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Scientific information on mycotoxins and natural[1] plant toxicants

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Summary

Undesirable substances such as mycotoxins and natural toxicants can be present in plants and derived products thereof. Depending on the nature and the concentration levels of the compound, these might be of concern for human and/or animal health. Risk assessments on the natural plant toxicant morphine in poppy seeds and the selected mycotoxins: alternaria toxins, moniliformin, diacetoxyscirpenol, sterigmatocystin and phomopsins in food and feed, ergot alkaloids in food and nivalenol in feed have not been carried out at the European level. Therefore it is expected that the European Commission may ask EFSA to assess the risks to human and animal health related to these substances in the near future. To carry out these risk assessments to the highest standards and in an efficient way scientific background information e.g. chemistry, occurrence, toxicokinetics and toxicity on these selected mycotoxins and plant toxicant are needed. The project CFP/EFSA/CONTAM/2008/01 “Scientific information on mycotoxins and natural plant toxicants” reports this information in the present report.

The report presents information regarding a plant toxicant, morphine, in poppy seeds, and some mycotoxins, in particular Alternaria toxins in food and feed, Ergot alkaloids in food, moniliformin in food and feed, nivalenol in feed, diacetoxyscirpenol in food and feed, sterigmatocystin in food and feed and phomopsins in food and feed. After a short introduction, fungi involved in the production of each specific mycotoxin are described. Physico-chemical characteristics, biosynthesis and chemical analisis are then discussed for all toxins. Occurrence data are presented, taking into account all products in which the mycotoxins have been reported. Mitigation, intended as all actions that can be taken in the pre- and post-harvest periods to reduce the contamination of the final products are discussed. Pharmacokinetic aspects (absorption, distribution and excretion, metabolism and carry-over), as well as toxicity (acute and chronic), are also discussed, based on available information in animals and in humans. This report represents the state-of-the-art knowledge on the toxic compounds included, and provides information useful to the scientific community to face the risks and challenges of mycotoxins and plant natural toxicants.

As a general conclusion it can be underlined that for all compounds considered there is a common lack of knowledge which can be summarised as follows:

- Detailed knowledge on fungi involved and their interaction with host crops
- Validated analytical methods and reference materials
- Detailed surveys in different countries and different years
- Specific sampling methods
- Detailed and updated knowledge on pharmacokinetics and toxicity

Keywords: scientific information, mycotoxins, natural plant toxicants, food, feed, morphine, alternaria toxins, ergot alkaloids, moniliformin, sterigmatocystin, diacetoxyscirpenol, nivalenol, phomopsins.

INDEX

Background

Undesirable substances such as mycotoxins and natural toxicants can be present in plants and derived products thereof. Depending on the nature and the concentration levels of the compound, these might be of concern for human and/or animal health. Risk assessments on the mycotoxins and natural plant toxicants listed below have not been done at the European level and therefore it is expected that the European Commission may ask EFSA to assess the risks to human and animal health related to these substances in the near future. To carry out these risk assessments to the highest standards and in an efficient way scientific background information on the following substances are needed.

Morphine in poppy seeds
Alternaria toxins in food and feed
Ergot alkaloids in food
Moniliformin in food and feed
Nivalenol in feed
Diacetoxyscirpenol in food and feed
Sterigmatocystin in food and feed
Phomopsins in food and feed

Terms of reference

The outcome of the project CFP/EFSA/CONTAM/2008/01 shall contain:

Collection and compilation of scientific information on the natural plant toxicant morphine in poppy seeds and the selected mycotoxins: alternaria toxins, moniliformin, diacetoxyscirpenol, sterigmatocystin and phomopsins in food and feed, ergot alkaloids in food only and nivalenol in feed only.

For each of the outlined mycotoxin/natural plant toxicant, a scientific report is requested. This report shall outline the key findings in the seven areas listed below, including a full reference list according to the EFSA citation standards.

All references cited in the report will be included in ProCite and the data base will be annexed to the report.

Scientific information shall be collected, compiled and synthesised for each substance for the following seven areas:

Area 1: Chemistry and biosynthesis
Area 2: Analytical methods recently used in food and feed monitoring and the state of the art in analytical methodology, and sampling techniques if available
Area 3: Occurrence data in food and feed commodities in Europe, particularly to identify food and/or feed commodities which are considered as susceptible for contamination by these compounds including co-occurrence data on other mycotoxins/natural toxicants if applicable.
Area 4: Factors, including environmental, agronomic and processing, (e.g. storing conditions), influencing the levels of these compounds in plant products used for food and feed production, including possible mitigation
Area 5: Transfer/carry over of these compounds from feed to products of food producing animals including fish
Area 6: Data on toxicokinetics (absorption, distribution, metabolism, excretion), toxicity (acute, subacute, long-term and developmental effects) in experimental and farmed animals
Area 7: Epidemiological data in humans if applicable

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Introduction

Undesirable substances such as mycotoxins and natural toxicants can be present in plants and derived products thereof. Depending on the nature and the concentration levels of the compound, these might be of concern for human and/or animal health. Risk assessments on the natural plant toxicant morphine in poppy seeds and the selected mycotoxins: alternaria toxins, moniliformin, diacetoxyscirpenol, sterigmatocystin and phomopsins in food and feed, ergot alkaloids in food and nivalenol in feed have not been carried out at the European level. Therefore it is expected that the European Commission may ask EFSA to assess the risks to human and animal health related to these substances in the near future. To carry out these risk assessments to the highest standards and in an efficient way scientific background information on these selected mycotoxins and plant toxicant are needed.

To fullfil the terms of reference of the project CFP/EFSA/CONTAM/2008/01 the partners of this project divided the entire theme into different issues such as:
1. Crops contamination: data collection on host plants and mycotoxins producing organisms, role of pre-and post-harvest on contamination and mitigation actions.
2. Characterisation and occurrence: collection of information about the chemistry and biosynthetic pathway of mycotoxins and plant natural toxins, analytical methods used to quantify the mycotoxins and plant natural toxicants and sampling protocols, occurrence in different matrices.
3. Contaminated products chain: data collection on food and feed contamination, carry-over included, with special attention to toxicokinetics and toxicity in experimental and farmed animals and epidemiological data related to food and feed.

The collected data were carefully evaluated, and elaborated when necessary, to obtain a data base and reports for different substances. The scientific information found in the project CFP/EFSA/CONTAM/2008/01 “Scientific information on mycotoxins and natural plant toxicants” is reported in this report.

The report presents information regarding a plant toxicant, morphine, in poppy seeds, and some mycotoxins, in particular Alternaria toxins in food and feed, Ergot alkaloids in food,

moniliformin in food and feed, nivalenol in feed, diacetoxyscirpenol in food and feed, sterigmatocystin in food and feed, and phomopsins in food and feed. After a short introduction, fungi involved in the production of each specific mycotoxin are described. Physico-chemical characteristics, biosynthesis and chemical analisis are then discussed for all toxins. Occurrence data are presented, taking into account all products in which the mycotoxins have been reported. Mitigation, intended as all actions that can be taken in the pre- and post-harvest periods to reduce the contamination of the final products are discussed. Pharmacokinetic aspects (absorption, distribution and excretion, metabolism and carry-over), as well as toxicity (acute and chronic), are also discussed, based on available information in animals and in humans. This report represents the state-of-the-art knowledge on the toxic compounds included, and provides information useful to the scientific community to face the risks and challenges of mycotoxins and plant natural toxicants.

As a general conclusion it can be underlined that for all compounds considered there is a common lack of knowledge which can be summarised as follows:

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Chapter 1 - Morphine in poppy seeds

Keywords: Morphine, Poppy seeds, Toxicity, Analytical methods, Alkaloids, Metabolism.

INTRODUCTION

The poppy (*Papaver somniferum*) is a natural source of opium. It has origins in Asia Minor but is now grown in locations with similar climates throughout the world, including Anatolia and Turkey. The annual production of poppy seeds exceeds 32.000 metric tons in Turkey, and 18.000 tons of this amount are produced in Afyon alone, a province in the central west of Turkey (Akinci and Bayram, 2003).

The opiate alkaloids present in poppy seeds intended for use in food recently have raised major concerns. The seeds of the opium poppy (*Papaver somniferum L*.) are commonly used in dishes and pastries in central Europe. The alkaloid content in the latex of the plant, with morphine as its major constituent, has raised major concerns. For this reason only the low-morphine variety Mieszko is certified for cultivation in Germany (Stolzenburg, 2006). However, Germany's annual poppy seeds requirement for baking and food use of up to 10000 t is so far almost exclusively covered by imported goods. Important producer countries are Turkey, the Czech Republic, Hungary, and Austria (Rochholz *et al.*, 2005).

Various toxicological studies report that consumption of large amounts of commercially available poppy seeds can lead to light-headedness and enteroparesis in sensitive individuals. The symptoms described are in agreement with the range of toxicological actions of morphine. In particular, reports indicate that morphine intake from poppy seeds may be on the scale of therapeutic morphine doses.

In a study published in 2004 Moeller and colleagues (Moeller *et al*., 2004) gave various poppy seeds-containing products (rolls, cake) to five individuals. The morphine content of the poppy seeds was 50 µg/g. Shortly after consumption these individuals were examined by a trained policeman using a checklist for routine tests for drug consumption in car drivers. The test proved negative. Only one of the individuals (55 kg), who had eaten the largest amount of poppy seeds cake, reported a slight drug effect involving light-headedness with reduced reaction time of the pupils. No free morphine or codeine was found in the serum of any of the five people. However, after hydrolysis of the morphine conjugates morphine could be detected using GH/MS (highest value: 24 ng/ml). Morphine and codeine could be detected in the urine of all test participants following hydrolysis.

In other relevant studies by Rochholz *et al*. (2005) and Westphal *et al*. (2006) twenty individuals (age 19 - 45) consumed between 25g and 250g of poppy seeds in different forms. They were instructed to eat as much as they could. The batches used in this study were analyzed for their

morphine levels, and concentrations of 72.4-114.3 µg/g were found. Serum collected 1, 2, 4, 8 and 24 hours after consumption was found to contain morphine and codeine. After the last blood sample was taken the individuals were asked to describe their subjective conditions since ingesting poppy seeds. Seven individuals indicated that they did not experience any effect. In contrast, the 13 other individuals reported one or more of the following symptoms: tiredness, lack of drive, difficulty concentrating, headache, dizziness, ongoing dry mouth, minor to severe nausea down to vomiting, heavy tongue, impaired field of vision.

Bjerver *et al*. (1982) described an experiment in which seven individuals consumed one or two pieces of a poppy seeds cake, which contained 5 mg morphine per portion. After intake of 5-10 mg morphine, constipation was the only effect observed.

Andresen and Schmoldt (2004) described a study on 12 persons (7 female/5 male, age: 23-58 years) who ate 1-4 pieces of poppy seeds cake within 30 minutes. The morphine content of the baking poppy seeds was 206 µg/g in batch 1 and 0.6 µg/g in batch 2. Each test person ingested between 9 – 55 g of poppy seeds. The blood concentration of free morphine was 8.5 ng/ml after 4 hours in one person and 13.5 ng/ml after 4.5 hours in another. The only adverse reaction which occurred in all female individuals was a major sensation of nausea. The authors suggest that these effects may be attributed to the content of alkaloids in the poppy seeds, or to other ingredients.

In other publications there have been reports of a feeling of and sensation of lethargy (Hagers Handbuch, 1998) or constipation (Rochholtz *et al*., 2004) as adverse reactions after consuming poppy seeds-containing pastry without morphine intake being quantified.

Hayes *et al*. (1987) carried out blood tests in four test persons after consumption of 25 g poppy seeds with 294 µg morphine/g and 14 µg codeine/g, equivalent to intake of 7.5 mg morphine and 0.4 mg codeine. After hydrolysis, they found 82-131 ng total morphine/ml serum (2 hours after consumption). However, none of the symptoms caused by morphine were observed.

A case report entitled “Morphine sans Morpheus” published in 1995 by Hanks (1995) described a 6-week old baby who suffered from severe health impairment including respiratory depression and had to be taken into intensive care after its mother had given him 75 ml of a milk preparation obtained after straining a mixture of 200 g poppy seeds and 500 ml milk. Morphine levels in the serum of the baby on the following day were 4.3 ng/ml. The poppy seeds used had a 0.1 % morphine and a 0.003 % codeine content.

Origin of alkaloids in poppy seeds

Besides the poppy variety, the method of harvest has the highest influence on morphine concentrations and leads to great variability of the alkaloid concentration (Moeller *et al.*, 2004). Poppy seeds harvest can take place in two ways. Traditionally, the ripe seed is manually shaken (especially where manual labour is cheap) so that the seed falls out of the holes below the many-rayed stigma. In contrast, the high-yield closed-capsule plants have to be crushed open using modified combine harvesters sealed against loss of fine seed (Feiffer, 1998; Rochholz *et al.*, 2005). The latter procedure contaminates the seed with the chyle of unripe capsules, which has to be removed afterwards. Otherwise, a higher alkaloid content will result (Andresen and Schmoldt, 2004). This is also proven by the fact that washing the poppy seeds can drastically reduce its morphine content. This study confirms the observations that poppy seeds morphine originates predominantly from external contamination.

High morphine concentrations in the seed can be attributed to insufficient harvest technology, leading to seed contamination with morphine rich latex or to inadequate cleaning and handling. Considering lot-to-lot differences and inhomogeneities in the sampling process, manufacturers were advised of their duty to exercise diligence and to use state-of-the-art measures to limit the seed morphine content. Washing, in combination with blending different batches, would be an adequate way for manufacturers to reduce the poppy seeds morphine content.

PHYSICO-CHEMICAL CHARACTERISTICS

The main physico-chemical characteristics of morphine are the following: molecular formula, $C_{17}H_{19}NO_3$; molecular weight 285.33; elemental analysis, C 71.56%, H 6.71%, N 4.91%, O 16.82%; boiling point 254°C, also a metastable phase with melting point 197°C; $[\alpha]_D^{25°C}$ = -132° (MeOH); pKa = 9.85; UV max in acid: 285 nm; in alkali: 298 nm. Usually found as monohydrate in the crystalline form, although the amorphous form is more soluble. Solubility: 1 gr in 5000 ml water, 210 ml alcohol, 525 ml ethyl acetate, 1220 ml chloroform; freely soluble in alkali and acids, phenol, cresols; slightly soluble in ammonia, benzene. Mostly available as salt with different inorganic or organic acids which show different physico-chemical behaviour and a generally higher solubility.

BIOSYNTHESIS

Morphine belongs to a class of vegetal alkaloids deriving from L-tyrosine (Facchini *et al.*, 2007). The PLP-dependent decarboxylation of L-Tyrosine affords the phenylethylamino derivative tyramine. Usually, molecules having phenylethylamino structure are 3,4-dihydroxylated or 3,4,5-trihydroxylated derivatives, formed starting from dopamine, the product of the decarboxylation of L-DOPA (L-dihydroxyphenylalanine). Among these molecules, most important are the catecholamines such as noradrenaline (norepinephrine) or adrenaline (epinephrine) (Page, 2005).

In the morphine biosynthetic pathways, the reaction between a phenylethyl and a phenylethylamino moiety gives rise to the benzyltetrahydroisoquinoline skeleton, which may undergo other modifications giving rise to a wide group of vegetal alkaloids, most of which are of high pharmacological interest (Facchini *et al.*, 2007) (Figure 1). Modification of this base skeleton increases the variety of compounds in this class, giving rise to modified tetrahydroisoquinolines. Many of these alkaloids contain an ortho dioxigenation in both the aromatic rings, whose origin is thus attributable to two units of L-DOPA. Although during biosynthesis two molecules of tyrosine are used, only the phenylethylamino fragment of the tetrahydroisoquinoline skeleton comes from DOPA, while the other carbon atoms derive from tyrosine via 4-hydroxyphenylpiruvic acid and 4-hydroxyphenylacetaldehyde (Rueffer and Zenk, 1987, Facchini *et al*., 2007).

The product of the Mannich reaction is the trihydroxyalkaloid norcoclaurine, stereospecifically formed as S-enantiomer (Stadler *et al.*, 1989) (Figure 1). The tetrahydroxy substitution pattern derives from another hydroxylation of the benzyl ring, although this biosynthetic step is preceded by an O-methylation (to afford S-coclaurine) and by a N-methylation (Figure 1). Finally, S-reticuline is obtained via another O-methylation (Frenzel and Zenk, 1990; Luk *et al.*, 2007).

Tebaine, codeine and morphine are obtained from R-reticuline: it has been demonstrated that the change of configuration is obtained via a redox process which involves the intermediate 1,2-dihydroreticulonium ion (Hirata *et al.*, 2004; Facchini *et al.*, 2007).

The next step, involving an oxidative phenolic coupling, is fundamental to understand the chemical transformation of the benzyltetrahydroisoquinoline skeleton to form alkaloids belonging to different classes (Figure 2). The main opium alkaloids morphine, codeine and tebaine derive from this kind of coupling, although the reduction of one of the aromatic rings partially masks

their benzyltetrahydroisoquinoline origin. R-reticuline has been identified as the precursor of these morphinanic alkaloids (Facchini *et al.*, 2007)

R-reticuline is the substrate of monoelectronic oxidation in each ring, via the phenol group, which at the end afford the biradicalic structure (Figure 2). The coupling in para of the phenol group of tetrahydroisoquinoline and in ortho to the hydroxyl group on the benzyl ring affords the dienone salutaridine, isolated as a minor component of the mixture of alkaloids in opium poppy *Papaver somniferum* (Papaveracee) (Gerardy and Zenk, 1993). Only in the original benzyl ring aromaticity can be regenerated, as the tetrahydroisoquinoline moiety is coupled in para to the phenolic function, an already substituted position. Alkaloid tebaine is obtained via salutaridinol, formed from salutaridine via stereospecific reduction of the carbonyl group (Grothe *et al*., 2001) (Figure 2). The ring closure to form the ether bridge in tebaine is the result of the nucleophilic attack on the dienol system with the following elimination of the hydroxyl group after activation of the leaving group with acetyl-CoA. The following reactions involve the conversion of tebaine in codeine and, then, in morphine, a process which modifies the oxidation state of the dienoic ring, but overall removes two o-methyl groups (Figure 2). One of this is present as enol ether and its elimination gives rise to neopinone, which gives codeinone and then codeine via allylic isomerisation and reduction, respectively. The last steps, both the demethylation of the enol ether and of the phenol ether, probably involves an initial hydroxylation followed by the loss of the methyl groups as formaldehyde (Lenz and Zenk, 1995; Unterlinner *et al.* 1999) (Figure 2).

In some poppy species the last steps are different: tebaine is converted into oripavine and morphinone, and in this case the O-methyl phenol group takes place before that of the enol ether, giving rise to a slight different order of these last steps.

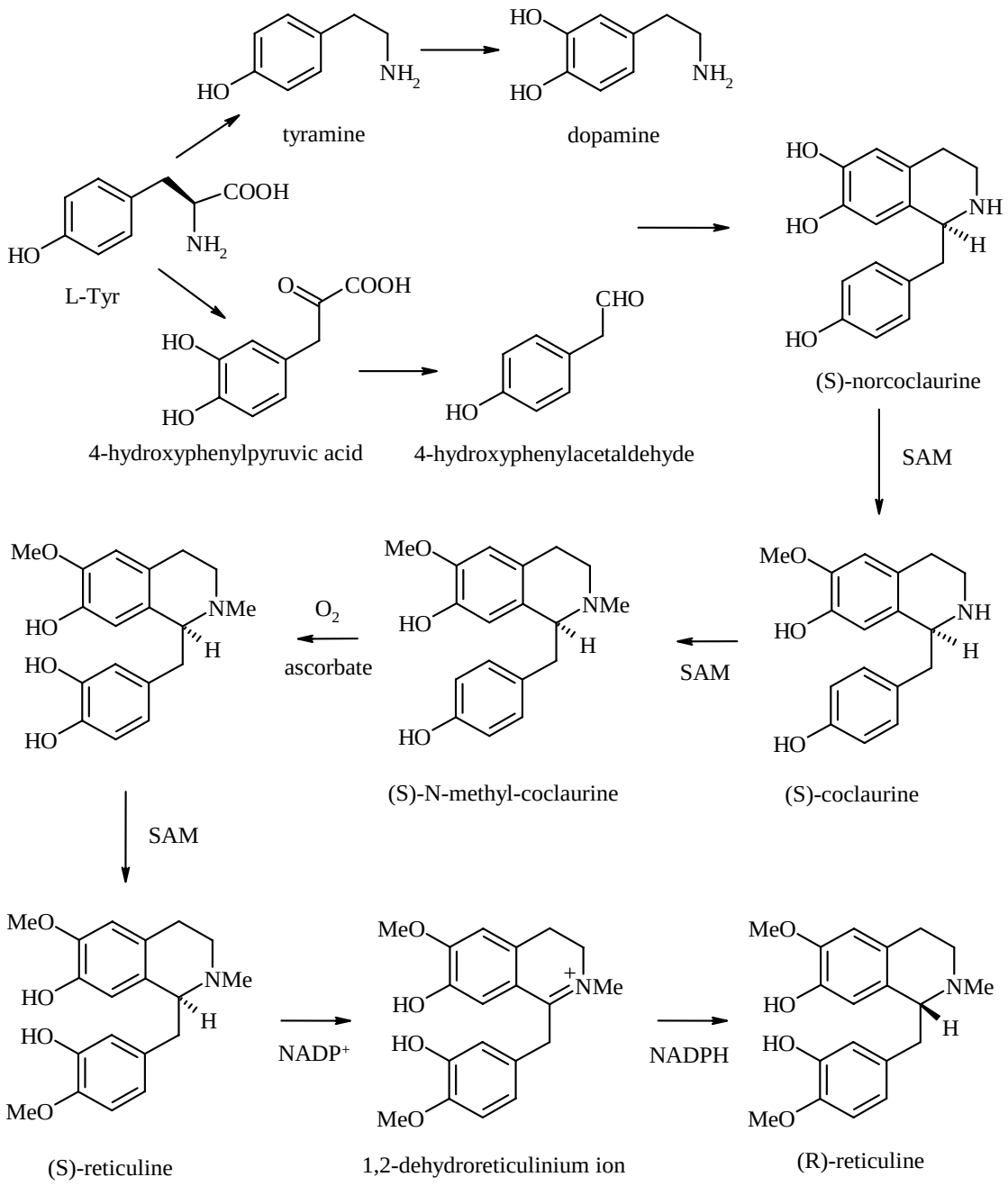

Figure 1. Morphine biosynthesis: biosynthetic steps from L-Tyrosine to (S)-reticuline.

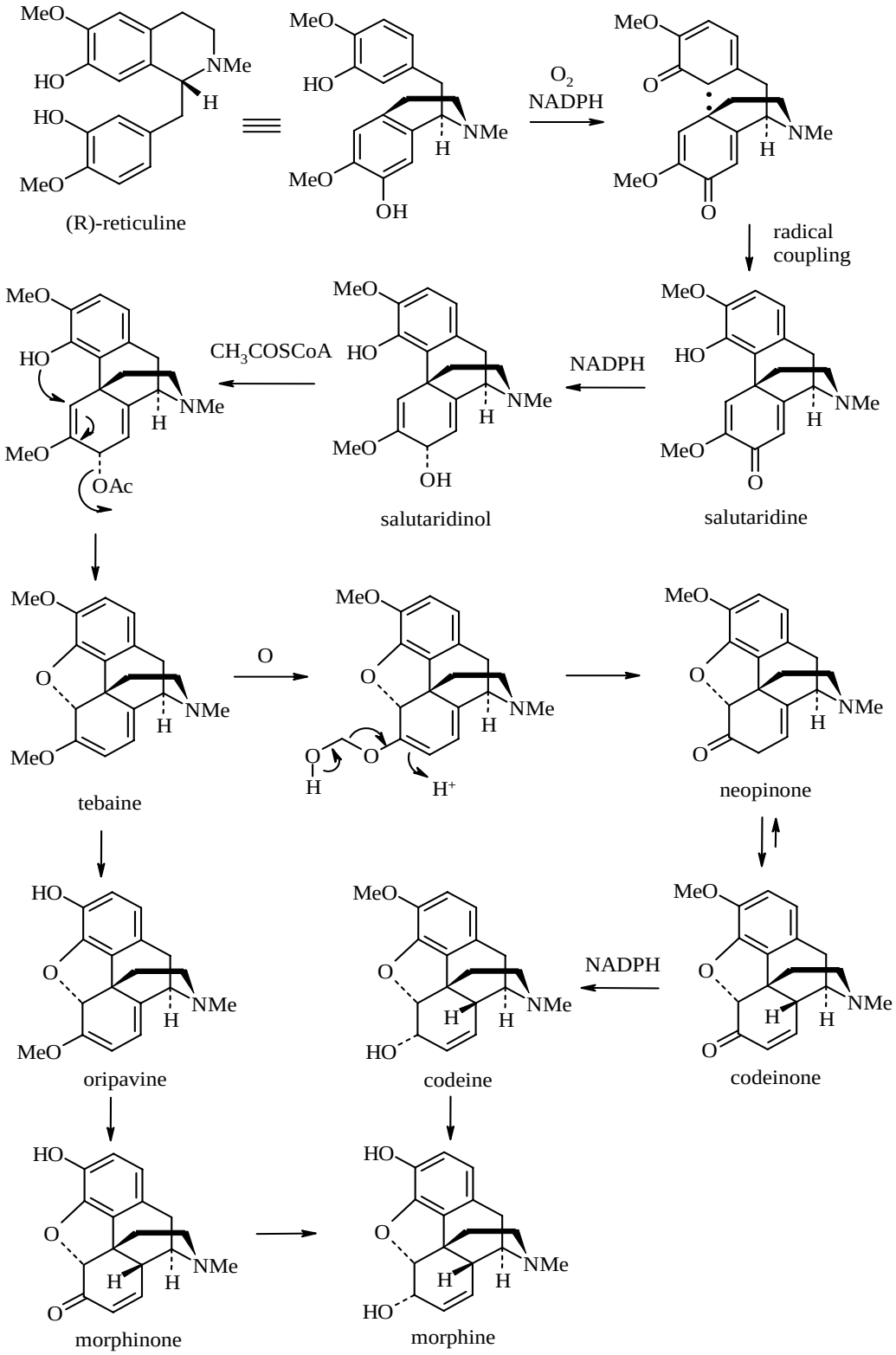

Figure 2. Morphine biosynthesis: biosynthetic steps from (S)-reticuline to morphine.

CHEMICAL ANALYSIS

Sampling

It can be presumed that the distribution of morphine in raw poppy seeds batches is predominantly inhomogeneous. Therefore, a suitable sampling procedure is inevitable. The effort needed for sampling can be reduced to one-third (5 instead of 15 individual samples per big bag, 20 instead of 60 individual samples per 4000 kg batch). The results show a very good correlation between the individual samples, the intermediate samples per big bag and the combined samples of the entire batch. Nevertheless, the strategy proposed by Moser and Kniel (2008), must be statistically secured by further tests and experiences gained. An effective sampling with reasonable effort for industry and official authorities as a basis for meaningful examination results would be desirable.

Extraction and clean-up

Several methods have been proposed for the extraction of morphine from poppy seeds: analytical procedures have been extensively reviewed in the last decade (Stockigt *et al*., 2002; Bosch *et al*., 2007).

The first attempt to quantitatively extract morphine from domestically grown opium poppy was performed in the 50's (Brekke *et al*., 1958a and b). The opium poppy was milled and treated with an aqueous ammonia solution to allow the release of alkaloids from their naturally occurring salts. Afterwards, an extraction step with water-saturated isobutyl alcohol in a continuos counter-current extractor was performed, allowing for a 95% recovery.

More recently, a warm 80% ethanol extraction was proposed (Schmidt *et al*., 2007). The extract was then evaporated under vacuum and the residue was dissolved in 1N chloridric acid. After a washing step with chloroform, the acidic layer was adjusted to pH 9.3 with 2N potassium hydroxide and back-extracted with chloroform. A similar approach was used also by Frick *et al*. (2005). In this case, the ethanolic extract was made alkaline with sodium bicarbonate and back-extracted with ethyl acetate.

Acidic methanol (0.1% acetic acid) was also used for morphine extraction (Sproll *et al*., 2006).

Since a good purification level can be achieved using a liquid-liquid approach, the use of Solid Phase Extraction (SPE) cartridges as well as immunoaffinity columns has never been reported in the literature until now. However, the extraction procedures commonly proposed are often

time-consuming and can be affected by low recovery, since several extraction steps are involved.

Standards

Morphine standards are commercially available from the main chemical reagents companies. In particular, Sigma-Aldrich may purchase a morphine standard solution (1mg/ml in methanol) and morphine sulphate salt pentahydrate as powder.

Isotopically labelled standard solutions are also available, to be used as internal standard for mass spectrometry based methods. In particular, morphine-d3 hydrochloride standard solution (100 μg/ml) is purchased by Sigma-Aldrich.

Availability of morphine standards is shown in Table 1.

Table 1. Sources of morphine standards.

Compound	Concentration	Quantity	Supplier	References
Standard Solution				
Morphine solution	1 mg/ml in methanol	1 ml	Sigma-Aldrich (Fluka)	
Morphine sulfate salt solution	1 mg/ml ± 5% in methanol	1 ml	Sigma-Aldrich (Fluka)	
Morphine hydrochloride solution	100 µg/ml	1 ml	Sigma-Aldrich (Fluka)	
6-Acetylmorphine solution	1 mg/ml in acetonitrile	1 ml	Sigma-Aldrich (Fluka)	
Solid Standard				
Morphine			Sigma-Aldrich (Taufkirchen, Germany) Lipomed (Arlesheim, Switzerland)	Sproll *et al*., 2007
Morphine sulphate		25/50/250 mg	Sigma-Aldrich Lipomed (Arlesheim, Switzerland) Macfarlan Smith - Johnson Matthey PLC (Edinburgh, Scotland)	
Morphine hydrochloride		5 mg	Sigma-Aldrich Lipomed (Arlesheim, Switzerland) Macfarlan Smith - Johnson Matthey PLC (Edinburgh, Scotland) Service Chemical Inc. (Regenstauf, Germany)	
6-Acetylmorphine hydrochloride			Lipomed (Arlesheim, Switzerland)	
Dihydromorphine			Lipomed (Arlesheim, Switzerland)	
Morphine tartrate			Macfarlan Smith - Johnson Matthey PLC (Edinburgh, Scotland)	

Analytical methods

The main methods used for morphine analysis in poppy seeds have been recently reviewed (Stockigt *et al*., 2002; Bosch *et al*., 2007). Most methods for the determination of alkaloids in poppy seeds apply gas chromatography-mass spectrometry (GC/MS), or liquid chromatography-diode array detection (HPLC/DAD) (Sproll *et al*., 2006).

All methods require an extensive extraction and cleanup step. The GC methods require an additional derivatization using bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane; moreover, an internal standard is often used for accurate quantitation (Popa *et al*., 1998; Meadway *et al*., 1998). HPLC-DAD is restricted by the similar chromatographic behavior of the minor alkaloids such as codeine and noscapine. Liquid chromatography in combination with tandem mass spectrometry on a triple quadrupole instrument (LC/MS/MS) is an efficient method for routine analysis because it commonly does not require extensive sample cleanup, and the high specificity of mass selective detection avoids matrix interferences and compensates for separation problems (Sproll *et al*., 2006; Stockigt *et al*., 2002). Usually, an Electrospray interface is preferred for allowing an optimal ionization of opium alkaloids.

Capillary electrophoretic (CE) methods have been also proposed for morphine analysis, using either an UV or a MS detection (Stockigt *et al*., 2002; Bosh *et al*., 2007): these methods are very common for drug analysis, since a very small sample amount is required, but their application in the food safety field is of growing interest. Alkaloid determination with capillary electrophoresis was performed several times using Micellar ElectroKinetic Chromatography (MECK) and Capillary Zone Electrophoresis (CZE).

The determination of opium alkaloids in poppy capsules was also performed by densitometric methods such as Thin Layer Chromatography (TLC) coupled with UV detection (Popa *et al*., 1998). In particular, TLC plates were developed with ethyl acetate:toluene:methanol:ammonia (68:17:10:5, v/v) and the densitometric analysis was performed at 275 nm allowing for a good linearity and a good accuracy of the method.

Opium poppy alkaloids have also been detected by chemiluminescence (Francis *et al*., 2008): in particular, liquid-phase chemiluminescent reagents such as tris(2,2'-bipyridyl(I)ruthenium(II) and acidic potassium permanganate exhibit remarkable sensitivity and selectivity towards opium alkaloids. These properties allowed for the development of a range of analytical procedures using flow analysis or microfluidic instrumentation.

Validation and outlook

Concerning the occurrence of morphine in poppy seeds, the analytical performance characteristics of most methods are not well known. None of the mentioned methods, including LC-methods, has been validated by interlaboratory studies and there are no certified matrix reference materials or proficiency studies available for the determination of opium alkaloids. Analytical techniques should aim to detect morphine and its major analogues as well as their corresponding biologically active metabolites formed in exposed animals. Particularly, in view of the ongoing discussions about the introduction of regulatory limits in poppy seeds, the use of LC-MS/MS for quantification and identification of opium alkaloids will become of increasing importance

OCCURRENCE DATA

There are significant variations in the morphine content in the variety of poppy grown for commercial use. When the capsules are cut and the seeds are put into a container, little opium juice will contaminate the seeds. If the capsules are squeezed mechanically and the seeds are rinsed with water, there seems to be a higher risk of contamination by opium juice causing higher morphine concentrations (Moeller *et al*., 2004).

Danish poppy capsules analyzed by Steentoft *et al*. (1988), contained 0.3-5 mg morphine per capsule and the content of morphine in opium exuded from the capsules amounted to 24%. This has resulted in misuse as both fresh and dried poppy capsules have been used for the production of “opium tea”. During the period 1982-1985, seven casualties occurred among drug addicts in Denmark, which were solely or partly caused by these opium poppies.

The morphine concentration of poppy seeds seems to be highly dependent on the kind of seed used and the type of harvesting. For example, it has been reported that white poppy seeds of *Papaver somniferum* contain morphine in the range 58.4-62.2 mg/kg and total codeine in the range 28.4-54.1 mg/kg (Lo and Chua, 1992). Hayes *et al*. (1987) state that black poppy seeds contain morphine from 17 to 294 mg/kg and codeine from 3 to 14 µg/g, respectively.

Several studies have shown the presence of these two major opium alkaloids, morphine and codeine in poppy seeds. Concentrations of these alkaloids have been reported to range from 1.5-294 mg/kg and 2.1-294 mg/kg of seed, respectively (Meadway *et al*., 1998).

The concentrations in morphine and codeine of dried mature capsules of Romanian *Papaver Somniferum* were determined by Popa *et al*. (1998), using two methods: thin layer

chromatography-UV densitometry and gas chromatography tandem mass spectrometry. The results obtained by these two methods were correlated between them and provided a high morphine content (2232-2322 mg/kg) and a low codeine content (210.4-219.8 mg/kg) of the Romanian poppy capsules.

In another study, poppy seeds from different shops and of different origins were tested for alkaloid residues by Thevis *et al*. (2003). Eight poppy seeds specimens from different suppliers or origins were obtained commercially in supermarkets, health food shops or bakeries and tested for traces of a possible content of morphine and codeine. In Table 2, the individual data concerning origin, country, and morphine concentration are summarized. Every charge contained morphine and one in particular (number 3) showed high amounts with 151.6 mg morphine/kg of poppy seeds. The analysis of a second package of the same batch number led to comparable results, while other batches of the same manufacturer contained only low amounts of 1.1 mg/kg. The codeine values of all products were negligible.

A poppy seeds study was performed by Hill *et al*. (2005), with Australian poppy seeds, because these were shown, at that time, to contain the highest morphine content of any on the American market, according to El Sohly *et al*. (1988). Figures from the American Spice Trade Association, New York, indicated that Australian, Dutch and Turkish poppy seeds varieties constituted 94% of the total American market. Because the morphine content of Australian poppy seeds ranged from 90 to 200 mg/kg seed and the Dutch and Turkish poppy seeds ranged only from 4 to 5 mg/kg seed, this investigation, therefore, was performed with Australian poppy seeds. A quantity of 50 lbs was purchased from the wholesale supplier McCormick & Co. (Hunt Valley, MD). The seeds were analyzed by LC-MS/MS and the average morphine concentration found of 325 mg/kg seed was substantially greater than that reported in the literature.

“Brown Mixture” is considered another source of morphine and codeine because it contains opium powder (10.0-10.5% morphine (w/w)), opium tincture (0.9-1.1% morphine), or camphorated opium tincture (0.045-0.055% morphine (w/w)), and is a popular cough remedy used particularly in Taiwan. In a study conducted by Liu *et al*. (2006), brown mixtures from seven different manufacturers, five tablets and two solutions, were analyzed for their morphine and codeine contents. The contents of these two alkaloids in the tablets were found to be very consistent, but with significant differences in the two brown mixture solutions. In particular, brown mixture tablets containing opium powder were found to include 263.05 to 285.59 µg of morphine and 30.17 to 32.41 µg of codeine per tablet. The [M]/[C] ratios in these brown mixture

tablets ranged from 8.62 to 8.98 with an average of 8.77 ± 0.15 (standard deviation). A brown mixture solution from manufacturer containing opium tincture was found to contain 134.91 µg of morphine and 46.85 µg of codeine per mL with [M]/[C] = 2.88. The other brown mixture solution from a manufacturer containing camphorated opium tincture, was found to contain 51.76 µg of morphine and 20.00 µg of codeine per mL with [M]/[C] = 2.59.

Table 2. Morphine and codeine concentrations present in poppy seeds specimens.

