, and ergosine, but the simpler lysergic acid derivatives agroclavine, chanoclavine, and ergometrine were not degraded. The conclusion from the study stated; “Degradation of ergopeptines to ergine is a previously unknown microbial reaction. The reaction end product, lysergic acid, has no or much lower vasoconstrictive activity than ergopeptines. If the genes encoding enzymes for ergopeptine catabolism can be cloned and expressed in recombinant hosts, application of ergopeptine and ergine degrading

enzymes for reduction of toxicity of ergot alkaloid-contaminated animal feed may be feasible." Therefore, further investigation in this area could be another way to mitigate the levels found in animal feeds, particularly using trial work on larger scales, laboratory and perhaps micro plant scale, with a view to possible industrial scale clean-up. However, cost implications may be prohibitive.

Coufal-Majewski *et al*. (2016) summarized and suggested various means to detoxify or mitigate ergot alkaloid exposure in animals, including investigation and identification of anaerobic bacteria that may be able to detoxify the alkaloids, chemical treatments or heat treatments, although they did note that the safety of any products produced would need to be thoroughly evaluated first. They also mentioned the possibility of use of 'binders' but remarked that binders sold for deoxynivalenol (DON) were not effective at dealing with alkaloids. This is not surprising as although some symptoms in the animals may appear similar (e.g. feed refusal) the compounds themselves are chemically very different and so it would not be expected that a binder active for DON would have any activity for alkaloids. At the moment, there are no products marketed specifically for the adsorption of alkaloids.

Other on farm mitigation measures were also suggested such as removal of dust, using high pressure air if necessary, to ensure this is efficient; soaking, dehulling, or roasting but these could introduce other problems.

9. Ergot alkaloids in Honeydew and Sclerotia

The only specific evidence found with respect to finding ergot alkaloids in honeydew associated with the growth cycle was reported at the recent World Mycotoxin Forum and published by Tittlemier *et al.,* (2016). Honeydew is sticky, a mixture of liquid and conidia, that is used to spread the spores of a fungus, e.g. by sticking to insects that are attracted by it.
Total concentrations for ten ergot alkaloids analysed ranged widely from between 16 and 5,459 µg/kg in honeydew to 1,798 mg/kg in sclerotia. Ergonovine and ergosine were the predominant alkaloids detected in honeydew whereas ergocristine and ergocryptine were the main alkaloids observed in sclerotia. One of the *Claviceps purpurea* isolates (EI2) used to infect the lines used, gave consistently higher alkaloid concentrations (695-1,010 mg/kg) in the associated sclerotia, regardless of the line infected. The other *Claviceps purpurea* (EI4) in induced sclerotia gave consistently lower levels (255-594 mg/kg).
Of interest is the reporting of low ergot alkaloid levels (4-33 µg/kg) from 34 analyses of a control CWAD line of grain. So even where there was no ergot in the grain, there were

occasional positives albeit near limits of detection for the MS/MS method used. More work on 'blank' or control samples needs to be done to confirm these findings. Since levels were determined in the honeydew (16 - 5,459 µg/kg), it is possible that this might be the source of this contamination. It should be noted however that this level of contamination was up to 1000 times lower than the concentrations measured in individual sclerotia. Further work would seem to be required to assess the impact of contaminated honeydew on grain levels.

The paper concluded: "Host plant lines and *Claviceps purpurea* isolates influenced the concentrations and patterns of the measured ergot alkaloids observed in sclerotia from infested wheat. Therefore, the identity and amount of ergot alkaloids found in wheat will vary according to variety cultivated, as well as environmental conditions shaping the populations of *Claviceps purpurea* in a growing area".

10. ECONOMIC EFFECTS

The economic impact of cereal ergot contamination results from reduced crop yields and quality, toxin production and lower growth of animals fed on the product, was described by Coufal-Majewski, *et al.,* (2016). Toxin production depends upon seasonal variations (possibly from climate change), the species and type of crops grown, geographical location and local agricultural practices combined with processing methods to give a complex landscape which is challenging to model. Whilst several papers discuss economic effects of ergot intoxication, few are able to give a monetary value for the losses that occur. Macroeconomic studies to support future research funding (cost vs potential benefits) would appear to be valuable.

10.1. Economic analysis of ergot alkaloid testing

Economic impacts of mycotoxins, including ergot alkaloids, are reported to range from the loss of human and animal life and subsequently lost productive capacity, increased costs of health or veterinary care, reduced livestock productivity, yield losses, the downgrading or disposal of contaminated foods and feeds, to expenditures for research and regulation (Zain, 2011; Bhat and Vasanthi, 2003; Schmale and Munkvold, 2016; Hussein and Brasel, 2001).

A simple framework that could be developed further to provide estimates of the economic (and other) impacts given potential changes in the testing regime is shown below. Data requirements are listed as well as the likelihood of its availability.

Firstly, the type of framework required is illustrated with the outline of a model used to assess the economic impacts on bread wheat and animal feed markets of Karnal bunt.

10.2. Example framework: the impact of Karnal bunt

The EC Fifth Framework Project QLK5-1999-01554: Risks associated with *Tilletia indica*, a newly-listed EU quarantine pathogen, the cause of Karnal bunt (KB) of wheat, produced an Excel based model to assess economic impacts. The model was developed by staff at NSW Agriculture (Australia) in conjunction with a team at Teagasc (Ireland). In determining the economic impact, three individual components of costs were identified:

a) Direct costs: Direct yield changes impacted on bread, durum, and feed wheats for the major producing areas of the EU. These occur when infected milling wheat is considered unsuitable for food use and is downgraded to feed wheat. Various assumptions can be made as to the size of the yield loss and the amount of crop affected.

b) Reaction costs: When the market reacts to the presence of KB there are indirect quality losses, loss of exports, and seed industry costs. The indirect quality losses result from the downgrading of unaffected grain – grain that is "guilty by association". In the KB model this is modelled as no wheat from the affected region (including buffer) being milled, an export ban on all wheat in affected region, and all bread and durum wheat produced in the region is downgraded to feed.

c) Control costs: Three elements: surveillance and testing (additional costs i.e. not normal border controls, including surveys), containment, and eradication. Costs for containment/eradication include those for produce quarantine and the imposition of growing restrictions in affected areas. The model assumes that: after the initial outbreak in year 1, fields on which KB was detected must be kept as bare fallow or be grassed down for five years, after which non-host crops can be grown; within the affected region (plus buffer) but excluding infected premises no wheat can be grown for the following nine years; all the affected region are subject to chemical controls in years 2-10.

The direct costs seem to be applicable to the ergot question and the reaction and control costs to varying but lesser degrees. A schematic of the model is shown in Figure 4. This nicely illustrates the flow of effects and impacts of the pest management process and is particularly useful given the complex linkages within the supply chain. The figure also shows where it could be adjusted to reflect the situation with ergot.

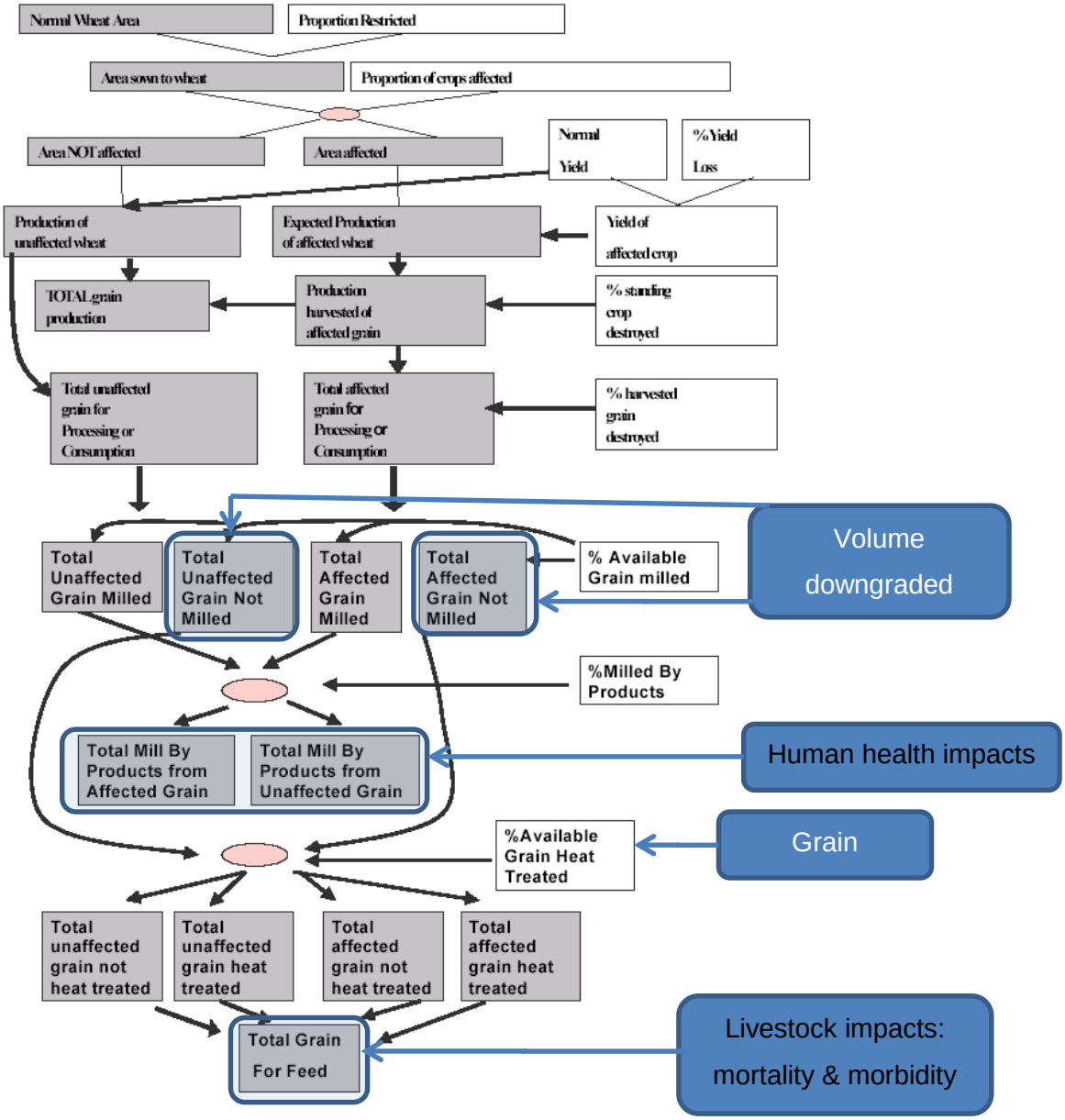


Figure 4: Flow diagram of the effects of an outbreak of Karnal bunt and links to ergot contamination (areas highlighted)

In simple terms, any downgrading will attract a lower price. Currently the gap between grades for human and animal feed wheat is not that great with the UK ex-farm price differing by only around £5/tonne (AHDB Market data centre). For relatively small levels of downgrading the price is unlikely to be affected as the supply to the food market declines and that to the feed market increases. Price changes in the KB model (reflecting a large outbreak of 50k ha) were very small for milling wheat at less than 0.5%. However, the feed market price initially dropped (in the UK) by over 25% in the first year to soak up the additional supply but returned to previous levels in subsequent years as markets adjusted and imports increased. For a simple graphical representation of the changes in supply and demand in these connected markets (Figure 5 below).

10.3. Our understanding of the current testing regime

The table below illustrates an initial, highly simplified, view of how batches of cereal pass through the current ergot testing regime. The downgrading impact is the most obvious example of increasing costs of positive detection of ergot but there are other potential costs, such as disposal costs.