Country	Commodity	Year	N° of samples	Alkaloid	LOQ (mg/kg)	*n* > LOQ	Mean (mg/kg)	Min/Max (mg/kg)	References	Sampling procedure
Singapore	White poppy seeds	NA	3 brands	Morphine Codeine	NA NA	NA NA	NA NA	58.4/62.2 28.4/54.1	Lo and Chua, 1992	NA
Portland (OR)	Black poppy seeds	NA	4 brands	Morphine Codeine	NA NA	NA NA	NA NA	17/294 3/14	Hayes *et al*., 1987	Samples obtained from retail stores
Denmark	Poppy seeds - Neuform®	NA	1 brand	Morphine	NA	NA	8.4	NA	Thevis *et al*., 2003	Commercially in supermarkets, health food shops or bakeries
Hungary	Poppy seeds - Neuform®		1 brand	Morphine		NA	6.9	NA		
Unknown	Poppy seeds - Müller's Mühle®		1 brand	Morphine		NA	151.6	NA		
Unknown	Poppy seeds - Insula®		1 brand	Morphine		NA	2.1	NA		
Unknown	Poppy seeds - FJD®		1 brand	Morphine		NA	4.1	NA		
Turkey	Poppy seeds - Rapunzel®		1 brand	Morphine		NA	0.8	NA		
Turkey	Poppy seeds - Davert®		1 brand	Morphine		NA	0.9	NA		
Unknown	Baking mixture		1 brand	Morphine		NA	0.6	NA		
Taiwan	Brown Mixture tablets Taiwan Biotech Co. Ltd.	2005	5	Morphine	NA	NA	281.11	NA	Liu *et al*., 2006	Commercially from different manufacturers
				Codeine	NA	NA	32.41	NA		
Taiwan	Brown Mixture tablets Astar Co. Ltd.		5	Morphine	NA	NA	271.47	NA		
				Codeine	NA	NA	31.49	NA		
Taiwan	Brown Mixture tablets Washington Pharmaceutical Co. Ltd.		5	Morphine	NA	NA	285.59	NA		
				Codeine	NA	NA	32.28	NA		
Taiwan	Brown Mixture tablets Center Laboratories Inc.		5	Morphine	NA	NA	285.23	NA		
				Codeine	NA	NA	31.77	NA		
Taiwan	Brown Mixture tablets Johnson Chemical Pharmaceutical Works Ltd.		5	Morphine	NA	NA	263.05	NA		
				Codeine	NA	NA	30.17	NA		
Taiwan	Brown Mixture solution Center Laboratories Inc.		5	Morphine	NA	NA	134.91 µg/ml	NA		
				Codeine	NA	NA	46.85 µg/ml	NA		
Taiwan	Brown Mixture solution Health Chemical & Pharmaceutical Co. Ltd.		5	Morphine	NA	NA	51.76 µg/ml	NA		
				Codeine	NA	NA	20.00 µg/ml	NA		

NA: NOT AVAILABLE

MITIGATION OF MORPHINE IN POPPY SEEDS

Prevention

The alkaloid content in the latex of the plant, with morphine as its major constituent. For this reason only the low-morphine variety Mieszko is certified for cultivation in Germany (Stolzenburg, 2006). Besides the poppy variety, the method of harvest has the highest influence on the morphine concentrations and leads to the great variability of the alkaloid concentration (Moeller *et al.*, 2004).

Poppy seeds harvest can take place in two ways. Traditionally, the ripe seed is manually shaken so that the seed falls out of the holes below the many-rayed stigma. In contrast, the high-yield closed-capsule plants have to be crushed open using modified combine harvesters sealed against loss of fine seed (Feiffer, 1998; Rochholz *et al.*, 2005). The latter procedure contaminates the seed with the chyle of unripe capsules, which has to be removed afterward, and a higher alkaloid content will result (Andresen and Schmoldt, 2004).

The reasons given for the high opiate contents of poppy seeds include the choice of less suitable botanical varieties, inopportune harvesting time and insufficient harvest technology and specific geographical origin, and alkaloid content depends also on locality (BfR Health Assessment, 2005).

Rochholz *et al.* (2004) argue that the poppy seeds themselves only contain very low levels of morphine and codeine and that these alkaloids only adhere as external contamination to the poppy seeds, with capsule fragments and latex both containing alkaloids (BfR Health Assessment, 2005). They base this on findings that the morphine content of seeds could be drastically reduced through washing.

The concentration of alkaloids in capsules is affected by many factors. Kadar *et al.* (2001) reported an increase of alkaloids in capsules as the result of N-fertilisation, while it generally decreased following P-fertilisation (Lachman *et al.*, 2006).

Poppy plants respond to the stress by an increase of alkaloid content, as narcotine, morphine and papaverine, in capsules (Lachman *et al.*, 2006). Szabo *et al.* (2003) observed that drought stress resulted in higher levels of the alkaloids whereas mycotoxin stress did not result in significant differences. As reported by Morimoto *et al.* (2001), in response to stress, morphine is quickly metabolised to bismorphine consisting of two morphine units. Bismorphine binds predominantly to pectins possessing high galacturonic acid residue contents through ionical bonds with higher ability as Ca^{2+} a cross-linker of these polysaccharides. Bismorphine could be thus evaluated as a defence response of the opium poppy (Lachman *et al.*, 2006).

Enhanced biosynthesis, or reversely, decrease of alkaloid contents in poppy capsules and seeds regarding the cadmium and zinc levels, could also be related to ability to form chelate complexes with hydroxyl groups. Cadmium is toxic for plants and induces an abiotic stress. On the contrary, higher Zinc content resulted in a decrease of narcotine, of codeine, and of papaverine. Zinc is a microbiogenic element for the poppy plant and its higher content could be correlated with a lower content of major alkaloids (Lachman *et al*., 2006).

An experiment was conducted during 2004-2005 on opium poppy and the influence of 10^{-6} M gibberellic acid (GA3) and 10^{-6} M triacontanol (TRIA) either alone or together, on growth, yield, quality and morphine content. Among the treatments, foliar spray of GA3 and/or TRIA significantly promoted the values of most of the physiological and biochemical parameters including the opium yield. The morphine content was also enhanced (Khan *et al*., 2007).

Lošák and Richter (2004) studied the efficiency of partial doses of nitrogen applied to the poppy. The ammonium nitrate was applied either in a single dose at the beginning of the growing season or in two split doses with the second application at the stage of flowering. With the increasing dose of nitrogen the number of capsules per plant during harvest and their volume and morphine content increased irregularly. The separated application of an optimum dose of nitrogen (i.e. 0.9 g N/pot) showed a statistically highly significant positive effect on the yield of poppy seeds compared to the same single dose of nitrogen made this figure increased by 25.6%. The morphine content in the capsules increased with the increasing supply of N from 0.85 to 1.01%. An increased dose of nitrogen increased its concentrations to 0.91–1.01% (i.e. by 7.1–18.8%) compared to controls. This finding corresponded with a number of literary data, which mentioned that the concentration of morphine increased with the increasing N supply (Yadav *et al*., 1984, Kadar *et al*., 2001). Prasad (2002) presented the increasing content of morphine according to the increasing dose of N from 50 to 100 kg N/ha in a form of ammonium sulphate nitrate. Kharwara *et al*. (1988) wrote that the content of morphine increased if the dose of 75 kg N/ha was doubled. Schrodter (1965) demonstrated a higher content of morphine at doses of 50–60 kg N/ha provided that the supply of P and K was sufficient. Laughlin and Chung (1992) observed, depending on the amounts of precipitation, an increase in morphine content of 10–24% in seeds and mentioned that, under conditions of a moisture deficit, the supply of N did not show to be statistically significant on the content of this alkaloid, however, under conditions of full water capacity, its content was increased by 8.8% in seeds.

Decontamination

Sproll *et al*. (2007) experiments allow the derivation of guidelines for consumers and bakeries about a correct treatment of poppy seeds and the study of the possibilities to reduce the morphine content of poppy seeds by simple operations applicable in households or in bakeries. Poppy seeds should be washed with water, afterwards the seed must be dried to prevent microbiological contamination, germination, and rancidity. The poppy seeds should then be ground using mills. If the poppy is used for baking purposes or for decoration of bakery products, the baking should be done at the highest possible temperatures (around 200-220°C) as experiments showed a significant thermal degradation of morphine during baking (Sproll *et al.*, 2006).

Sproll *et al*. (2007) experiments were performed using different treatments: fifty grams of untreated or ground poppy seeds were washed with 200 ml of water or citric acid solution (5 g/l) at different temperatures (cold, hot (60°C) and boiling) and at different times (30 s, 2 min, and 30 min).

Several seed-washing experiments significantly reduced the morphine content (Grove *et al.*, 1976; Bjerver *et al.*, 1982; Meadway *et al.*, 1998). Bjerver *et al.* (1982) and showed that 40% of the total morphine can be removed by a single washing with slightly acidified water. Soaking poppy seeds in water for 5 min was found to remove about 45.6% of their free morphine and 48.4% of their free codeine (Lo and Chua, 1992).

All washing treatments led to a significant reduction of the morphine content with a minimal removal of 48% and a maximum removal of the total morphine content (100%). The temperature of the washing solution had a significant influence. With cold water (i.e., water directly from the tap around 15°C), only about 60% of morphine is removed. At temperatures above 60°C, significantly higher reductions, around 90%, was observed. Interestingly, there was no difference between 60°C and water at boiling point. A significant effect was also determined for the washing time. Longer times of rinsing the poppy seeds led to a higher morphine reduction with a nearly complete removal after 30 min.

Using the proposed treatment consisting of washing, drying, and grinding, not only the morphine content is significantly reduced, but also the organoleptical quality of the poppy seeds is notedly enhanced.

Pretreatment had a significant influence: round poppy seeds showed lower content than untreated seed. The poppy bun experiment proved the significant quadratic influence of baking temperature, which means that at first the reduction is relatively low up to 135 °C (around 30%), but at 220 °C a reduction of 80-90% was determined (Sproll *et al*., 2006).

The degradation of morphine could be accelerated in the presence of oxygen and at higher pH, whereas temperature and light have only a minor influence on the degradation rate. The influence of oxygen, leading to the formation of pseudomorphine and morphine-N-oxide, can be assumed especially in the case of grinding that leads to large active surfaces (Sproll *et al*., 2006; Vermeire and Remon, 1999).

The stability of morphine in aqueous solutions had been extensively investigated and it was generally accepted that oxygen of air, sunlight, UV irradiation, iron and organic impurities catalyse the degradation of morphine. Quantitative studies on the influence of these different factors on the degradation rate however, had never been conducted until Yeh and Lach (1961) studied the influence of oxygen, temperature, molarity of the buffer, ionic strength and morphine concentration on the degradation kinetics of morphine. From their experiments it can be concluded that in the presence of excess of oxygen, the degradation rate and extent increased with increasing pH of the solutions (Vermeire and Remon, 1999). Thus the degradation of morphine could be accelerated in the presence of oxygen and at higher pH, whereas temperature and light have only a minor influence on the degradation rate. The influence of oxygen, leading to the formation of pseudomorphine and morphine-N-oxide, can be assumed especially in the case of grinding that leads to large active surfaces (Sproll *et al*., 2006; Vermeire and Remon, 1999).

Role of food processing

Meadway *et al.* (1998) reported the first observation of differences in opiate concentration between cooked, sieved, and untreated seed specimens. They concluded that the method of seed preparation influenced the alkaloid concentrations. Brenneisen and Borner (1985) reported the results of a single baking experiment. In black poppy seeds from the food trade they measured 0.002% morphine before processing and only 0.0002% morphine after baking the seed used as topping on a poppy bun. During the baking process, at first the reduction of the morphine is relatively low up to 135 °C (around 30%), but at 220 °C a reduction of 80-90% was determined (Sproll *et al.*, 2006).

Mechanical pretreatments such as grinding, as well as heat treatment, were found to have the greatest influence on morphine reduction. The cake recipes bakeries use demand grinding to improve the aroma of the product. Cakes and buns are then baked at high temperatures around 200 °C. These processes make a decrease of the morphine content by at least 80% possible. Kniel (2006) reported results of analyses made at different stages during the manufacture of baking mixes. A median of 6.8 mg/kg was determined in the original poppy seeds. A significant reduction was found in the convenience baking mixes that

are manufactured including the grinding and heating steps (median 3.9 mg/kg). In the finished products found in bakeries and supermarkets, morphine could no longer be detected. (<1 mg/kg).

PHARMACOKINETICS

Poppy seeds (Semen papaveris) need to be assessed for their content of pharmacologically active alkaloids. Even if morphine is present in the largest amount (12%) other important opium alkaloids that could be identified are codeine (approximately 2%), thebaine (approximately 0.5%), noscapine (5%), narceine (approximately 0.5 %) and papaverine (approximately 1%) (Hager, 2003; Kommentar zum Europäischen Arzneibuch, 2004).

Some toxicological data on these alkaloids are available.

For codeine in adults the lethal dose is 0.5 to 1.0 g (Gosselin, 1984). This dose may cause convulsions and unconsciousness, and death from respiratory failure may result within 4 hours. In a man who self-administered 900 mg of codeine intravenously serum concentrations over 5 mg/L were detected; he regained consciousness only after 3 days when serum levels reached 1.3 mg/L (Huffman and Ferguson, 1975). Drug concentrations in codeine death are approximately 2.8 mg/L in blood and 103.8 mg/L in urine (Baselt and Cravey, 1989). In toddlers, Von Muhlendahl *et al*. (1976) demonstrated that an acute ingestion of less than 5 mg/kg of codeine is non-toxic. Significant respiratory symptoms developed in 8 of 430 children with ingestions of ≥5 mg/kg.

Thebaine is far more toxic than morphine. The LD_{50} in mice is 31 mg/kg s.c. and 20 mg/kg i.p.; other authors have reported an intraperitoneal LD_{50} of 42 mg/kg in mice. In rabbits, the intravenous LD_{50} is 3-4 mg/kg and is dependent on the age of the animals (Corrado and Long, 1961). For dogs the lethal doses are reported to be 10-30 mg/kg sub-cutaneous and 5-7 mg/kg intravenous (Kruger *et al*., 1941).

Little is known on the toxicological effects of noscapine and narceine on humans.

Some studies have been performed on papaverine: following a 15 g oral dose, metabolic acidosis with hyperventilation, hyperglycemia and hypokalemia has been reported (AHFS, 2003). Moreover doses as high as 1 g by mouth produce only minimal side effects. IV doses of 30 and 65 mg produced rapid death of 2 adults preceded by hyperpnea, tachypnea and eventually apnea. Heart sounds were not audible after onset of respiratory symptoms. Death may have resulted from either cardiac or respiratory disturbance (Gosselin *et al*., 1976).

Absorption

After oral administration, morphine is variably absorbed from the gastrointestinal tract, mainly from the upper small intestine and, to a lesser degree, from the stomach. The low bioavailability (20% - 40%) in the case of oral morphine administration can be attributed to elimination through metabolism in the intestinal mucosa and liver (Goodman & Gilman's, 1996; Forth *et al*., 2001; Martindale, 2005).

The plasma half-life of morphine is 2-3 hours. About 30% of morphine is bound to plasma proteins. After subcutaneous or intramuscular injection morphine is readily absorbed into the blood. Morphine is distributed throughout the body, mostly (65%) found in the kidneys, liver, gastrointestinal tract, lungs and spleen, while low levels (20%) are present in the brain and muscles (Martindale, 2005). Though the brain is its primary site of action, morphine does not cross the blood-brain barrier easily, since 80% is present in the ionised form. Indeed, when morphine sulphate is administered by iv injection, systemic plasma concentrations of the drug remain higher than the corresponding CNS concentration, because of the presence of the blood-brain barrier (AHFS, 2007). Morphine diffuses across the placenta and traces also appear in milk and sweat (Martindale, 1982).

Distribution

Following intravenous administration, morphine has an apparent volume of distribution ranging from 1-4.7 L/kg. Protein binding is reported to be 36% and muscle tissue binding reported to be 54%. Following epidural administration as the conventional injection, the absorption half-life of morphine across the dura is approximately 22 minutes. After intrathecal administration of morphine, there is a rapid initial distribution phase lasting approximately 15-30 minutes. Approximately 4% of an epidurally injected dose of conventional morphine sulphate injection distributes into cerebrospinal fluid. Distribution across the dura is slow, with peak CSF concentrations occurring 60-90 minutes after an epidural dose (AHFS, 2007).

Metabolism

Morphine is metabolized mainly in the liver and undergoes conjugation with glucuronic acid principally at the 3-hydroxyl group. Secondary conjugation also occurs at the 6-hydroxyl group to form the 6-glucuronide, which is pharmacologically active, and to a limited extent the 3,6-diglucuronide.

Morphine is excreted in urine mainly as morphine-3-glucuronide. In addition to the 3,6-diglucuronide, other minor metabolites that have been described include normorphine and the 3-ethereal sulfate. (AHFS, 2007)

Excretion

Up to 2-12% of the administered dose of morphine is found in unchanged form in urine. About 90% of total urinary execretion occurs within 24 hours of the last dose given. Approximately 7-10% of a dose of morphine is excreted in feces; most of the metabolites are excreted via the kidneys (80%), but also via the liver and gall bladder. The fact that lower morphine doses are recommended for older patients has to do with the lower distribution volume and reduced renal function (Martindale, 2005; Forth *et al*., 2001).

A relevant aspect of morphine excretion is represented by milk. Five lactating mothers who were at least 1 month post partum were given 1 to 2 doses of either epidural 4 mg or parenteral (intravenous or intramuscular) 5 to 15 mg morphine every 4 to 6 hours for postoperative analgesia. Milk was sampled from each mother 10 times over the 8 hours after her dose. The peak milk level after epidural morphine was 82 µg/L and occurred 30 minutes after a second dose. The peak milk level after parenteral morphine was about 500 µg/L and occurred about 45 minutes after a single 15 mg (10 mg intravenous plus 5 mg intramuscular) dose (Feilberg *et al*., 1989).

Nevertheless there are no reports of harm to babies following therapeutic administration of morphine to the lactating mother. The American Academy of Pediatrics has stated that the administration of morphine is normally compatible with lactation. Moreover, it also points out that there are no reports of adverse reactions in babies although measurable morphine levels may occur in their blood (Martindale, 2005).

Carry over

There are no studies on animals about the carry-over of morphine from poppy seeds.

Only in humans morphine transfer into maternal milk has been described after administration of single doses by oral, intramuscular end epidural routes (Eisenach *et al*., 1988; Sinatra *et al*., 1989). Morphine is widely used for Patient-Controlled Analgesia (PCA) after caesarean delivery, despite the potential deleterious influence of this opiate on neonatal behaviour. However, evidence that morphine has prolonged plasma elimination half-lives in neonates, compared to adults, does suggest that neonates are at increased risk for opioid toxicity (Wittels *et al*., 1990).

Morphine and its active metabolite M-6-G were measured by high-performance liquid chromatography in plasma and colostrum or breast milk by Baka *et al*. (2002) of seven informed and consenting mothers, receiving morphine via PCA after caesarean delivery. Samples were obtained at 12, 24, 36 and 48h. In plasma, morphine concentrations ranged from 1 to 274 ng/mL, M-6-G ranged from 5 to 974 ng/mL. In milk, opioids were found in only

3 patients in whom morphine concentrations ranged from 1 to 48 ng/mL and M-6-G from 5 to 1084 ng/mL. In conclusion, very small morphine and M-6-G concentrations were observed in colostrum during PCA with morphine. Under these conditions, the amounts of drug likely to be transferred to the breast-fed neonate are negligible.

Only one previous study has evaluated this transfer in similar clinical conditions, and the results are very close to these. Wittels *et al*. (1990) found the same large interindividual variation between the morphine concentrations in colostrum. In that study, the maximal mean concentration of morphine in colostrum was 60 ng/mL over a 24h period in 5 women undergoing PCA. However, the opioid plasma concentrations were not known.

TOXICITY

Opium *per se* and morphinane alkaloids alone are addictively narcotic and have been abused for centuries. Now, with the easy chemical conversion of morphine into heroin, the opium linked evils have become a menace of global proportions. This trend is a dangerous one unless effective control over the production of opium and morphine is exercised (Sharma *et al.*, 1999).

Starting in the 1980s, it has been well documented that ingestion of products containing poppy seeds, e.g., raw seed, in cakes, in cokies, in pastes or in rolls or bagels, can result in the excretion of detectable concentrations of morphine and codeine in urine and produces positive urine tests in routine drug abuse screens (Hayes *et al*., 1987; Struempler, 1987; Zebelman *et al*., 1987; Lo and Chua, 1992; Meadway *et al*., 1998; Rohrig and Moore, 2003; Thevis *et al*., 2003).

This unfortunate situation has created the “poppy seeds defence”, a dilemma for all concerned in drug abuse testing for opiates (Mule and Casella, 1988). In 1991 the US Department of Defence expressed concern over the reliability of screening results obtained using the universally accepted 300 ng/ml cut-off limit for opiate assays. To try to eliminate this defence, in November 1998, the federal governmental raised this cut-off concentration for urine drug testing from 300 ng/ml to 4000 ng/ml and 2000 ng/ml for morphine and codeine, respectively (Rohrig and Moore, 2003).

Consumption of strongly contaminated poppy seeds can lead to detectable contents of free morphine in blood as well as measurable concentrations in urine, sometimes for many days (Rochholz *et al.*, 2004). Until now, the idea that poppy seeds could serve as the source of appreciable amounts of morphine was not given much credence despite the old European custom that recommended quieting a noisy baby with a poppy seeds filled pacifier (Fritschi and Prescott, 1985). In fact, older literature from the 19th century reported isolated cases of

accidental morphine poisonings of infants (Lodge, 1858). A recent case reported by the Federal Institute for Risk Assessment confirms that old home remedies to encourage infants to sleep through the night are still used today (Federal Institute for Risk Assessment). A mother had given her six-month-old infant the strained milk of baking poppy seeds with the very best intentions of helping it sleep better. She had taken the recipe from a cookbook. Just a few hours later the infant had to be taken by ambulance to a hospital. The child was suffering from breathing disturbances; it was not fully conscious and scarcely reacted at all to pain stimulus. Because of the threat of respiratory arrest, the infant had to be ventilated with an oxygen mask. Because of the suspicion of opiate poisoning, the child was given an antidote. A urine test revealed high levels of the alkaloids morphine and codeine, confirming the suspicion. The mother had given her child 75 mL of strained milk made from a mixture of 200 g of poppy seeds containing 1000 mg/kg of morphine in 500 mL of milk. This home remedy had even recommended the 2-fold amount of 400 g of poppy seeds. This case prompted the Federal Institute for Risk Assessment to warn against using home remedies with poppy seeds. Because of their qualitative fluctuations, baking poppy seeds may contain differing amounts of the alkaloids morphine and codeine. These alkaloids may lead to serious health damage in infants, ranging from breathlessness to respiratory arrest.

Morphine is a potent opioid analgesic which is used for short-term treatment of postsurgical and traumatic pain as well as for long-term treatment of severe pain in cancer patients (Leis *et al*., 2000). Besides these clinical applications, morphine is a common drug of abuse.

Morphine has a major effect on the central and peripheral nervous system by acting on the opioid receptors. The effects include analgesia, sedation, hypotension, drowsiness, respiratory depression, nausea, vomiting and alterations of the endocrine and autonomic nervous system; potentially fatal effects are respiratory depression and cardiovascular instability (Jaffe and Martin, 1996).

Considering animals, the release of oxytocin and milk ejection during manual stimulation of the teats and machine milking are important for rapid and complete milk removal in dairy cows (Schams *et al*., 1984; Gorewit *et al*., 1992; Tančin *et al*., 1995). Milk ejection occurs when oxytocin release results in concentration above a threshold level of 3-5 ng/l. Without milk ejection, only cisternal milk is available and this represent 5-25% of the total milk in the udder (Knight *et al*., 1994). It has been shown by Tančin *et al*. (2000) that opioids, and particularly morphine, have a suppressive effects on oxytocin release during milking. Besides the effects of opioids on the release of cortisol, prolactin and luteinizing hormone under basal

and stress conditions are well documented in cows (Peck *et al*., 1988; Nanda *et al*., 1989 and 1992).

Another study carried out by Akinci and Bayram (2003) considered the effects of poppy seeds meal on the egg production and hatching of quail. The results of the experiment showed that the addition of poppy seeds meal to the feed ratios increased egg production, feed consumption and feed conversion per kg of eggs and per dozen eggs. However, this addition significantly reduced hatchability, mainly because of increased infertility and embryonic deaths, even if the responsible mechanisms are not clearly understood.

Until now, the idea that poppy seeds could serve as the source of appreciable amounts of morphine was not given much credence despite the old European custom that recommended quieting a noisy baby with a poppy seeds filled pacifier (Fritschi and Prescott, 1985). In fact, older literature from the 19th century reported isolated cases of accidental morphine poisonings of infants (Lancet, 1838; Lodge, 1858). A recent case reported by the Federal Institute for Risk Assessment confirms that old home remedies to encourage infants to sleep through the night are still used today. A mother had given her six-month-old infant the strained milk of baking poppy seeds with the very best intentions of helping it sleep better. Just a few hours later the infant was suffering from breathing disturbances; it was not fully conscious and scarcely reacted at all to pain stimulus. A urine test revealed high levels of the alkaloids morphine and codeine, confirming the suspicion of opiate poisoning.

Symptoms such as “dim feelings in the head”, vomiting, and hangover-like feelings the next day were reported in a recent case. The consumer had eaten a pasta dish strewn with a mixture of poppy seeds and sugar. Approximately 75g of poppy seeds containing 210 mg/kg of morphine and 39 mg/kg of codeine were consumed, corresponding to dosages of 16 mg of morphine and 3 mg of Codeine (Sproll *et al*., 2006).

Acute toxicity

Acute morphine intoxication normally causes miosis, respiratory depression and unconsciousness (coma). Respiratory depression is the most important risk in conjunction with opioid overdose as the direct cause of death is respiratory arrest (Forth *et al*., 2001). In individuals who do not show any tolerance development, serious toxic symptoms may already occur after the oral administration of 40 to 60 mg morphine (Goodman & Gilman’s, 1996). For adults, doses from 200 mg morphine may be acutely lethal (Frohne and Pfänder, 1987). Other sources give a range of 300-1500 mg for morphine hydrochloride trihydrate (equivalent to 228 mg-1139 mg morphine) for the oral lethal doses in the case of non-opiate-

dependent adults, while babies and infants are far more sensitive (Kommentar zum Europäischen Arzneibuch, 2004).

Chronic toxicity

Morphine has an analgesic effect by targeting the central nervous system (CNS). At various levels; in addition to supraspinal there are also spinal points of attack. By increasing the release of dopamine in the nucleus accumbens, morphine creates an unrealistic feeling of well-being (euphoria).

Morphine increases the tone of the gastrointestinal tract and reduces motility with the consequence of spastic obstipation. Stomach motility is already reduced at relatively low doses of morphine. As a consequence of the contraction of the pylorus, the chyme only leaves the stomach slowly. In the ileum, in addition to increased tone, there is inhibition of peristalsis. Colon peristalsis is slowed down and the defecation reflex is suppressed. Morphine leads to a contraction of the gall bladder musculature and the sphincter oddi, leading to bile congestion. Therapeutic doses of morphine can increase the tone and amplitude of urethral contraction. After administration of therapeutic doses of morphine, urinary retention may occur and catheterisation may be necessary. Therapeutic morphine doses can lead to a widening of blood vessels in the skin. Furthermore, pruritus can also occur (Goodman & Gilman's, 1996; Forth *et al*., 2001; www.bfarm.de).

Developmental and reproductive toxicity

Morphine crosses the placenta barrier. Adequate data are not available for human beings which would permit an assessment of the possible teratogenic risk (Federal Institute of Risk Assessment, 2006). However, there have been reports of a possible association with an elevated incidence of hernias. Respiratory depression and withdrawal symptoms occur in neonates particularly of dependent mothers. Furthermore, foetal cardiac frequency may be reduced. Other clinical signs in neonates are muscular hypertonus, hyperactivity, cramps, shrill crying, tremor, vomiting, diarrhoea, sneezing and tachypnoae. Delayed language development may also be a sequelae (Hager, 2003).

In published developmental toxicology studies, morphine was not administered orally but only subcutaneously or intraperitoneally (Lin *et al*., 1975; Zagon and McLaughlin, 1977a and b; Khera, 1984; Fujinaga and Mazze, 1988; Fazel and Jalali, 2002). There were reports of CNS malformations (Zagon and McLaughlin 1977b; Fazel and Jalali, 2002), retarded growth, reduced litter size, a higher number of stillbirths, increased mortality of offspring (Zagon and McLaughlin, 1977a), rib and vertebrae coalescence and cryptorchismus (failure of the testes

to descend into the scrotum) (Khera, 1984). Furthermore, morphine affected male sexual behaviour and female fertility in various animal species (www.bfarm.de).

Genotoxicity/carcinogenicity

According to published investigations the in vivo administration of morphine to mice led to an increased incidence of chromosomal aberrations in bone marrow cells (Swain *et al*., 1980) and to the induction of micronuclei in bone marrow cells and lymphocytes (Das and Swain, 1982; Li and Lin, 1998). Since attempts failed to induce chromosomal aberrations or micronuclei in vitro, it is assumed that there is metabolic activation in vivo. In one in vitro test in human HUT-78 cells, morphine increased the mutation frequency and DNA damage (Li and Lin, 1998). Tests on Drosophila, Salmonella and yeast cells were negative (Knaap and Kramers, 1976; Li and Lin, 1998).

No long-term studies in animals are available concerning the tumorigenic potential of morphine.

Morphine serum levels

The detection of free morphine in serum is interpreted as an indication of exposure to specific pharmaceuticals or drugs, i.e. morphine itself or heroin and codeine, which can be metabolised into morphine (Forth *et al*., 2001). According to some studies (Westphal *et al*., 2006) the detection of morphine in serum may also be linked to the consumption of poppy seeds containing higher levels of morphine. In an official document by the Bavarian State Office for Health and Food Safety, poppy seeds with a morphine content of more than 10 mg/kg was regarded as not safe if consumed in usual quantities (Lepper, 2005). This value could create problems with the consumption of poppy seeds-containing food. According to the official document of the German Federal Institute of Risk Assessment (2006) a threshold dose of morphine in food upwards of which relevant health effects are expected in humans cannot be defined on the basis of existing data because of the diverse pharmacological and toxicological effects of morphine and the major variations in individual sensitivity. Moreover this situation is complicated by the fact that data from pharmaceutical applications of morphine can only be transferred to a limited degree to the consumption of poppy seeds, as healthy test persons react differently from pain patients with respect to toxicologically relevant morphine effects.

The pharmacological scientific literature shows that in adults a single oral effective dose of morphine is normally given as 7.6 mg/person (equivalent to 127 µg/kg body weight for an adult weighing 60 kg) (www.bfarm.de) and in the lowest case as 1.9 mg/person (equivalent

to 31.7 µg/kg body weight for an adult weighing 60 kg) (Martindale, 2005). At these therapeutic doses healthy individuals can be expected to develop at least nausea, retching, light-headedness, dizziness, difficulties in carrying out intellectual tasks, apathy and reduced physical activity as well as other adverse reactions to normal therapy doses.
In line with this, forensic studies by Westphal *et al*. (2006) showed that morphine intakes of 11.4 mg (equivalent to 135 to 191 µg/kg body weight) from the matrix of poppy seeds also led to the above-listed symptoms typical for morphine.

Observations in humans
In a study conducted by Struempler (1987), the morphine concentrations determined in the urine of some volunteers after the ingestion of poppy seeds bagels, were 2797 ng/ml at 3h and 676 ng/ml at 22h, over the cut-off limit of 300 ng/ml proposed.
Hayes *et al*. (1987) detected morphine and codeine in serum and urine following ingestion of four brands of poppy seeds, and urine levels greater than 300 ng/ml were reported for as long as 48h after ingestion.
The study of Lo and Chua (1992) showed that in ingesting curry meal containing various amounts of washed seeds with a morphine intake from 200.4 to 1002 µg and a codeine intake from 95.9 to 479.5 µg, the urinary morphine levels were found to be in the range 120 to 1270 ng/ml urine and urinary codeine levels in the range 40 to 730 ng/ml urine.
Urine samples of seven volunteers ingesting 25g of poppy seeds baked into bundt cakes, were screened by Meneely (1992). All of the urine specimens were found to be opiate positive shortly after consuming the cake.
In the study of Rohrig and Moore (2003), the results for urine specimens are similar to those previously reported in the literature. However the morphine concentrations detected in all volunteers were above 2000 ng/ml for at least 4h after ingestion of poppy seeds.
This shows that the detection of morphine in urine does not necessarily indicate an illegal drug use and that the presence of urinary alkaloids may arise from consuming food containing poppy seeds.

On the other hand publications dealing with blood concentrations of morphine after poppy seeds consumption are rare. Hayes *et al*. (1987) found concentrations in the range 82-131 ng/ml morphine 2h after the subjects ingested 25g of poppy seeds containing 294 mg morphine/kg poppy seeds. In contrast, Sachs *et al*. (1994) found no free morphine, but they reported a limit of detection of only 10 ng/ml.

Moeller *et al*. (2004) tested serum samples by immunological tests for morphine and found all these tests negative even if a cake with approximately 20-30g of poppy seeds and a concentration of 50 mg morphine/kg poppy seeds was consumed by the volunteers.

LEGISLATION ON MORPHINE IN POPPY SEEDS

The goal of poppy breeding and cultivation is, besides the extraction of alkaloids for pharmaceutical products, the use of poppy oil and seeds in food.

Because poppy seeds are usually consumed in small quantities, for example poppy buns contain around 3g of poppy seeds, and previously they were believed to be nearly morphine-free (Sproll *et al*., 2006), it is not surprising that the European Union has so far established no maximum limits for morphine in poppy seeds (Rochholz *et al*., 2005). Today, especially for babies, infants, and old or ill people, the consumption of large quantities of poppy seeds can represent a health risk. Imported seed must be tested by manufacturers and official food authorities foralkaloid content using efficient analytical methods. Cases of intoxication after consumption of strongly contaminated poppy seeds have even led to a discussion about maximum limits. In Germany, the cultivation of poppies is limited by restrictions of the Federal Health Agency which fixed the maximal morphine content in *Papaver Somniferum* at 0.01% in the dry capsules of cultivars admitted for cultivation (Schulz *et al*., 2004).

In a toxicological study by the Bavarian State Office for Health and Food Safety, poppy seeds with a morphine content of less than 10 mg/kg was regarded as safe if consumed in usual quantities (Lepper, 2006).

The Federal Institute for Risk Assessment derived, from the lowest pharmaceutically active dosage of 31.7 µg of morphine/kg of body weight under inclusion of a safety factor of 5, a provisional tolerable daily upper intake level of 6.3 µg/kg of body weight. Taking into account the estimated maximum consumed amount of 100 g/day, a provisional guidance value for poppy seeds of 4 mg/kg was derived (2006).

Since 2005, the official food surveillance in Baden-Wurttemberg, Germany, has continuously been analysing the contents of opiate alkaloids in poppy seeds and food made thereof. Ready-made bodies, poppy seeds from bakeries and poppy seeds dedicated directly to the consumer were investigated. In 2005, morphine content was high enough to cause adverse health effects in several cases, whereas average findings in 2006 were somewhat lower. Of 110 samples evaluated, 76% exceeded the guidance value of 4 mg morphine/kg poppy seeds proposed, but fewer than 30 % showed a higher content than 20 mg/kg, a value estimated as tolerable by the Federal Institute for Risk Assessment, if warning notices are given (Perz *et al*., 2007). However, these studies totally disregarded the influences of seed

preparation or food processing in their establishment of guidance values, although such influences were previously reported (Meadway *et al*., 1998).

For raw, untreated poppy without any warning notice labels, the guidance value of 4 mg/kg has to be used because direct consumption, e.g., as milk extract, cannot be ruled out. Morphine ingestion via poppy buns, the most frequent application of poppy seeds in Germany, makes only a small contribution to the total morphine exposure due to food, as only 1-4 g of poppy seeds is used per bun. Based on this very small consumed amount and the reduction during baking, the poppy seeds could contain up to 100 mg/kg of morphine without even nearing the tolerable daily intake (Sproll *et al*., 2007). Appropriate labelling for the intended use (e.g., “only for decoration” or “not for direct consumption”) would be required in this case. Relevant morphine quantities can be consumed only in products with poppy fillings. For cake making, ground poppy seeds are cooked in milk and this mixture is baked with additional ingredients. The usual recipes prescribe between 10 and 30% of poppy seeds in the cake. One piece of cake in German bakeries and pastry shops weighs 150-200g. Considering the morphine elimination during baking and a maximum consumed amount of 2-4 pieces of cake, a guidance value of 20 mg morphine/kg can be estimated for poppy seeds intended for baking (Sproll *et al*., 2006).

CONCLUSIONS

Morphine is a potent opioid analgesic which is used for short-term treatment of postsurgical and traumatic pain as well as for long-term treatment of severe pain in cancer patients. Besides these clinical applications, morphine is a common drug of abuse.

Morphine has a major effect on the central and peripheral nervous system by acting on the opioid receptors. The effects include analgesia, sedation, hypotension, drowsiness, respiratory depression, nausea, vomiting and alterations of the endocrine and autonomic nervous system; potentially fatal effects are respiratory depression and cardiovascular instability.

Alkaloids are present in the latex of poppy (*Papaver somniferum*), with morphine as its major constituent. There is scientific evidence that poppy seeds themselves contain very low levels of morphine. An increased opiate content depends on the botanical variety, inopportune harvesting time and insufficient harvest technology and geographical origin..

It has been demonstrated by studies that some substances are capable of increasing the content of morphine in poppy seeds, i.e. nitrogen, cadmium, abiotic stress, gibberellic acid and triacontanol. In fact, poppy plants respond to stress by an increase of alkaloid content, as narcotine, morphine and papaverine. On the other hand the use of P-fertilisation and zinc

could reduce morphine content. It is also possible to reduce the morphine content of seeds through washing.

Using the proposed treatment consisting of washing, drying and grinding, not only the morphine content is significantly reduced but also the organoleptical quality of the poppy seed is notably enhanced. In particular, the poppy seed should be washed with water, then seeds must be dried to prevent microbiological contamination, germination and rancidity. The seed should then be ground using poppy mills. If the poppy is used for baking purposes or for decoration of bakery products, the baking should be done at the highest possible temperatures.

The fear mongering by tabloid press, stating that all foods containing poppy seeds are “toxic” and have to be banned from the market, should be disregarded. The use of uncontaminated seed with morphine contents below 4 mg/kg from controlled producers should be enforced so that morphine reduction can become superfluous in future. However, at the current levels of contamination, actions to reduce morphine levels during food processing are necessary and vital, so that the proposed limits can easily be maintained in most cases.