Currently we are unaware of the proportions of material in the pass/fail categories and the degree to which false positives and false negatives could be a problem. For failing batches there are two other potential costs: the batches downgraded incorrectly (false positives) and the effect on livestock of infected grain in terms of increased mortality and reduced performance. For batches that pass the test, if there are false negatives there are potential costs to human health.

Table 7. Decisions about batches of cereal that pass through the current ergot testing regime

Batch sampling – mechanical/visual inspection – thresholds for pass/fail	
Fail – what proportion? Any false positives?	Pass – what proportion? Any false negatives?
Batch goes to animal feed market – for a particular ergot threshold or all that fail the inspection, i.e. for high levels, are any destroyed? Any impacts of ergot in livestock or costs associated with dilution of failed batches?	Batch goes to food processing sector Any issue re false negative and human health?

This can be represented in a similar flow diagram to the KB model (Figure 5) and highlights some of the major data gaps (red).

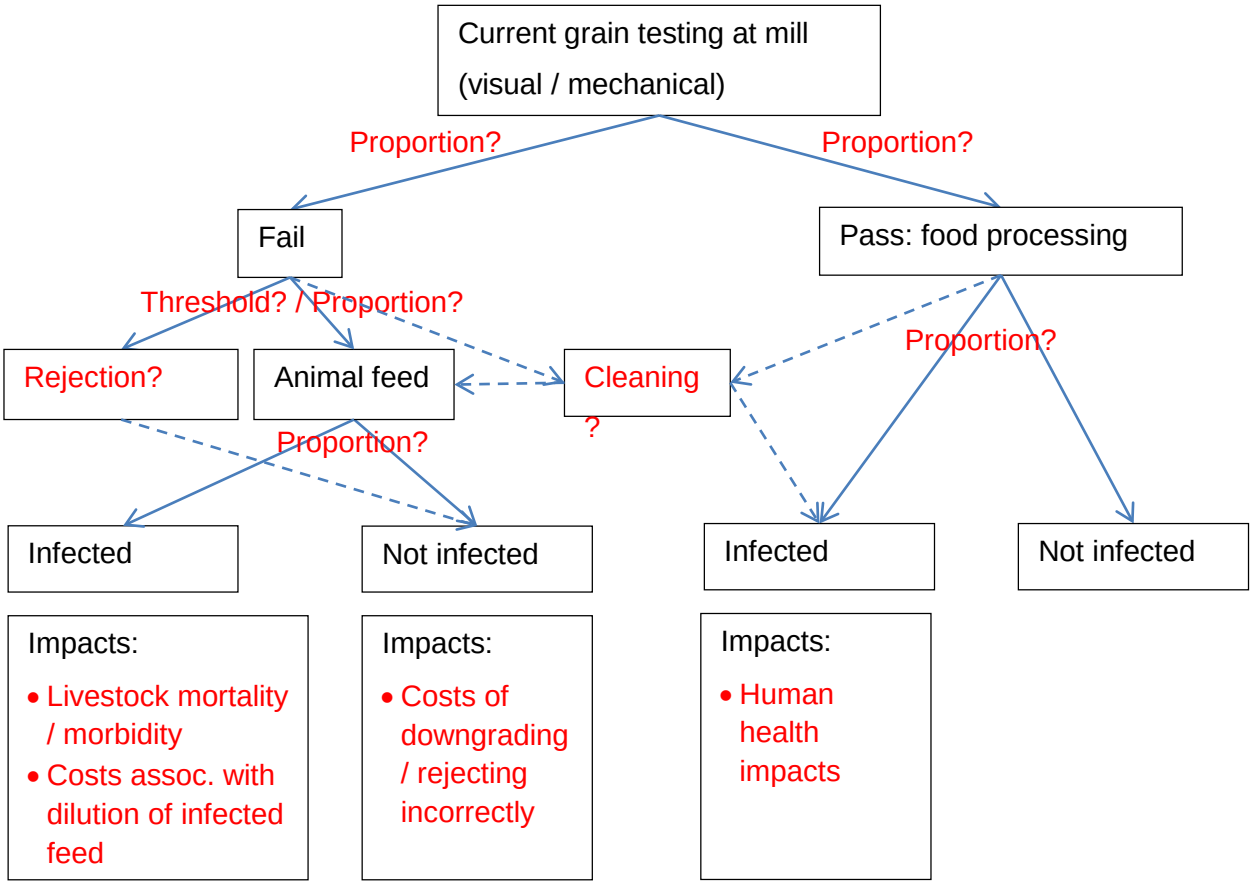


Figure 5. Flow diagram of the Karnal Bunt model (applicable to ergot alkaloid contamination), showing supply and demand in connected markets and highlighting data gaps

10.4. Data needed to complete an economic model of the consequences of ergot contamination

For the current regime, the following data sets are required to model the consequences of ergot contamination:

- Production volume and market price of each commodity
- Proportions that are downgraded and to what new market price level noting that downgrades will not all be to the animal feed price
- Commodity price elasticities to ascertain additional price movements from downgrading (if any). Elasticities describe the way in which prices react to changes in

volumes. For example, if more batches are downgraded, the supply of feed cereals will increase leading to a price decrease. The level of the price change is determined by the price elasticity of that cereal and these vary across different food types.

- Current activities related to grain cleaning – volumes processed, cost per tonne, price post cleaning etc.
- Value of livestock impacts that will require mortality and morbidity data for each sector with estimates of the number of animals affected by contaminated feed
- Value of human health impacts

Any new testing regime has potential to affect all of these data needs (apart from the elasticities), assuming that it will change the flow of batches through the system. Those effects might be exacerbated by market reactions, i.e. a reduction in price for feed may lead to substitution of other feed with infected material thus increasing the impact on the animal husbandry sector. Whether or not it does will depend upon how it is deployed and whether the same thresholds will be used. If there is a change in threshold for pass/fail, it would make comparison of the new system to the current system more complex as we would not be comparing like with like.