The country of origin and any pre-treatment of seeds prior to ingestion will influence the likelihood of obtaining a positive screening result. The positive results obtained following the ingestion of a relatively small quantity of seeds demonstrates the plausibility of the poppy seed defence. Therefore, until a suitable marker of seed ingestion has been isolated, each case must be considered on its own merits, and great care should be taken when interpreting the data obtained from drugs of abuse screens.

Perhaps no medicinal plant possesses such a high food value together with an excellent pharmaceutical property as does the opium poppy. The processing of opiate alkaloids in the pharmaceutical industry has increased during the last two decades and further increase is expected. Therefore, since 1992 some operations have been initiated to generate poppy forms with low morphine content, following the development of new low-morphine poppy lines, especially for food products. In opposition to that, the pharmaceutical industry demands that poppy cultivars present high amounts of pharmacological active substances and with a special spectrum of alkaloids (Michalski, 2004). So, in the last few years, breeding has been oriented in three main directions: creating cultivars accumulating high morphine (1.5-2.0%) or special alkaloid content (narcotine, codeine, thebaine etc.) for industrial extraction; selection of cultivars for seed and oil production, accumulating low level of morphine in the capsules (below 0.01%); producing ornamental types with special flower or capsule form, accumulating restricted amounts of alkaloids.

No maximum limits for morphine in poppy seeds have beeen establish in the European Union

FUTURES

- The preparation of guidelines for pre- and post-harvest poppy and its seeds management to minimise morphine content is strongly suggested.
- The development of analytical methods and their validation for morphine in poppy is strictly necessary.

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Chapter 2 - Ergot alkaloids in food

Keywords: Ergot, *Claviceps*, Metabolism, Infection cycle, Pharmacokinetics, Toxicity.

INTRODUCTION

In this report only *Claviceps* purpurea is considered as the other *Claviceps* species of this fungal family are not relevant for food. More detailed information on other *Claviceps* are found in the EFSA opinion (EFSA, 2005), which reports these species in various feeds.

Ergot is derived from the old French word 'argot', meaning the cock's spur (Van Dongen and De Groot, 1995) and this term is related to a disease caused by fungi belonging to the genus *Claviceps* on plants classified as *Graminacee*. Ergot alkaloids are mycotoxins produced by fungi of all the species of *Claviceps* genus, most notably by *Claviceps purpurea*, which parasitize the seed heads of living plants at the time of flowering. Although fungi are the primary source of ergot alkaloids, they are also synthesized by some plants, mainly of the morning glory family (Wilkinson *et al.*, 1987).

Other important sources of these ergot alkaloids are grasses infected with endophytes, for example, tall fescue (*Festuca arundinacea*) infected with *Claviceps* spp. or *Acremonium coenophialum* (Powell and Petroski, 1992).

The fungus replaces the developing grain or seed with the alkaloid-containing wintering body, known as ergot, ergot body or sclerotium. Recently, ergot contamination on sorghum has also been signalled (Bandyopadhyay *et al.*, 1998), which is caused by *Claviceps africana*. *Sorghum* species, principally *Sorghum bicolor*, are an important food and fodder crop in Africa, Central America, and South Asia.

In sorghum, *Claviceps* spores germinate and grow into the unfertilized seed producing a sclerotium. Fertilized flowers are resistant to infection. *C. africana* produces primarily dihydroergosine with lesser amounts of dihydroelymoclavine and festuclavine. The most important species *C. purpurea* seldom causes serious yield losses, but the alkaloids which its sclerotia ('ergots') contain are toxic both to humans and to animals and ergot contamination of grain lots greatly reduces their value (Yarham, 1996). In the Middle Ages epidemics of ergotism, derived from the ingestion of rye bread contaminated by *C. purpurea*, occurred frequently and they were popularly known as "St. Anthony's Fire"; they often resulted in gangrene, neurological diseases and death.

In 1582, for the first time, a medical application of ergot was reported: a delivery could be hastened by administering a few spurs of ergot, but the dosage was very inaccurate causing uterine ruptures. Therefore, after 1828 the ergot alkaloids were no longer used during delivery but only to prevent post-partum haemorrhage (van Dongen and de Groot, 1995). Since 1875 many derivatives of ergot alkaloids have been found; in particular the most famous was ergometrine isolated by Dudley and Moir in 1932. This substance has a very specific uterotonic action, and is able to prevent or to treat postpartum haemorrhage, but its

instability produces sometimes unpredictable effects making it unable to be used in medicine (van Dongen and de Groot, 1995; Krska and Crews, 2008). However, the beneficial pharmacological effects of ergot alkaloids stimulated research into ergot compounds, and natural and semisynthetic drugs have been produced from them (Flieger *et al*., 1997). The long list of pharmacological effects includes prolactin inhibition, treatment of Parkinsonism, cerebrovascular insufficiency, migraine, venous insufficiency, thrombosis, embolites, stimulation of cerebral and peripheral metabolism, uterine stimulation (Berde and Schild, 1978).

No discussion of ergot drugs would be complete without mentioning the semisynthetic psychomimetic drug, diethillysergamide (LSD-25) (Stoll and Hoffmann, 1943). Originally produced for experimental use as a treatment for such disorders as schizophrenia, the drug eventually became popular as a drug of abuse, due to its hallucinogenic effects. The drug was never found to have any profound beneficial effect, but continues to be made and consumed illegally to this day.

In general, the main groups of natural ergot alkaloids are simple lysergic acid derivates such as ergometrine (ergonovine), peptide alkaloids or ergopeptines (e.g. ergotamine, ergovaline and ergocornine), clavines such as agroclavine and lactam ergot alkaloids (e.g. ergocristam) (Flieger *et al*., 1997). The main ergotoxins produced by the *Claviceps* species which are contained in the sclerotia are ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine and the group of agroclavines, the latter being less toxic. In endophyte-infected grasses clavines and the ergopeptine ergovaline may be detected at major concentrations.

The amount and pattern of ergot alkaloids vary between fungal strains, depending on the host plant and the geographical region (Krska and Crews, 2008). For example, different surveys have suggested that in central Europe the total alkaloid concentrations in sclerotia of cereals vary between 0.09 and 0.21% (w/w) (Wolff, 1999), and sclerotia originating from grass seeds may contain 0.16 to 0.23% (w/w) of alkaloids (Wolff and Richter, 1989).

CLAVICEPS AND ITS HOSTS

The genus *Claviceps* includes 40 species, spread all over the world, parasitic on around 600 plants mainly belonging to *Graminacee*. The main species that grows on cultivated *Graminacee* is *C. purpurea* with rye (*Secale cereale*) and triticale (x Triticosecale Wittmack), that have open florets, as the most important hosts (Dabkevicius and Semaskiene, 2001), but also wheat (*Triticum spp*.), sorghum (*Sorghum vulgare*), pearl millet (*Pennisetum spp*.) and barley (*Hordeum vulgare*) infected (Lorenz, 1979; Kobel and Sanglier, 1986; Rehacek

and Sajdl, 1990; Flieger *et al.* 1997). The fungus can grow on many other minor *Graminacee*, such as *Agrotis*, *Alopecurus*, *Bromus*, *Festuca*, found in field as weeds or grown for forage. *C. purpurea* is widespread all over the world and is signalled on over 400 plant species (Muhle and Breuel, 1977).

Strains of the pathogen well adapted to a species or groups of species are known, while a geographic variation between strains is not known (Yarham, 1996).

Infection cycle

In spring, the first round of infection by *C. purpurea* is initiated by wind-borne ascospores derived from flask-shaped perithecia in germinating sclerotia. Insects contribute to the dispersal of ascospores to the seed heads of living plants at the time of flowering. Early flowering grasses can act as alternative hosts for the fungus allowing it to build up within or around a field before the cereal crop flowers. Ergot sclerotia, buried in the soil with seed, overwinter and early in the summer, before anthesis, ergots give rise to ascospores, which are forcefully ejected and land on stigma (female part of the flower) of florets (CAST, 2003), forming ascocarps in which ascospores ripen and are dispersed at cereals anthesis. When ascospores land on dehiscent plant florets, they start the infection (Dabkevicius and Mikaliunaite, 2006).

Ascospores give rise to hyphae that invade the ovary producing a white mould and originate the anamorph *Sphacelia segetum* Lev. A mycelial stroma, the sphacelium, develops in the ovary and produces masses of conidiospores that are exuded into a sugar-rich fluid derived from floem sap. This honeydew is the first visible symptom of a successful infection; it is produced for about 2 weeks and allows insects to transfer spores from floret to floret, spreading the disease in the field. The growing mycelium infects florets very close to the time of pollination, grows into the ovary, and surrounds (largely killing) the female structures of the florets. Fungal growth continues until the mycelium become organised in a compact body, oblong, strait or curved, between 5-6 to 12-20 mm long and 1.5-7 mm wide, initially white, then pink and purple at maturity. This resting structure is termed sclerotium, or ergot. Mature sclerotia can vary in number and size from a few millimetres to more than 4 cm according to the host plant (Kamphues and Drochner, 1991), and differ in mass from a few grams to 25 g per 100 sclerotia.

Before or during harvest time sclerotia fall to the ground and remain intact during the winter and then fruit sexually the following spring around host flowering.

The sclerotia are also harvested together with the cereals or grass and this can therefore lead to contamination of cereal-based food and feed products with ergot alkaloids.

Sclerotia are the most characteristic and noticeable signs of ergot. They are purple-black, horn-like and replace one or more seeds in the head. They protrude from the glumes as cereals mature and are up to four times larger than normal seeds. Intact or broken sclerotia are easily seen among harvested seed. In minute quantities, however, they are best detected by chemical assays for ergot alkaloids (Wiese, 1977).

The ergot sclerotium contains up to 40% of fatty oils (Komarova and Tolkachev, 2001a) and a variety of alkaloids, mainly those with the clavinet or ergoline ring system. These include lysergic acid, lysergic acid amide (precursor of the illegal narcotic, lysergic acid diethylamide or LSD), and ergopeptines such as ergotamine (CAST, 2003). Sclerotia show significant differences in their total alkaloid content that varies between 0.01 and 0.5% (w/w) (Lorenz, 1979; Schoch and Schlatter, 1985; Wolff, 1999) and show large differences in the patterns of alkaloids produced that are determined by the individual fungal strain in a geographical region and the host plant.

Ecology

C. purpurea is favoured by mild climate with high humidity, but ecological needs vary during the infection cycle.

Ergot sclerotia overwinter in the field and temperatures around 0°C are suitable for vernalisation; 9-10°C is the minimum temperature for germination, with 18-22°C as optimal. This moderately warm temperature is optimal also for the infection by conidia, favoured by high air humidity or drizzling rain (Ruokola, 1972) and for mycelium growth. Warm and windy weather limits infections both because of conidia drying and shortening of the flowering period. Scletotia formation is optimal in dry and moderately warm conditions; in hot weather, the sclerotia growing period is shorter, their mass is limited and alkaloid content is lower.

Plant-pathogen interaction

C. purpurea is a biotrophic pathogen of cereals and grasses. The infection process starts with a directed growth in the host's style and ovarian tissue into the vascular system without forming any appressoria or other penetration structures. Penetration occurs via the stigma and ovary wall and colonization is intercellular; within a week, the ovary is replaced by the sphacelium.

The cell wall is degradated, including the middle lamella, meaning that *C. purpurea* has a specific degradation system of thwe host tissue. It is reasonable to suppose that ergot synthesizes xylanase, cellulase and pectinase because the cell wall of grasses is mainly

composed of glucurono-arabino-xylans, cellulose and pectins (Cramer, 1998; Tenberge, 1999).

Studies on the molecular aspects of host-pathogen interaction have been made in the last twenty years and most of them were conducted on rye.

Muller *et al.* (1997) found cellobiohydrolase cell being induced during early infection of rye and hypothesized that it could be involved in ergot pathogenicity allowing hyphae penetration in ovarian tissue (Tudzynski and Tenberge, 2003). Xylanase production was observed during *in situ* rye ovaries infection and two endo-β-1,4-xylanase genes were characterized (Giesbert *et al.*, 1998) and were expressed during all phases of ergot infection (Cramer, 1998). Xylanases were detected in host ovaries infected with *C. purpurea* and the presence of arabinoxylans was observed in cell walls of rye ovaries (Giesbert *et al.*, 1998; Tenberge, 1999) Pectinolytic activity was described by Shaw and Mantle (1980) and then by Tenberge *et al.* (1996) in relation to hyphal growth. Two endo-polygalacturonase (PG) genes were found by Tenberge *et al.* (1996)*;* they were homologues with endo-PGs of other filamentous fungi and probably coded for proteins of 343 and 344 amino acids. During *C. purpurea* infection of rye, they caused the modification and degradation of homogalacturonan of the plant wall, so they could be used in the infection process.

Ergot secreted a beta-1,3-glucanase during all infection phases that was found mainly in honeydew and in the host-pathogen interface. It might suppress host defence, reaching the stock sites of callose and hydrolysing it, maintaining a nutrient supply to the pathogen (Brockmann *et al.*, 1992; Tenberge *et al.*, 1999) and preventing its further deposition since none is found in infected ovaries (Dickerson *et al.*, 1978).

Another study on rye revealed a hydrophobin mRNA that is produced during *C. purpurea* early infection and colonization in the external mycelia and in hyphae that penetrate the host epidermal layer (Tenberge *et al.*, 1998). Hydrophobins are known to be fungal extracellular proteins involved in pathogenicity, that produce amphipathic films at interfaces mediating contact to hydrophobic surfaces.

C. purpurea also produces four different catalases (CAT A,B,C,D) that were detected in honeydew and infected rye tissue, although studies on CAT1 showed that it is secreted in the honeydew of infected rye ears and in rye ovaries, but it is not essential for the colonization of host tissue (Garre *et al.*, 1998).

Ergot has developed a signal transduction pathway that regulates the infection process. Nowadays several studies are underway to try to clarify the entire pathway, because there are still unknown parts (Tudzynski and Scheffer, 2004).

Three mitogen-activated protein (MAP) kinases encoded by gene cpmk1-3 are produced during ergot infection of rye (Mey *et al.*, 2002a and b). They are respectively homologous to fus3, slt2, hog1 of *Saccharomyces cerevisiae* and to pmk1, mps1, psm1 of *Magnaporthe grisea*, all genes essential for pathogenicity. Signal transduction pathways of *C. purpurea* and *M. grisea* were found to be highly conserved, suggesting that MAP kinases are involved in the pathogenicity process (Mey *et al.*, 2002a).

GTPases regulate the signal transduction pathway as regards cell polarity in eukaryotic cells as well as in *C. purpurea*. In particular Cdc42 and Rho-type are known to be key enzymes. Scheffer *et al.* (2005) found that the GTPase Ctcdc42 was involved on vegetative differentiation and pathogenicity by stimulating branching and conidiation. Further studies are underway to assess the role of histidine kinase and cAMP pathway in ergot pathogenicity (Tudzynski and Scheffer, 2004).

Beta-fructofuranosidase is an enzyme localized at all stages during the growth of the ergot, but not in the spachelium or the sclerotia; its hypothetical role could be to mobilise sucrose as the C source for growth (Pollard and Dickerson,1984).

PHYSICO-CHEMICAL CHARACTERISTICS

Ergot alkaloids is a group of natural biologically active compounds belonging, according to the general alkaloid classification, to the class of indole derivatives. The structure of ergot alkaloids is built around a tetracyclic ring system of ergoline (Figure 1). Depending on the structure of ring D in the ergoline nucleus and the types of substituents at C8, all ergot alkaloids can be divided into three biogenetically related classes: clavine ergot alkaloids, simple lysergic acid derivatives and peptide ergot alkaloids (ergot peptides) (Krska and Crews, 2008)

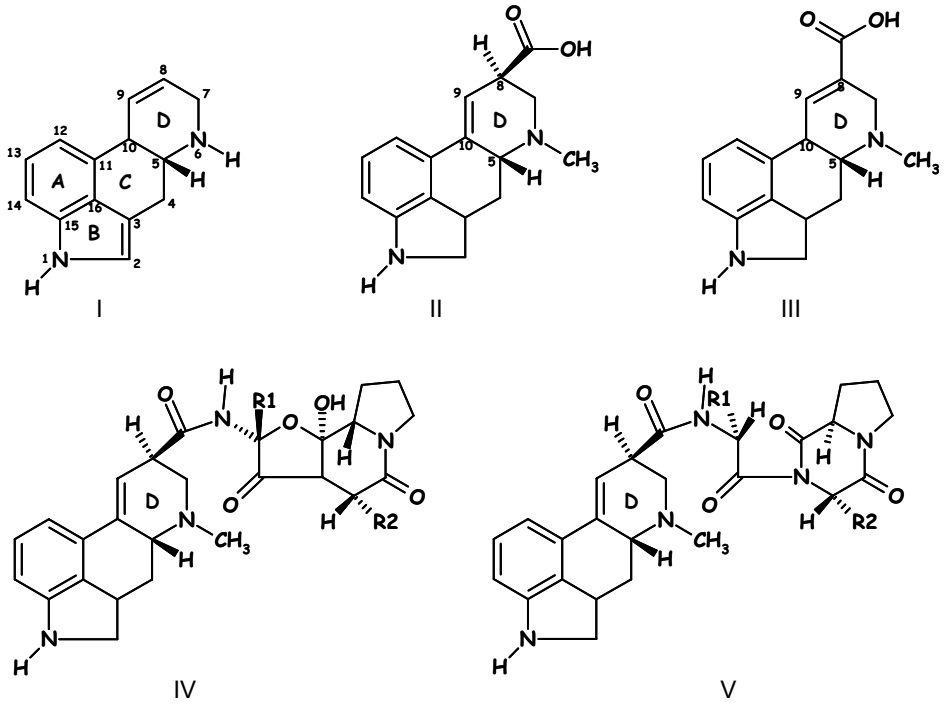


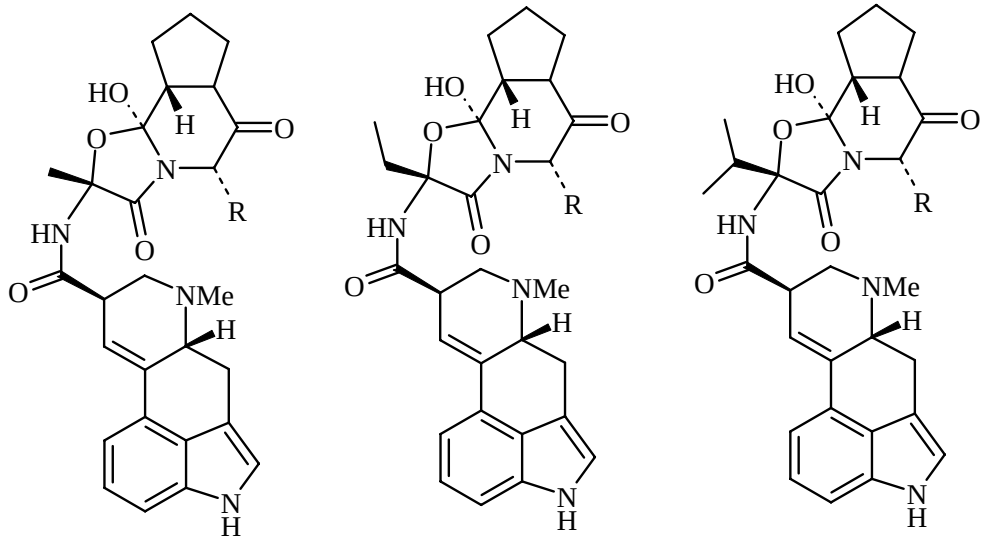
Figure 1. Chemical structures of ergoline (I), lysergic acid (II) and paspalic acid (III) ergopeptines(IV) and lactam ergot alkaloids – ergopeptams (V).

The classes of simple lysergic acid derivatives and peptide ergot alkaloids are based on the lysergic acid (LA) fragment. The two classes are distinguished by the type of substituents on the acidic group. The LA molecule shows two chiral centers (C5 and C8): these centres have a 8R,5R stereoconfiguration in the bioactive molecule. Thus, all the natural pharmacologically active ergot alkaloids are derivatives of this compound, representing a series of left-hand rotation isomers (Krska and Crews, 2008).

Peptide ergot alkaloids contain the LA nucleus and a tripeptide group (Figure 2) linked through an amidic bond. The classification of the ergot peptides is based on the tripeptide structure. The LA fragment is responsible for the basic biological activity of the compounds, while the substituent imparts certain specificity to this general activity.

Ergot peptides can be divided into two main groups (Figure 1). Ergopeptines represent the classical cyclic ergot alkaloids (cyclopeptides) with the oxazolopyrrolopyrazine system (cyclole) as substituent in the LA radical. Ergopeptines are the most widely spread natural type of peptide ergot alkaloids. Lactam ergot alkaloids are called ergopeptams and are characterized by a diketopiperazine peptide substituent (Figure 1) (Krska and Crews, 2008).

Peptide ergot alkaloids



	ergotamine group	ergostine group	ergocristine group
R = CH_2Ph	ergotamine	ergostine	ergocristine
R = CH_2CHMe_2	ergosine	ergoptine	α-ergocriptine
R = CH(Me)Et	[β-ergosine]	[β-ergoptine]	β-ergocriptine
R = $CHMe_2$	ergovaline	ergonine	ergocornine
R = Et	ergobine	ergobutine	ergobutirine

[ ] never found in nature.

Figure 2. Peptide ergot alkaloids.

C8-epimerization of ergot peptides

Lysergic acid derivatives readily exhibit epimerization, especially in the presence of alkalialkaline media, with respect to the centre of symmetry C8 with the formation of a series of right-hand rotation (S)-isomers representing isolysergic acid (*iso*-LA) derivatives (Komarova and Tolkachev, 2001a and b). This group of ergot alkaloids exhibits a relatively weak pharmacological activity. The epimerization is due to the presence of a double bond C9=C10, which favors enolization of the carboxyl group (Figure 3). The *iso*-LA derivatives represent LA diastereomers with a 8S,5R stereoconfiguration. According to the international classification, the left-hand rotation isomers of ergot alkaloids representing LA derivatives are termed ergopeptines and ergopeptams, while the right-hand rotation diastereomers representing the *iso*-LA derivatives are termed ergopeptinines (Table 1).

The conditions for and the rate of epimerization in ergopeptines depend on the structure of substituent R in the carboxamide group of the LA fragment (Figure 3). No (S)-isomers were

reported for ergopeptams, which is related to a high lability of the lactam ergot alkaloids: in the presence of bases, these compounds readily decompose into simpler derivatives.

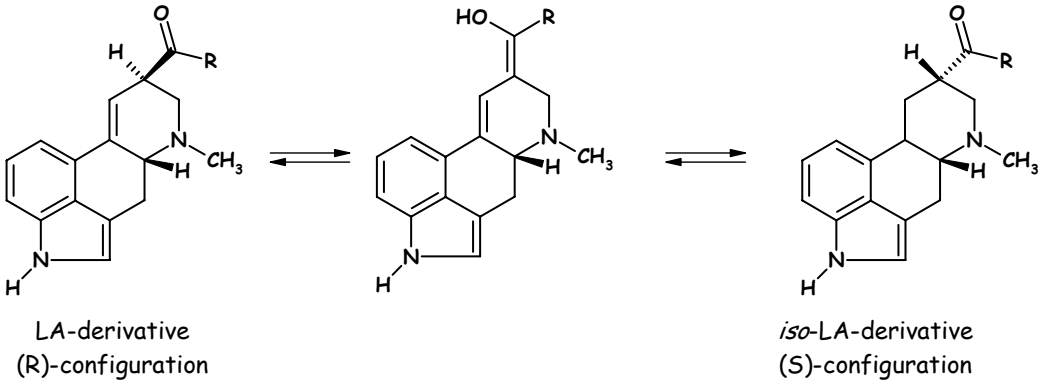


Figure 3. Epimerization of LA-derivatives to iso-LA ergot alkaloids involving C8 position.

Epimerization may easily occur in media containing an organic solvent (ethanol, methanol, acetone, etc.), water (10 to 95%), and acid (pH 1.5 – 5) and in alkaline media. The most pronounced epimerization activity was observed for mixtures containing phosphoric acid. The rate of isomerization and the isomer ratio depend on the conditions (temperature, pH) and the ergot alkaloid structure (Komarova and Tolkachev, 2001a and b).

The optimum storage of solid standards implies low temperature (not above 5°C), inert gas atmosphere and hermetic and light-tight package (Komarova and Tolkachev, 2001a and b).

The presence of a double bond in C9–C10 position explains a number of features characteristic of ergot alkaloids (including both (R) and (S) derivatives) and facilitates some chemical transformations. This bond constitutes a conjugated system of bonds with the indole fragment of a molecule, which is manifested in a typical UV spectrum of ergot peptides with λmax = 313 nm and λmin = 270 nm. The ergot peptides are also characterized by a bright-blue fluorescence (Krska and Crews, 2008)

The physico-chemical properties of the main ergot alkaloids are reported in Table 1.

Table 1. Physico-chemical properties of ergot alkaloids.

	Molecular Formula	m.p. (°C)	MW	Elemental analysis	$[\alpha]_D^{20}$ (chloroform)	UV max (methanol)	log ε	Solubility
Ergotamine	C33H35N5O5	212 – 214	581.65	C 68.14%, H 6.07%, N 12.04%, O 13.75%	- 160°	312	3.93	Methanol, acetone, chloroform, pyridine, ethyl acetate.
Ergometrine	C19H23N3O2	162	325.39	C 70.13%, H 7.12%, N 12.91%, O 9.83%	+ 90 ° (H_2O)	312	3.92	Methyl and ethyl alcohols, ethyl acetate, acetone, water (more than the other ergot alkaloids), chloroform (slightly)
Ergosine	C30H37N5O5	228	547.64	C 65.79%, H 6.81%, N 12.79%, O 14.61%	- 161°	312	3.94	Chloroform, acetone, methanol; ethyl acetate (slightly)
Ergocristine	C35H39N5O5	155-157	609.74	C 68.94%, H 6.45%, N 11.49, O 13.12%	- 183°	312	3.94	Ethyl and methyl alcohol, acetone, chloroform, ethyl acetate; ether (slightly)
α-Ergocriptine	C32H41N5O5	212	575.69	C 66.76%, H 7.18%, N 12.17%, O 13.90%	- 198°C	241 nm, 312 nm	4.31, 3.95	Alcohol, chloroform
β-Ergocriptine	C32H41N5O5	173	575.69	C 66.76%, H 7.18%, N 12.17%, O 13.90%	-179°	312 nm	3.93	Alcohol, chloroform
Ergocornine	C31H39N5O5	181	561.66	C 66.29%, H 7.00%, N 12.47%, O 14.24%	-175°C	311 nm	3.91	Acetone, chloroform, ethyl acetate; ethyl-methyl alcohol (slightly)

BIOSYNTHESIS

The most pharmacologically interesting alkaloids are derivatives of **(+)-lysergic acid**, which forms an amidic bond with an amino alcohol, as in **ergometrine**, or with a small polypeptide moiety, as in **ergotamine**.

Base synthons for the formation of lysergic acid are tryptophan (Petroski *et al.*, 1978) (excluding the carboxylic group) and an isoprenic unit derived from mevalonate (Arigoni *et al.*, 1970; Abou-Chaar *et al.*, 1972). Tryptophan alkylation with dimethylallyl pyrophosphate gives 4-dimethylallyl-tryptophan (Robbers and Floss, 1968; Pachlatko *et al.*, 1975; Gebler *et al.*, 1992; Li and Unsoeld, 2006), which then undergoes to N-methylation (Plieninger *et al.*, 1967; Groeger *et al.*, 1991; Rigbers and Li, 2008).

Formation of the tetracyclic system of lysergic acid takes place via **chanoclavine-I** and **agroclavine**, although the mechanistic details of the reactions are not completely clear (Floss *et al.*, 1974). Isotopic labelling studies (Fehr *et al.*, 1966; Groeger *et al.*, 1966) revealed that the double bond of the dimethylallyl substituent has to change to a single one in two different steps, thus allowing for the rotation needed for the formation of new rings (Plieninger *et al.*, 1978). The entire process thus seems a double *cis-trans* isomerisation (Floss *et al.*, 1968), the first one involved in 4-dimethylallyl-L-tryptophan-chanoclavine-I transformation and the second one in the cyclization of chanoclavine-I aldehyde (Tudzynski *et al.*, 1999) in agroclavine (Ogunlana *et al.*, 1970; Naidoo *et al.*, 1970). In the last steps, agroclavine is hydroxylated to **elymoclavine** (Floss *et al.*, 1967; Kobayashi and Floss, 1987), which then undergoes oxidation of the primary alcoholic group to carboxylic acid to obtain **paspalic acid**, which then spontaneously isomerizes to **lysergic acid** (allylic isomerisation) (Figure 4).

Also the simplest derivatives of lysergic acid show the formation of an amidic bond: i.e., **ergine**, isolated from species of *Rivea* and *Ipomea* is the amide of lysergic acid, whereas **ergometrine** from *Claviceps purpurea* is the amide with 2-aminopropanol (Groeger and Erge, 1970).

The most complex structures which contain a polypeptide fragment, like **ergotamine**, are probably formed via the sequential addition of amino acids to the lysergyl-CoA (Agurell, 1966; Basmadjian *et al.*, 1969), thus giving a linear tripeptide covalently linked to the enzymatic complex (Figure 5).

The cyclised tripeptide moiety in the ergometrine structure can be easily obtained via formation of another amidic bond and an emiketalic bond.

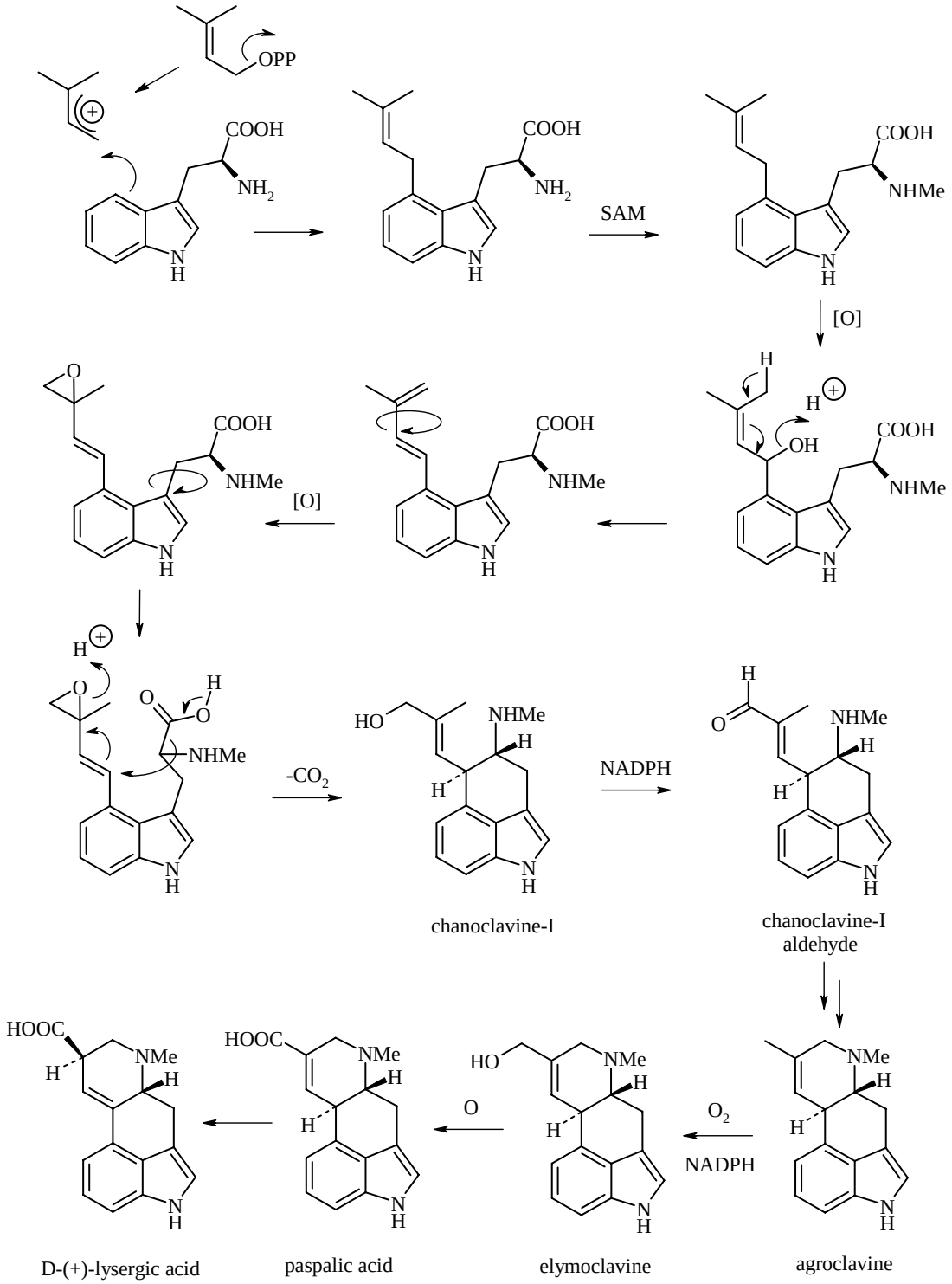


Figure 4. Biosynthesis of ergot alkaloids: lysergic acid.

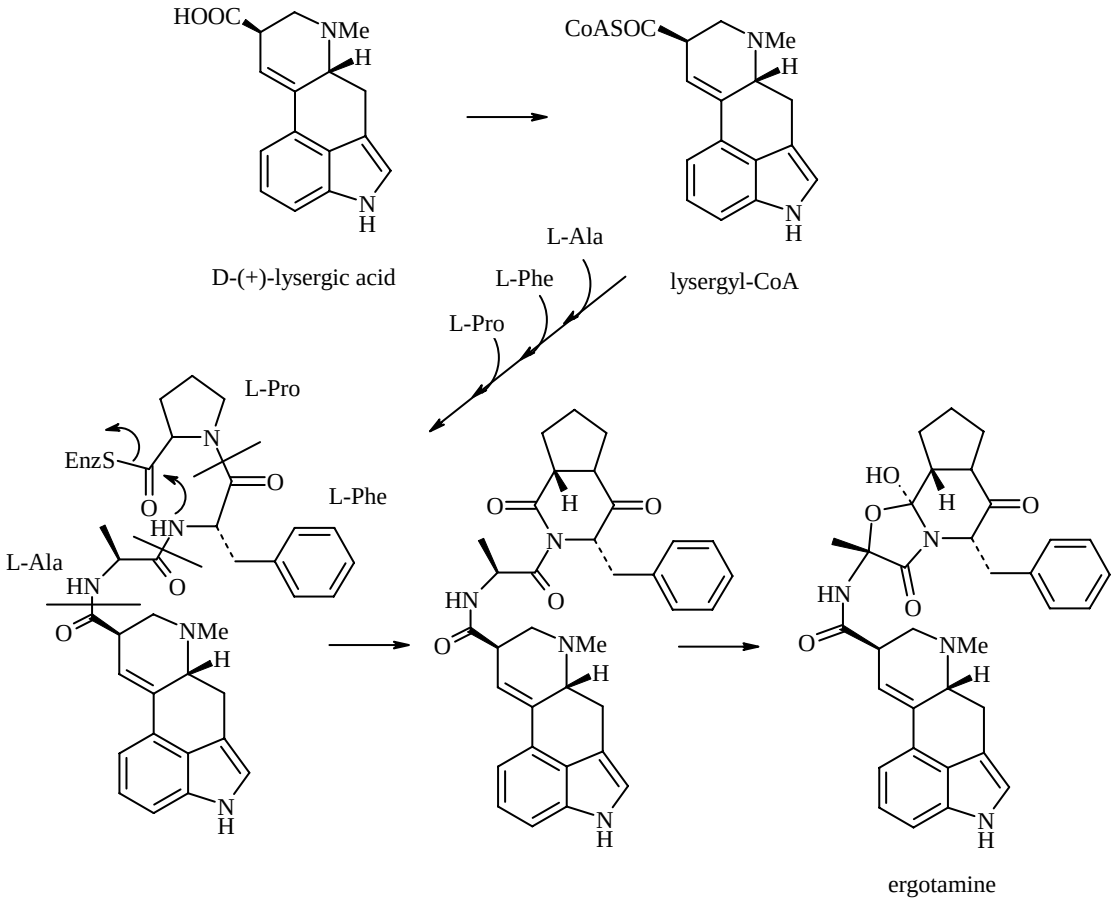


Figure 5. Biosynthesis of ergot alkaloids: ergotamine.

In the last decade, several genetic studies have been performed, in order to identify the genes involved in the ergot alkaloid biosynthesis.

In particular, L-tryptophan dimethylallyl transferase (DMAT synthase) was found to regulate dimethylallyltryptophan formation and seemed to be the first pathway specific step in alkaloid biosynthesis. DMAT synthase was purified from *Claviceps fusiformis* and characterized it as a homodimer of 52-kDa subunits. The cloning of the dmaW gene encoding this enzyme, allowed confirmation of the biochemical activity of the gene product (Wang *et al*., 2004). All of the enzymatic steps after DMAT formation concern modifications and rearrangements leading to the formation of rings C and D.

Its is most noteworthy that the pathway of ergot alkaloid formation may end at different stages, such as at chanoclavine-I. The differences in the alkaloid biosynthesis pathway found in nature may be the result of natural mutations in the downstream genes of the clavine assembly genes or a complete lack of the relevant genes.

The recent cloning of the ergot peptide alkaloid gene cluster will allow a functional analysis of all genes involved in ergoline ring synthesis and identification of the corresponding steps by

enzymatic analysis of the gene products (Tudzynski *et al*., 2001). The characterization of the various enzymes in terms of substrate specificity and structure will facilitate the development of control strategies. With the availability of the ergot alkaloid biosynthesis gene cluster from *C. purpurea*, it will become possible to determine the regulatory mechanisms directly at the molecular level.

CHEMICAL ANALYSES

Sampling

There has been no research on sampling plans for grains or grain products to be analysed for ergot alkaloids. However, to determine ergot bodies as a percentage of the net weight of a grain sample (e.g. wheat, rye or barley), the minimum representative portion is 500 g and the optimum is 1000 g in Canada (Scott, 2007). Due to the inhomogeneous distribution of ergots in the grain, a sample size of 1000-5000 g is recommended for optical assessment of the presence of ergot bodies (Lampen and Klaffke, 2006).

Extraction and clean-up

In most methods for the qualitative and quantitative determination of ergot alkaloids in cereals, extraction has either been performed with non polar organic solvents under alkaline conditions or with polar solvents under acidic conditions. A mixture of methylene chloride, ethyl acetate, methanol and 25% ammonium hydroxide (50 : 25 : 5 : 1 v/v) was used by Müller *et al.* (2006); whereas methanol:0.25% aqueous phosphoric acid (40 : 60 v/v) was used by Ware *et al.* (2000).

Clean-up is carried out either by liquid-liquid partitioning by exploiting the acid/base properties of N-6 (Scott and Lawrence, 1980), or more recently by similar acid-base partition on solid-phase extraction columns (SPE) (Ware *et al*., 1986; Fajardo *et al*., 1995). Other clean-up procedures include partition using Extrelut® columns (Baumann *et al*., 1985), use of strong cation exchange particle-loaded membrane extraction disks (Ware *et al*., 2000), and silica gel columns (Rottinghaus *et al*., 1993), which did not retain the inactive -inine isomers. All those methods gave satisfactory recoveries of individual ergot alkaloids from spiked matrices.

Recently, a method has been developed and validated (Krska *et al*., 2008), for ten different cereal and food samples, which enabled a rapid and simultaneous quantification of the six major ergot alkaloids defined by EFSA (ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine) and their corresponding epimers. The method involves

extraction under alkaline conditions (acetonitrile and ammonium carbonate buffer) followed by a rapid clean-up using dispersive solid-phase extraction with PSA (primary secondary amine).

Standards

Analyses of agricultural commodities and foods for ergopeptide alkaloids should be carried out in subdued light to minimize the formation of 'lumiergopeptines', which are water addition products. It should also be noted that epimerization to ergopeptinines can occur in solution, particularly at room temperature, leading to equilibrium mixtures (Smith and Shappell, 2002). The degree of epimerization depends on the solvent, so stock standard solutions should be prepared in aprotic solvents such as chloroform and stored at less than 0°C in amber vials (Scott, 2007). Calibrants should be freshly prepared or immediately evaporated to dryness after preparation, stored deep frozen at -18°C and reconstituted just before use (Lauber *et al*., 2005).

Ergot alkaloid standards are less readily available than other common mycotoxins. Meanwhile, the major ergot alkaloids ergometrine, ergotamine, ergosine, ergocristine, ergostine, ergocryptine and ergocornine are all commercially available as naturally occurring α-isomers. Ergocryptine and ergocryptinine might, however, also occur as β-isomers, which are not yet available commercially (Krska and Crews, 2008). Availability of standards is shown in Table 2.

Reference substances are not available for all naturally occurring alkaloids. Therefore many laboratories only calibrate the most common alkaloids, ergometrine and ergotamine and assign a response factor to other alkaloids. Thus the total alkaloid concentration is calculated on the basis of guide substances (Lauber *et al*., 2005).

Table 2. Sources of ergot alkaloid standards.

Compound	Concentration (µg/ml)	Quantity	Supplier	References
Standard Solution				
Ergometrine	100 dried down	5 ml	Romer Labs Diagnostic (Tulln, Austria)	
Ergosine	100 dried down	5 ml	Romer Labs Diagnostic (Tulln, Austria)	
Ergotamine	100 dried down	5 ml	Romer Labs Diagnostic (Tulln, Austria)	
Ergotaminine	25 dried down	5 ml	Romer Labs Diagnostic (Tulln, Austria)	
Ergocornine	100 dried down	5 ml	Romer Labs Diagnostic (Tulln, Austria)	
Ergocorninine	25 dried down	5 ml	Romer Labs Diagnostic (Tulln, Austria)	
α-Ergocryptine	100 dried down	5 ml	Romer Labs Diagnostic (Tulln, Austria)	
α-Ergocryptinine	25 dried down	5 ml	Romer Labs Diagnostic (Tulln, Austria)	
Ergocristine	100 dried down	5 ml	Romer Labs Diagnostic (Tulln, Austria)	
Ergocristinine	25 dried down	5 ml	Romer Labs Diagnostic (Tulln, Austria)	
Solid Standard				
Ergometrine maleate salt		100/500 mg	Sigma-Aldrich (Taufkirchen, Germany) Novartis-Sandoz Pharma (Basel, Switzerland) EDQM (Strasbourg, France) Andard-Mount Company Ltd. (London, UK) Service Chemical Inc. (Regenstauf, Germany)	Muller *et al*., 2006 Reinhard *et al*., 2008
Methylergometrine maleate salt		50/250 mg 25/100 mg	Tocris Bioscience (Bristol, UK) Sigma-Aldrich Andard-Mount Company Ltd. (London, UK) Carbone Scientific Co. Ltd. (London, UK)	
Ergometrine tartrate			Sigma-Aldrich (Buchs, Switzerland) Service Chemical Inc. (Regenstauf, Germany)	Mohamed *et al*., 2006a
Ergometrinine			Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008
Ergosine			Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008
Ergosinine			Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008
Ergotamine			Andard-Mount Company Ltd. (London, UK) Service Chemical Inc. (Regenstauf, Germany)	
Ergotamine tartrate		250 mg/1g	Sigma-Aldrich (Taufkirchen, Germany) Novartis-Sandoz Pharma (Basel, Switzerland)	Muller *et al*., 2006 Reinhard *et al*., 2008
		1g	Fluka (Buchs, Switzerland) Tocris Bioscience (Bristol, UK) Andard-Mount Company Ltd. (London, UK) Boehringer Ingelheim (Ingelheim, Germany) Service Chemical Inc. (Regenstauf, Germany)	Ruhland and Tischler, 2008 Storm *et al*., 2008
Dihydroergotamine tartrate salt		50/500 mg	Sigma-Aldrich Andard-Mount Company Ltd. (London, UK)	
Dihydroergotamine methanesulfonate salt		100 mg/1g	Sigma-Aldrich	
Dihydroergotamine mesylate		100 mg 100 mg	Tocris Bioscience (Bristol, UK) Biotrend Chemicals AG (Zurich, Switzerland)	

		EDQM (Strasbourg, France)	
		Service Chemical Inc. (Regenstauf, Germany)	
		Carbone Scientific Co. Ltd. (London, UK)	
		Andard-Mount Company Ltd. (London, UK)	
		Boehringer Ingelheim (Ingelheim, Germany)	
Ergotaminine		Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008
Ergostine		Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008
Ergostinine		Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008
Ergocornine	25/100 mg	Sigma-Aldrich (Taufkirchen, Germany)	Muller *et al*., 2006
		Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008
		Service Chemical Inc. (Regenstauf, Germany)	
Ergocorninine		Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008
α-Ergocryptine	25/100 mg	Sigma-Aldrich (Taufkirchen, Germany)	Muller *et al*., 2006
		Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008
		Service Chemical Inc. (Regenstauf, Germany)	
		Carbone Scientific Co. Ltd. (London, UK)	
α-Ergocryptinine		Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008
Ergocristine		Andard-Mount Company Ltd. (London, UK)	
Dihydroergocristine mesylate		Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008
		Andard-Mount Company Ltd (London, UK)	
	100 mg	Tocris Bioscience (Bristol, UK)	
	100 mg	Biotrend Chemicals AG (Zurich, Switzerland)	
Dihidroergocristine methanesulfonate salt		Sigma-Aldrich	
Ergocristinine		Novartis-Sandoz Pharma (Basel, Switzerland)	Reinhard *et al*., 2008

Analytical methods

A wide variety of methods have been explored for the final determination of ergot alkaloids in grains, grasses, feeds and grain foods. They incorporate simple detection procedures, such as colorimetry (Robbers *et al*., 1975; Young, 1981), thin-layer chromatography (TLC) (Agurell, 1965; Lobo *et al*., 1981), and immunoassays (ELISA) (Shelby and Kelley, 1990 and 1992), or instrumental procedures such as capillary zone electrophoresis (Frach and Blaschke, 1998), or gas chromatography (GC), usually with mass spectrometric detection (MS) (Scott, 1993).

Reviews of available analytical methods for the determination of ergot alkaloids, including the most frequently employed LC methods, have been published by Scott (1995), and Komarova and Tolkachev (2001b), and have recently been updated by the former author (Scott, 2007).

ELISA techniques seem to be an attractive option for the screening of ergot alkaloids in agricultural crops and grain flour, but it is difficult to identify a marker toxin for monitoring the extent of the contamination. Cross reactivity might be high for one group of ergot alkaloids and low for another (Schnitzius *et al*., 2001).

Total ergot alkaloid content in tall fescue can also be quantified by near-infrared spectroscopy (NIR) with calibration developed from immunochemical reference data, though it may not be possible to include samples of stockpiled tall fescue, if they are infected with a toxic endophyte. Such a calibration can be robust and precise, reliably predicting an entire class of compounds in a diverse population of tall fescue samples (Roberts *et al*., 2005).

GC is not very useful for the determination of ergopeptide alkaloids as they decompose in a hot injector (Scott, 1993). The resulting peptide fragments can be separated by capillary GC with MS identification, but this procedure only identifies the peptide portion of the molecule and epimers such as ergotamine and ergotaminine are not differentiated (Scott, 2007). GC-MS has not been applied to the determination of ergot alkaloids in foodstuffs and has only been used in pharmaceutical and forensic areas and, in one instance, for confirmation of the alkaloids identified in grain foods (Klug *et al*., 1988).

Capillary zone electrophoresis was applied to the qualitative and quantitative analysis of ergot alkaloids and their epimers, but only for rye ergot sclerotia and not yet to grain foods (Frach and Blaschke, 1998).

In the 1960's and 1970's TLC was the major technique. The main criterion of usefulness of TLC for determining ergot alkaloids in agricultural products is that the solvent system should separate the alkaloids of interest. Comprehensive studies by Agurell (1965), Fowler *et al*. (1972), and Lobo *et al*. (1981), using silica gel and alumina thin layers and several solvent systems, illustrated the difficulty in achieving this objective. Of the 12 main alkaloids usually

found in rye ergots, such as ergometrin(in)e, ergosin(in)e, ergotamin(in)e, ergocornin(in)e, ergocryptin(in)e and ergocristin(in)e, ergocryptine and ergocristine were particularly inseparable even with two-dimensional TLC. This technique could be used in developing countries, preferably using extraction and clean-up procedures developed for LC methods (Scott, 2007).

TLC screening methods have mostly been replaced by HPLC procedures with normal phase columns and subsequent ultraviolet light detection (UV), at one or more of several wavelengths from 225 nm to 320 nm. However, reversed phase LC with fluorescence detection (FLD) is the usual mode for determining ergot alkaloids in grain ergots, grain foods and endophyte infected tall fescue. Excitation wavelengths are in the range 235-250 nm or 310-360 nm (Rottinghaus *et al.*, 1993; Ware *et al*., 2000; Komarova and Tolkachev, 2001a and b; Lombaert *et al*., 2003). Ergot alkaloids frequently analysed together by HPLC include ergometrine, ergotamine, ergocornine, ergocryptine, ergocristine, ergosine and their respective -inine isomers; the sum of the ergot alkaloids determined is often referred to as the total alkaloid content (Mainka *et al*., 2005). Reported detection limits for individual ergot alkaloids in grains and grain foods were of the order of 0.01-0.5 µg/kg (Müller *et al*., 2006), or 1-2 µg/kg (Ware *et al*., 2000). The limit of quantitation reported by Lombaert *et al.* (2003) for infant cereals was 4 µg/kg.

C18 (Mohamed *et al*., 2006a) and Phenomenex Gemini C18 column materials (Lehner *et al*., 2005a), have mainly been employed for the LC separation of ergolines. Various isocratic mobile phases and gradient systems were used for reversed phase LC (Scott, 2007). Acetonitrile mixed with either aqueous base (Scott and Lawrence, 1980; Baumann *et al*., 1985), or acidic solutions (Ware *et al*., 2000), have often been used. Acidic mobile phases are often preferred because many silica based LC phases are degraded at high pH.

LC coupled to mass spectrometry (LC-MS) and LC tandem MS (LC-MS/MS), usually with electrospray ionization operated in the positive mode ESI(+), has been employed for the quantification of ergot alkaloids as an alternative to FLD. The use of this technique provides in addition an unequivocal identification of the alkaloids. Shelby *et al*. (1997) used ESI(+)LC-MS to identify ergot alkaloids including ergine, ergovaline, ergosine and ergonine in endophyte-infected tall fescue; ergonovine and ergotamine were not found. More recently, Stahl and Naegele (2004) have reported nano-LC-MS/MS analysis of fungal extracts, with ion trap detection enabling MS^n experiments which enabled the identification of three unknown ergot alkaloid derivatives. Mohamed *et al*. (2006b) studied the fragmentation mechanism of six major ergot alkaloids by triple quadrupole and ion-trap mass spectrometers operated in ESI(+). Characteristic product ions at m/z 223 and 208 were observed for

peptide-type and lysergic acid derivatives. As a result precursor ion scanning of the most abundant m/z 223 ion was employed for survey studies of rye samples. Lehner *et al*. (2005a) demonstrated the facility of using ESI(+) mass spectrometry with selected reaction monitoring (SRM) for screening of grass and forage samples for novel ergot alkaloids. The same authors (Lehner *et al*., 2005b) have thoroughly studied the fragmentation patterns of selected ergot alkaloids by LC-MS/MS which allows the prediction of mass spectra of related compounds for which standards are not readily available.

Mohamed *et al*. (2006a) used LC-MS/MS with SRM after C18 clean-up for the quantification of five ergot alkaloids (ergotamine, ergosine, ergocristine, ergocryptine, ergometrine and ergocornine) in rye flour and obtained recoveries from 24% (ergonovine) to 92% (ergocryptine) and limits of quantification of 11-37 µg/kg. Bürk *et al*. (2006) reported an LC-MS/MS method capable of quantifying five ergot alkaloids down to 0.1-1 µg/kg (LOQ) with mean recoveries from 65 to 82% without the need for any clean-up. These methods do not include both -ines and -inines, possibly because of the lack of available standards.

Krska *et al*. (2008), obtained the simultaneous determination and separation of the six major ergot alkaloids defined by EFSA (ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine) with a short chromatographic run (14 min) and SRM in ESI(+) mode with LOQs of 0.17-2.78 µg/kg. Sulyok *et al*. (2007) integrated 25 ergot alkaloids including five ergopeptides and their epimers in a multi-analyte LC-MS/MS method without any prior clean-up which in total covers 87 different mycotoxins. Though low LODs of 0.02-1.2 µg/kg have been achieved, a high degree of epimerization was observed in that study which strongly influenced the recoveries obtained for the individual ergot alkaloids.

Validation and outlook

Apart from LC methods, the performance characteristics of most methods are not well known.

None of the methods mentioned, including LC-methods, has been validated by interlaboratory studies and there are no certified matrix reference materials or proficiency studies available for the determination of ergot alkaloids. Recently, EFSA (2005) concluded that validated analytical methods for the quantification of ergot alkaloids in feed materials are needed as a prerequisite for a survey on the occurrence of ergot alkaloids in feed materials in Europe. Analytical techniques should aim to detect the major ergot alkaloids as well as their corresponding biologically active metabolites formed in exposed animals. Particularly, in view of the ongoing discussions about the introduction of regulatory limits for individual ergot alkaloids in food and feed, the use of LC-MS/MS and most probably also of time-of-flight

(TOF)-MS for quantification and identification of ergot alkaloids and their derivatives, will become of increasing importance (Krska and Crews, 2008).

OCCURRENCE DATA

Ergot is ubiquitous, yet the prevalence of the species is dependent on climatic conditions and is especially pronounced in seasons with heavy rainfall and wet soils (Craig and Hignight, 1991).

Investigations in Germany indicate an increase in the occurrence of *Claviceps purpurea* infections in the last 10 years. This increase seems to be associated with the more extensive use of hybrid varieties of rye and perennial rye breeds (Amelung, 1995). There are published articles on the content of ergot alkaloids in European cereal products. Occurrence data are summarized in Table 3.

Normally, the frequency of ergot on wheat is low, but the disease is a constant threat (Wiese, 1977). A Danish survey was conducted in 1987-1988 (Rasmussen, 1991), on 55 samples of cereal products including 15 rye flour samples. Rye flour is particularly interesting in Denmark, because rye is the most susceptible cereal and Danes consume on average 72 g of rye per day. This study refers an average of ergot alkaloid content in organic rye flour of 205 µg/kg, compared with 3 µg/kg for conventional rye flour.

An inverse relationship was seen by Lauber *et al.* (2005). Since June 2004, 51 samples of rye grains and rye meals of the harvests 2003 and 2004 (conventionally as well as organically grown) were analyzed for their total alkaloid content. Sampling was done at random, normally in mills.

The aim was to sum up the special climate situation in 2003, to compare the data with those of the harvest of 2004 and to determine whether there was a difference between conventionally and organically grown products. In all rye samples analyzed ergot alkaloids were detected. The maximum allowable impurity with ergot (0.05% = 1000 µg alkaloids/kg) was exceeded, however, exclusively in samples of harvest 2003 (23%). All exceeding samples, except one, were detected in conventionally grown products. To give a possible reason for the generally significant lower concentrations in organically grown products, it should be mentioned that, according to the guidelines of some associations of organic cultivation, hybrid species (which are more susceptible to ergot) should not be grown.

Because of the very hot and dry summer in 2003, ergot of that harvest was comparatively limited, hence it could not be separated from healthy grain or only with extraordinary expenses. On the other hand, from the visual control of rye samples of harvest 2004 emanates in fact a high impurity rate with ergot as well, but because of the better growing

conditions for the fungi, exceptional big sclerotia were observed, which could be separated more easily.

In a recent Danish survey (Storm *et al*., 2008), 34 rye flour samples were collected at mills all over Denmark between 2000 and 2005 and analyzed for their content of ergot alkaloids, in particular for ergocornine, α-ergocryptine, ergocristine, ergometrine, ergotamine and the relative –inine isomers. In the 34 tested samples there was no significant difference in ergot alkaloids content between organic and conventional rye flour. A total of 30 samples were positive and the most common ergot alkaloids were ergotamine and α-ergocryptine, including their isomers. The distribution between individual ergot alkaloids was, on the other hand, highly variable, with some samples containing all five types and their epimers, while others contained only a few types; thus it is consistent with the variations between ergot sclerotia from different locations.

The incidence of ergot was also observed in 13-49 commercial fields of winter rye, winter triticale and spring barley in different regions of Lithuania, annually, over the period of 1996-2000 (Dabkevičius and Semaškiené, 2001). During this period, 65.9, 40.3 and 11.6% of the total area of the three cereals under observation were affected by ergot, respectively. On average 4.0, 4.7, and 4.0% of ears in the affected area were with sclerotia. This study suggested that ergot occurs to some extent every year on cereals in Lithuania. The disease is generally more prevalent in rye and triticale than in other cereals.

A paper by Lombaert *et al*. (2003) describes the analysis of 363 infant cereal foods obtained from the Canadian retail market over 3 years (1997-1999) and the results of those analyses for targeted mycotoxins. Some of these samples, categorized as oat-, barley-, soy-, rice-based or multi-grain infant cereals according to their common name, teething biscuits (baked wheat products), creamed corn and soy-based infant formulas, were analyzed for five ergot alkaloids (ergosine, ergotamine, ergocornine, α-ergocryptine and ergocristine). Ergot alkaloids were detected in 25% of all samples (41/162). The incidence and overall mean level of ergot alkaloids was highest in the barley-based samples (56%, 18 µg/kg).

In a more recent study (Reinhard *et al*., 2008), bread, flour, infant formula and baby food samples (n=109, from which n=54 made of or containing rye), collected in Switzerland in 2001, 2003 and 2005 were analysed for ergot alkaloids. The ergot alkaloid content of a sample was defined as the sum of the 8 ergolines ergometrine, ergosine, ergotamine, ergostine, ergocornine, α-ergocryptine, β-ergocryptine, ergocristine and the relative 8 -inine isomers.

Compared with earlier data (Baumann *et al*., 1985) for median levels of ergot alkaloids in rye flour and bread from Switzerland, the median values for ergot alkaloids in rye flour collected

in 2001and in 2005 were slightly elevated. For rye bread, a noteworthy increase in 2001, 2003 and 2005 was observed, but ergot alkaloid patterns did not significantly differ between the three years. Low levels of ergot alkaloids were also found in wheat products and in some infant formulae and baby foods containing rye.

Table 3. Results of surveys for ergot alkaloids, showing concentrations and distribution of contamination in food commodities.

Country	Commodity	Year	Mycotoxin	N° of samples	LOQ (µg/kg)	*n* > LOQ	Mean (µg/kg)	Median (µg/kg)	Min/Max (µg/kg)	References	Sampling procedure
Denmark	Cereal products	1987/88	Total EA[1]	40	NA	NA	NA	NA	NA	Rasmussen, 1991	-
	Organic rye flours			2		NA	205	-	-		
	Conventional rye flour			13		-	3	-	-		
Germany	Conventional rye grains and meals	2003	Total EA[2]	18	NA	-	1147	850	NA/3280	Lauber *et al*., 2005	Randomly, normally in mills
	Organic rye grains and meals			12		-	324	196	NA/1490		
	Conventional rye grains and meals	2004	Total EA[2]	15	-	-	281	220	NA/974		
	Organic rye grains and meals			6		-	208	256	NA/363		
Denmark	Organic rye flour	2000/05	Total EA[3]	17	(LOD: 0.2-1.1 range)	15	32	-	NA/100	Storm *et al*., 2008	Samples from Danish mills. Sampling of 100 subsamples of 100 g from lots of 50-300 tons, homogenated
	Conventional rye flour			17		15	60	-	NA/230		
Canada	Oat-based cereals	1997/99	Total EA[4]	6	4	2	2	-	NA/5	Lombaert *et al*., 2003	Samples collected from retail outlets across Canada
	Barley-based cereals			55		31	18	-	NA/108		
	Soy-based cereals			7		ND	ND	-	ND		
	Rice-based cereals			9		ND	ND	-	ND		
	Multi-grain cereals			75		6	1	-	NA/47		
	Teething biscuits			9		2	1	-	NA/4		
	Soy formulas			1		ND	ND	-	ND		
	Creamed corn			0		ND	ND	-	ND		
Switzerland	Wheat type 400 flour	2001	Total EA[5]	6	-	-	-	83	9/133	Reinhard *et al*., 2008	-
	Wheat type 720 flour			5		-	-	103	76/211		
	Wheat type 1100 flour			4		-	-	100	27/186		
	Spelt flour			2		-	-	20	19/20		
	Rye flour			13		-	-	172	18/519		
	Rye whole grain meal			7		-	-	124	60/186		
	Durum wheat semolina			2		-	-	55	46/64		
	No rye bread			3		-	-	18	5/25		
	Rye bread			14		-	-	87	17/248		
	Infant formulae and baby food with rye			9		-	-	1	ND/26		
	No rye bread	2003	Total EA[5]	3	-	-	-	37	13/45		
	Rye bread			7		-	-	20	46/477		
	Wheat type 400 flour	2005	Total EA[5]	4	-	-	-	6	2/17		

	Wheat type 720 flour			4		-	-	25	19/29		
	Wheat type 1100 flour			2		-	-	64	51/77		
	Spelt flour			1		-	-		51		
	Rye flour			2		-	-	160	47/273		
	Durum wheat semolina			1		-	-		8		
	Rye bread			2		-	-	156	110/201		
Switzerland	Rye flour	1985	Total EA[5]	-	-	-	-	140	-	Baumann *et al.*, 1985	-
	Rye bread			-		-	-	21.3	-		

NA= Not Available; ND= Not Detected
[1] Sum of: Ergometrin (EM), α-ergocryptine (EY), Ergocristine (ET), Ergotamine (EA), Ergocornine (EC).
[2] Sum of: Ergometrin (EM), Ergometrinine (EMI), Ergotamine (EA), Ergotaminine (EAI), Ergocornine (EC), Ergocorninine (ECI), α-ergocryptine (EY), α-ergocryptinine (EYI), Ergocristine (ET), Ergocristinine (ETI), Ergosine (ES), Ergosinine (ESI).
[3] Sum of: : Ergometrin (EM), Ergometrinine (EMI), α-ergocryptine (EY), α-ergocryptinine (EYI), Ergocristine (ET), Ergocristinine (ETI), Ergotamine (EA), Ergotaminine (EAI), Ergocornine (EC), Ergocorninine (ECI).
[4] Sum of: Ergosine (ES), Ergotamine (EA), Ergocornine (EC), α-ergocryptine (EY), Ergocristine (ET).
[5] Sum of: Ergometrin (EM), Ergometrinine (EMI), Ergosine (ES), Ergosinine (ESI), Ergotamine (EA), Ergotaminine (EAI), Ergostine (EO), Ergostinine (EOI), Ergocornine (EC), Ergocorninine (ECI), α-ergocryptine (EY), α-ergocryptinine (EYI), β-ergocryptine (BY), β-ergocryptinine (BYI), Ergocristine (ET), Ergocristinine (ETI).

MITIGATION OF ERGOT ALKALOIDS

There are several strategies for the control of ergot including both good cultural practices and the use of chemical fungicides or biological control agents.

The reduction of ergots in field could be obtained using different approaches based principally on the use of uncontaminated seed, a deep ploughing to bury the sclerotia, the rotation of crops, the control of weed grasses (Yarham, 1996; Filatova, 2004), nitrogen fertilization, herbicide application (Naylor and Mundro, 1992; Pageau *et al.*, 1994a) and the use of resistant cultivars.

Mature ergots may fall to the ground or be harvested with the grain. In the latter case, they may serve to introduce the pathogen into other fields if the contaminated grain is used as seed (Yarham, 1996). Experimental evidence suggests that winter rye is one of the cereals most susceptible to ergot and a cleaned winter rye seed can contain up to 26% of ergot sclerotia formed in the ears (Nemkovich, 1999). For this reason, in order to prevent the spread of ergot, it is important to reduce the number of sclerotia from getting into the soil with the seed and, if the seed clearing is not perfect, it is important to ensure that ergot sclerotia produce as few ascospores as possible, treating seeds with fungicides (Dabkevicius and Mikaliunaite, 2006).

So, the use of uncontaminated seeds is the first preventive action strongly suggested.

Spring fertilization with calcium cyanamide was shown to reduce germination of ergots laid on the ground by 40-50% (Mielke, 1993).

Ergots from some of the many grass species found in the arable environment are highly infective towards cereals. It is likely that ergot in wheat originates as much from the general field environment (paths, gateways, hedge bottoms) as from sown margins. Sowing margins with grass species which consistently produce ergots with low infectivity may help to reduce the proportion of infective inoculum in the environment. It is important to remember that grass species that flower after winter wheat could be considered safer than earlier flowering species (Gladders *et al*., 2001).

The use of resistant cultivars

The content of ergot sclerotia in grain is strictly dependent on the varietal susceptibility to ergot, in particular, interspecific and intraspecific hybrids and tetraploid cereals are heavily affected by this disease (Mielke, 1993; Betz and Mielke, 1996; Fuchs and Voit, 1996).

Some wheat varieties possess partial resistance to ergot which makes them less prone to infection but there is no evidence of an association between a high degree of another extrusion in varieties and susceptibility to ergot (Gladders *et al*., 2001).

Claviceps purpurea is a pathogen that affects numerous cereals and many studies have been carried out to try to find new resistant or less susceptible genotypes over the last three decades.

The resistance ratio can be measured by looking at the three characteristic factors of *C. purpurea* infection: honey-dew production, size and frequency of sclerotia, that is the number of sclerotia/kg of grain (Gregory *et al.*, 1985b).

As regards wheat, one of the first studies concerned cultivars *Triticum aestivum* Kenya Farmer and *T. durum* Carleton, the former moderately resistant, showing no highly virulent *C. purpurea* isolate, and the latter susceptible. Platford *et al.* (1977) showed some genes on chromosome 3B in Carleton that could interfere with *C. purpurea* infection by reducing sclerotial frequency and honey-dew production. Chris and Cajeme spring wheat are known as two very resistant cultivars (Schmidt and Lucken, 1976). Larter (1974) described tetraploid and hexaploid wheat as resistant to ergot. Vance wheat, a 1989 cultivar (Busch *et al.*, 1990), Wheaton wheat (Busch *et al.*, 1984) and Marshall wheat (Busch *et al.*, 1983) are moderately resistant cultivars, while Angus wheat (Elsayed *et al.*, 1979) and Kitt red spring wheat (Crops and Soils, 1975) are tolerant to *C. purpurea*. One accession of *T. zhukovskyi* has been reported free of sclerotia from 14 ergot isolates and also some accessions of *T. aestivum, T. timopheevii* and *T. orientale* were resistant to most of the ergot isolates tested (Bernier, 1978). Victorian wheat cultivars Halberd, Kalkee and Zenith were moderately resistant to ergot even under favourable environmental conditions (Bretag and Merriman, 1980).

Male sterility is a factor that confers resistance to wheat (Darlington and Mathre, 1976) but not to rye (Mauszynska *et al.*, 1998) and barley plants. Most of the male-sterile barley cultivars in the world collection were susceptible to ergot, except a few that showed low susceptibility both to natural and artificial infection (Cunfer *et al.*, 1974). This susceptibility has been overcome in some male-sterile cultivars by inoculating with conidial suspensions of *C. purpurea* at various times after hand pollination instead of inoculating before pollination (Cunfer,1975). Barley resistance to *C. purpurea* also varies a lot among cultivars (Pageau *et al.*, 1994b; Darlington *et al.*, 1977). Susceptibility could also be exacerbated by viruses infecting the plant at the same time as *C. purpurea* (e.g. BSMV, Darlington *et al.*, 1976).

Susceptibility to ergot in triticale has been one of the most important problems of triticale breeding since the 70's (Saulescu and Eustatiu, 1972). Newton winter triticale, a cultivar realized in 1977, showed a moderate resistance to *C. purpurea* in the field (Plant Breeding Institute, 1984) and Minskaya 2 and Moskovskaya 35 are two resistant spring triticale cultivars (Turbin *et al.*, 1982). Hexaploid triticale was first reported as susceptible (Kiss, 1971), but later the cultivar Welsh was described as resistant (Larter *et al.*, 1978). Many

Russian lines of triticale were tested to ergot resistance and found to be not susceptible because of their high ear fertility (Shevchenko and Karpachev, 1985). Resistance in triticale is also correlated to selection for high yield and against regrowth (Gregory *et al.*, 1985a). Pageau *et al.* (1994a) compared different cultivars of triticale, durum wheat (*Triticum durum*) and soft wheat (*Triticum aestivum*) finding that soft wheat cultivars were more resistant than durum wheat and triticale. Studies about ergot resistance were also conducted making crosses of *T. timopheevii* with rye or *T. durum* Carleton (Krakar, 1980). Trispecific triticale was found to be highly resistant to ergot (Shulyndin, 1974).

Poludennyi *et al.* (1979) conducted one of the first studies on rye resistance to *C. purpurea* and found that cultivars with small caryopses, such as Chishminskaya 4, were more resistant than large caryopses, such as Belta and Khar'kov 55. They also observed that tetraploids cultivars were less resistant than diploids and Vyatka 2 was the most resistant in field experiments.

Claviceps purpurea virulence towards rye is very variable among isolates (Jungehulsing, 1995) as much as rye cultivars susceptibility to ergot (Singh *et al.*, 1992). Accordingly, Larter (1974) found some susceptible rye cultivars. Sastry *et al.* (1978) observed that yield loss was greater in rye and barley than in wheat and oats.

The genus *Pennisetum* was studied for ergot resistence as regards two species: *P. glaucum* and *P. americanum*. *Pennisetum glaucum* cultivar SDMV 89004 showed moderate resistance to *C. purpurea* (International Crops Research Institute for the Semi Arid Tropics, 1987) like cultivar ICMV1 (International Crops Research Institute for the Semi Arid Tropics, 1987) while *P. americanum* cultivar CO.6 was more resistant (Appadurai *et al.*, 1978). Ergot susceptibility was found to be moderately heritable among different *P. americanum* lines (Gupta *et al.*, 1988). Some mutant pearl millet lines were cross combined to male sterile cultivars and found to restore ergot resistance (Rawat and Tyagi, 1997).

Only a few studies have been carried out on sorghum, although some resistant sorghum lines were identified by Musabiymana *et al.* (1995).

Chemical and biological control

Seeking to limit the spread of ergot, it is vital to prevent sclerotia from getting into the soil with the seed and, if the seed cleaning is not perfect, it is important to ensure that ergot sclerotia produce as few ascospores as possible. One of the ways to suppress sclerotial germination is through the application of fungicidal seed treaters (Dabkevicius and Mikaliunaite, 2006).

Experimental evidence suggested that various active ingredients had a different effect on the suppression of sclerotial germination and ascocarp formation. The impact of chemical formulations was diverse and depended on the chemical composition, origin and size of sclerotia, and environmental conditions (Nemkovich, 1999).

The results of two experiments managed in the laboratory and two experiments conducted under field conditions are similar and confirm the suppressive effect of most seed fungicides on ergot sclerotia; they also delayed germination and ascocarp formation. The most effective seed treaters contain prochloraz, an imidazole, and triticonazole or fluquinconazole, which are triazoles (Dabkevicius and Mikaliunaite, 2006).

Sclerotial germination and stroma formation were effectively suppressed by the seed treater Panoctine 35 LS, a guanidine, containing the active ingredient guazatine. The seed treaters containing triazoles, diphenoconazole, cyproconazole and triticonazole were less-effective than the above mentioned ones (Dabkevicius and Mikaliunaite, 2006). Significant reductions (up more than 95%) in the percentage of ergot germination and the number of stromata were recorded after treating seeds with a combination of prothioconazole and fluoxastrobin (included in the commercial product EfA) and galmano (Puhl *et al.*, 2007).

In general, fungicides also delayed the germination of ergot sclerotia and only incomplete fruit bodies emerged in later germinating sclerotia unable to form normal ascocarps. This could be of great relevance, since later-formed ascocarps and ascospores would not be able to infect plants post-anthesis. Moreover, abnormal ascocarps produce fewer or no spores at all. Such a reduction in sclerotial germination and ascocarp formation, and a delay in their germination, could markedly reduce the infection rate at the rye anthesis stage (Dabkevicius and Mikaliunaite, 2006; Dabkevicius and Semaskiene, 2002).

The use of fungicides sprayed onto the ground to prevent infection does not yet have a practical application (Yarham, 1996). Baitan [triadimenol]-Universal 19.5 WS and Divident Star 036 FS suppress sclerotia germination showing a better performance in vitro (till 100%) than under field conditions (Mikaliunaite and Dabkevicius, 2006). Fungicides performance was tested in glasshouses and field. The former studies showed a significant decrease of ergot infection, while the latter were effectively deterrent only if azole products (e.g. bromuconazole, epoxiconazole, fluquinconazole and tebuconazole) and strobilurins were used (Gladders *et al*., 2001).

Claviceps purpurea control in the field with ear spray was demonstrated to be difficult (Evans *et al.*, 2000). Only double ear treatments with tebuconazole + triadimenol and cyproconazole + prochloraz gave effective pre-inoculation control, but no fungicide treatments gave successful post-inoculation control (Mielke, 1993).

Prevention of ergot (caused by *C. Africana*) in sorghum was obtained mixing acibenzolar-S-methyl (as Bion 50WG) and mancozeb (as Penncozeb 750DF), but only applications carried out at the right time assures control of the pathogen (Ryley *et al.*, 2003).

On inoculated barley, sprays of benomyl applied through the flowering period were efficient, but under natural conditions, using germinating ergots as a source of ascospore inoculum, the effectiveness of benomyl was reduced. The timing of the fungicide application was critical as none of the fungicides tested showed marked eradicant properties. Benomyl sprays just before and at maximum flower opening gave the best results (Wood and Coley-Smith, 1980).

Ergot control was carried in pearl millet by managing pollination. Only bagging after pollination and no pollination- no inoculation treatments gave good results (Rakesh *et al.*, 2002).

Regarding biological control, few studies are available. *In vitro* studies showed that certain forms of *Fusarium roseum* could be efficient in the biological control for ergot of wheat (Mower *et al.*, 1975).

Later studies on the fungus *Trichoderma lignorum* and the bacterium *Pseudomonas aureofaciens* used as seed coating did not show any significant effect on sclerotial germination and ascocarp formation (Dabkevicius and Semaskiene, 2002).

Decontamination

Ergot bodies remain intact during grain storage, although they tend to rise to the top of the transporting vehicle during transit and to break into smaller fragments during loading, transportation and unloading (Gilles *et al.*, 1972). Ergots are largely removed with the dockage during conventional grain cleaning with about 82% of ergots removed from dirty wheat (Shuey *et al.*, 1973).

Ergot sclerotia are often larger in size than cereal grains and thus they can be removed by mechanical means with conventional grain cleaning equipment such as sieves and separators used during the harvesting process. These modern cleaning techniques are able

to remove up to more than 80% of ergot present after harvest, thus reducing the risk of exposure (Posner and Hibbs, 1997).

The mechanical separation methods mostly used become less reliable, however, when a drier season results in the production of sclerotia smaller than usual which are similar to cereal kernels in size, or when larger ergot bodies break up during transport (Lauber *et al*., 2005). So, despite cleaning efforts, ergot toxins have been detected in German, Danish, Swiss and Canadian surveys of cereals and cereal products, with total ergot alkaloid toxin levels of up to 7.255 µg/kg in German rye flours (Scott and Lawrence, 1980; Baumann *et al*., 1985; Lombaert *et al*., 2003; Dusemund *et al*., 2006).

Gravity separation and electronic separators are possible physical methods of ergot particles removal since ergot bodies are less dense than wheat grains (Gilles *et al*., 1972). Also flotation in 20% sodium chloride or 32% potassium chloride solutions could be efficient to remove ergot particles from rye (Champlin and Mac Ewan, 1942; Gilles *et al*., 1972).

Today effective clearing techniques at mills enable the removal of up to 82% of ergots from grain (Posner and Hibbs, 1997).