Some key parts of the data required do not appear to be readily available to make a quick assessment of the impacts of ergot i.e. volumes downgraded and the prices obtained for this material. A more thorough analysis would also need to account for countermeasures that might be applied such as mixing or grain cleaning that become economic responses at certain price levels.

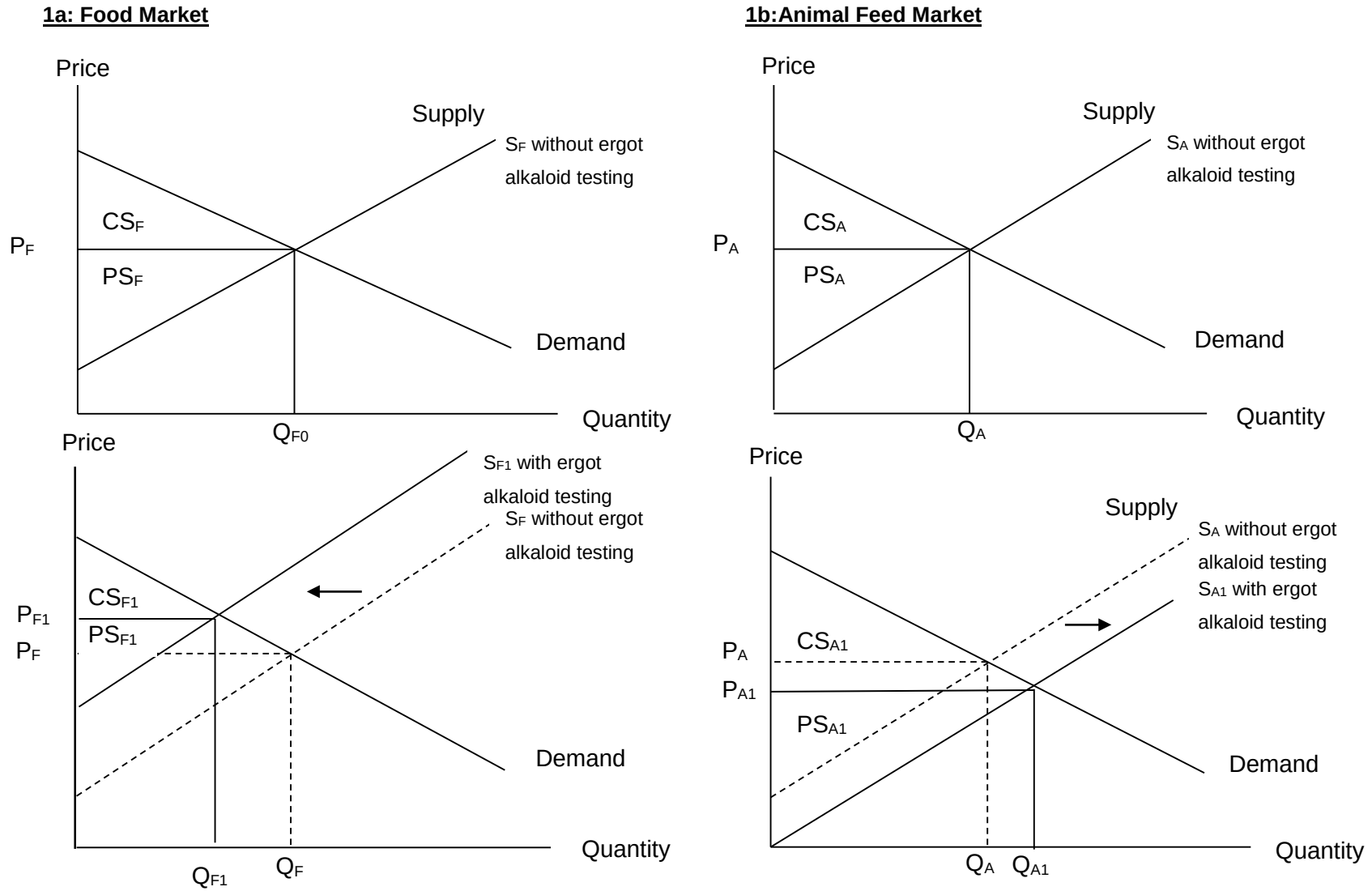


Figure 5. Change in Producer and Consumer Surplus for Down-grading from Food to Animal Feed. Where, PF & PA =price of food & animal feed, respectively; QF & QA=quantity of food & animal feed, respectively; SF & SA=supply of food & animal feed, respectively; CS=Consumer surplus; PS=producer surplus.

11. Knowledge Gaps

Menzies and Turkington (2015) commented on the relatively few active plant pathologists that have worked extensively with the *Claviceps purpurea* pathogen and improving understanding the biology of ergot production is an obvious requirement. Menzies goes on to mention that if molecular markers for different types of resistance could be developed, then finding crop lines which were most resistant to infection would be a lot more effective and efficient control measures and speed up ergot contamination reduction considerably.

The impact of geographical location has also not been extensively studied. This includes the impact of climatic conditions (especially local microclimates), predominating forms of the infective agents, preferred crop species and nutritional status of the plants grown as all of these may be important.

Effective long-term management of ergot requires investment in research into this disease.

One of the largest knowledge gaps that still needs to be addressed are the factors that influence the concentration of total ergot alkaloids in raw grain samples. Concentrations vary largely between those found for example by Malysheva *et al.* (2014) in EU grains (mean level 311 µg/kg) and Straumfors *et al.* (2015) in Norwegian grain mills (low µg/kg levels). There is still a need to better understand levels of all of the 12 ergot alkaloids in grain samples to support pending EFSA requirements. Canty *et al.* (2014) goes further and says that a national sampling, testing and reporting system for ergot alkaloid in foods and feeds needs coordinating and there is a need for inclusion of ergot alkaloid testing within the EU seed certification regulatory framework.