Ergot alkaloids are commonly found in flour, particularly rye flour, in low concentrations (Scott and Lawrence, 1980) and hence ergot fragments survive commercial cleaning and milling of grains to a small extent. Fortunately, the pharmacologically active alkaloids are unstable to heat and considerable losses would be expected after processing of flour to bread and other foods (Scott, 1991).

Ultraviolet radiations had no effect on alkaloid content of ground ergot sclerotia, while treatments with chlorine can reduce the total alkaloid content of wheat of about 90%. Hydrogen chloride and sulphur dioxide caused much less decomposition (<20% after 4 hours) of ergot alkaloids in sclerotia, while ammonia and ozone had no significant effects on alkaloid levels (Young *et al*., 1983).

Fate of ergot alkaloids during food processing

During milling ergots pass into flour fractions used for human consumption with relatively little in the break flours and 18-36% in the bran fractions (Wolff *et al*., 1988). These fractions are regularly destined for animal feed, but there is no available data on contamination levels. Care also needs to be taken that grain ‘dockage’ does not ultimately get routed into animal feed.

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In the baking of bread and pancakes using grains that contained naturally occurring ergot, a 59-100% reduction in the individual ergolines (ergosine, ergocornine, ergometrine, ergotamine, alpha-ergocryptine, ergocristine) was observed in whole wheat bread, a 50-86% reduction in all-rye flour bread, and a 25-74% reduction in triticale pancakes (Scott and Lawrence, 1982). When bread made of rye flour spiked with finely ground sclerotia of C. purpurea (containing a total ergoline concentration of 312.8 µg/kg) was baked, there was an overall reduction of 50% in the ergot alkaloid concentration, as measured by HPLC (Baumann *et al*., 1985).

PHARMACOKINETICS

Absorption

Hydrophilic amides, like ergometrine, are rapidly absorbed, whereas the less water-soluble alkaloids of the ergotamine group have a lower oral bioavailability, approaching 62 % in humans (Aellig and Nüesch, 1977).

The ergot alkaloids are variably absorbed following oral administration. The amine alkaloids are also absorbed from the rectum and the buccal cavity and after administration by aerosol inhaler. Absorption after intramuscular injection is slow, unpredictable and often delayed. The effective oral dose is approximately 10 times the intramuscular dose (Goldfrank, 2002; Katzung, 2006).

Oral administration of ergotamine results in low or undetectable systemic drug concentration. Ergonovine and methylergonovine are rapidly absorbed after oral administration. A uterotonic effect in postpartum women can be observed within 10 minutes after oral administration of 0.2 mg of ergonovine (Brunton *et al*., 2007). Bioavailability after sublingual administration is poor, and is often inadequate for therapeutic purposes. Bioavailability after administration of rectal suppositories is greater, and maximal plasma concentrations of ergotamine of over 400 pg/ml can be achieved following a 2-mg dose, compared to peak plasma concentrations of approximately 20 pg/ml in plasma 70 minutes after a 2-mg dose taken orally (Madlon, 2002). Ergotamine suppositories increase bioavailability 20 times compared to orally administered doses (Goldfrank, 2002).

Dihydroergotamine mesylate is poorly bioavailable following oral administration (Novartis, 2001).

Distribution

The volume of distribution is 2 L/Kg and the half-life varies from 1.4-6.2 hours (Goldfrank, 2002). While some ergot alkaloids can cross the blood-brain barrier and also the placental barrier, comparative investigations in rabbits, with dihydroergocryptine, dihydroergotamine and dihydroergocornine given intravenously showed that none of these dihydro-compounds were able to cross the blood-brain barrier (Filipov *et al*., 1999).

Dihydroergotamine mesylate is 93% bound to plasma protein. The apparent steady-state volume of distribution is approximately 800 litres (Novartis, 2001).

Metabolism

There is little information available on the metabolism of ergot alkaloids, even for those used as therapeutic agents. Human and small animal experiments suggest that ergot alkaloids are rapidly absorbed and disappear equally rapidly from blood and tissues with a high first-pass clearance by the liver. The cytochrome P450 enzyme system (particularly CYP3A4) plays a significant role in their elimination by extensive biotransformation (Moubarak *et al.*, 2002; Settivari *et al.*, 2008). P450 enzymes can hydroxylate or dealkylated ergot alkaloids (Hussein and Brasel, 2001). Ergot alkaloids have also been shown to affect the cytochrome P450 system by binding to the isoenzyme as a substrate (Moubarak *et al.*, 2002). The structure of many metabolites has not been elucidated yet, although it is likely that these retain biological activity and their physiological effects persist for lengthy periods of time (Moubarak *et al*., 1996). For example, despite an absorption of about 66% in humans, after oral application, ergotamine (a peptide alkaloid), undergoes first-pass metabolism by the liver and only shows an oral bioavailability of less than 2% (Aellig and Nüesch, 1977). Another example is methysergide, which has an oral bioavailability of approximately 13 % and is converted in the liver into the active methylergometrine. Interestingly, the lysergic acid amides had at least 20 times higher plasma levels at similar oral doses as the ergopeptine alkaloids. This suggests that these alkaloids have a higher bioavailability and thus a higher toxic potential than the ergopeptine alkaloids (Hill, 2005). Ergotamine is metabolized in the liver by largely undefined pathways (Brunton *et al*., 2007)

Four dihydroergotamine mesylate metabolites have been identified in human plasma following oral administration. The major metabolite, 8´-β-hydroxydihydroergotamine, exhibits affinity equivalent to its parent for adrenergic and 5-HT receptors and demonstrates equivalent potency in several venoconstrictor activity models, in vivo and in vitro. The other metabolites, i.e., dihydrolysergic acid, dihydrolysergic amide, and a metabolite formed by oxidative opening of the proline ring are of minor importance. Following nasal administration,

metabolites represent only 20%-30% of plasma AUC. Quantitative pharmacokinetic characterization of the four metabolites has not been performed (Novartis, 2001).

Excretion

The excretion of ergot alkaloids was found to be dependent upon the molecular weight of the compound investigated. Generally molecular weights <350Da were excreted in the renal tubules with the urine; compounds with a molecular weight of 350 to 450 Da often were excreted in roughly equal portions in the urine and the bile; those above 450 Da were excreted in the bile (Roberts *et al*., 2005) 90% of the metabolites are excreted through the bile, while unmetabolized alkaloids are eliminated through the urine and the feces (Madlon, 2002; Dart, 2004).

Ergotamine's plasma $t_{1/2}$ is 2 hours, while methylergonovine is 0.5-2 hours. Dihydroergotamine and ergonovine are eliminated more rapidly than ergotamine: for the first, this is presumably due to its hepatic clearance (Brunton *et al*., 2007)

The major excretory route of dihydroergotamine is via the bile in the feces. The total body clearance is 1.5 L/min which reflects mainly hepatic clearance. Only 6%-7% of unchanged dihydroergotamine is excreted in the urine after intramuscular injection. The renal clearance (0.1 L/min) is unaffected by the route of dihydroergotamine administration. The decline of plasma dihydroergotamine after intramuscular or intravenous administration is multiexponential with a terminal half-life of about 9 hours (Novartis, 2001).

Stuedemann *et al*. (1998) incubated endophyte infected tall fescue in autoclaved rumen fluid and realised that the aqueous concentration of ergot alkaloids increases with time when viable ruminal microbes decompose the plant tissue. Conversely, the total alkaloid concentration in the fescue pellet remained the same in the autoclaved ruminal fluid, but decreased when viable ruminal microbes were present. The microbes serve to liberate the toxins from the plant tissue which is a disadvantage for the host, but, on the other hand, the toxins might be metabolised by the microbes. This metabolism again might result in less active products but also in a higher toxicity of the substances. Furthermore, increased water solubility as a result of microbial action might increase the rate of extraction, but might also facilitate absorption from the intestine (Kiessling *et al*., 1984). Further research is needed to understand the exact role of the rumen in ergot alkaloid metabolisation.

Carry-over

As with recent available data on the toxicokinetics of ergot alkaloids in target animal species, information regarding a potential carry-over into edible tissues is scarce .

Kalberer (1970) administered 1mg ergotamine/kg BW to rats and observed an accumulation of this alkaloid after 2 hours in a decreasing order from the liver, kidneys, lung, heart and brain to the blood. Similar results were described by Acramone *et al*. (1972), who administered rats with 20 mg nicergolin/kg BW and found higher amounts of this alkaloid 30 minutes later, in a decreasing order from the liver, lung and kidneys to the heart, blood, fat and brain. Twelve hours after oral administration the residues analyzed rapidly decreased.

Young and Marquardt (1982) fed poultry chicken with various concentrations of ergotamine tartrate. But residual amounts of ergotamine in muscle (5 µg/kg) and liver (4 µg/kg) could only be detected at the highest concentrations of 810 mg/kg feed. The possible presence of metabolites was not analysed.

Carry-over research in pigs was conducted by Whittemore *et al*. (1976 and 1977), who did not detect any residues after natural exposure to ergot alkaloids, although in this study quantitative data on total exposure and limit of quantification for individual alkaloids in animal tissues are lacking. Furthermore, Mainka *et al*. (2005) did not find any carry-over into edible tissue of growing-finishing pigs fed with concentrates of 1 and 10 g ergot/kg diet.

The literature concerning carry-over in cattle and dairy cows is scarce and in the few published studies mostly only milk residues were analysed. Wolff *et al.* (1995) administered 3 µg ergot alkaloids/kg BW of the animals (as natural grown ergot), over a period of two weeks, to two dairy cows, but no residues could be detected in the milk. In another study, where very high and practically not relevant amounts of 125 mg ergot/kg BW were fed to dairy cows, a milk contamination with alkaloids was found (Parkheava, 1979). The authors concluded that the carry-over rate into milk is less than 10% of the applied dose.

Some authors analysed the potential carry-over of endophyte alkaloids into meat (Realini *et al*., 2006), but unfortunately the only time that alkaloids have been detected in beef tissue, the exact data on alkaloid intake was not available.

Thus, although Gareis and Wolff (2000) considered a carry-over of ergot alkaloids into edible tissues as negligible, further research seems to be necessary. Additionally, kinetics, metabolism and tissue deposition might depend on a variety of factors which have not been considered so far.

TOXICITY

Ergot alkaloids fall loosely into that class of compounds produced by fungi which are referred to as secondary metabolites. This means that they are not directly necessary for the life process of the producing organism, but may have profound influences on other organisms in the environment which may ingest them or come into casual contact with them.

The earliest recorded effects of ergot alkaloids are related to the toxic effects of ingestion of cereal products infested with ergot sclerotia (mostly *C. purpurea*). In particular, the human disease acquired by eating cereals infected, usually in the form of bread made from contaminated flour, is called ergotism or St. Anthony's fire.

The ingestion of ergots has been associated with diseases since antiquity (Krska and Crew, 2008; Bennett and Klich, 2003). Numerous epidemics in Europe occurred between the 9th and the 18th century. They can cause pronounced peripheral vasoconstriction of the extremities, disruption in functions of the CNS, contraction of the uterus, gangrene (induced by vasoconstriction), and even death (Schiff, 2006; Richard, 2007).

Two forms of ergotism are usually recognized: gangrenous and convulsive. In gangrenous ergotism tingling effects are felt in the fingers and toes followed in many cases by dry gangrene of the limbs, and finally loss of the limbs. In convulsive ergotism the tingling is followed by hallucinations, delirium and epileptic-type seizures (Mohamed *et al*., 2006b). The gangrenous type was mostly seen in France and the convulsive one in Germany. The two distinct types of ergotism may be considered as acute and chronic varieties (Van Dongen and De Groot, 1995).

Human ergotism was common in Europe in the Middle Ages. For example, a three-volume work entitled *Handbook of Geographical and Historical Pathology* published in London by August Hirch between 1883 and 1886, recorded 132 epidemics of European ergotism between the 6th and 18th centuries. Matossian (1981) suggested that the 'slow nervous fever' described by the 18th century English physician Jon Huxham may be another example of human ergotism.

The epidemics decreased due to changes in farming practices including deep ploughing, which resulted in the sclerotia being buried. In addition, wheat replaced rye as the major grain crop and was much less susceptible to ergot infection.

Nevertheless human poisoning from ergot has occurred in more recent times in France (Fuller, 1968), India (Bhat *et al*., 1976), and Ethiopia (Demeke *et al*., 1979). This last episode occurred in the Wollo region, following two years of drought. During this time, the locally grown barley, the staple food, had become dominated by wild oats heavily contaminated with *C. purpurea* sclerotia. A total of 93 cases of ergotism was reported during the spring of 1978. More than 80% of affected persons were between 5 and 34 years of age. In addition to the 93 cases, 47 deaths were reported as having been due to ergotism. Examination of 44 patients out of the 93 registered revealed ongoing dry gangrene of the whole or part of one or more limbs (7.5%), feeble or absent peripheral pulses (36.4%), swelling of limbs (11.2 %), desquamation of the skin (12.8%), and loss of one or more limbs (21.5%). It was noted that

88% of patients had involvement of the lower extremities. The most common general symptoms were weakness, formication, burning sensation, nausea, vomiting, and diarrhoea. In addition, 50-60 infants and young children died from starvation due to failure of the mothers to lactate.

In India, several outbreaks have occurred since 1958 as a result of ingesting pearl millet containing clavine-type ergot from *C. fusiformis.* Symptoms included nausea, vomiting, and giddiness. Pearl millet, containing 15-26 mg/kg of ergot alkaloids, caused the toxic symptoms. Also in 1975 in the state of Rajasthan different diseases were reported, following ingestion of ergot in pearl millet infected by *C. fusiforrnis (*symptoms are less severe and there were no cases of death) (Palle, 1989; Peraica *et al.*, 1999). Since autopsies were not performed in either of the episodes, no information is on record of the pathological effects on human viscera.

Recently, a severe outbreak of gangrenous ergotism was again reported in Ethiopia (Urga *et al*., 2002). It was attributed to the ingestion of barley containing ergotized wild oats. All the grain samples collected contained ergot alkaloids with a maximum concentration of ergotamine of 2.51 mg/100 g. Acute toxicity studies were also conducted by feeding male, non pregnant and pregnant Swiss albino mice, with the collected grain samples. A high mortality rate among mice was observed (55%), and cases of abortion were noted after 3 days of feeding, in all pregnant mice. Despite these reports, ergotism has nowadays practically been eliminated as a human disease, but it remains an important veterinary problem, particularly in cattle, horses, sheep, pigs and chicken (Bennet and Klich, 2003).

Acute toxicity

Ergot alkaloids vary in their acute toxicity, as demonstrated by the comparison of LD50 values for individual compounds in rabbits, rats and mice. For example, following intravenous injection, the LD50 for ergometrine was 3.2 mg/kg b.w. in rabbits, 120 mg/kg in rats and 160 mg/kg in mice. The LD50 for ergotamine varied between 3.0 mg/kg in rabbits, 38 mg/kg in rats and 265 mg/kg b.w. in mice, respectively (Griffith *et al*., 1978). The acute form of ergotism, which is seldom observed, is characterized by lethargy, convulsions and depression, followed by death due to paralysis of the respiratory centre (Lorgue *et al*., 1987).

In pigs a high level (level not specified) of toxin intake results in vasoconstriction and subsequently dry gangrene of hooves, ears and tails (Bryant, 2008).

The acute effects of ergot alkaloid (no details provided) poisoning in humans include tachycardia, hypertension, confusion, thirst, abdominal colic, vomiting, diarrhea, and hypothermia of the skin (Deshpande, 2002; Patnaik, 2007).

A study evaluated the subacute toxicity in rats with ergotamine ergometrine and ergocryptine and subchronic toxicity with ergotamine. Sprague-Dawley (10/rats/group/sex) received a 0, 5, 20 and 80 mg ergotamine tartrate (EAT)/kg diet for 13 weeks. The subchronich toxicity study with ergotamine revealed a no observed adverse effect level (NOAEL) of 0.9 mg ergotamine/kg body weight/day. The results of the subacute studies indicated toxic effects including vasoconstriction effects of peripheral arterial vessels, effects on kidney function, decreased thyroid function (decresed serum thyroxin levels), effects on carbohydrates and on ovaries (Deshpande, 2002).

The subacute toxicity of one of these alkaloid, ergometrine maleate, was studied by Peters-Volleberg *et al.* (1996). Rats (6/group/sex) received a diet containing 0, 2, 10, 50 or 250 mg/kg ergometrine maleate diet for 4 wk. The results indicated that plasma glucose levels were decreased in females at 50 and 250 mg/kg. Thyroxin levels were decreased at 50 (males only) and 250 mg/kg. The weights of the heart, liver, ovaries and kidneys were increased at the high dose level. In male rats, a slight dose-related increase in the incidence of enlarged mediastinal lymph nodes and, to some extent, of enlarged parathymal lymph nodes, was seen. Histopathological examination revealed evidence of increased glycogen storage in the liver of animals treated with 250 mg/kg. The no-observed-effect level in this study was 10 mg/kg.

Another study (Janssen *et al.*, 2000 a, b) described the metabolic changes observed in a dietary subacute toxicity experiment with the ergot alkaloid α-ergocryptine in Sprague-Dawley rats.The rats were fed a 0, 4, 20, 100 or 500 mg ergocryptine/kg diet for 28-32 days (equal to 0, 0.36, 1.7, 8.9 and 60 mg ergocryptine/kg body weight/day for females and 0, 0.34, 1.4, 6.6 and 44 mg ergocryptine/kg body weight/day for males). Total cholesterol and high-density lipoprotein (HDL)-cholesterol were decreased dose dependently in females but the ratio HDL-cholesterol/total cholesterol was only decreased at 20 mg/kg body weight. Triglycerides and glucose concentrations were decreased in the highest dose groups of both sexes. Serum urea concentrations were increased and insulin, glucagon and liver glycogen were increased . Prolactin, T4 and FT4 were decreased in both sexes. Follicle-stimulating hormone (FSH) was decreased and luteinizing hormone (LH) was increased. It is concluded that in rats fed ergocryptine for 28 days the dose-effect curve is rather steep and that the NOAEL is 4 mg/kg diet.

A study (Oresanya *et al*., 2003) investigated the effect of ergot alkaloids on performance and clinical symptoms in 192 weaned pigs for 28 d. Wheat ergot sclerotia (1880 mg alkaloid kg-1; ergocristine, ergotamine, ergosine, ergocryptine, and ergocornine constituting 40, 36, 11, 7, and 6% of the total, respectively) were added on a weight basis to a basal diet at 0.05, 0.10,

0.25, 0.50, and 1.00%. Ergot alkaloids decreased serum prolactin and urea nitrogen concentrations. The maximum tolerable ergot level in the diet was 0.10 and 0.05% based on average daily gain and daily feed intake respectively, corresponding to a 2.07 mg and 1.04 mg alkaloid kg-1 diet

Chronic toxicity

Symptoms of chronic poisoning include disturbances in the GI tract, angina pectoris, hypotension or hypertension, and the characteristic symptoms seen in ergotism, i.e., gangrene or mental confusion and /or convulsions (Patnaik, 2007).

In one study (Fitzhugh *et al.*, 1944) female rats were started to feed at three weeks of age on doses of 1, 2 and 5 percent of ground crude ergot in a diet containing high quantities of protein. A second series of animals was started to feed with the same levels of ergot but a low protein diet. Ergot retarded the growth rate of the rats and this effect was more pronounced in male animals during the early growing period. With an adeguate diet, the 1 and 2 percent of ergot did not retard the growth of the female animals.

Histologically neurofibromas were produced on the ears of a high percentage of rats fed 5 percent of ergot. Two other lesions, neurosis and calcification of the lower ends of the renal pyramids, and corpus luteum hyperplasia of the ovaries, were frequently caused by feeding of the ergot. No cutaneous gangrene and no vascular lesions attributable to ergot were observed.

Ergot alkaloids from endophyte-infected tall fescue affect the vascular tissues and blood of grazing animals by acting on α-adrenergic, serotonin and dopamine receptors to cause a vasoconstrictive response. As α-adrenergic agonists, ergot alkaloids may decrease respiratory rates in sheep and cattle. The ergopeptides also suppress prolactin secretion by binding to dopamine receptors; prolactin concentration is, therefore, often used as an indicator of fescue toxicosis in livestock. Depressed serum prolactine in prepartum cattle can result in a reduced milk yield but has negligible effects once lactogenesis occurs. Conception and calving by heifers feeding on endophyte-infected hay is also decreased, leading to reproductive efficiency problems (Schnitzius *et al*., 2001).

Chronic exposure to moderate amounts of ergot alkaloids results in reduced weight gain, low reproductive efficiency, agalactia and central vasoconstriction and renal insufficiency caused by tubulo-interstitial nephritis (Floss *et al*., 1973; Döcke, 1994; Janssen *et al*., 2000c). An article (Janssen *et al*., 2000c) described a case of a 48-year-old female who had taken Cafergot® (ergotaminetartrate 6–12 mg/day) for >10 years; she was admitted to the hospital

for tiredness, pressing retrosternal pain, painful legs, weakness in her arms fever up to 38.9°C and a tubulo-interstitial nephritis

Other clinical symptoms of ergotism in animals include gangrene, abortion, convulsions, suppression of lactation, hypersensitivity and ataxia (Bennet and Klich, 2003).

Most of the outbreaks of ryegrass staggers and fescue foot have been reported predominantly from the USA where tall fescue (*Festuca arundinacea*) is the major forage grass, and from Australia and New Zealand, where perennial ryegrass is the most common pasture species (Galey *et al*., 1991; Easton and Tapper, 2005; Reed *et al*., 2005). Similarly, in Africa outbreaks of fescue toxicosis have been recorded (Botha *et al*., 2004).

Endophyte infected swards may occur also in Europe.

The interaction between thermal stress and fescue toxicosis, as recently reviewed by Spiers *et al*. (2005), might be a reason for the scarcity of fescue toxicosis in Europe. Environmental conditions are less stressful for farm animals as compared to the USA, for example.

Zabalgogeazcoa and Bony (2005) explain the lower incidence of clinical cases by the fact that herbivourous animals in most European production systems are not likely to be as heavily exposed as in the USA or New Zealand. This is, according to their opinion, because extensive grazing systems are not as widely used, and when they are, pastures are mainly natural or artificial mixtures of many grass species and clover (*Trifolium spp.*), thus toxins contained in meadow fescue and perennial rye grass are diluted.

Recently, gangrenous ergotism has also been reported among free-living moose and roe deer in Norway (Uhlig *et al*., 2007). In that study, seven of the ten moose and the roe deer came from areas in northwestern Norway with no grain production, and the intoxication was thought to be a result of ingestion of ergots that were present in the seed heads of wild grasses.

Intake of ergot alkaloids found in entophyte-infected tall fescue grass is also associated with decreased feed intake and reduction in body weight gain. A study conducted by Settivari *et al*. (2008) on rats, demonstrated that even short-term exposure to ergot alkaloids can potentially induce hepatic oxidative stress which can contribute to the pathogenesis of fescue toxicoses. In fact, it was found that the expression and activity of antioxidant enzymes were reduced. This could lead to increased oxidative stress, which might be responsible for the decrease in hepatocellular proliferation after exposure to ergot alkaloids.

Developmental and reproductive toxicity

Ergot alkaloids have been associated with decreased livestock reproductive rates. It is (empirically) know that some compounds can cross the blood-brain barrier (see the hallucinogenic effect of lysergic acid derivatives and the use of ergot alkaloids in the treatment of Parkinson patients) and the placenta barrier. Ergot can induce abortion, stillbirth, agalactia, malnutrition, or clinical disorders in progeny. Ergot toxicity in the newborn usually manifests itself as respiratory depression, cyanosis, oliguria, and seizures. Death is usually caused by respiratory failure.

A study (Sharma *et al.*, 2002) has indicated that ingestion of ergot alkaloids at 3 ppm or higher resulted in reproductive toxicity in mink.

.An outbreak of bovine abortion associated with the ingestion of ergot was reported by Appleyard (1986): eleven out of 36 suckler cows, all in late pregnancy, aborted in 7-11 days following introduction to a rye grass pasture heavily infested with ergot.

Data from from O'Neill and Rae (1965) suggest that adult poultry are less sensitive compared to chicks, when exposed to a diet with 0.3 % ergot sclerotia; a concentration of 9 % ergot in the feed did neither increase mortality nor affected egg size, eggshell quality or hatchability. A study (Schuenemann *et al.*, 2005) reported that extended exposure of bulls to ergotamine tartrate appeared to reduce fertilization potential of sperm.

In an experiment with Canadian cereals a reduced birth weight of piglets was recorded. Already at levels of 0.2 % ergot sclerotia rye ergot reduces lactation in sows (Bryant, 2008). This may by caused by a reduction in the plasma concentration of hormones such as prolactin. Ergot poisoning (no details provided) may also induce spontaneous abortions. As such, ergot infected grain should never be fed to brood sows. Pigs become exposed to ergot alkaloids following the ingestion of infected rye, wheat and barley or by sorghum ergot . The clinical sign is agalactia in sows, feed refusal, reduced weight gain and eventually abortion (Holden and Zimmermann, 1998; Oresanya *et al*., 2003; Kopinski *et al.*, 2004 and 2008).

In addition, ergot alkaloids are toxic for the reproductive tract and mammary gland of the mare. These compounds have been associated with depression of serum prolactin and progestagens , a longer gestation, a thickened edematous placenta and agalactia. In fact alkaloids interfere with the normal rise of progestagens and prolactin in the last days of gestation. Foals born without the normal increases in maternal progestagens suffer hypoadrenocortical function and are small, weak or stillborn (Wright and Kenney, 2009).

Ergot alkaloids reportedly influence pregnancy, perhaps by affecting progesterone metabolism (Newberne, 1974).

The uterine environment appears to be suitable in maintaining pregnancy in animals administered ergot, but the development of embryos is retarded.

For humans methylergometrine is often used in the management of the third stage of labour and for treatment of prevention of puerperal hemorrhage . Intoxication in newborns is rare but may lead to severe complications. A study (Aeby *et al.*, 2003) described 34 cases of methylergometrine poisoning that occurred in Belgium between 1969 and 1999, where 40 patients were newborns and 20 were older children. Twenty-nine patients were exposed by the oral route, 3 by the intranasal route, and 2 by the intramuscular route. Oral exposure was associated mostly with gastrointestinal symptoms, but one newborn required mechanical ventilation for apnea. Intramuscular exposure was associated with severe complications, including apnea, coma, and convulsions. A study (Bangh *et al.*, 2005) described a limited number of neonatal cases where neonatal vitamin K was exchanged with maternal methylergonovine. A full-term male infant was inadvertently given methylergonovine instead of naloxone at birth. Several hours later, he required intubation for respiratory failure. Peripheral perfusion, ventilation, and renal function improved rapidly with nitroprusside infusion, and he was extubated on the third hospital day. Even asymptomatic newborns should be transferred to a neonatal intensive care unit for close observation after methylergonovine administration because toxicity can be life threatening. Rapid recognition of the therapeutic error, ventilatory support, and prompt administration of sodium nitroprusside should lead to a good outcome.

Another study (Baum *et al*., 1996) described a 2977-g female product of a full-term pregnancy with altered splanchnic arterial blood flow after accidental substitution of an ergot alkaloid, methylergonovine maleate (0.5 mL (0.1 mg)), for phytonadione (vitamin K1) via intramuscular injection. The error was recognized during administration of a 1-mL premeasured adult dose of the ergot derivative, intended for control of postpartum uterine hemorrhage

Genotoxicity/carcinogenicity/cytotoxicity

One of the semi-synthetic derivatives of the ergot alkaloids is lysergic acid diethyl amide (LSD). It was first synthesized by Albert Hofmann of Sandoz in 1938, but its hallucinogenic properties were not known until 1943.

Data present in literature are very old and scarse. A paper by Finley (1975) described the clastogenic effects of lysergic acid diethylamide (LSD), like a chromatid break found in a chromosome obtained from cultured leukocytes treated with LSD. However studies on patients treated with LSD under clinical conditions, that did not provide similar results,

suggest that LSD is unable to induce structural chromosomal aberrations *in vivo*. Mutagenic activity of LSD was also observed in *Escherichia coli* and in barley and in ICR mice.

A study by Shappell (2003) shows that ergovaline's effect on Caco-2 cells was dependent on concentration, exposure period, and differentiation status of cells; undifferentiated Caco-2 cells are more sensitive to ergovaline than differentiated cells.

In another study (Dighe and Vaidya, 2005) five ergot alkaloids were tested for the induction of sister chromatid exchange (SCE) frequencies in cultured Chinese hamster ovary cells (range of concentration: 10^{-5} and 10^{-8} M). The results indicated that ergotamine, ergonovine, and methylergonovine are effective inducers of SCE, while ergocristine is a weak inducer, and α-ergocryptine has no effect.

Health Canada is, at present, evaluating ergot alkaloids, with a view towards establishing a group TDI, based on either animal toxicity data or on human data, and a full toxicological database is under development. Health Canada has investigated the occurrence of ergot alkaloids in a variety of foods,including infant cereals, and we are at present estimating Canadian exposure.

In the 2005, during the Meeting of the Standing Committee on the Food Chain and Animal Health, a proposal was made by Germany and EFSA to focus on the ergot alkaloids ergometrine, ergotamine, ergocristine and ergocryptine for control purposes guidance values. In fact these ergot alkaloids correspond to the current maximum level for sclerotia.

Ergot alkaloids as pharmaceutical drugs

Ergot alkaloids are an important group of pharmaceutical drugs. Among the most important drugs derived from ergot alkaloids, there are methysergide, ergotamine, ergometrine and bromocriptine. Methysergide is a non-selective 5-HT2 serotonin receptor antagonist (Johnson *et al*., 2003). It is used as a prophylactic medication against migraine, but its metabolites have some adverse effects which limit its clinical use (Muller-Schweinitzer and Tapparelli, 1986; Johnson *et al.*, 2003).The acute toxicity (LD50) for methysergide in rats is 200 mg/kg, and in mice 581 mg/kg.

Ergotamine is a non-selective 5-HT1 agonist. Its oral absorption is 60-70% (Bulow *et al*., 1986). It is metabolized in the liver by cytochromes P450 and is excreted in the faeces and the urine. Ergotamine can constrict coronary blood vessels and inhibit the trigeminal sensory nerves through prejunctional 5-HT1d receptors (Silberstein and McCrory, 2003). It is widely used for the treatment of severe migraine attacks (Tfelt-Hansen *et al*., 2000). The combination of ergotamine and caffeine is used to prevent and treat migraine headaches. Ergotamine can cause adverse effects, like ergotism, stroke, gangrene, diarrhea, swollen

fingers, generalized weakness, and peripheral and coronary vasoconstriction and also death. The recommended starting dose for oral ergotamine is 2 mg and the maximum dose is 6 mg. For rectal ergotamine the recommended starting dose is 1mg and the recommended maximum dose is 4 mg (Tfelt-Hansen *et al*., 2000; www.medscape.com). In humans, the minimum lethal dose of ergotamine ranges from 15 to 20 mg (www.ask.novartispharma.ca). By 1925 ergotamine was beginning to be used to treat migraine attacks. Unfortunately, because of ergotamine's chemical and pharmacokinetic properties, extracts of ergot of rye were incapable of producing consistent therapeutic results, so for migraine treatment patients had to wait until pure ergotamine was available for administration (Eadie, 2004).

Bromocriptine is a dopamine agonist and is a derivative of D lysergic acid. It acts as D2 dopamine receptors agonist (Pijl *et al.*, 2000). It directly stimulates ovarian dopamine receptors and directly activates lactotrope dopamine receptors. It is also a free radical scavenger (Yamashita *et al*., 1995). Bromocriptine is taken orally, absorbed rapidly and excreted in the faeces and in the urine. It is used in hyperprolactinemia, prolactinomas, inhibition of lactation, benign breast disease, premenstrual symptoms, in Parkinson's disease and in type-2 diabetes (Pijl *et al*., 2000). Bromocriptine's adverse effects are nausea, vomiting, hypotension, fatigue, and nasal congestion and sometimes also diarrhea, dyspepsia, severe hypotension, arrhythmia and angina (Parfitt, 1999). Generally, oral bromocriptine is supplied in 2.5 mg tablets and in 5 mg capsules. Also, standard oral bromocriptine tablets can be placed in the vagina. The lethal dose has not been established.

Ergometrine is an amide derivative of d-lysergic acid and it is used in the treatment of postpartum hemorrhage. It is reported (Boobis *et al*., 1991) to be rapidly absorbed after oral or intramuscular administration. The total daily dose of ergometrine is usually less than 1 mg (Boobis et al., 1991). The main adverse effects of ergometrine are nausea, vomiting, abdominal pain, diarrhoea, headache, chest pain, palpitations, bradycardia, hypertension, dyspnoea, leg cramps and hematuria. The acute toxicity of ergometrine has been reported as 8.3 mg/kg (LD50, intravenous, in mice) and 93 mg/kg (LD50, oral, in rats; methylergometrine maleate) (British Pharmacopoeia Chemical Reference Substance data sheet).

LEGISLATION ON ERGOT

Limits for ergot bodies

In the European Union no regulatory limits apply to ergots in grain for human consumption (Egmond and Jonker, 2004). According to EU Commission Regulation 1572/2006, amending Regulation EC 824/2000, a maximum value of 500 mg/kg ergot bodies in grain (0.05% w/w)

has been set for intervention grain, but not for consumption of grain. The European Union has established an intervention system in order to stabilize the markets and ensure a fair standard of living for the agricultural community in the cereals sector (EU Council Regulation 1784/2003). Through this system, the EU dictates certain standards in grain for intervention affairs within the common market, which individual countries can also accept for consumption grain (Bürk *et al.*, 2006).

A maximum limit of 1000 mg/kg ergot bodies (0.10% w/w) has been introduced for feed products containing unground cereals (EU Council Directive 2002/32/EC). At present, the maximum permissible level in the USA and Canada, is 300 mg ergot per kg grain. In Australia feed materials exceeding this limit are labelled as ergoty and are discarded or mixed with non-contaminated batches. Australia has a limit for ergot of 0.05% in cereal grains. Besides the guideline limits mentioned, the ergot content is to some extent controlled by good agricultural practice, including segregation of sclerotia by cleaning machinery.

Limits for ergot alkaloids

The common method to determine ergot in unprocessed cereals is to sort it out and count it, which is not applicable for processed grain. Another drawback of the ergot counting method is that no direct information is obtained concerning the alkaloid composition, thus the toxic potential of ergot and consequently its impact on animal and human health cannot be described by this method.

Since the total ergot alkaloid content within each single ergot shows significant variations between 0.01 and 0.5% w/w (Schoch and Schlatter, 1985), no limiting value for maximum ergot alkaloid level can be derived from the maximum value for ergot bodies (Bürk *et al.*, 2006). However, assuming an average alkaloid content of 0.2% w/w in the ergot, a level of 0.05% ergot equals a total ergot alkaloid content of 1000 μg/kg (Wolff *et al.*, 1988). Based on this consideration and available toxicological data, guideline limits for ergot alkaloids in cereals for human consumption of 400-500 μg/kg and 100 μg/kg in Germany and in Switzerland, respectively, have recently been discussed (Bürk *et al.*, 2006). Guideline limits for the total ergot alkaloid content in feed exist only in Canada and Uruguay, where they vary from 450 to 9000 μg/kg depending on the animal (Egmond and Jonker, 2004).

No country has set limits for individual ergot alkaloids in food or feed yet. Chemical analyses of each ergot alkaloid would be more reliable and more suitable to indicate a potential health risk and to control exposure to ergot alkaloids than determination of number and weight of ergot particles. As soon as a suitable method is available, more data will be obtainable for risk assessment and the setting of limits (Ruhland and Tischler, 2008).

CONCLUSIONS

Available data indicate that adverse effects may occur in agricultural animals, particularly in pigs after the intake of feed with ergot at levels close to the current EU level. The limited data available do not provide any evidence that ergot alkaloids accumulate in edible tissues, including milk and eggs and thus food from animal origin is unlikely to be an important source of human exposure. Only low levels of ergolines remain in prepared foods as cleaning and milling processes remove the sclerotia (Wood, 1992; Environmental Health Criteria, 2005). The published information from only one survey of cereals and cereal products indicates a total daily human intake of ergolines in Switzerland of approximately 5.1 µg per person, the contents of certain commodities being up to 140 µg/kg. Baking reduces the ergolines present in contaminated flour by 25-100%. Primary food preparation, such as bread-baking, reduces the total alkaloid contents by about 50% (Kainulainen, 2003). If the ergot has been soaked in water before ingestion, hydrophilic ergot alkaloids, such asergometrine, tend to dissolve. In addition, the ergot alkaloids may undergo substantial presystemic elimination after oral administration (Eadie, 2004).

To limit the risk for exposure to ergot alkaloids the authorities intend to minimise the ergot content of grain. According to EU regulation 824/2000 a limiting value of 0.05% w/w of ergot is valid within the EU for interventional grain, but not for consumption grain (with exception for Germany) (Bürk *et al.*, 2006). However, since the total alkaloid content within each single ergot shows significant variations (between 0.01 and 1% w/w), no limit value for maximum ergot alkaloids can be derived from the maximum value for ergot. However, if one assumes an average alkaloid content of 0.2% w/w in the ergot, the legislative ergot value of 0.05% w/w in grain leads to a guidance level of about 1000 µg/kg ergot alkaloids in cereal. In Germany there are no limit values for ergot alkaloids in cereal and cereal products, while in other countries, for example Switzerland, a limit value of 100 µg/kg ergot alkaloids in cereal has been discussed recently. New limits will probably also be established in the years to come for ergot alkaloids in foods (van Egmond *et al.*, 2007; Burk *et al.*, 2006).

FUTURES

- Data is needed to define the variability of ergot alkaloid patterns in feed and food materials.
- Methods of analysis and validation for ergot alkaloids should be developed.
- Epidemiological studies should be performed on the possible effects of low levels of ergolines on the human population.

- Pharmacological and toxicological studies should be performed using individual and combined ergot alkaloids on experimental animals.
- The distribution and the possible transmission of ergot alkaloids to edible tissues, milk and eggs should be elucidated.