Levels of ergot alkaloids determined in honeydew as reported by Tittlemier *et al.* (2016) and their presence in some of the clean grain samples are important if alkaloid contamination is to be understood and therefore controlled. However, considering that the levels determined in sclerotia are 1000x times higher than honeydew, it is more likely that contamination stems from physical exchange between sclerotia and the cereal grains. However, this needs to be confirmed through additional research.

As noted in the ‘Exposure’ section above, cocktail effects arising from combinations of mycotoxins, including ergot alkaloids, are not well understood. In Research Review No. 71282, section 5.17, MacDonald *et al.* 2016, considered such synergies. If one ergot alkaloid is looked for in grain as a marker then it would be prudent to screen for all potential mycotoxin contaminants, suggesting the need for multi-analyte approaches to analysis.

Further to work done by Oregon State University as reported by Craig *et al.* (2014), feeding trials need to be conducted to establish threshold of toxicity levels in livestock species for the collection of ergot alkaloids that impact on animal health. Dänicke (2015), reported that ducks are more sensitive to TEA content than some other avian species but more information is required on other animals, e.g., horses, camels, pigs etc. Similarly, there have been no specific studies looking at 'individual' alkaloid effects other than physiometry results documenting vasoconstriction. One of the most recent and thorough reviews of this topic comes from Coufal-Majewski, *et al.* (2016). In their conclusions, and in highlighting the knowledge gaps they identified, the authors also recommend that *in vitro* and *in vivo* animal studies would be of benefit. The authors also noted that alkaloid 'binders' and antioxidants could limit the effects of alkaloid poisoning if effective binders could be identified. In summary the use of a two pronged attack of reducing ergot in cereals and reduce the toxicity of alkaloids for livestock should be further investigated.

In terms of methodology for the analysis of ergot alkaloids, immediate analytical chemistry development should focus on multi-analyte approaches such as LC-MS/MS, e.g., as currently being trialled by Fera Science Ltd. as part of a CEN method testing protocol (Krska, et al., 2008). However, with a view to the future, MIP and aptamer based solutions for both total and individual ergot alkaloid analyses may lead to a quick screening method for grains and foodstuffs and so should be investigated further. Neither of these is suitable for use outside the laboratory environment and more robust approaches such as hyperspectral imaging may have some utility in grain processing facilities.

One area that may also prove fruitful is the investigation of breakdown of alkaloids by use of bacteria or other microorganisms. This is a strategy that has been proven for other mycotoxins such as DON, as a small number of products have completed the EFSA approval process and are now on the market. Further research to isolate and identify suitable organisms for ergot alkaloid degradation is needed.

This review has emphasised the need to better understand variability in ergot alkaloid levels in food and feed but has shown that analytical approaches are available to detect the key toxins at low levels. Further research to better understand and prevent ergot alkaloid production and contamination and to mitigate the impact of contaminated feed on animal production is also required. To this end, multi-faceted approaches as described below and in Section 8.1 should be considered. Good agricultural practices;

- Using the most resistant varieties of seed for which a grading system for the seed is utilised.
- Optimised seed planting times to inhibit sclerotia growth to a small window of optimal opportunity, i.e., early seeding with flowering earlier.

- Control of crop bordering grasses (appropriate and accurate use of herbicides), and types of grasses (perhaps hedges used instead), to reduce inoculum source (disease reservoirs) and minimise honeydew transfer to adjacent crops during flowering.
- Minimise harvesting of crop edges near grass field margins where contamination is highest or establishing quarantine zones at field margins.
- Deep ploughing and a minimum two yearly crop rotation to allow sclerotia to rot away.

To post agricultural practices:

- Cleaning and / or sorting of grains to remove sclerotia by the most effective methods, e.g. mechanical means, scouring or high pressure air systems to remove dust, or using hyperspectral imaging for sorting in combination with sclerotia removal to optimise efficiency.
- Possible dilution of poorer grain grades with higher graded grain harvests, where this is allowed, to achieve a product that is perhaps more applicable for animal feed use.
- Possible inclusion of ‘binders’ or feed additives to animal feed to reduce toxicity or bioavailability of ergot alkaloids in feed.
- The potential for breaking down ergot alkaloids in feed and in possibly some foodstuffs, e.g. by hydrothermal treatment, needs to be investigated.

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13. Appendix 1 – Occurrence data for ergot alkaloids in cereal products published since 2013.