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Chapter 3 - Alternaria mycotoxins in food and feed

Keywords: *Alternaria*, Alternariol, Monomethyl ether, Tenuazonic acid, Altenuene, Altertoxins, Biosinthesys, Agricultural commodities, Fruits, Vegetables, Methods of analysis, Validation, Toxicity, Mitigation.

INTRODUCTION

Alternaria is a common genus of microfungi, and contains numerous species that are both saprophytic on organic materials and pathogenic on many plants (Sinha and Bhatnagar, 1998). A number of species can contaminate a wide variety of crops in the field and cause post-harvest decay of various fruits, grains, and vegetables causing considerable losses due to rotting of fruits and vegetables (Chelkowski and Visconti, 1992; Weidenborner, 2001; Deshpande, 2002; Visconti *et al*., 1986). Fungi of the genus *Alternaria* grow at moderate temperatures on different materials, e.g., soil, wallpapers, and textiles, and they are commonly isolated from an indoor air environment (Montemurro and Visconti 1992; Ren *et al.*, 1998; Scott, 2001). Due to their growth even at low temperature they are also responsible for spoilage of these commodities during refrigerated transport and storage (Ostry, 2008). In addition to causing economic losses to growers and commercial marketers, *Alternaria* also threaten consumers' health (Kralova *et al*., 2006). In fact, under suitable conditions it may lead to production of a range of metabolites, some of which are powerful mycotoxins with mutagenic and teratagenic properties, and have been linked to certain forms of cancer. As such, the occurrence of *Alternaria* secondary metabolites in foodstuffs such as grains, peanuts, tomato products, apple sauce, olive oil, fresh fruits and vegetables, is becoming an increasing environmental concern. Perhaps more importantly, *Alternaria* spores are one of the most common and potent indoor and outdoor airborne allergens. Additionally, *Alternaria* sensitization has been determined to be one of the most important factors in the onset of childhood asthma.

The principle *Alternaria* mycotoxins that have been shown to occur naturally are tenuazonic acid, alternariol monomethyl ether, alternariol, altenuene, and altertoxin I (Weidenborner, 2001).

A. alternata strains also produce host-selective toxins that are selectively toxic to certain plants or certain genotypes of a plant species (Kohmoto *et al.*, 1995); and these toxins are designated as pathotypes of *A. alternata*. For example, specificity in the interaction between rough lemon (*Citrus jambhiri Lush*) and the fungal pathogen *A. alternata* rough lemon pathotype is determined by a host-selective toxin, ACR-toxin.

ALTERNARIA AND ITS HOSTS

The fungus

Taxonomic Classification

Fungi of the genus Alternata belong to the following Taxonomic Classification:

- Kingdom: Eumycota;
- Phylum: Ascomycota;
- Class: Euascomycetes;
- Order: Pleosporales;
- Family: Pleosporaceae;
- Genus: *Alternaria*.

Description and natural habitats

Alternaria is a dematiaceous (phaeoid) fungus and production of melanin-like pigment is one of its major characteristics (Takano *et al.*, 1997). Its teleomorphic genera are called *Clathrospora* and *Leptosphaeria*.

The diagnostic characteristics of the genus include the production in chains of dark-coloured multi-celled conidia with longitudinal and transverse septa (phaeodictyospores), and a beak of tapering apical cells.

Species

The genus *Alternaria* was originally described in 1816 with *A. tenuis* as the type and only member of the genus (Nees, 1817), and currently contains around 50 species.

As decomposers of foodstuffs, *Alternaria* spp. are ubiquitous postharvest pathogens and contribute to the spoilage of 20-40% of agricultural output. In contrast, several *Alternaria* spp. have shown promise as beneficial biocontrol agents of certain weeds and as mycoparasites of other fungal pathogens. The main *Alternaria* species of phytopathological interest are listed in Table 1.

Table 1. Main species of *Alternaria* relevant in plant pathology, disease caused and hosts.

Alternaria species	**Disease**	**Host plant**
Alternaria alternata	early blight	potato
A. arborescens	stem canker	tomato
A. arbusti	leaf lesions	Asian pear
A. blumeae	lesions	*Blumea aurita*
A. brassicae		vegetables and roses
A. brassicicola		
A. brunsii	bloosem blight	cumin
A. carotiincultae	leaf blight	carrot
A. conjuncta		parsnip
A.dauci		carrot
A. euphorbiicola		cole crops
A. gaisen	ringspot disease	pear
A. infectoria		wheat
A. japonica		cole crops
A. molesta	skin lesions	porpoises
A. panax	Blight	ginseng
A. petroselini	leaf blight	parsley
A. selini	crown decay	parsley
A. solani	early blight in	potatoes and tomatoes
A. smyrnii		alexanders and parsleys

Macroscopic Features

Alternaria spp. grows rapidly and the colony size reaches a diameter of 3 to 9 cm following incubation at 25°C for 7 days on potato glucose agar. The colony is flat, downy to woolly and is covered by grayish, short, aerial hyphae in time. The surface is greyish white at the beginning, but later it darkens and becomes greenish black or olive brown with a light border. The reverse side is typically brown to black due to pigment production (St-Germain and Summerbell, 1996).

Microscopic Features

Alternaria spp. have septate, brown hyphae. Conidiophores are also septate and brown in colour, occasionally with a zigzag appearance. They bear simple or branched large conidia (7-10 x 23-34 µm) which have both transverse and longitudinal septations. These conidia

may be observed singly or in acropetal chains and may produce germ tubes. They are ovoid to obclavate, darkly pigmented, muriform, smooth or roughened. The end of the conidium close to the conidiophore is round while it tapers towards the apex. This gives the typical beak or club-like appearance of the conidia (Collier *et al.*, 1998; Larone, 1995; St-Germain and Summerbell, 1996).

Histopathologic Features

Dark coloured filamentous hyphae are observed in the sections of infected tissue stained with hematoxylin and eosin stain. If the pigment formation is not obvious, Fontana-Masson silver stain, which is specific to melanin, may be applied (Collier *et al.*, 1998).

Infection cycle

Alternaria fungi overwinter on infected crop debris, seeds, and on weeds, and may be spread by insects or wind, and by rain/irrigation. It is particularly prevalent on stinkweed. Wind-blown spores are produced on crop residues in the spring, and are able to infect leaves during favourable (moist) environmental conditions. New spores are produced on diseased plants, which may cause more leaf, stem and pod or fruit infections. Seeds may become infected following the development of black spots on the pods. Infection occurs through natural plant openings if water remains on plant tissue for more than a couple of hours. After 2-3 days, the first symptoms appear. The optimum temperature for infection is 15-24°C with high humidity ($a_w \geq 0.84$).

Conidia

Conidia grow *in vitro* on PCA (plate count agar) medium, and they are mature conidia typically 10-30 x 5-12 µm, short conical beak or beakless, narrowly ellipsoid to ovoid and elongated on branching chains. Jjuveniles are narrowly elliptical, with punctate roughened ornamentation especially when old, dull olive in colour, 3-7 transepta, 1-5 longisepta. Individual chains of 5-15 conidia. A complex of branching chains may contain up to 50-60 conidia.

Conidiophores

Conidiophores grow *in vitro* on PCA medium. Individual conidiophores arise directly from substrate forming bushy heads consisting of 4-8 large catenate conidia chains. Secondary conidiophores are generally short and 1-celled.

Diseases

Alternaria is a fungus which causes leaf spotting, fruit rot, bulb rot, blossom rot, blight. Leaf spots progress from older leaves to younger. Spots are circular, target-shaped, and often surrounded by a slight yellow area. Entire leaves may die and drop from the plant. They may also be found around stem ends on fruits and on stem.

Brown spot, the most important disease of tobacco in North Carolina, is caused by *A. alternata* (Fries) Keissler (previously *A. tenuis*) (Lucas *et al.*, 1971). The fungus attacks plants before harvest, causing extensive necrosis and chlorosis of leaf tissues.

Alternaria is common in split pits of fruit, rain-split fruits and in insect feeding injuries. It also infects aborted fruit, and can spread into attached healthy fruit. *Alternaria* usually occurs in post-harvest as a dark green to black mould on the nose of the fruit; i.e,.early infections on apricot appear as red rings on the fruit and can occur when the fruit is still green. As the fruit matures the centre of these rings becomes sunken and brown. In these cases fruit is culled at packing or not picked.

Alternaria spp. have emerged as opportunistic pathogens particularly in patients with immunosuppression, such as the bone marrow transplant patients (Vartivarian *et al.*, 1993; Morrison *et al*., 1993).

Alternaria produces large spores of between 20 - 200 microns in length and 7 - 18 microns in width, suggesting that the spores from these fungi are deposited in the nose, mouth, and upper respiratory tract. Indeed *Alternaria* is the causal agent of phaeohyphomycosis and other respiratory allergies in humans considered as caused by *Alternaria* spores. Cases of onychomycosis, sinusitis, ulcerated cutaneous infections, and keratitis, as well as visceral infections and osteomyelitis due to *Alternaria* have been reported (Garau *et al.*, 1977; Goodpasture *et al.*, 1983; Manning *et al.*, 1991; Schell, 2000; Pastor and Guarro, 2008). It represents one of the main fungal causes of allergy, being a common type I & III allergen. In particular, *A. alternata* can cause both allergic rhinitis (hay fever), in which inflammation of the mucous membrane of the nose occurs, and allergic asthma. It is the most common species isolated from human infections. In immunocompetent patients, *Alternaria* colonises the paranasal sinuses, leading to chronic hypertrophic sinusitis. In immunocompromised patients the colonization may end up with the development of invasive disease (Vennewald *et al.*, 1999). It is among the causative agents of otitis media in agricultural field workers (Wadhwani and Srivastava, 1984).

Alternaria hosts

Over 380 *Alternaria* hosts have been recorded in the USDA Systematic Botany and Mycology Fungus-Host Distribution Database. (http://nt.arsgrin.gov/fungaldatabases/index.cfm).

Cereal grains are frequently infected by species of *Alternaria*, particularly *A. alternata* which can cause a disease called “black point”, which consists of a discolouration of the germ and the seed due to mycelial and conidial masses (Patriarca *et al.*, 2007).

The *Alternaria* conidia are usually present on many commercially important fruits and vegetables which may become visibly infected, particularly after the tissues are weakened by prolonged storage or chilling. Fruits that are affected by *Alternaria* rot include apples (King and Schade, 1984), grapes, blueberries, peaches, cherries, and citrus fruits such as lemons and oranges (Stinson *et al.*, 1980,1981), red delicious apples (Robiglio and Lopez, 1992), mandarin oranges (Logrieco *et al.*, 1988).

Vegetables that are affected by *Alternaria* include cucurbits (cucumber, squash), Solanaceous crops such as eggplant, pepper, potato, tomato (Harwig *et al.*, 1979, Mundt and Norman, 1982; Andersen and Frisvad*,* 2004) peas, onion, cabbage. *Alternaria* products have been found in olives in Apulia (southern Italy) (Bottalico and Logrieco, 1993), sunflower seeds (Logrieco *et al.*, 1988; Dalcero *et al.*, 1989). In carrots, *A. alternata* is found mainly on the seeds and the carrot roots. *A. radicina* is a root pathogen that causes damping off of carrot seedlings, leaf blight, and black rot of the roots (Tylkowska, 1992; Pryor *et al.*, 1998, 2001; Konstantinova *et al.*, 2002; Coles and Wicks, 2003).

Alternaria mycotoxins

The *Alternaria* genus produces 71 known mycotoxins and phytotoxins but only a few occur naturally in food or are of major toxicological significance. *A. alternata* is probably the most important mycotoxin producing species and occurs e.g. on cereals, sunflower seeds, oilseed rape, olives and various fruits (King and Schade, 1984; Otani and Kohmoto, 1992; Scheffer, 1992; Weidenbőrner, 2001).

Alternaria toxins are divided into three main structural classes: dibenzo-α-pyrones which include alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altenuisol (AS); the tetramic acid class, which includes tenuazonic acid (TeA); and a third class of compounds that includes altertoxins I, II and III (ATX-I, -II and -III) (Weidenbőrner, 2001; Deshpande, 2002).

Important *Alternaria* toxins which contaminate food are: alternariol, alternariol monomethyl ether, tentoxin, tenuazonic acid, altertoxins, stemphyltoxin III, *Alternaria alternata* f. sp.

lycopersici toxins, and minor toxins (as shown in Table 2). These toxins are produced in relatively large amounts usually at the pre-harvest crop stage. (Weidenbőrner, 2001).

When it is known that certain *Alternaria* toxins are toxic for animals and humans if inhaled (De Lucca, 2007), their toxicity in food and feed is also cause of concern, and regulatory limits are still lacking (Lawley, 2009; Moss, 2008).

Table 2. Important and minor alternaria toxins and producing *Alternaria* species.

Mycotoxin	**Acronym**	**Species Producing**
Alternariol	AOH	*Alternaria alternata*
Alternariol monomethyl ether	AME	*A. alternata*; A. solani;
Tentoxin	TA	*A. alternata*
Tenuazonic acid	TeA	*A. alternata*, *A. tenuissima*
Altertoxins	ATXs	*A. alternata*
Alternaria alternata f. sp. *lycopersici* toxins	AAL-toxins	*A. alternata* f. sp. *lycopersici*
Stemphyltoxin III	Stemphyltoxin III	*A. alternata*
Altenuene	ALT	*A. alternata*

Alternariol

Alternariol (AOH) is a dibenzo-α-pyrone derivative, and is cytotoxic, fetotoxic and teratogenic. The lethal dose is 200 mg AOH/Kg body weight (bw) in mice (Weidenbőrner, 2001).

Dibenzo-a-pyrones are abundant, comprising up to 10% of the dry weight of the mold mycelia, and have been isolated from naturally infected grain sorghum (Sauer *et al.*, 1978) and pecans (Schroeder and Cole, 1977).

The natural occurrence of AOH is reported in apples, barley, mandarin fruits, oats, pecans, pepper, rye, sorghum, sunflower seeds, tomatoes, triticale, and wheat (Weidenbőrner, 2001).

Alternariol monomethyl ether

Alternariol monomethyl ether (AME) is a dibenzo-α-pyrone derivative, and it has necrotic, fetotoxic, teratogenic and mutagenic effects. The lethal dose is 400 mg AME/Kg bw mice (Weidenbőrner, 2001).

It naturally occurs on apples, barley, mandarin fruits, melon, oats, olives, pecans, pepper, rye, sorghum, sunflower seeds, tomatoes, triticale, and wheat (Weidenbőrner, 2001).

When grain sorghum, *Sorghum bicolour* (L.) Moench, is exposed to wet weather before harvest, the seeds are often discoloured by fungal growth. Two Alternaria metabolites, AME and AOH, were found in Kansas sorghums (Seitz *et al.*, 1975a,b).

Alternaria species are capable of producing AOH and AME in apples, tomatoes, and blueberries (Stinson *et al.*, 1980).

The natural occurrence of AOH and AME has been reported in wheat, barley, and oats in Germany (Gruber-Schley and Thalmann, 1988), sorghum (Seitz *et al.*, 1975a,b) and pecans (Schroeder and Cole, 1977) in the US, and apple juice concentrates in Spain (Delgado and Gómez-Cordovés, 1998).

Tentoxin

Tentoxin (TA), a natural cyclic tetrapeptide produced by phytopathogenic fungi from the *Alternaria* species affects the catalytic function of the chloroplast F_1-ATPase in certain sensitive species of plants (Santolini *et al.*, 1999).

Tenuazonic acid

Tenuazonic acid (TeA) is one of the most important *Alternaria* mycotoxins found in nature. Tenuazonic acid (TeA) is a 3-acetyl-5-*sec*-butyltetramic acid ([5^S-[5*R*(*R*)]]-3-acetyl-5-(1-methylpropyl)-2,4-pyrrolidinedione). It was first isolated in 1957 from the culture filtrates of *A. tenuis* (Rosett *et al.*, 1957; Schobert *et al.*, 2004), and probably possesses the highest toxicity of all *Alternaria* mycotoxins. The LD_{50} (po) is 81/168 mg/Kg bw female/male mice (Weidenbőrner, 2001).

TeA is a phytotoxin produced by several fungi including *A. alternata*, *A. longipes* and *A. tenuissima* (Chelkowski and Visconti, 1992; Da Motta and Soares, 2000a).

The acid has been detected in many vegetables, fruits and some cereals in different countries (Visconti and Sibila, 1994).

TeA have been found in olives, peppers (Chelkowski and Visconti, 1992), tomatoes (Stack *et al.*, 1985), tomato paste (Weidenbőrner, 2001).

TeA in fruits have been found in tangerines, melons (Chelkowski and Visconti, 1992; Logrieco *et al.*, 1988), and in apples (Weidenbőrner, 2001).

TeA in cereal grains have been found in sorghum, rice (Chelkowski and Visconti, 1992); wheat (Weidenbőrner, 2001). In Argentina, TeA has been detected at high concentrations in sunflower seeds (Torres *et al.,* 1993, Chulze *et al.,* 1995). TeA has been detected in domestic grown rapeseed meal and imported sunflower meal in the UK (Nawaz *et al.*, 1997),

in weather-damaged wheat and sorghum in Australia (Webley *et al.*, 1997), in weathered wheat in China (Li and Yoshizawa, 2000).

TeA has been found in tobacco (Mikami, 1971).

It showed significant phytotoxic activity when tested on monocotyledonous and dicotyledonous plants. The toxin induced chlorosis and necrosis on leaves of *Datura innoxia*, *D. stramonium*, *D. metel*, belladonna, cowpea, wheat, rye, cabbage, cauliflower and maize at 200 µg/ml and wilting of seedlings of *D. innoxia* at 100 µg/ml concentration. It also causes complete inhibition of root and shoot elongation of germinating seeds of *D. innoxia*, wheat, rye, green gram and lettuce at 100 µg/ml concentration. It is a nonspecific phytoxin and appears to have a significant role during pathogenesis.

Tenuazonic acid did not cause any significant change in the rate of respiration or in the sugar, carbohydrate, total phenol and nitrogen contents of *D. innoxia* leaves, but its treatment on the host plant induced a 64% reduction of chlorophyll content in leaves after 72 hours and a 40% reduction in protein content after 24 hours (Janardhanan and Husain, 2008).

Argentina sunflower seeds are susceptible to *Alternaria* contamination (Logrieco *et al.*, 1988; Dalcero *et al.*, 1989). AOH, AME and TeA, have been detected in freshly harvested sunflower seeds, with contamination frequencies of 85%, 45% and 66%, respectively (Chulze *et al*., 1995).

The toxins were found in meal, with 88–92% of AOH, 44–48% of AME and 64–47% of TeA in the samples tested (Montemurro and Visconti, 1992; Torres *et al.*, 1993; Chulze *et al*., 1995).

The natural occurrence of AOH, AME, TeA, and, in some cases, other *Alternaria* toxins (ALT, ATX-I) has been reported in various fruits and vegetables visibly infected by *Alternaria* rot, including tomatoes, olives, mandarins, melons, peppers, apples, and raspberries. They have also been found in processed fruit products such as apple juice, other fruit beverages and tomato products, wheat and other grains, sunflower seeds, oilseed rape meal, and pecans (Scott, 2001).

High levels of toxins have been found in infected fruits and vegetables: apples, up to 58,800 ng AOH/g but only up to 500 ng TeA/g (Stinson *et al.*, 1981); tomatoes, up to 5,300 ng AOH/g and 139 000 ng TeA/g (Stinson *et al.*, 1981); mandarins, up to 5,200 ng AOH/g and 173,900 ng TeA/g (Logrieco *et al.*, 1988); peppers, up to 440,000 ng AOH/g, 294,000 ng AME/g, 103,000 ng ALT/g, and 342,000 ng TeA/g (Chung *et al.*,1998); and olives, up to 2,300, 2,900, and 1,400 ng/g AOH, AME, and ALT, respectively (Visconti *et al.*, 1986).

Altertoxin I, II and III

Altertoxin I, [1^S-(1α, 12aβ, 12bα)] 1,2, 11, 12, 12a, 12b-hexahydro-1,4,9,12a-tetrahydroxy-3,10-perylenedione; altertoxin II, [7aR-(7aα,8aα,8bα,8cα)]-7a,8a,8b,8c,9,10-hexahydro-1,6,8c-trihydroxyperylo[1,2-*b*]oxirene-7,11-dione; and altertoxin III, [la*R*-(laα,lbβ,5aα,6aα,6bβ,10aα)]- la,1b,5a,6a,6b,10a-hexahydro-4,9-dihydroxyperylo[1,2-*b*:7,8-*b*']bisoxirene-5,10-dione. They are 4,9.difydroxyperylene-3,10-quinons (Weidenbőrner, 2001).

Altertoxin I (ATX I) is one of the common mycotoxins produced by genus *Alternaria* (Dejun *et al.*, 2008).

ATX-I, AOH and AME are mutagenic, although there is evidence that mutagenicities of AME and ATX-I decreased after purification (Chelkowski and Visconti, 1992; Woody and Chu, 1992; Zhen *et al.*, 1991; Liu *et al.*, 1992; An *et al.*, 1989; Scott and Kanhere, 1980; Stack *et al.*, 1986; Stack and Prival, 1986).

Alternaria alternata f. sp. lycopersici toxins

Alternaria alternata f. sp. *lycopersici* toxins (AAL-toxins) are produced by the tomato pathogenic fungus *Alternaria alternata* f. sp. *lycopersici* (Xu and Du, 2006).

AAL-toxins possess a "sphingosine-like" structure. They are host-specific pathotoxins, active at cell level (Weidenbőrner, 2001).

They are primary determinants for stem canker disease on tomatoes and induce apoptosis in animals and plants (Xu and Du, 2006). AAL toxins and fumonisins comprise a family of highly reactive, chemically related mycotoxins that disrupt cellular homeostasis in both plant and animal tissues (Winter *et al.*, 1996).

In leaves of susceptible genotypes, AAL-toxins cause severe necrosis, while in leaves of resistant genotypes necrosis has never been observed. Inhibition effects of toxins have been observed at all other levels in susceptible and resistant genotypes: AAL-toxins inhibited shoot induction on leaf discs, root growth and growth of calli, suspension cells and protoplasts (Witsenboer *et al.*, 1988).

Alternaria alternata (Fr) Keissler f.sp. *lycopersici* has been shown to be the cause of a serious stem canker disease of tomatoes in several parts of the world (Grogan *et al.*, 1975).

Stemphyltoxin III

Stemphyltoxin III, (6aR, 6bS, 7R, 8R)-3,6a,10-trihydroxy-4,9-dioxo-4,6a,6b,7,8,9-hexahydro-7,8-epoxyperylene, a known metabolite of *Stemphylium botryosum* var. *lactucum*, has been

identified as a mutagenic metabolite of *A. alternata* by Ames *Salmonella typhimurium* assay (Stack and Mazzola, 1989).

Stemphyltoxin III was tested for mutagenicity in the Ames *Salmonella typhimurium* plate incorporation assay with and without Aroclor 1254-induced rat S-9 metabolic activation. A positive response was noted with and without metabolic activation in *S. typhimurium* TA98 and TA1537, and there was a marginal response in strain TA100 (Davis and Stack, 1991, 1994).

Minor Alternaria toxins

Limited knowledge is available on minor *Alternaria* toxins such as dehydroaltenusin, altenuene (ALT), isoaltenuene (iALT), epialtenuene (2',3',4',5'-tetrahydro-3,4'β,5'β-trihydroxy-5-methoxy-2'β-methyldibenzo-α-pyrone), and neoaltenuene (2',3',4',5'-tetrahydro-3,3'β,4'β-trihydroxy-5-methoxy-6'-methyldibenzo-α-pyrone), which have been isolated from infested fruits in sub-milligram amounts.

PHYSICO-CHEMICAL CHARACTERISTICS

Physico-chemical characteristics are reported in the literature only for the main Alternaria mycotoxins. In particular, Tenuazonic acid is a colourless, viscous oil and is a monobasic acid with pKa 3.5. It is soluble in methanol and chloroform. On standing, heating or treatment with a base, optical activity is lost and crystallisation may occur as a result of the formation of isotenuazonic acid. It forms complexes with calcium, magnesium, copper, iron and nickel ions.

Alternariol and alternariol monomethyl ether crystallise from ethanol as colourless needles, and melting points with decomposition are 350°C and 267°C respectively. They are soluble in most organic solvents and give a purple colour reaction with ethanolic ferric chloride. Altenuene crystallises as colourless prisms melting at 190-191°C.

Altertoxin I is an amorphous solid melting at 180°C, which shows a characteristic bright yellow fluorescence under UV light.

BIOSYNTHESIS

The structures of Alternaria metabolites are of several types. The dibenzo-α-pyrone group includes alternariol (AOH), alternariol methyl ether (AME), altenuisol (ASL), altertenuol, altenuene (ALT) and the dibenzopyrone derivatives altenusin and altenuic acid II. The nitrogen-containing group includes tenuazonic acid (TeA), cyclic petides (tentoxin and AM-toxins), amides (AK-toxins) and zwitter ions (Al-toxins). TeA and tentoxin are non-specific

phytotoxins, whereas AM-, Ak- and AL-toxins are host-specific phytotoxins. The anthraquinones are primarily phytotoxins, but these compounds are often used and classified as antibiotics.

Alternaria also produce a number of metabolites of varied structure, including altertoxin-I (ATX-I), a partially saturated perylene.

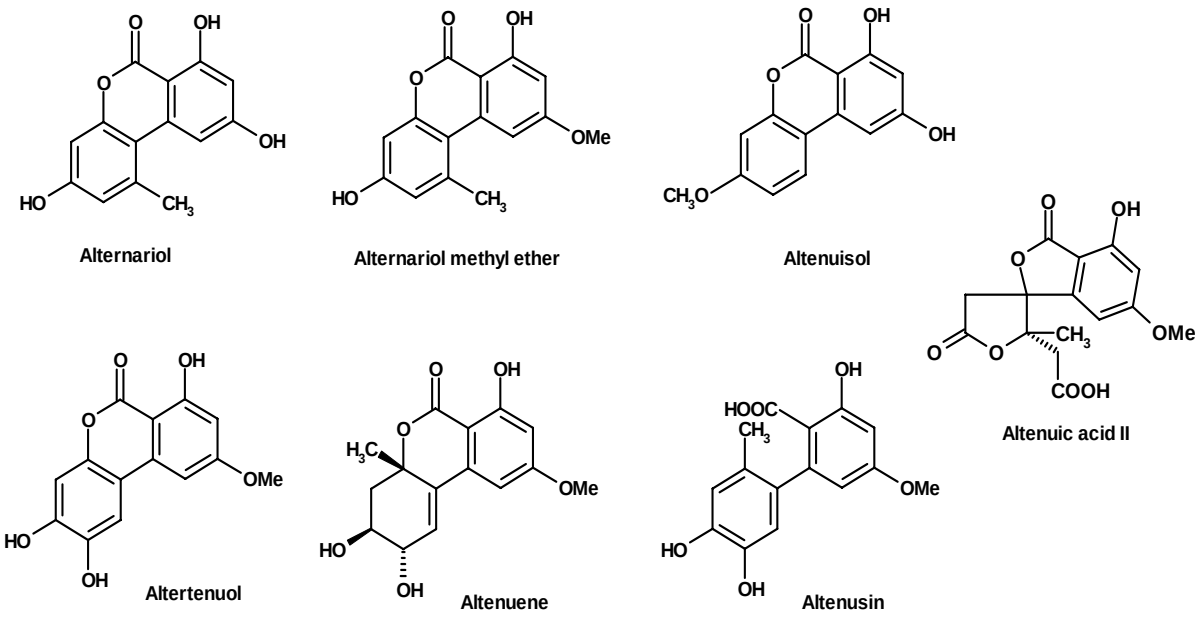
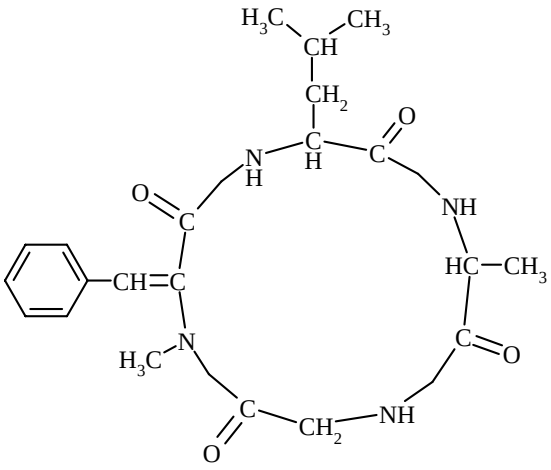
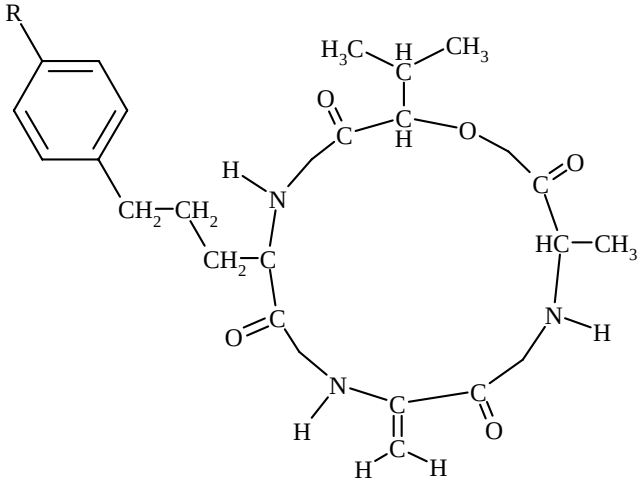


Figure 1. Alternaria dibenzo-α-pyrone metabolites.

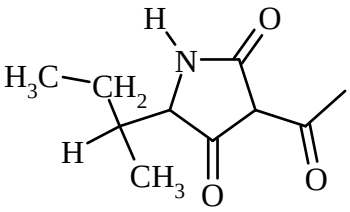


Tentoxin

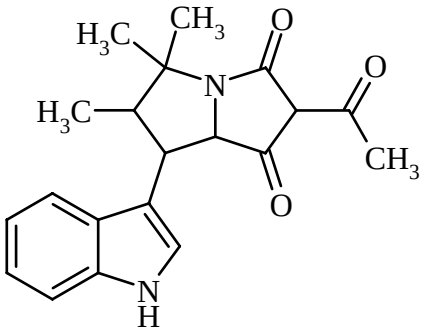


AM-Toxin

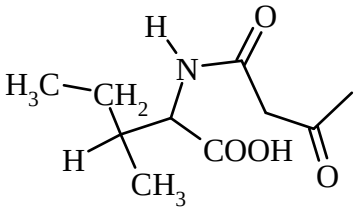
AM-Toxin I, R = OCH_3
AM-Toxin II, R = H
AM-Toxin III, R = OH



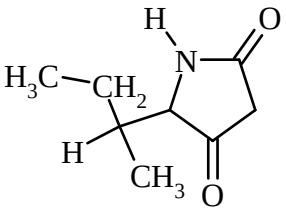
Tenuazonic acid



α-Cyclopiazonic acid



Acetoacetylisoleucine



5-sec-Butyltetramic acid

Figure 2. Alternaria nitrose-containing metabolites.

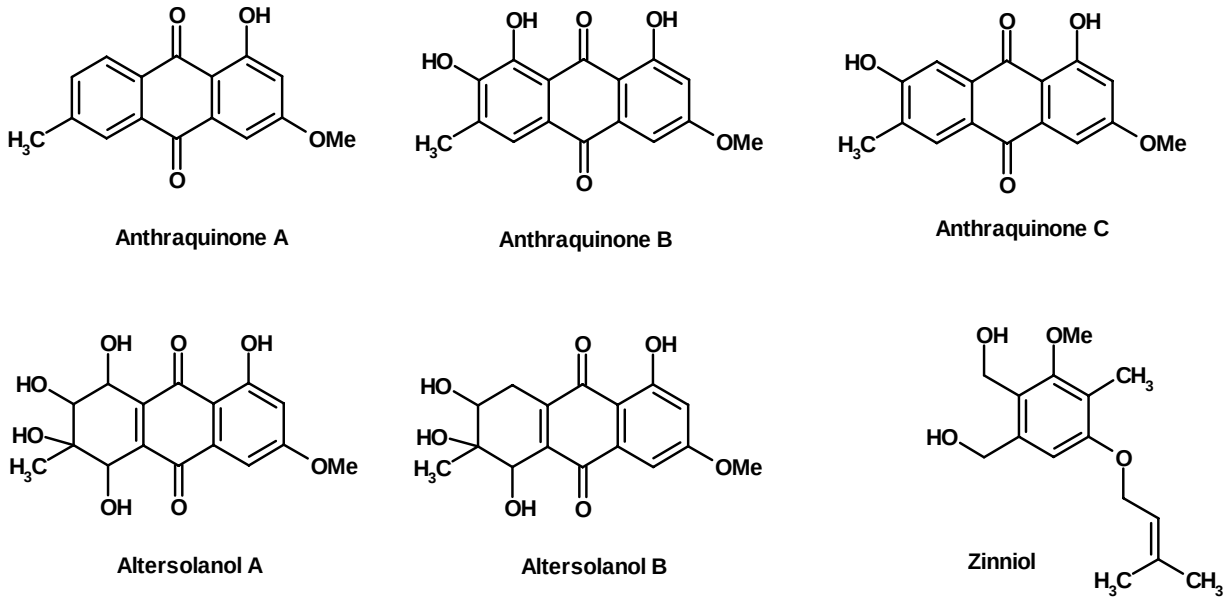


Figure 3. Alternaria anthraquinone metabolites.

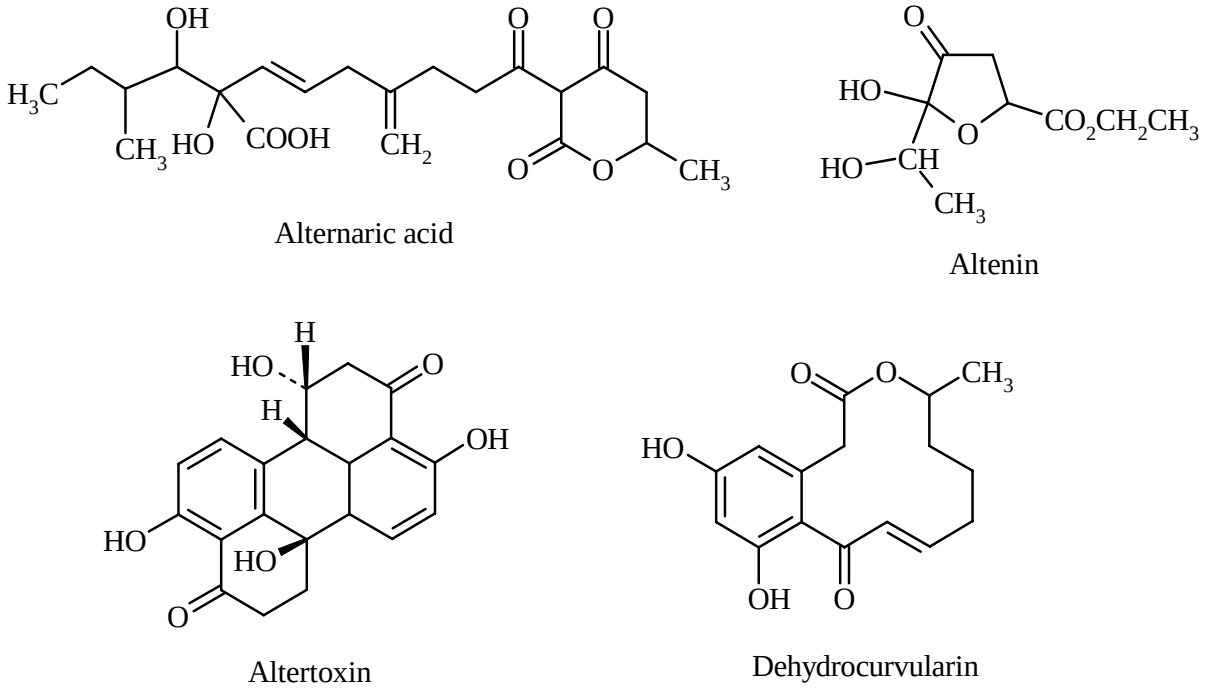


Figure 4. Alternaria varied structure metabolites.

Concerning the production of the main Alternaria metabolites, biosynthetic pathways have been elucidated only for alternariol and other main dibenzo-α-pyrone compounds (Stinson *et al.*, 1982), whereas there is a lack of information about the formation mechanisms involved in nitrogen containing metabolite synthesis.

AOH and AME, as well as the other dibenzopyrones produced by Alternaria, belong to the large group of secondary metabolites classified as polyketides. Polyketide synthesis involves condensation of acetyl-CoA and malonyl-CoA units with the simultaneous release of the terminal carbon of the malonyl unit. The condensations continue thus without hydrogenation until a poly-β-ketomethylene chain of the required length is assembled. At this point, the activated methylene units of the polyketides react spontaneously with carbonyl groups by a Claisen condensation to yield the required aromatic compounds, which are then released from the enzyme surface. Cyclization is enzymatically controlled in order to bring the correct reactive sites into proximity to form the desired aromatic product. For AOH it has been proposed (Abell *et al.,* 1986) that the compound shifts to form a *cis*-double bond at the chain-folding site so that the three bonds are in a *syn* position.

Several experiments have suggested that the AOH crucial double bond is formed from keto-enol equilibrium when oxygen is retained (Abell *et al.,* 1982).

Several experiments (Thomas, 1959, 1961) have suggested that the AOH biosynthesis proceed through an orsellinic acid-type arrangement. Alternariol is considered the precursor of most of the dibenzo-α-pyrones produced by Alternaria. In particular, the formation of AME from AOH in the presence of S-adenosyl-methionine (SAM) has been established (Sjoland and Gatenberck, 1966).

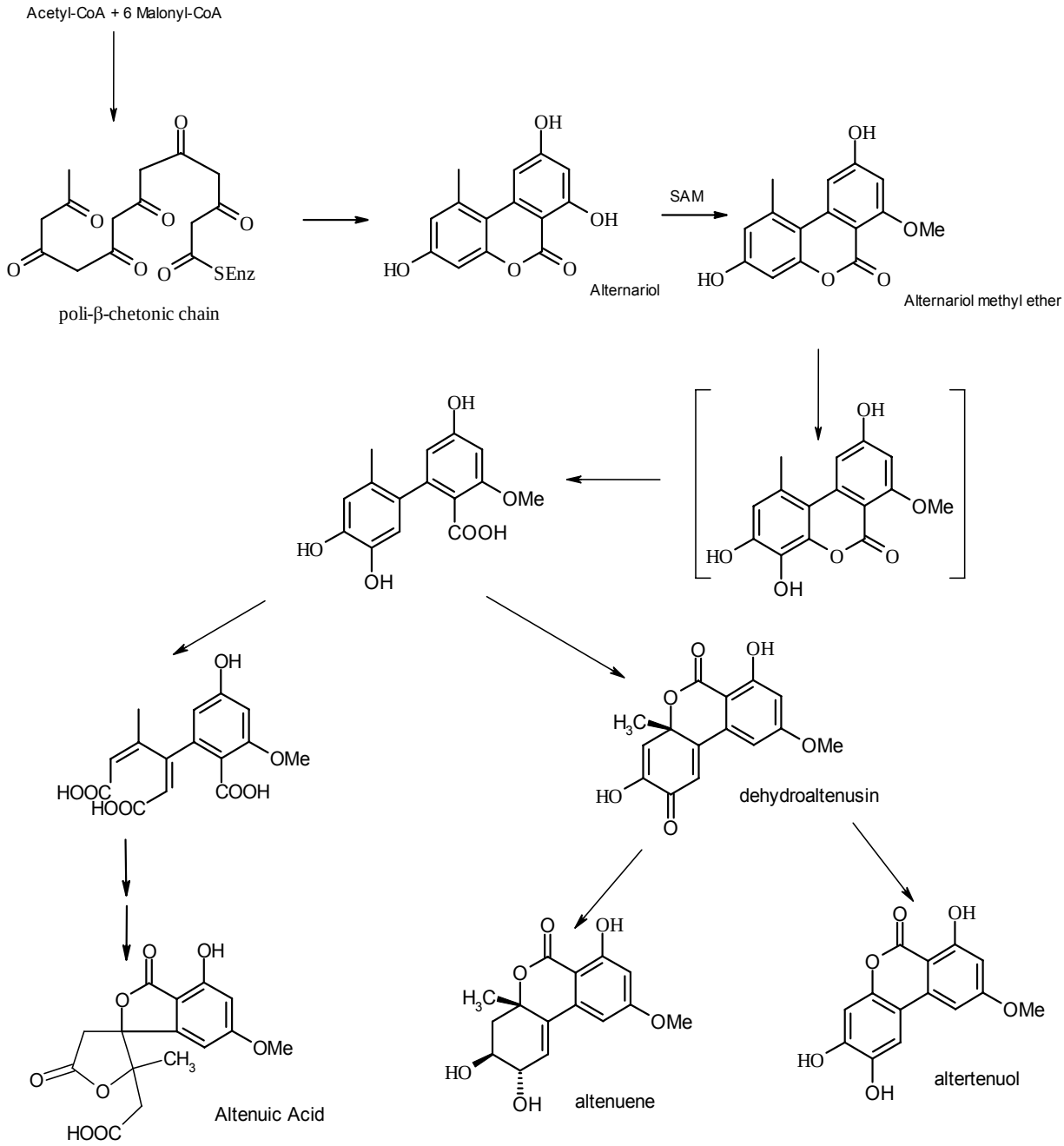


Figure 5. Main biosynthetic pathway for Alternaria toxin formation.

An alternative biosynthetic pathway suggested the formation of AOH from anthraquinone metabolites, as reported in Figure 6. The scheme started with altersolanol A, a prearomatic β-methylanthraquinone, and requires the loss of the β-methyl group, which could give rise to altertenuol. Then, the 6-methyl group of AOH and AME is introduced. Accordingly, altenuene, dehydroaltenusin and altenusin could be originated by an α-methylanthraquinone

analog of altersolanol A. This alternative biosynthesis was disproved by an experiment involving incorporation of [1-13C,18O_2]acetate into alternariol in *Alternaria tenuis*, establishing that all the oxygen atoms are acetate-derived (Dasenbrock and Simpson, 1987).

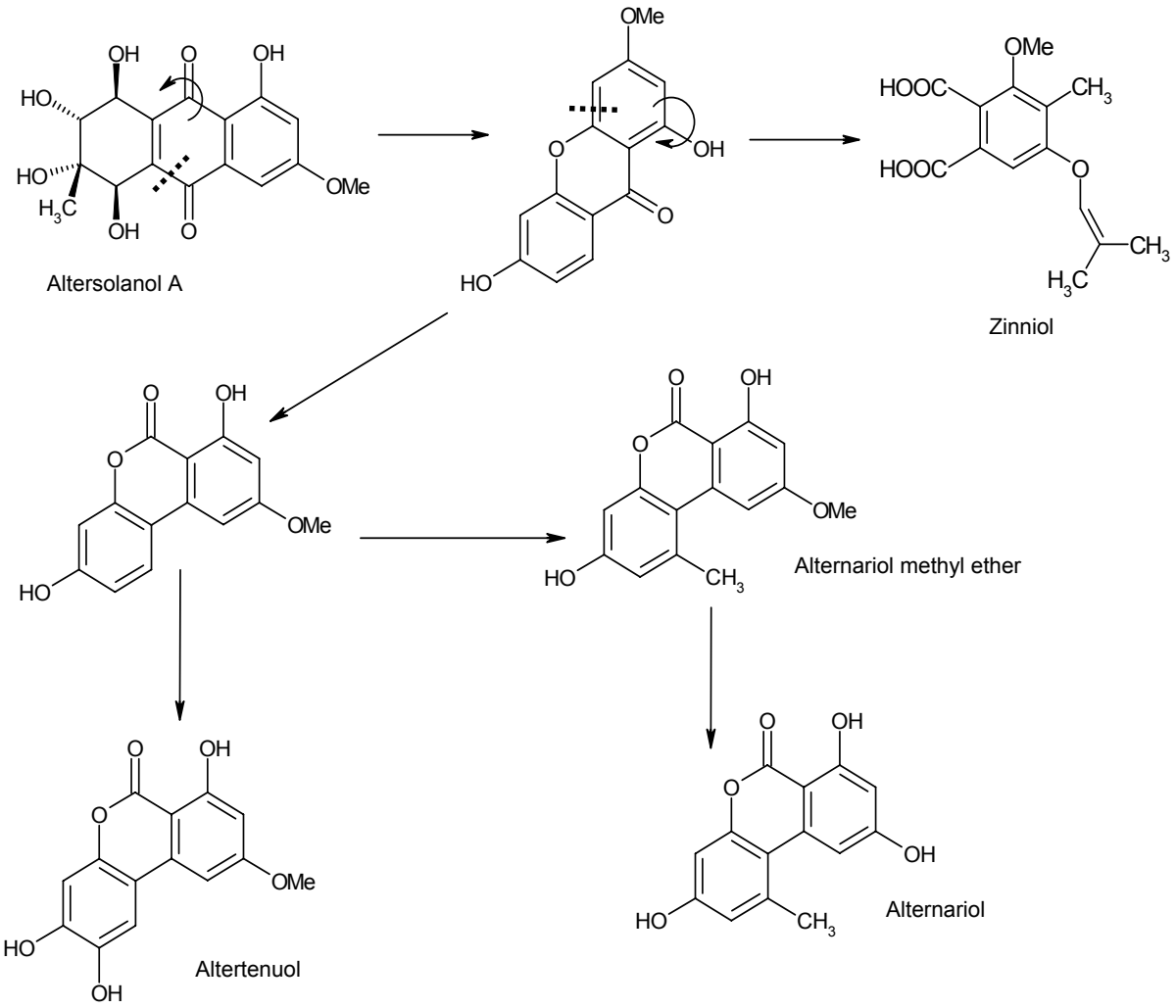


Figure 6. Alternative biosynthetic pathway for Alternaria toxin formation.

Tenuazonic acid is one of the most common metabolites produced by Alternaria fungi. It is a tetramic acid formed by condensation of a L-isoleucine and two acetate units. Details of the polyketide assembly and final condensation with the amino acid to form the tetramic acid ring system have not been established.

CHEMICAL ANALYSIS

Source of Standards

The main mycotoxins produced by Alternaria are commercially available as standard material for method development and quantification, as reported in Table 3.

Standard solutions should be prepared in acetonitrile and stored at +4 - +8°C.

Certified reference materials for method development and validation are not commercially available.

Table 3. Sources of Alternaria toxins standards.

Compound	Concentration or Purity (µg/ml) %	Quantity	Supplier
Solid standard			
Tenuazonic acid copper salt from *Alternaria alternata*	-	10 mg	Sigma-Aldrich
Tentoxin from *Alternaria tenuis*	-	250 µg/1 mg	Sigma-Aldrich
Alternariomethylether from *Alternaria alternata (tenuis*)	-	5 mg	Sigma-Aldrich
Alternariol from *Alternaria* sp.	~ 96%	5 mg	Sigma-Aldrich
Altenuene from *Alternaria* sp.	-	1mg/5 mg	Sigma-Aldrich
Altertoxin I	-	10 mg	Chemos GmbH (Regenstauf, Germany)
Standard solution			
Alternariol	100 dried down	1 ml	Romer Labs Diagnostic (Tulin, Austria)
Alternariomethylether	100 dried down	1 ml	Romer Labs Diagnostic (Tulin, Austria)
Tentoxin	100 dried down	1 ml	Romer Labs Diagnostic (Tulin, Austria)

Sampling

No specific research has been undertaken on sampling plans for Alternaria toxins in food products, a topic which should be faced in the near future.

Extraction and Clean up

Several methods are reported in literature for the extraction and clean up of Alternaria mycotoxins from food commodities. In particular, sample preparation usually involves a clean up step on Solid Phase Extraction columns, using an aminopropyl stationary phase.

The determination of Alternaria toxins from raw tomatoes or tomato-based products such as puree and sausages is often reported: the procedure started with a solid/liquid extraction step using methanol (Da Motta and Valente Soares, 2000a, 2000b, 2001; Terminiello *et al*., 2006). After filtration and addition of a 10% ammonium sulphate solution, the sample was defatted with hexane and then extracted into a separatory funnel with chloroform. Afterwards, the organic layer containing AOH and AME was collected, whereas the aqueous layer containing TA was acidified to pH 2 with HCl and further extracted with chloroform. The

chloroform layers were then pooled, evaporated and redissolved in methanol for analysis. Recovery for TA were in the range 83 – 98% (Da Motta and Valente Soares, 2000a).

When only AOH and AME were considered, the extraction step was performed using ethyl acetate containing 1% formic acid. After evaporation, the residue was dissolved in methanol and analysed for mycotoxin detection (Andersen and Frisvad, 2004).

Alternariol and alternariol methyl ether have also been detected in flavedo and albedo tissues from Tangerines Citrus (Magnani *et al*., 2007). The samples were extracted with acetonitrile containing 1% acetic acid in an ultrasonic bath. After filtration and solvent evaporation, the residue was dissolved in ethyl acetate and purified on an aminopropyl SPE cartridge. The elution step was performed using acetonitrile containing 1% acetic acid.

Alternaria mycotoxin occurrence in juices is also often reported in the literature. Juices are usually purified on a C18 SPE column, followed by a further purification on aminopropyl SPE column (Delgado and Gomez-Cordoves, 1998). In order to avoid a double purification step, the direct application of juice sample on an aminopropyl SPE column was proposed (Lau *et al*., 2003; Scott *et al*., 2006). After elution with methanol acidified with 1% acetic acid, the eluate was analysed for mycotoxin detection. When cloudy juices are considered, such as apple or pear juice, a clarification step with pectinase is added before SPE clean up (Lau *et al*., 2003). Recovery was 80 – 88 % in red grape juice, 93 – 94% in white grape juice, 88 – 92% in red wine and 78 – 88% in cranberry juice (Scott *et al*., 2006).

The occurrence of Alternaria mycotoxins has also been evaluated in vegetables such as sweet pepper: samples were extracted with ethyl acetate containing 1% formic acid, then purified on a strong anionic exchange (SAX) column followed by a second clean up on an aminopropyl SPE column (Monbaliu *et al.,* 2009).

Analytical methods

Several methods have been reported in the literature for the analytical determination of the main Alternaria mycotoxins and the most important methodologies have been reviewed by several authors (Shephard *et al*., 2009; Ostry, 2008, Pinto, 2008, Scott and Kanhere, 2001). In particular, the most common technique for AOH, AME or TA quantification is RP-HPLC with UV or Diode Array detection. In the last decade, some methods involving mass spectrometry have been proposed as well as several methodologies based on biosensing.

AOH and AME in vegetables and juices have usually been detected by RP-HPLC using a C18 column and a methanol-water elution (Scott *et al*., 2006; Da Motta and Valente Soares 2000a, 2000b and 2001; Delgado *et al*., 1996, 1998). On account of the acidic properties of the analytes, both eluents are usually acidified with formic or acetic acid. UV detection was

usually performed at 254 nm. For peak purity and confirmation, a Diode Array detector was used scanning in the range 200 – 300 nm. For TA, which is characterized by a stronger acidic behaviour, chromatographic separation was performed on a C18 column using $ZnSO_4$ as eluent modifier (Da Motta and Valente Soares, 2000a).

The occurrence of Alternaria toxins has also been evaluated in carrots by RP-HPLC with UV diode array detection (Solfrizzo *et al*., 2004). In particular, two consecutive isocratic mixtures of acetonitrile-sodium dihydrogen phosphate solutions were used for separation. Mean recoveries of TA, ATX-I, AME and AOH from carrots were 69, 71, 90 and 78%, respectively. Limits of detection for TA, ATX-I, AME, and AOH were 0.02, 0.02, 0.01, and 0.005 mg/kg, respectively.

Since alternariol and alternariol methyl ether are naturally fluorescent, several HPLC methods based on fluorescence detection have been developed. In particular, fluorescence detection is usually performed at λex = 230 nm and λem = 333 nm or 450 nm (Andersen and Frisvad, 2004; Fente *et al*., 1998). The use of HPLC-FLD for the determination of Alternaria toxins in tomato paste allowed for good recoveries (min. 77.2%) and detection limits (1.93 µg/kg for AOH) (Fente *et al*., 1998). In the last decade, several mass spectrometry based methods have been proposed, not only for analyte identification and confirmation but also for quantification. In particular, LC-MS/MS analysis has usually been performed using electrospray ionisation (ESI) interface in the negative mode. For quantification a Multiple Reaction Monitoring mode is usually choosen; in particular, common transitions monitored for AOH are m/z 257 ➔ m/z 215 and m/z 257 ➔ m/z 213 (collision energy: 25 eV); for AME are m/z 271 ➔ m/z 256 and m/z 271 ➔ m/z 228 (collision energy: 30 eV). Quantification of AOH and AME in Tangerines Citrus tissues by means of LC-MS/MS allowed for a quantification limit of 0.5 µg/kg for both AOH and AME (Magnani *et al*., 2007).

The use of an APCI interface in the negative ion mode has also been proposed (Lau *et al*., 2003), allowing for a sensitivity comparable to that reported for ESI ionization.

Tenuazonic acid is poorly ionisable, thus it is usually detected by UV. However, very recently its quantification by HPLC-ESI-ion trap-MS after derivatization with 2,4-dinitrophenylhydrazine (DNPH) was proposed (Siegel *et al*., 2009). DNPH is usually used as an LC-MS derivitization agent for poorly ionizing carbonyl compounds in environmental analysis. This method allowed for a quantification limit of 50 µg/Kg.

APCI-MS techniques seemed to be very effective for the determination of tentoxin and its related compounds, isotentoxin and dihydrotentoxin from Alternaria porri. In particular, APCI-MS provided not only [M + H]+ but also [M + H-Gly-(N-Me-Ala)]+ for the three analytes, allowing for a good sensitivity (Horiuchi *et al*., 2003, 2004).

Gascromatography has been applied for Alternaria mycotoxin identification after trimethylsilyl- or heptafluorobutyril-derivatization (Scott *et al*., 1997). Since the derivatization step is usually troublesome and time-spending, identification is nowadays preferred by means of LC/MS/MS techniques.

TLC has also been used for Alternaria mycotoxin analysis (Fabrega *et al*., 2002). In particular, separation was achieved using silica gel plates and elution based on toluene:methanol:acetic acid 92:6:2 v/v/v. Retention factors (Rf) were 0.11 for AOH, 0.23 for AME, 0.08 for ALT, 015 for ATX-I and 0.09 for tentoxin. Detection was performed under UV-lamp at 254 nm. Limits of detection were 1.25 ng for AME, 2.5 ng for AOH, ALT and ATX-I and 50 ng for tentoxin.

The electroactive characteristics of Alternaria mycotoxins allowed the use of electrochemicaltechniques for their detection. Molina *et al.,* (2002) showed the electro-oxidation of AOH and AME at glassy carbon and platinum electrodes. However, the high potentials required compromised the specificity of the assay. In order to solve this problem, Moressi *et al*., (2004) exploited the affinity of mushroom tyrosinase for these two mycotoxins. This enzyme allowed working in mixed aqueousorganic media, which is of great interest when analytes of low aqueous solubility have to be detected. However, the limit of detection for AME was 7.5 · 10_6 M, higher than that obtained by direct ECD.

A common problem of these two approaches was the fouling of the electrode surface due to the accumulation of oxidized products. This shortcoming was circumvented by using gold electrodes coated with a dodecanethiol self-assembled monolayer (SAM), which not only decreased electrode passivation but also overpotential. This approach attained an LOD for AME of 9.1 · 10_8 M, about three times smaller than in direct ECD on bare electrodes and about 50 times smaller than in the enzymatic approach.

Although this approach seems to be promising, the selectivity problem does not allow the application of the method to routine analysis.

Validation and Outlook

None of the mentioned methods, including LC-methods, have been validated by interlaboratory studies and there are no certified reference materials or proficiency studies available for the determination of Alternaria toxins. In the near future, certainly, validated analytical methods for the quantification of Alternaria toxins are needed as a prerequisite for a survey on their occurrence in feed and food materials in Europe.

OCCURRENCE DATA

Many species of *Alternaria* commonly cause spoilage of various food crops and occurrence results were presented with reference to groups of products

Occurrence data, with concentrations and distribution in the commodities are presented in Table 4.

Agricultural commodities

Alternaria toxins have been found in several agricultural commodities, including grains, sunflower seeds, oilseed rape, sorghum, and pecans.

Seitz *et al.* (1975b) found AOH and AME in weathered, discolored sorghum, but none to barely detectable levels in normal-colored, good quality sorghum. Later Seitz *et al*. (1975a) found AOH and AME in some commercial sorghum hybrids grown at Kansas experiment stations. Metabolite levels were correlated with the degree of grain discoloration and number of rainy days during September and October. Sauer *et al*. (1978) surveyed 12 field samples of weathered sorghum: 3 samples were positive for ALT and ATX-I (>100 μg/kg), TeA was not found (< 100 μg/kg).

In another study (Ansari and Shrivastava, 1990), the natural occurrence of major *Alternaria* mycotoxins i.e. AOH, AME, ALT, ATX-1 and TeA was investigated in sorghum (*Sorghum vulgare* [*S. bicolor*]) and ragi (*Eleusine coracana*) collected from North Bihar. Nine out of 20 sorghum samples, and three out of eight ragi samples, were found to be contaminated with one to three *Alternaria* mycotoxins.

Thalmann *et al*. (1987) investigated the occurrence of AOH and AME in wheat, barley, rye, oats and mixed cereals by HPLC with a detection limit in a range of 6-160 µg/kg. Barley samples from northern Germany were more frequently contaminated than those from southern Germany, whereas the reverse was the case for oat samples. No clear trend was observed for wheat. Culture medium containing rice supported greater production of AME and AOH than that containing wheat or oats.

There are reports of AOH and AME in “black point” wheat in Poland (Grabarkiewicz-Szczęsna and Chelkowski 1993); AOH and AME in German wheat (Gruber-Schley and Thalmann, 1988); AOH, AME, and TeA in weather-damaged wheat in Australia (Webley *et al*., 1997); AOH, AME, ATX-I, ALT, and TeA in Egyptian wheat (Abd El-Aal, 1997); and of AOH, AME, and TeA in weathered wheat from China (Li and Yoshizawa, 2000). In the Chinese wheat, there were good linear correlations between concentrations of AOH and AME (r=0.850) and between concentrations of [AOH + AME] and TeA (r = 0.796), indicating coproduction of the toxins in the field (Li and Yoshizawa, 2000).

Skarkova *et al*. (2005) analyzed 129 wheat samples from the Czech Republic and detected AOH (46.5% positive samples) and ALT (91.5% positive samples).

The natural occurrence of *Alternaria* mycotoxins in Argentinean wheat from zone 5 South during the 2004 to 2005 harvest was investigated in 64 wheat samples (Azcarate *et al*., 2008). Three mycotoxins produced by species of *Alternaria* were determined in wheat: AOH (6%) AME, (23%) and TeA (19%). AME was the predominant toxin, but TeA was detected in higher concentrations. AOH was present in fewer samples and at lower levels than were the other toxins.

As another example, sunflower seeds have been shown to contain *Alternaria* toxins in Argentina (up to 792 µg AOH/kg, 836 µg AME/kg, and 31600 µg TeA/kg; Dalcero *et al*., 1997; Torres *et al*., 1993; Chulze *et al*., 1995) and in Italy (up to 1840 µg AOH/kg and 129 µg AME/kg; Logrieco *et al*., 1988).

Analyzing hay and other samples, including corn silage and mixed feed, Yu *et al*., (1999) found that the frequency of A. *alternata* (AAL) TA toxin in the feed was 96.8 %. For hay alone, the frequency was 100%.

AME was detected in 20 flax seed samples; ALT and AOH were present in only 2 and 4 samples, respectively. A more frequent incidence of *Alternaria* toxins was recognised in fibre flax seeds as compared to linseed samples. Compared to crops from conventional farming, the concentrations of these mycotoxins found in positive organic samples were higher. No *Alternaria* mycotoxins were detected in pea samples, probably due to the presence of antifungal compounds in the respective crop.

Häggblom *et al*., (2007) analyzed the presence of *Alternaria* mycotoxins in whole grain and straw samples. The results showed different levels of AOH and AME. TeA was detected in all 18 analysed samples, 4 out of 18 samples contained TeA, in the range 980 – 4310 µg/kg. Interestingly, 2 of the 4 samples where the highest levels of TeA were detected were oats.

Fruits and vegetables

The production of *Alternaria* under natural infection conditions or following inoculation has been studied in a variety of fruits and vegetables. However, various qualitative and quantitative studies clarified that *Alternaria* toxigenicity in fruits may vary not only with fungal species or strains, but also with the host fruit species and cultivar and with the conditions under which the mycotoxin may be synthesized in the host fruit.

Inoculation studies

Studies by Ozcelik *et al*. (1990) indicated that AOH and AME were the main mycotoxins formed in tomatoes and apples following inoculations with A. *alternata*. They compared the toxin levels in tomatoes and apples after 3 weeks at 25°C, and recorded maximum AOH concentrations of 1161 and 372 mg/kg, respectively, and maximum AME concentrations of 323000 and 32000 µg/kg , respectively, under the same conditions.

In contrast with the findings of Özcelik *et al*. (1990), who did not detect any TeA in *Alternaria*-inoculated tomatoes and apples, earlier studies by Harwig *et al*. (1979) in ripe tomatoes inoculated with toxigenic isolates of A. *alternata* found TeA at levels up to 106000 µg/kg , and studies by Stinson *et al*. (1980, 1981) in naturally infected and in inoculated tomatoes reported TeA levels up to 139000 and 1373000 µg/kg fruit tissue, respectively. In fact, these studies found TeA to be the major mycotoxin in tomatoes, in spite of the differences between the findings of the three studies.

As a result of inoculation studies, the potential for the occurrence of *Alternaria* mycotoxins in various fruits has been established. Thus, following inoculation of blueberries with *A. alternata* isolated from decayed blueberries, the production of TeA (up to 2025000 µg/kg) was demonstrated (Stinson *et al*., 1980). The potential for ATX-I production in tomatoes was demonstrated, following inoculation with A. *alternata* (Stinson *et al*., 1980), although no ATX-I was recorded in tomatoes under natural infection conditions (Stinson *et al*., 1981).

The production on TeA in oranges and lemons, at levels up to 61100 and 48800 µg/kg, respectively, was recorded, following inoculation of the fruits with A. *citri*. Levels up to 41000 and up to 2800 µg/kg of AOH and AME, respectively, were also produced in inoculated oranges and lemons (Stinson *et al*., 1981). Studying the relative importance of the mycotoxins produced in inoculated oranges and lemons, Stinson *et al* (1981) found similar patterns of production in the two fruits, although the overall mycotoxin contents were higher in oranges than in lemons.

Two different strains of *A. alternata*, which were isolated by Logrieco *et al*. (1990) from a natural black or grey “heart rot” of mandarins, were found to differ, not only in their pigmentation, but also in their abilities to produce mycotoxins. The black rot samples contained TeA, AOH and AME, of which TeA was the most abundant, whereas TeA was the only toxin to be found in the grey rot sample, at up to 173900 µg/kg.

Examining the mycotoxin-producing potential of A. *alternata* in “Red Delicious” apples, Singh and Sumbaly (2004) found that 83% of the A. *alternata* isolates produced TeA as the main mycotoxin in the inoculated apples, with the highest level being 96000 µg/kg. No detectable amounts of AOH and ATX-I were found in the rotten apple tissues or their surroundings.

Working with two toxigenic strains of *A. alternata*, Tournas and Stack (2001) found that the fungus and its mycotoxins were not a major problem in strawberries, because of the presence of fast-growing moulds, such as *Rhizophus* and *Botrytis*, which outgrow *Alternaria* strains and inhibit its growth. The growth of both *Alternaria* strains on apples was limited, even without the presence of fast-growing moulds. Both AOH and AME were produced, at up to 5000 and up to 14000 µg/kg , respectively, by one strain of *A. alternata* in inoculated "Golden delicious" and "Gala" apples. Moderate *Alternaria* growth, along with the production of AOH and AME, at up to 3336000 µg/kg and up to 1716000 µg/kg , respectively, were recorded by Tournas and Stack (2001) in grapes.

The toxigenic profiles of *A. alternata* and *A. radicina* from carrots were determined by growing the fungi on rice and carrot discs. Most of the A. *alternata* isolates produced TeA, AOH, AME and ATX-I when cultured on rice, but only AOH and AME when cultured on carrot discs (Solfrizzo *et al*., 2005).

Evaluation of the potential for mycotoxin production by fungi in dried vine fruits indicated that several A. *alternata* strains were producers of TeA, AOH and AME (Romero *et al*., 2005).

Natural occurrence

AOH and AME were detected in Japanese pears (Tirokata *et al.*, 1969).

In a survey on moldy tomatoes collected from the processing lines of catsup manufacturers, Stack *et al*. (1985) found that whereas 69 of 142 samples analyzed were negative for TeA, 28 contained up to 1900 µg/kg, and 45 up to 70000 µg/kg .

Tenuazonic acid was the major *Alternaria* mycotoxin produced in naturally infected tomatoes collected in southern Italy. Lower levels were recorded for AOH and AME (Bottalico and Logrieco, 1998). Altenuene was found in smaller amounts in each of the fruits tested, and frequently at trace levels only.

Wittkowski *et al*., (1983) described a reversed-phase HPLC method for the determination of AOH, AME, ALT and ATX-I in fruits and vegetables. Forty seven commercial samples of fruit products (juices, purees, jams) were screened by this method; none contained any of the 4 toxins; 22 samples of mouldy and rotten fruit and vegetables were analysed; 2 apple samples were contaminated, one with AME at 250 µg/kg and the other with AOH at 160 µg/kg.

Although the potential for mycotoxin production by A. *alternata* in carrots has been demonstrated, no *Alternaria* mycotoxin were detected in carrot roots during the production and storage of carrots and their products (Solfrizzo *et al*., 2005). Another *Alternaria* species, *A. radicina*, which infects carrot roots in the field and postharvest (Snowdon, 1990), is known

to produce radicinin (RAD) and radicinols, which exhibit phytotoxic activity (Solfrizzo *et al*., 2005). Radicinin, produced by *A. radicina*, was detected in three out of 266 carrot samples produced under organic conditions in European locations, whereas A. *alternata* mycotoxins were not found in any of the samples (Solfrizzo *et al*., 2004).

The natural occurrence of AOH was reported in pecans (Schroeder and Cole, 1977). However, their production was limited to discolored pecan kernels, which are removed from shelled pecans during processing, which led to the suggestion that such pecans would be rejected by buyers of in-shell pecans (Schroeder and Cole, 1977).

The natural occurrence of the major *Alternaria* mycotoxins in olives and related processing products (oil and husks) was first reported by Visconti *et al*. (1986). None of the undamaged olives properly harvested in several areas of Apulia (Italy) were found to contain mycotoxins, despite the presence of *Alternaria* on the olive surface; of the 13 olive samples collected, 4 were contaminated with 2-4 *Alternaria* mycotoxins. Heavily damaged olives contained considerable amounts of *Alternaria* mycotoxins: TeA, AME, AOH and ALT. ATX-I was not detected in any sample at levels higher than 200 µg/g. These studies indicated that toxin production in olives is probably associated with physical damage to the fruit surface, which would allow fungal penetration into the fruit pulp, with subsequent mycelia growth and toxin synthesis.

A. *alternata* isolated from seeds of Brassica (important source of oil) collected from different localities of Pakistan were analyzed for *Alternaria* toxins i.e., AOH, AME, TeA, ATX-I and ATX-II. AME was detected in all isolates of A. *alternata*, whereas TeA and ATX-I were detected in 73 and 67% of the isolates, respectively, and none of the isolates produced ATX-II and AOH (Mehdi *et al*., 2004).

Kocher (2007) analyzed edible oil samples (safflower, olive, rape, sesame and sunflower oils). All sunflower oil samples, except one, were positive for *Alternaria* toxins (tentoxin, TeA and AME). *Alternaria* toxins were also investigated in 14 sunflower seeds and 3 sesame seeds samples. One sunflower seeds sample was positive for: AOH, AME, TeA and tentoxin, while 2 sesame seeds samples contained TeA, and one was also positive for AOH and AME.

Logrieco *et al*., (1990) analyzed mandarin fruits naturally infected by *Alternaria alternata* with 2 different symptoms: black and gray heart rot. The causal agents isolated from the 2 types of heart rot differed in toxin production: AOH, TeA and AOH were all present in black rot samples, while gray rot sample contained only TeA.

Magnani *et al*. (2007) developed a method for the quantification of AOH and AME on tangerines with and without symptoms of *Alternaria* brown spot disease. In general, the amounts of AOH were 5 times greater than the amounts of AME in flavedo obtained from

fruits with symptoms. In addition, both toxins were detected in all flavedo samples obtained from fruits with symptoms of *Alternaria* brown spot. Here the levels of AOH and AME varied from 13.1 to 17.40 µg/kg and from 2.5 to 3.5 µg/kg, respectively. The levels of AOH and AME on the flavedo of fruits without symptoms varied from not detected to 3.7 µg/kg and from not detected to 0.9 µg/kg, respectively. However, neither AOH nor AME was detected in albedo samples, even on those obtained from fruits with symptoms of Alternaria brown spot. This indicates that these toxins are not accumulated inside tangerine fruits, suggesting that flavedo tissues might act as a barrier for fungal penetration.

High levels of toxins (AOH, AME, ALT and TeA) were also found in peppers (Chung *et al*., 1998).

In a study by Ostry *et al*. (2004), commercial samples of poor quality lentils collected in Canada (a crop 2002) were tested. The determination of *Alternaria* toxins was conducted in non sorted and in sorted (non damaged and damaged) lentils. The average concentration of AOH in non sorted lentils was 220 µg/kg, while after sorting in non damaged and damaged beans the concentrations were 110 and 290 µg/kg, respectively. There were only traces of ALT, AME and TeA.

Various food products may be profusely supplemented with various amounts of fresh or dried fruits and vegetables, which may be contaminated by diverse fungi, and become good substrates for the production of mycotoxins. Such supplements may subsequently cause slow toxicoses (Lugauskas *et al*., 2005).

Studying mycotoxin production in moldy core disease in “Red Delicious” apples in storage, Robiglio and Lopez (1995) isolated 11 strains of A. *alternata*. Most of the isolates produced AOH and AME in the whole fruits. Studies on the possible transfer of *Alternaria* mycotoxins from the rotten part of an inoculated fruit to the surrounding sound tissues indicated that toxins were not restricted to the rotted area; they could also be isolated from the surrounding tissue, although comparative evaluation showed that toxin levels were lower (Robiglio and Lopez, 1995).

Fruit-derived products

Scott *et al*. (1997) confirmed the presence of AOH in apple juice samples. The natural occurrence of AOH and AME was reported in commercial apple juice samples in Spain, which were analyzed by Delgado and Gómez-Cordovés (1998). A different rate of contamination was found between samples processed from apples of two different harvests. Concentrates from apples harvested in 1993 showed a higher percentage of contamination

(72%) with concentration levels of AOH ranging from 1.35 to 3.56 ng/ml in 14 out 18 samples analyzed. Concentrates from apples harvested in 1994 showed higher levels of AOH ranging from 2.99 to 5.42 ng/ ml, but in only 4 out of 14 samples (28%). AME was frequently present, but only at low or trace levels (1.71 µg/l the highest concentration). They concluded that AOH and, to a lesser extent, AME occurred naturally in apple juice concentrates used in the production of commercial reconstituted apple juices (Delgado and Gómez-Cordovés, 1998).

Lau *et al*. (2003), analyzing apple juice samples and single samples of some other fruit beverages, including grape juice, raspberry juice, cranberry nectar, prune nectar and red wine, found AOH and also AME.

Analyzing wines, and grape and cranberry juices for *Alternaria* mycotoxins, Scott *et al*., (2006) found AOH in 13 of 17 local Canadian red wines, and in all seven imported red wines; it was usually accompanied by lower concentrations of AME. White wines contained only sublevels of the two mycotoxins (≤1.5 µg/l), both of which were found stable in the wine (Scott and Kanhere, 2001). Sublevels of the two mycotoxins were also reported in 5 of 10 samples of red grape juice, except for one sample that contained AME at 39 µg/l, and in one of five samples of cranberry juice. No toxins were recorded in white grape juices.

The occurrence of AOH has also been reported in raspberry drink (Giryn and Szteke, 1995).

TeA was found by Scott and Kanhere (1980) in tomato, and AOH was recorded in tomato paste by Fente *et al*. (1998). Co-occurrence of TeA and cyclopiazonic acid, in samples of tomato purée and in samples of tomato pulp was recorded by Da Motta and Soares (2001); neither AME nor AOH was detected in the various tomato samples. It was suggested that the presence of TeA and cyclopiazonic acid in tomato products may indicate the use of infected tomatoes in the processing procedure

The occurrence of *Alternaria* mycotoxins was investigated in 80 samples of tomato puree processed and sold in Argentina (Terminiello *et al*., 2006). AOH, AME and TeA were investigated by liquid chromatography. Thirty-nine of the 80 samples showed TeA, AOH and AME contamination. Co-occurrence of two of these toxins was detected in 10 samples. Hajslova analysing some tomato products (tomato puree, peeled tomato, tomato juice and tomato ketchup) in the Czech Republic found AOH, AME and ALT.

The possible transfer of *Alternaria* mycotoxins in olive fruits was studied by Visconti *et al*. (1986) and Bottalico and Logrieco (1993). No mycotoxins were detected in olive oil destined for human consumption or in olive husks collected from oil mills after the first pressing of olives. However, an olive oil sample produced in the laboratory by processing the most contaminated olive sample contained AOH and AME. The estimated percentages of

mycotoxins transferred into the oil were about 4% for AME, 1.8% for AOH and 0% for ALT and TeA.

Table 4. Summary tables for *Alternaria* toxins occurrence and concentration in most exhaustive surveys.