Reference	Matrix		Ergometrine	Ergometrinine	Ergotamine	Ergotaminine	Ergosine	Ergosinine	Ergocristine	Ergocristinine	Ergocryptine	Ergocryptinine	Ergocornine	Ergocorninne	Σ Ergot
Uhlig, Eriksen *et al.*. 2013. Faces of a Changing Climate: Semi-Quantitative Multi-Mycotoxin Analysis of Grain Grown in Exceptional Climatic Conditions in Norway. (note. Chanoclavine also listed but not covered in this review).	Barley (n=20)	Positive (%)	10		nr	nr	nr	nr	25		[a]15		12		
		Median (µg/kg)	0.6						20.2		[a]25.5		2.5		
		Max (µg/kg)	0.6						68.3		[a]58.7		10.3		
	Oats (n=28)	Positive (%)	0		nr	nr	nr	nr	<		[a]4		4		
		Median (µg/kg)	-						-		[a]0.3		0.2		
		Max (µg/kg)	-						-		[a]0.3		0.2		
	Wheat (n=28)	Positive (%)	18		nr	nr	nr	nr	4		[a]21		21		
		Median (µg/kg)	0.1						44.2		[a]8.6		4.2		
		Max (µg/kg)	0.9						44.2		[a]78.9		55.0		
Craig, Blythe *et al.*. 2014. The Role of the Oregon State University Endophyte Service Laboratory in Diagnosing Clinical Cases of Endophyte Toxicoses. (Note: Table does not include the ergovaline or lolitrem B results)	Case 2 (1500 steers)	Silage, wheat and barley grain (µg/kg)	nr	nr	545	nr	262	nr	1299	nr	[a]613	nr	753	nr	3
	Case 3 (330 steers)	Fescue straw + pellets (µg/kg)	nr	nr	[b]820	nr	nr	nr	nr	nr	nr	nr	nr	nr	
	Case 4 (40 cattle)	Pellets, seed screenings and haylage	nr	nr	[c]36858	nr	[c]4684	nr	[c]8016	nr	[ac]3494	nr	nr	nr	[c]5
(Kononenko and Burkin 2014). Mycotoxin contaminations in commercially used haylage and silage.	Haylage and silage	Minimal	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	
		Average													5
		Maximal													2
(Malysheva, Larionova *et al.*. 2014). Pattern and distribution of ergot alkaloids in cereals and cereal products from European countries.	Rye Food[1] (µg/kg)	Mean	3	1	12	3	10	3	11	4	17	8	10	5	
		Maximum	71	14	334	11	193	53	242	49	234	121	276	62	
	Wheat food[2] (µg/kg)	Mean	4	3	10	5	11	7	7	4	4	2	4	3	
		Maximum	74	145	139	62	142	136	76	151	52	32	74	42	
	Multigrain food[3] (µg/kg)	Mean	0.3	0.3	1	0.7	0.7	1	1	1	0.5	0.6	0.3	0.4	
		Maximum	4	15	36	10	17	37	10	13	6	6	5	8	
	Rye feed[4] (µg/kg)	Mean	1	8	48	13	43	34	91	7	57	10	38	13	
		Maximum	997	447	3270	1092	1567	1046	934	258	1748	480	1375	267	
	Wheat feed[5] (µg/kg)	Mean	2	0.2	0.1	0.02	5	5	1	0.2	3	4	3	1	
		Maximum	102	7	5	1	251	66	106	16	138	41	165	67	
	Triticale feed[6] (µg/kg)	Mean	1	0.2	8	2	9	2	22	8	5	1	2	1	
		Maximum	19	6	113	32	180	25	568	219	74	19	24	16	

Appendix 1. Contd.

Reference	Matrix			Ergometrine	Ergometrinine	Ergotamine	Ergotaminine	Ergosine	Ergosinine	Ergocristine	Ergocristinine	Ergocryptine	Ergocryptinine	Ergocornine	Ergocornine	Σ Ergot
(Zachariasova, Dzuman *et al.*. 2014). Occurrence of multiple mycotoxins in European feeding stuffs, assessment of dietary intake by farm animals.	non-fermented feedingstuff (µg/kg)	Hay (n=4)	Mean	nr	nr	nr	nr	nd	nd	nd	nd	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	nd	nd	10	10	10	nr	10	nr	
		Feeding wheat (n=21)	Mean	nr	nr	nr	nr	nd	nd	4	nd	6	nr	4	nr	
			Median	nr	nr	nr	nr	nd	nd	1.3	nd	1.3	nr	1.3	nr	
			Max	nr	nr	nr	nr	nd	nd	81	nd	118	nr	54	nr	
			LOQ	nr	nr	nr	nr	nd	nd	2.5	2.5	2.5	nr	2.5	nr	
		Feedin Barley (n=16)	Mean	nr	nr	nr	nr	nd	nd	nd	nd	2.5	nr	nd	nr	
			Median	nr	nr	nr	nr	nd	nd	nd	nd	1.3	nr	nd	nr	
			Max	nr	nr	nr	nr	nd	nd	nd	nd	33	nr	nd	nr	
			LOQ	nr	nr	nr	nr	nd	nd	2.5	2.5	2.5	nr	2.5	nr	
		Feeding Maize (n=8)	Mean	nr	nr	nr	nr	nd	nd	nd	nd	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	nd	nd	2.5	2.5	2.5	nr	2.5	nr	
		Feeding oat (n=3)	Mean	nr	nr	nr	nr	nd	nd	nd	nd	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	nd	nd	2.5	2.5	2.5	nr	2.5	nr	
		Soya meal (n=10)	Mean	nr	nr	nr	nr	nd	nd	5	3	nd	nr	nd	nr	
			Median	nr	nr	nr	nr	nd	nd	3	3	nd	nr	nd	nr	
			Max	nr	nr	nr	nr	nd	nd	39	30	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	nd	nd	5	5	nd	nr	nd	nr	
		Sugar beet pulps (n=6)	Mean	nr	nr	nr	nr	nd	nd	nd	nd	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	nd	nd	5	5	5	nr	5	nr	
		Extracted oil seeds (n=14)	Mean	nr	nr	nr	nr	nd	nd	11	nd	nd	nr	nd	nr	
			Median	nr	nr	nr	nr	nd	nd	5	nd	nd	nr	nd	nr	
			Max	nr	nr	nr	nr	nd	nd	60	nd	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	nd	nd	10	10	10	nr	10	nr	
	fermented feedingstuff (µg/kg)	Maize Silage (n=11)	Mean	nr	nr	nr	nr	nd	nd	nd	nd	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	nd	nd	10	nd	10	nr	10	nr	
		Clover, grass, alfalfa silages (n=12)	Mean	nr	nr	nr	nr	nd	nd	nd	nd	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	nd	nd	10	nd	10	nr	10	nr	
		Malt sprouts (28 samples)	Mean	nr	nr	nr	nr	nd	nd	nd	nd	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	nd	nd	5	nd	5	nr	5	nr	
		Brewer's grains (n=28)	Mean	nr	nr	nr	nr	nd	nd	nd	nd	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	nd	nd	2.5	nd	2.5	nr	2.5	nr	
		Maize-based dried distiller's grains with solubles (n=71)	Mean	nr	nr	nr	nr	nd	nd	2.8	nr	2.7	nr	3	nr	
			Median	nr	nr	nr	nr	nd	nd	2.5	nr	2.5	nr	2.5	nr	
			Max	nr	nr	nr	nr	nd	nd	90	nr	24	nr	14	nr	
			LOQ	nr	nr	nr	nr	nd	nd	5	nr	5	nr	5	nr	
		Wheat based dried distiller's grains with solubles (n=16)	Mean	nr	nr	nr	nr	nd	nd	12	nr	4	nr	3	nr	
			Median	nr	nr	nr	nr	nd	nd	3	nr	3	nr	3	nr	
			Max	nr	nr	nr	nr	nd	nd	146	nr	45	nr	37	nr	
			LOQ	nr	nr	nr	nr	nd	nd	5	nr	5	nr	5	nr	
	complex compound feed (µg/kg)	Feed for pigs (n=26)	Mean	nr	nr	nr	nr	6	nd	9	nr	3	nr	7	nr	
			Median	nr	nr	nr	nr	3	nd	3	nr	3	nr	3	nr	
			Max	nr	nr	nr	nr	116	nd	129	nr	53	nr	22	nr	
			LOQ	nr	nr	nr	nr	5	nd	5	nr	5	nr	5	nr	
		Feed for chickens and laying hens	Mean	nr	nr	nr	nr	nd	nd	nd	nr	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	5	nd	5	nr	5	nr	5	nr	
		Feed for cows (n=19)	Mean	nr	nr	nr	nr	nd	nd	nd	nr	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	5	nd	5	nr	5	nr	5	nr	
		Feed for birds and rodents (n=6)	Mean	nr	nr	nr	nr	nd	nd	nd	nr	nd	nr	nd	nr	
			LOQ	nr	nr	nr	nr	5	nd	5	nr	5	nr	5	nr	

Appendix 1. Contd.

Reference	Matrix			Ergometrine	Ergometrinine	Ergotamine	Ergotaminine	Ergosine	Ergosinine	Ergocristine	Ergocristinine	Ergocryptine	Ergocryptinine	Ergocornine	Ergocorninne	Σ Ergot
(Miskimins, Neiger *et al.*. 2015). Case report - ergot alkaloid poisoning in weaned beef calves.	Creep feed (µg/kg; cracked corn, rolled oats, alfalfa, brewer's grain or any combination of these four)			nr	nr	30	nr	25	nr	95	nr	35	nr	20	nr	2
(Mulder, Pereboom-de Fauw *et al.*. 2015). Tropane and ergot alkaloids in grain-based products for infants and young children in the Netherlands in 2011-2014. (Note: LODs were no higher than 0.15 µg/kg for all analytes)	Cereal Based products (µg/kg)	2011	No of positives	18		18		19		19		22		18		
			Mean	0.81		2.73		1.61		2.29		1.66		1.39		
			Max	7.8		23.9		14.9		11.7		8.9		12.5		
		2012	No of positives	13		14		15		15		16		15		
			Mean	0.54		1.45		1.06		1.11		1.42		0.58		
			Max	6.2		39.9		18/0		24.9		18.4		8.4		
		2014	No of positives	7		6		8		9		8		8		
			Mean	0.83		2.11		1.18		2.36		1.11		0.84		
			Max	8.7		14.4		10.4		26.8		10.2		10.6		
(Bauer, Gross *et al.*. 2016). Investigations on the occurrence of mycotoxins in beer.	Beer (n=44)		Positive (%)	93	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	
			Mean	0.19												
			Median	0.07												
			Minimum	0.15												
			Maximum	0.47												

n - number of samples
nd – not detected
nr - not reported
uk - unknown
[a] - Where only the α form is stipulated
[b] - One bin of pellets
[c] - Pellet contamination
[1] - Flour, n=202 samples; crispbread, n=6; bread, n=16; grain, n=2.
[2] - Flour, n=151; biscuit, n=4; bread, n=32; bran, n=137; germs, n=2; grain, n=6.
[3] - Flour, n=18; biscuit, n=42; crispbread, n=11; bread, n=81; flakes, n=34.
[4] - Grain, n=157
[5] - Grain, n=137
[6] - Grain, n=27
< - < LOQ.

14. Appendix 2 - Recent Methods Published for determination of ergot alkaloids

#	Paper Title	Compounds	Foodstuff	Limits (µg/kg)	Validation	Advantages/ Dissadvantages	Publication
1	Developments in mycotoxin analysis: an update for 2010-2011 Example of series of papers updated each year, from approximately 2008.	Review – all mycotoxins, includes section on ergot alkaloids	Review	Review	Review	Section 6 deals with Ergot Alkaloids.	Shephard, G. S., *et al.*. (2012). World Mycotoxin Journal 5(1): 3-30.
2	Developments in mycotoxin analysis: an update for 2015-2016	Review – all mycotoxins, includes section on ergot alkaloids	Review	Review	Review	Methods for ergot alkaloids include, LC-MS, LC-HRMS and	Berthiller et al. 2017, World Mycotoxin Journal,10 (1) 5-29. open access on line.
3	Validation and transferability study of a method based on near-infrared hyperspectral imaging for the detection and quantification of ergot bodies in cereals.	Specific to ergot bodies in grains.	Cereal grains.	Not µg/kg but could detect ergot body content as low as 0.02% in grain, less than the EC intervention limit of 0.05%.	Reported repeatability and robustness. Described in Review given below.	Screening method for assessing grain in situ. Not specific to the ergot compounds.	Vermeulen, P., *et al.*. (2013). Analytical and Bioanalytical Chemistry 405(24): 7765-7772.
4	Holistic approach based on high resolution and multiple stage mass spectrometry to investigate ergot alkaloids in cereals.	ergometrine, ergosine, ergotamine, ergocornine, α-Ergokryptine, ergocristine, ergometri- nine, ergosinine, ergotaminine, ergocorninine, α-ergokryptinine, ergocristinine	Rye samples.	Not reported.	Not specific to sample; tuning of instrument etc.	Largely about discovering ergot pathways. Expensive technique, Orbitrap MS.	Arroyo-Manzanares, N., *et al.*. (2014). Talanta 118: 359-367.
5	Developments in mycotoxin analysis: an update for 2012-2013	Review	Review	Review	Review	References range to accommodate most of those discussed here in years 2012 - 2013. Section 7 most relevant to ergot alkaloids.	Berthiller, F., *et al.*. (2014). World Mycotoxin Journal 7(1): 3-33.
6	Aptamer-based extraction of ergot alkaloids from ergot contaminated rye feed.	Ergosine, ergocryptine, ergocornine.	Rye Feed.	None provided.	Comparative test results to different methodology to test robustness used. Triplicate results only.	Highly specific DNA aptamer almost to the ergot, as opposed to less animal friendly antibodies with specificity for ergots in general. SPE/ LC-QTof-MS. Expensive technology for analyses.	Martin, E. R *et al.* (2014). Advances in Bioscience and Biotechnology 5(8): 692-698.

Appendix 2. Contd.

#	Paper Title	Compounds	Foodstuff	Limits (µg/kg)	Validation	Advantages/ Dissadvantages	Publication
7	Macroscopic and microscopic spatially-resolved analysis of food contaminants and constituents using laser-ablation electrospray ionization mass spectrometry imaging (LAESI-MSI).	Ergine, erginine, ergometrine ergometrinine, ergotamine ergotaminine, and ergocristine ergocristinine	Rye.	Not relevant as analysis of specific ergot bodies rather than, for example, a rye flour.	Not specific to sample; tuning of instrument etc.	No sample preparation or chromatography. Direct analysis of physical sclerotia ergot bodies. Expensive technology	Nielen, M. W. F. and T. A. van Beek (2014). Analytical and Bioanalytical Chemistry 406(27): 6805-6815.
8	Liquid Chromatography/Ion Trap Mass Spectrometry Technique to Determine Ergot Alkaloids in Grain Products.	Ergosine, Ergometrine, ergotamine, ergocornine, ergocryptine and ergocristine including the relative inines of each.	Rye based food products; Flour 34 samples, Bran 12 samples, Rye 18 samples and one flake sample.	LOQs: Ines; inines; 3 and 1 µg/kg resp. LODs: Ines; inines; 0.5 and 0.2 µg/kg resp.	Robust data inc. Recoveries range 63 – 104.6%, RSD < 18%; wide linear ranges and correlations > 0.98.	Alumina SPE clean up. Thorough validation. Expensive equipment. Samples need to be very clean to stop matrix interferences and loss of sesitivity.	Bryla, M., K. *et al.* (2015). Food Technology and Biotechnology 53(1): 18-28.
9	Simultaneous separation of ergot alkaloids by capillary electrophoresis after cloud point extraction from cereal samples.	Ergotamine and Ergonavine	Cereal; Flour; Rye, Wheat, oat, rice, soybean and corn. Grain; Wheat and oat. Infant food; Composite of rice, corn, wheat, barley, sugar and additives	2.6 and 2.2 µg/kg respectively	Extraction and clean up recoveries > 92%.	Low solvent and environmentally friendly. Relatively cheap compared with MS. Still specialised equipment required.	Felici, E., *et al.* (2015). Electrophoresis 36(2): 341-347.
10	Multi-mycotoxin Analysis of Finished Grain and Nut Products Using Ultrahigh-Performance Liquid Chromatography and Positive Electrospray Ionization-Quadrupole Orbital Ion Trap High-Resolution Mass Spectrometry.	Ergocornine, ercocristine, ergocryptine, ergometrine, ergosine, ergotamine. No inines.	Corn, rice, wheat, almond, peanut, and pistachio.	Matrix dependant but ranging from 0.03 µg/kg LOD to 0.6 µg/kg LOQ. Very low.	Thorough and according to EPA acceptance criteria.	Simple solvent extraction technique. Very expensive instrumentation.	Liao, C.-D., *et al.* (2015). Journal of Agricultural and Food Chemistry 63(37): 8314-8332.
11	Target analysis and retrospective screening of veterinary drugs, ergot alkaloids, plant toxins and other	ergosine, ergosinine, ergocornine, ergo- corninine, ergocryptine, ergocriptinine,	32 poultry, swine, cattle, horse and lamb feed samples.	LOQ 20 µg/kg.	Some validation including Trueness and RSD values with	Solvent extraction with salt packet. Data can be extracted retrosectively. Expensive detector.	Leon, N., A. *et al.* (2016). Talanta 149: 43-52.

	undesirable substances in feed using liquid chromatography-high resolution mass spectrometry.	ergocristine and ergocris-tinine.			R^2 over dynamic range.		

Appendix 2. Contd.

#	Paper Title	Compounds	Foodstuff	Limits (µg/kg)	Validation	Advantages/ Dissadvantages	Publication
12	Screening for total ergot alkaloids in rye flour by planar solid phase extraction-fluorescence detection and mass spectrometry.	Total ergot alkaloid content expressed as ergocristine.	Rye flour.	LOD and LOQ were: 0.07 and 0.24 mg/kg respectively.	Relates 'near 100% recoveries'.	Screening for total ergot alkaloid content. Not ergot specific but relatively cheap instrumentation.	Oellig, C. and T. Melde (2016). Journal of Chromatography A 1441: 126-133.
13	The different conformations and crystal structures of dihydroergocristine.					This is relating dihydroergocristine (DHEC) as a suitable compound to be used as a molecularly imprinted polymer (MIP) rather than for use as an analytical method in a conventional sense.	Moench, B., W. *et al.* (2016). Journal of Molecular Structure 1105: 389-395.
14	Rapid Screening of Ergot Alkaloids in Sclerotia by MALDI-TOF Mass Spectrometry.	Ergosine, ergocornine, ergocryptine and ergocristine.	Specific to ergot analysis in sclerotia.	Not reported.	Not reported.	Rapid screening method. Minimal sample prep. Requires expensive instrumentation. Not used in food or feedstuffs.	Sivagnanam, K., *et al.* (2016). Journal of AOAC International 99(4): 895-898.