Micotoxin	*N° samples*	*N > LOQ*	*Min/Max (μg/kg)*	*Commodity*
Agricultural commodities				
AOH	875	227	6/1388	Wheat, barley, oats, whole grain, straw
AME	774	249	1/7451	Sorghum, ragi,, wheat, barley, oats, whole grai, straw
TeA	132	60	nd/8814	Sorghum, ragi, wheat, whole grain, straw
ALT	169	127	nd/1500	Sorghum, ragi, wheat
Fruits and vegetables				
AOH	62	16	nd/5200	Tomatoes, olives, sunflower seeds, mandarins, tangerines
AME	62	16	nd/2870	Tomatoes, olives, sunflower seeds, mandarins, tangerines
TeA	174	81	24/173900	Tomatoes*, olives, sunflower seeds, mandarins*
ALT	13	1	1400	Olives
Derived products				
AOH	85	54	nd/19.4	Fruit derived products
	213	48	0.1/8756	Vegetables derived products
AME	53	30	nd/1.71	Fruit derived products
	133	68	0.06/1734	Vegetables derived products
TeA	162	43	<11/4021	Vegetables derived products
nd: not detected * : moldy samples				

Table 4. Results of surveys for *Alternaria* toxins showing concentrations and distribution of contamination in food and feed commodities

Country	Commodity	Year	Sampling procedure	Mycotoxins	N°. of samples	LOQ (μg/kg)	*n* > LOQ	Mean (μg/kg)	Min/Max (μg/kg)	References
Agricultural commodities										
Kansas	Grain sorghum	1973	Na	AME+AOH	60	na	na	na	0-7900	Seitz *et al*., 1975a
Kansas	Weathered discolored sorghum	1973	Na	AME+AOH	na	na	na	na	0-3000 or 5000	Seitz *et al*., 1975b
	Good quality sorghum			AME+AOH	na	na	na	na	0 to trace	
Kansas	Sorghum		Na	AME+ AOH	12	na	na	4800	2500/7900	Sauer *et al*., 1978
				ALT	12	na	3	673	120-1500	
				ATX-1	12	na	3	na	trace	
North Bihar (India)	Sorghum	1987-1988	Randomly from market and flour mills. Samples of 3-4 kg, blended and subdivided to a 1 kg sample	TeA	20	na	5	na	nd/5600	Ansari *et al*., 1990
				AME	20	na	7	na	nd/1800	
				ALT	20	na	5	na	nd/700	
	Ragi			TeA	8	na	3	na	2030/5700	
				AME	8	na	2	na	nd/1400	
				ALT	8	na	1	na	nd/30	
Germany	Wheat, barley, rye, oats, mixed cereals	na	Na	AME	642	na	25 (barley), 38 (wheat), 134 (oats)	na	6/160	Thalmann *et al*., 1987
				AOH	642	na	6 (barley), 12 (wheat), 109 (oats)	na	6/160	
Poland	Wheat	na	Na	AOH	na	na	na	na	na./600	Grabarkiewicz-Szczęsna and Chelkowski; 1993
				AME	na	na	na	na	na400	
Germany	Wheat	na	Na	AOH	na	na	na	na	na/200	Gruber-Schley S and Thalmann A, 1988
				AME	na	na	na	na	na/12	
Australia	Wheat	na	Na	AOH	na	na	na	na	na224	Webley *et al*., 1997
				AME	na	na	na	na	na/15	
				TeA	na	na	na	na	na./90	
Egyptus	Wheat	na	Na	AOH	na	na	na	na	na./2300	Abd el-Aal, 1997
				AME	na	na	na	na	na./1900	
				ATX-I	na	na	na	na	na./1700	
				ALT	na	na	na	na	na/1500	
				TeA	na	na	na	na	na/700	
China	Wheat	1998	samples (about 250 g each) representing three geographical locations were btained by visiting the farmers houses	AOH	22	50 [a]	20	335	116/731	Li and Yoshizawa, 2000
				AME	22	50 [a]	21	443	52/1426	
				TeA	22	100 [a]	22	2419	260/6432	
Czech Republic	Wheat	2003-2005	Na	AOH	129	5	60	7.7	6.3/44.4.	Skarkova *et al*., 2005
				ALT	129	5	118	18.7	6.3/41	

Country	Commodity	Year	Sampling procedure	Mycotoxins	N°. of samples	LOQ (μg/kg)	*n* > LOQ	Mean (μg/kg)	Min/Max (μg/kg)	References
Agricultural commodities (contd)										
Argentina	Wheat	2004-2005	na	TeA	64	80 [a]	12	2313	1001/8814	Azcarate *et al*., 2008
				AOH	64	50 [a]	4	1054	645/1388	
				AME	64	50 [a]	15	2118	566/7451	
na	Hay and others (including corn silage and mixed feed)		na	AAL TA	63 (25 hay and 38 others)	na	25/hay 96.8 % (feed)	720/hay 560/feed		Yu *et al*., 1999
Pennsylvania	Maize silage	2001-2002	na	AAL-TA	na	na	na	170	200/2010	Mansfield *et al*., 2007
				AAL-TB	na	na	na	50	30/900	
Czech Republic	Fibre flax, linseed,	2002-2003	Fibre flax and linseed samples obtained from small-plot field	ALT	79 fibre flax, 43 linseed	3	na	na	nd/9	Kralova *et al*., 2006
				AOH	na	8	na	na	nd/104	
				AME	na	6	na	na	nd/30	
Sweden	Whole grain, straw	2006	Collected at farm level	AOH	18	na	16	na	9/335	Haggblom *et al*., 2007
				AME	18	na	7	na	1/184	
				TeA	18	na	18	na	980/4310	
Fruits and vegetables										
Canada	Lentils	2002	Commercial samples	AOH	na	na	na	220	na	Ostry *et al*., 2004
na	Moldy tomatoes	na	Collected from processing lines of catsup manufacturers	TeA	142	na	73	na	na/70000	Stack *et al*., 1985
na	Fruit and vegetables	na		AME	22	na	1	na	250	Wittkowski *et al*., 1983
				AOH	22	na	1	na	160	
Italy	Tomatoes	na		TeA	2	na	na	na	24/7200	Bottalico and Logrieco, 1998
				AOH	2	na	na	na	traces/1274	
				AME	2	na	na	na	37/268	
France, The Netherlands, Denmark	Carrots	na	na	RAD	266	6 [a]	3	na	16/13900	Solfrizzo *et al*., 2004
				TeA	266	20 [a]	0	na	-	
				ATX-I	266	20 [a]	0	na	-	
				AME	266	10 [a]	0	na	-	
				AOH	266	5 [a]	0	na	-	
Apulia (Italy)	Olives	na	Some samples collected from the ground, some other from the tree.	TeA	13	100 [a]	2	na	109/262	Visconti *et al*., 1986
				AME	13	30 [a]	4	na	30/2870	
				AOH	13	50 [a]	4	na	109/2320	
				ALT	13	100 [a]	1	na	1400	

Country	Commodity	Year	Sampling procedure	Mycotoxins	N°. of samples	LOQ (μg/kg)or (μg/l)	*n* > LOQ	Mean (μg/kg)or (μg/l)	Min/Max (μg/kg)or (μg/l)	References
Fruits and vegetables (contd)										
Germany	Sunflower seeds			AOH	14	na	1	na	13	Kocher , 2007
				AME	14	na	1	na	4	
				TeA	14	na	1	na	1000	
				Tentoxin	14	na	1	na	880	
Basilicata (Italy)	Mandarins affected with hearth rot	1987	Mandarin fruits divided into 3 groups (2 of black and 1 of gray heart rot). Internal tissues of mandarin of each group collected up to a 100 g sample	TeA	3	na	3	94033.3	21000/173900	Logrieco *et al*., 1990
				AME	3	na	2	320	500/1400	
				AOH	3	na	2	2650	100/5200	
Brazil	Tangerines	2004	Collected in a farm in Aguai and in a market in Recife. Flavedo was manually separated from albedo. Dried at 45°C for 3 days then ground to a powder	AME	8 (3 replicate/sample)	na	6	na	nd/3.5	Magnani *et al*., 2007
				AOH	8 (3 replicate/sample)	na	6	na	nd/17.4	
na	Peppers	na	Na	AOH	na	na	na	na	nd/440000	Chung *et al*., 1998
				AME	na	na	na	na	nd/294000	
				ALT	na	na	na	na	nd/103000	
				TeA	na	na	na	na	nd/342000	
Fruits derived products										
na	Apple juice	na	Na	AOH	8	na	2	na	2.0/4.1	Scott *et al*., 1997
Spain	Apple juice concentrates	1993-1994	Samples of 2 different harvest campaign taken at random, supplied by a processing plant	AOH	32	na	16	na	nd/5.42	Delgado and Gómez-Cordovés, 1998
				AME	32	na	17	na	nd/1.71	
na	Apple juice	na	Na	AOH	11	na	11	na	0.04/2.4	Lau *et al*., 2003
				AME	11	na	10	na	nd/0.43	
	Fruit beverage			AOH	10	na	5	na	nd/5.6	
				AME	10	na	3	na	nd/1.4	
Canada	Red wine	na	Na	AOH	17	na	13	na	0.03/5.02	Scott *et al*., 2006
	Imported red wine	na		AOH	7	na	7	na	0.27/19.4	

Country	Commodity	Year	Sampling procedure	Mycotoxin	N°. of samples	LOQ (µg/kg)or (µg/l)	*n* > LOQ	Mean (µg/kg)or (µg/l)	Min/Max (µg/kg)or (µg/l)	References
Fruits derived products (contd)										
na	Tomato paste	na	Na	AOH	na	na	na	na	na/100	Scott and Kanhere, 1980
na	Tomato paste	na	Commercial samples	AOH	na	1.93 [a]	na	na	na/196	Fente *et al*., 1998
Camoinas (Brazil)	Tomato pulp	1994/1995	Acquired in local market and supermarket. A minimum of 500 g or ml taken for each sample	TeA	22	11 [a]	7	na	<11/111	Da Motta and Soares, 2001
	Tomato puree			TeA	22	11 [a]	4	na	<11/76	
Argentina	Tomato puree	2006	Acquired in local market and supermarket	TeA	80	11	23	na	<11/4021	Terminiello *et al*., 2006
				AOH	80	2	5	na	<2/8756	
				AME	80	5	21	na	<5/1734	
Czech Republich	Tomato puree	2006	Na	AOH	10	0.08 [a]	10	7.9	1.2/14.1	Hajslova, personal communication
				AME	10	0.05 [a]	10	1.3	1.2/2.7	
				ALT	10	0.27 [a]	1	0.4	na	
Czech Republich	Peeled tomato	2006		AOH	5	0.08 [a]	5	1.2	0.5/1.9	
				AME	5	0.05 [a]	5	0.2	0.2/0.3	
				ALT	5	0.27 [a]	0	-	-	
Czech Republich	Tomato juice	2006		AOH	2	0.08 [a]	2	0.4	0.1/0.7	
				AME	2	0.05 [a]	1	0.8	na	
				ALT	2	0.27 [a]	0	-	-	
Czech Republich	Tomato ketchup	2004		AOH	8	0.08 [a]	8	6.9	0.3/27.4	
				AME	8	0.05 [a]	8	1.6	0.2/5.8	
				ALT	8	0.27 [a]	1	1.2	na	
Czech Republich	Tomato ketchup	2006		AOH	21	0.08 [a]	17	1.0	0.1/3.7	
				AME	21	0.05 [a]	21	0.4	0.06/1.2	
				ALT	21	0.27 [a]	0	-	-	
Apulia, (Italy)	Olive oil	na	Na	AME	6+1	30	1	-	794	Visconti *et al*., 1986
				AOH	6+1	50	1	-	286	

[a] = LOD
na = not available
nd = not detected

MITIGATION OF *ALTERNARIA*

Alternaria diseases are best controlled by using several integrated strategies:

- Chemical control
- Biological control
- Cultivar Resistant
- Physical methods

Chemical control

Chemical control against *Alternaria* is usually carried out in the field with fungicides. Several families of fungicides, such as dicarboximide, carbamates, benzimidazoles, and triazoles are used as seed or foliar treatments in the protection of cruciferous crops against pathogenic *Alternaria* spp. in many countries.

Dicarboximide iprodione is the most effective fungicide to control *Alternaria* on citrus, where copper and mancozeb are very effective as well. Approaches that are based on using iprodione, as a mixture or in alternation with other fungicides, have been proposed by Solel *et al.* (1997), to control *Alternaria* brown spot of *Minneola tangelo*.

Azoxystrobin, pyraclostrobin, trifloxystrobin, difenoconazole, bromoconazole, and polyoxin B reduced the *in vitro* spore germination and growth of *A. alternata*.

The usage of fungicide-treated seed may help to increase stand establishment when infected seed is planted.

Sulfur and copper can be sprayed when temperatures are between 12 and 30°C and the weather is wet, to protect leaves from infection. These "least toxic" options are less effective when overhead irrigation is applied.

The fungicides azoxystrobin and boscalid are registered for control of *Alternaria* black spot on canola. Iprodione is registered for suppression of black spot.

Four fungicides alone or in combination were tested in the field for activity on *Alternaria mali.* No satisfactory control was achieved except with iprodione at 0.30 g/L. Captan and mancozeb alone and in combination with benomyl failed to control *Alternaria* blotch after artificial inoculation, whereas up to 65.1% disease reduction was achieved under conditions of natural infection compared with the nontreated control. Iprodione provided 69.1–79.8% and 71.6–75.1% disease reduction for the higher (0.30 g/L) and lower rate (0.15 g/L), respectively, under conditions of natural infection. After artificial inoculation, disease reduction ranged from 53.7 to 68.9% and 34.1 to 59.8% at the higher and lower rates of iprodione, respectively. The EC_{50} of iprodione for three isolates of *A. mali* was 1.04 mg/ml. No resistance of *A. mali* to iprodione was detected in the field (Filajdi and Sutton, 1992).

Azoxystrobin, difenoconazole, Polyoxin B, and trifloxystrobin fungicides are effective in apple orchards to control the moldy-core development produced by *Alternaria* in Red delicious apple fruit (Reuveni *et al*., 2002; Reuveni, 2006).

Different postharvest treatments to control *Alternaria* rot are recommended in fruits. The combination of prochloraz in hot water brushing further reduces the incidence of decay of mango fruit.

In some cotton growing regions of the world where disease is a problem and causes yield losses, fungicides such as maneb, mancozeb, difenoconazole and tebuconazole are used as protectant sprays (Shtienberg and Dreishpoun, 1991). Olsen and colleagues (1998), demonstrated that the number of lesions per leaf of Pima cotton was reduced significantly by the application of mancozeb and sulfur compared to the urea treatment or untreated control. Mancozeb was the most effective treatment and reduced lesions by more than 50%.

Alternaria tenuis causes black core rot of mandarin oranges (*Citrus reticulata*). It is possible to use copper sulphate, zinc sulphate, and ferrous sulphate as spray materials for trees and as a dip for ripe fruits to prevent black core rot. Concentrations of 20 ppm or more of copper sulphate, boric acid, and ammonium molybdate, however, caused significant reduction of growth. Conidia of the fungus were very sensitive to even low amounts of copper, zinc, and iron (Singit and Ki-Ianna, 1969).

A delay in fungal growth and toxin production could be observed at concentrations above 10mg/kg of sodium propionate. It is important to note that even when fungal growth was partially inhibited, toxin production was more strongly affected at low concentrations (10± 25 ppm) of this preservative (Combina *et al.*, 1999a).

Black spot disease in persimmon fruit, caused by *A. alternata*, develops primarily in the high humidity environment beneath the calyx. Three treatments with gibberellic acid (GA_3, 20 µg/ml) applied during fruit development at 30, 20, and 10 days before harvest, reduced decay more effectively than the single commercial treatment of GA_3 (50 µg/ml) applied 10 days before harvest. As a result of GA_3 treatment, the calyx of the fruit remained erect till harvest (Perez *et al.*, 1995).

Biological control

Biological control has been considered as one of most promising alternatives to fungicides, which employs microorganisms to protect fruits and vegetables from infection by phytopathogens (Wilson *et al.*, 1991; Janisiewicz and Korsten, 2002; Spadaro and Gullino, 2004).

Antagonists can be used for the biological control of crop: *A. alternata* and other pathogens cause root rot complex in peas (PRRC). A strain of *Clonostachys rosea* (syn. *Gliocladium roseum*), ACM941 (ATCC 74447), was identified as a mycoparasite against these pathogens. Results of this study suggest that ACM941 is an effective bioagent in controlling PRRC and is an alternative to existing chemical products.

An ecological approach was used to select fungal antagonists effective against the seedborne pathogens *A. dauci* and *A. radicina* on carrot. Fungal isolates belonging to *Clonostachys rosea* (biopriming) controlled pre- and postemergence death caused by *A. dauci* and *A. radicina* as effectively as the fungicide iprodione. On highly infected seeds, biopriming reduced the incidence of *A. radicina* to <2.3% and that of *A. dauci* to <4.8% while the level of both pathogens was <0.5% on bioprimed seed with a low initial infection rate. In this study, Jensen and colleagues (2004) demonstrated that biopriming with the biocontrol strain *C. rosea* IK726 facilitates priming of infected seeds without risking adverse effects on seedling establishment.

A strain of *Metschnikowia pulcherrima* (MACH1) was studied for its efficacy as a biocontrol agent against *Alternaria alternata* on apples; MACH1 outcompetes through iron depletion. This study illustrated that iron depletion by the yeast strain MACH1 under low iron conditions could reduce the growth of some postharvest pathogens *in vitro* and *in vivo* (Saravanakumar *et al.*, 2008).

Biocontrol activity of *Cryotococcus laurentii* or *Trichosporon pullulans* against postharvest decay caused by *Pennicillum expansum* and *A. alternata* in pear fruits was significantly increased when *C. laurentii* or *T. pullulans* combined with 2% (w/v) sodium bicarbonate (Hongjie *et al.*, 2004).

Elicitors such as salicylic acid (SA), oxalic acid, calcium chloride, and antagonistic yeast *C. laurentii* could significantly enhance defense related enzyme activities, such as β-1,3 glucanase, phenylalanine ammonia lyase, peroxidase, and polyphenol oxidase activity, and reduce the disease incidence caused by *A. alternata* in pear fruits (Shiping *et al.*, 2006).

The main postharvest fungal diseases of cherry tomato fruit caused by various pathogenic fungi include *A. alternata* (Feng and Zheng, 2007), which is a saprophytic pathogen of tomatoes causing postharvest black rot at high frequency (Akhtar *et al.*, 1994). Currently, several promising biological approaches that include 1-MCP (Guillen *et al.*, 2007), chitosan (Liu *et al.*, 2007), essential oils (Feng and Zheng, 2007; Tzortzakis, 2007) and microbial antagonists (Schena *et al.*, 1999; Xi and Tian, 2005) have been advanced as potential alternatives to synthetic fungicides to control postharvest decay of cherry tomato fruit.

Wang *et al.* (2008) demonstrated that the marine yeast *Rhodosporidium paludigenum* isolate had great potential in controlling postharvest disease caused by *A. alternata* on cherry tomatoes for the first time.

Essential oils can be used for biological control of the crop: both the essential oils cassia and thyme exhibited antifungal activity against *A. alternata*.

Cassia oil completely inhibited the growth of *A. alternata* at 300–500 ppm. Thyme oil exhibited a lower degree of inhibition 62.0% at 500 ppm. Cassia oil at 500 ppm reduced the percentage of decayed tomatoes. Therefore, essential oils could be an alternative to chemicals for the control of postharvest phytopathogenic fungi on fruits or vegetables (Feng and Zheng, 2007).

Resistant cultivars

There are examples in literature of the use of resistant cultivars. Saxena *et al.* (2008), report experiments in which *Pelargonium graveolens* cv. *Hemanti* somaclones, resistant to leaf blight caused by a fungal pathogen *A. alternata*, have been isolated by regenerating calli from stem nodes, selected against the fungal toxin followed by early assessment of resistant phenotype among regenerating shoots, using a leaf phytotoxicity bioassay. The calli were subjected to various concentrations of culture filtrate (0%, 4%, 8%, 12%, 16% and 20%) obtained from *A. alternata*. Resistant calli were selected and placed on the Murashige and Skoog regeneration medium) supplemented with 0.5 mg l^{-1} 6-benzyl aminopurine and 0.1 mg l^{-1} naphthalene acetic acid. The regenerants were confirmed for *A. alternata* resistance by exposing their leaves to the same concentrations of culture filtrate.

Chemical control against stem canker disease of tomatoes caused by *A. alternata* f.sp *lycopersici* is ineffective and the use of tomato disease-resistant cultivars is the most effective and reliable means of control (Vakalounakis, 1988).

Resistance of durum wheat cultivars to black point caused by *A. alternata* was evaluated in the field in northern New South Wales by Southwell and colleagues, (1980). The cultivars tested showed a range of infection levels. The most susceptible cultivars were Duramba and Gaza, while those showing the greatest resistance were Wandell, Wascana and aus 15350.

Physical methods

Postharvest cooling has given the most consistent control of postharvest decay. Cooling the fruit after harvest retains quality and prolongs shelf life. Cool as quickly as possible to 5°C or lower, but not below 0°C.

McDonald and colleagues found that by dipping rockmelons in 50, 55, 60 and 65°C water for one minute, the development of postharvest disease such as *Alternaria* spp was dramatically inhibited at all temperatures in comparison to the control (22°C) (McDonald *et al*., 2006).

Gamma-irradiation, water activity (a_w) and incubation temperature were found to affect the production of tenuazonic acid by *A. alternata* in tomato paste and juice. By increasing the irradiation doses, the dry weight as well as TeA decreased greatly until complete inhibition at 4 kGy. The greatest production of TeA occurred at 0.98 a_w (57.5 g/g and 26.3 g/g) for both tomato paste and juice, respectively, at 25°C. Changing temperature and aw altered the relative amounts of TeA produced in tomato paste und juice by unirradiated and irradiated conidia of *A. alternata*. Only a trace amount of TeA was detected at 0.98 a_w (1.50 g/g) by 3 kGy-irradiated conidia in tomato paste, while it was inhibited completely in juice. Increasing gamma-irradiation doses and decreasing water activities decreased greatly or inhibited TeA production in both tomato paste and juice (Aziz *et al.*, 2006).

The investigation of Gorawar and colleagues (2006) indicated that fungicides viz., penconazole, hexaconazole, propiconazole and mancozeb proved to be effective against *A. alternata* under *in vitro* conditions.

Processing

Some studies on the effects of processing on AOH, AME, and TeA in sunflower products have been performed. Levels of AOH and TeA decreased during ensiling sunflower seeds (Dalcero *et al*., 1997); about half the AME, but no AOH and only 2% of the TeA, was transferred from sunflower seed meal into oil (Combina *et al*., 1999b). On heating sunflower flour at 100°C for 90 min, AOH and AME were stable but half the TeA was lost (Chulze *et al*., 1995). Because sunflower seeds are commonly eaten with minimal processing, their analysis as packaged for the consumer is needed.

Mansfield *et al*. (2007) wanted to detect and quantify fumonisins B_1 and B_2 and *Alternaria* toxins (AAL toxins) AAL-TA and AAL-TB and determine how agronomic practices, weather conditions, and ensiling affected the occurrence and levels in maize silage. Silage was collected in 2001 and 2002 at harvest and after ensiling from 30 to 40 dairies, from four regions in Pennsylvania. *Alternaria* toxins AAL-TA and AAL-TB were found with a frequency of 23% and 13%, respectively. Temperature during maize development was negatively correlated with AAL-TA occurrence and levels, while moisture events were positively correlated with AAL-TA. Ensiling did not affect toxin concentration nor did agronomic practices (tillage system, inoculant use, or silo type) or silage characteristics (dry matter, pH,

or organic acid concentration). In the years 2002–2003, Kralova *et al*. (2006) analysed fibre flax, linseed and pea seed samples grown under organic and/or conventional farming conditions.

Prevention practices

Mycotoxin-producing mold species are extremely common, and they can grow on a wide range of substrates under a wide range of environmental conditions. For agricultural commodities, the severity of crop contamination tends to vary from year to year based on weather and other environmental factors.

Preharvest

Alternaria is spread by splashing water and by walking through plants when wet, so good agricultural practise include the following strategies:

- keep water off leaves,
- water with soaker hoses or drip irrigation,
- maintain adequate nitrogen levels,
- remove and destroy infected leaves.
- use disease-free seeds of high germinability. If seeds from an infected crop must be used, they should be cleaned thoroughly to remove shrunken, severely infected seed before planting.
- rotate with non-host crops for at least three years. Control volunteers and weeds (e.g. shepherd's-purse, wild mustard), and especially stinkweed during the rotation.
- incorporate infected stubble into soil thoroughly.
- swath badly infected crops in time to prevent serious losses from shattering and seed shrinkage.
- Focus on nutrient programs that improve fruit quality. Apply calcium sprays to improve fruit resistance to disease.
- Control insects that may act as vectors of disease spores.
- Prune out cankers in varieties or blocks prone to bull’s eye rot.
- Prune for increased air flow to promote faster drying and better spray penetration.
- Practice orchard sanitation and “soft handling” of fruit.

Postharvest

Post-harvest decay control is an integrated pest management strategy. It is advisable to:

- Harvest fruit at proper maturity.
- Not put windfalls into bins.
- Use clean bins. As much as practical, minimize the amount of soil and plant debris brought into the packinghouse on the bottom of bins.
- Keep fruit cool after harvest, i.e. keep bins in the shade.
- Minimize time between harvest and delivery of fruit to the packinghouse.

PHARMACOKINETICS

Only a few studies in the peer-reviewed literature have examined the toxicokinetics and metabolism of *Alternaria* toxins.

Absorption, Distribution and Excretion

Pollock *et al*. (1982) reported that in adult male rats, upon oral administration of ^{14}C-alternariol monomethyl ether (AME) in olive oil, total excretion of radioactivity in feces and urine was 94.1% of the dose within three days. AME concentration was also measured in several rat tissues: it was mainly concentrated in the gastrointestinal tract (1.5-5.8 ppm), in fat (3.4 ppm), and in the kidney and liver (2-3 ppm). AME levels in other tissues were <1ppm. This study also showed that AME is extensively metabolized; in particular, AME is o-demethylated to alternariol (AOH).

Metabolism

Recently, Pfeiffer *et al*. (2007a) incubated AOH e AME with microsomes from rat, human and porcine liver and detected several oxidative metabolites. AME, as well as AOH, were monohydroxylated and AME was also demethylated to AOH. Furthermore, these investigations showed that both toxins undergo extensive metabolism and conjugation with glucuronic acid *in vitro*. AOH was glucuronidated with higher efficiency than AME, whereas the opposite was observed for hydroxylation. The majority of the oxidative metabolites are catechols, which are highly reactive and can cause cell damage by redox cycling or reaction with critical nucleophiles. Additionally, Pfeiffer *et al*. (2007b) have reported that AOH and AME caused a dose–dependent induction of DNA strand breaks in human liver cell line HepG2 cells but not in human colon HT-29 cells, due to the fact that HT-29 cells exhibited much higher enzymatic activity than HepG2 cells, giving rise to a higher concentration of glucuronides.

The same authors have also shown that altenuene (ALT) and isoaltenuene (iALT), when incubated with microsomes from rat, pig and human liver in the presence of the NADPH-

generating system, exhibited a propensity to metabolic hydroxylation reactions, suggesting that the same oxidative metabolites may be formed under *in vivo*-like conditions (Pfeiffer *et al*., 2008).

Carry over

Although *Alternaria* mycotoxins contamination has been naturally found in food, such as tomatoes, carrots, wheat and other grains, and fruits, also in their processed products (Scott, 2001), and in feedstuffs, such as poultry feed (Benkerroum and Tantaoui-Elaraki, 2001, Labuda and Tancinova, 2006), there are no available studies about their carry over and their effects on humans.

TOXICITY

Alternaria toxins is of concern, because some of the toxins are fetotoxic and teratogenic towards mice and hamsters, and cytotoxic towards bacterial and mammalian cells (Woody and Chu, 1992; Visconti and Sibilia, 1994; Bottalico and Logrieco, 1998). Culture extracts of *A. alternata* were mutagenic in various microbial and cell systems and tumorigenic in rats (Liu and Cheng, 1984; Dong *et al.*, 1987).

Acute toxicity

TeA administered to mice caused diarrhea, muscle tremor and convulsion, with a LD50 between 125-225 and 81-115 mg/kg, for male and females, respectively. TeA is a acutely toxic for other animal species, and the following LD50 have been reported: day-old chicks 37.5 mg/kg (oral); dogs 2.5 to 10 mg/kg (oral); rats 168 mg/kg or 157 mg/kg (oral or intravenous). Monkeys (up to 50 mg/kg) were not affected, but at higher doses emesis, diarrhea and gastrointestinal hemorragies were observed (Woody and Chu, 1992; Yekeler *et al*., 2001).

ALT was the most toxic dibenzo-α-pyrones when given in single dose for three consecutive days orally (100 mg/kg/day) or administered intraperitoneally to mice. The LD50 values were found to be 50, 400 and 400 mg/kg for ALT, AOH and AME, respectively (Pero *et al*., 1973).

Chronic toxicity

Animals

Sauer *et al*. (1978) fed diets high in AME and AOH and ALT (24, 39 and 10 µg/g, respectively) to chicks and rats for 21 days and did not observe any toxicological effect. Feeding studies on day-old chicks with a diet supplemented with AME at doses up 100

mg/kg, showed no loss of weight gain or mortality, confirming the very low toxicity of this mycotoxin in poultry (Griffin and Chu, 1983).

Humans

Alternaria produces spores of between 20-200 μm in length and 7-18 μm in width. Therefore, if inhaled, they can be deposited in the nose, mouth and upper respiratory tract. Exposure to airborne *Alternaria* spores, above all on immunocompromised patients, has been associated to different airway diseases: asthma, pneumonitis, allergies, sinusitis and some skin pathologies: deratomycosis, onychomycosis, subcutaneous phaeohyphomycosis, invasive infection. Acute symptoms include edema and bronchiospasms, chronic cases may develop pulmonary emphysema (De Lucca, 2007; www.mould.ph/alternaria_mould.htm). However, *Alternaria* is not as commonly found in infections of healthy individuals as are other fungi (De Lucca, 2007). Finally, signs of neurotoxicity (weakness and numbness in legs, dizziness, loss of memory, light-headedness, vertigo, fatigue, getting lost in familiar territory, and confused thoughts) were observed in 12 white office female workers chronically exposed to a toxicogenic mold including different fungi spores, including Alternaria Tenuis (Kanu *et al*., 2006).

Developmental and reproductive toxicity

Only a few data are available on the developmental toxicity of *Alternaria* mycotoxins. Griffin and Chu (1983) have determined the influence of TeA on chicken embryonic development and have showed that TeA induced embryonal death (LD_{50} = 548 μg per egg), but elicited no teratogenic effect at either lethal or sublethal dose levels. Studies in pregnant mice showed that AOH, but not AME, was able to induce a teratogenic/fetotoxic effect (100 mg/kg). Both alternariols caused a significant increase in dead and resorbed fetuses, as well as a decrease in mean fetal weight and fetal malformation (Pero *et al*., 1973). Furthermore, alternariol, AME and ALT assayed on 7-day-old chicken embryos, caused no teratogenicity or mortality at single doses up to 1, 0.5 and 1 mg per egg, respectively (Griffin and Chu, 1983).

Tiemann *et al*. (2009) have reported that the mycotoxins AOH, AME, but not TeA, decreased progesterone (P_4) synthesis in porcine ovarian cells *in vitro*. In view of the fact that granulosa cells directly influence the metabolic and structural growth of the oocytes (Tiemann *et al*., 2009), exposure to AOH and AME may affect reproductive performance by interfering with follicular development in swine and possibly other mammalian species.

Genotoxicity and carcinogenicity

Exposure to *Alternaria* toxins has been related to esophageus cancer. Liu *et al*. (1991) indicated that *A. alternata* had tumorigenic effects and argued that *A. alternata* contamination in regions where high esophageal cancer incidence is observed was higher than in regions with low esophageal cancer incidence. Thus, the investigator had established a possible link between exposure to *A. alternata* and human esophageal cancer. Yekeler *et al*. (2001) have shown that in animals fed with mycotoxins (e.g. AOH, AME and TeA) for 10 months, precancerous changes were evident in aesophageal mucosa, suggesting that progression to esophageal cancer might occur, when exposure to mycotoxins occurs for a long period of time (Yekeler *et al*., 2001). Even if esophageus cancer could be induced by deposition of Alternaria spores along the airways, a possible role of them introduced in food cannot be completely excluded.

Scott and Stoltz (1980) reported a mutagenic activity of several *Alternaria* metabolites determined on *S. typhimurium* strains by the Ames test with the following findings: AOH, TeA and iso-TeA, nonmutagenic; AME, weak mutagen; ATX-I and ATX-II, significant mutagenic activity. These earlier findings have been confirmed by several successive studies.

ATX-I had mutagenic activity in TA98, and ATX-II in TA100 (Woody and Chu, 1992). Dibenzo-α-pyrones, and in particular AS and AOH, were mutagenic to TA100; AME was mutagenic to TA98, while TeA and its derivatives were neither mutagenic nor toxic to TA100 (Woody and Chu, 1992).

With regard to the mutagenic activity of altertoxins, Stack *et al*. (1986) have compared the activity of ATX-I, ATX-II and ATX-III to that of aflatoxin B1, the well-known mutagenic and carcinogenic mycotoxins of *Aspergillus flavus*. Data from this study indicated that all altertoxins were mutagenic in the following, decreasing order: ATX-III, ATX-II and ATX-I; however, the potency of ATX-III was 10-fold lower than that of aflatoxins B1 (Stack *et al*., 1986; Stack and Prival, 1986).

Osborne *et al*. (1988) assayed the activities of ATX-I and ATX-II in the Raji cell Epstein-Barr virus early antigen (EBV-ES) induction system and in the murine fibroblast cell transformation system. Both altertoxins induced fibroblast transformation, and increased the activation of EBV-EA expression, suggesting a mutagenic/carcinogenic role of these molecules.

It has been reported that AOH induce DNA single-strand breaks in closed circular double-stranded supercoiled DNA (Xu *et al*., 1996) and in cultured primary rat hepatocytes (Liu *et al*., 1982). Furthermore Brugger *et al*. (2006) have demonstrated that AOH (10 μM) gave rise to a significant and concentration-dependent induction of HPRT and TK mutations in V79

cells and in mouse lymphoma L5178Y cells, confirming the mutagenicity of this mycotoxin. Finally, Fehr *et al*. (2009) have identified AOH as a topoisomerase I- and II- poison, which might cause, or at least contribute to, the impairment of DNA integrity in human colon carcinoma HT-29 cells.

Cytotoxicity

Altertoxins

Among the alternariolic mycotoxins, the most toxic in HeLa cells was found to be ATX-II (ID_{50} = 0.5 mg/ml), followed by ATX-I (ID_{50} = 20 mg/ml). A similar pattern of toxicity was observed in Chinese hamster lung fibroblast (V79); however, toxicity was 100-fold higher than in HeLa cells, and noncytotoxic levels were found to be < 0.02, 0.2 and 5 µg/ml for ATX-II, ATX-III and ATX-I, respectively (Sinha and Bhatnagar, 1998; Pero *et al*., 1973).

Dibenzo-α-pyrones

Dibenzo-α-pyrones were found to be less toxic in HeLa cells than ATX-II, with ID_{50} values of 6, 8-14 and 28 µg/ml for AOH, AME and ALT, respectively (Pero *et al*., 1973). The same ID_{50} was reported for AOH on mouse lymphoma cells (L5178Y) (Sinha and Bhatnagar, 1998).

It has been also reported that AOH competitively inhibits rat brain acetylcholinesterase *in vitro* (Mohammed *et al*., 1974). Another reported *in vitro* effect of these toxins is induction of lipid peroxidation in the epithelium of foetal esophagus *in vitro* (Liu *et al*., 1992).

Recently, Lehmann *et al*. (2006) have described the estrogenic potential of AOH in the human endometrial adenocarcinoma Ishikawa cell line. The same investigator also reported a modest induction of micronuclei in cultured human Ishikawa cells, and a pronounced induction of micronuclei in Chinese hamster lung fibroblast (V79). In V79 cells treated with AOH, an arrest of cell cycle in the G2 and S-phases was reported, as a defence response to a genotoxic challenge (Lehmann *et al*., 2005).

The reported estrogenic activity of AOH (Lehmann *et al*., 2006) was not in agreement with data reported by Wollenhaupt *et al*. (2008), who could detect neither the binding of AOH to cytosolic ER, nor an affect of AOH on the levels of ERα. The same investigators, in porcine endometrial cells cultured with AOH, also described a decrease of phosphorylation of eIF4E, a protein fundamental to form a molecular complex which mediates the cap function during the translation initiation process. Additionally, they have showed that AOH (3.12 µM) was able to inhibit the viability of porcine endometrial cells and decreased the levels of α-tubulin. At higher concentrations (12.5 µM) AOH caused a significant reduction of cells in the S phase together with the arrest of the cells in the G_0/G_1 phase.

Due to the fact that some aspects of the frog skin physiology can be considered homologous to mammalian kidney nephron and in particular frog skin actively transports sodium ions from the external environment of the animal into the blood (Sinha and Bhatnagar, 1998), many studies carried out with AOH showed that that this mycotoxin decreased sodium transport through frog skin, suggesting a toxic inhibition of AOH on cell metabolism (Sinha and Bhatnagar, 1998).

Tenuazonic acid (TeA)

The main mode of action of TeA appears to be the inhibition of protein synthesis, by suppressing the release of newly formed proteins from the ribosomes into the supernatant fluid (Lawley, 2009).

Tenuazonic acid has significant antitumoral activity when assayed in human adenocarcinoma-1 (Had-1) grown in embryonated eggs (Sinha and Bhatnagar, 1998).

Zhou and Qiang (2008) examined the cytotoxicity of tenuazonic acid in three mammalian cell lines. TeA inhibited the proliferation, and decreased total protein content in 3T3 mouse fibroblasts, Chinese hamster lung cells (CHL cells) and human hepatocytes (L-O2 cells) at concentrations ranging from 12.5 – 400 μg/ml. Of the three cell lines, 3T3 cells were the most sensitive to the toxin (EC_{50} = 34.75 μg/ml), followed by CHL cells (EC_{50} = 63.44 μg/ml) and L-O2 cells (EC_{50} = 117.77 μg/ml).

Alternaria alternata f. sp. lycopersici toxins (AAL-toxins)

Alternaria alternata f. sp. lycopersici toxins are plant specific phytotoxins, which are structurally similar to fumonisin B_1, and cause necrotic lesions in genetically susceptible tomato lines (De Lucca, 2007). Fumonisin B_1 and AAL-toxins are sphinganine-analog mycotoxins (SAM_S) and competitively inhibit *de novo* sphingolipid (ceramide) biosynthesis *in vitro*, which leads to a variety of cellular responses, including the accumulation of sphingoid bases in animal cells and in plants (Brandwagt *et al*. 2001). It has been reported that AAL-toxins induce apoptosis in some mammalian cell lines and in sensitive tomato lines (Brandwagt *et al*. 2001).

LEGISLATION ON ALTERNARIA

There are no regulatory limits set for *Alternaria* metabolites (Moss, 2008).

CONCLUSIONS

Alternaria is a common genus with a number of species that can invade crops at the pre- and post-harvest stage and cause considerable losses due to rotting of fruits and vegetables. Under suitable conditions it may lead to production of a range of mycotoxins as well as other less-toxic metabolites. *Alternaria* toxins exhibit both acute and chronic effects, and they have been implicated in animal and in human health disorders. Most *Alternaria* mycotoxins exhibit considerable cytotoxic, carcinogenic, foetotoxic, teratogenic, antitumoral, antiviral and antibacterial activity.

Most *Alternaria* species are saprophytes that are commonly found in soil or on decaying plant tissues. Some species are (opportunistic) plant pathogens that, collectively, cause a range of diseases with economic impact on a large variety of important agronomic host plants including cereals, ornamentals, oilcrops, vegetables such as cauliflower, broccoli, carrot and potato, and fruits like tomato, citrus and apple. *Alternaria* spp. are also well known as post-harvest pathogens. Some *Alternaria* spp. are of clinical significance as they are well known for the production of toxic secondary metabolites, some of which are powerful mycotoxins that have been implicated in the development of cancer in mammals. *A. alternata* in particular is gaining prominence as an emerging human pathogen, especially in immunocompromised patients. In addition, *Alternaria* spores are one of the most common airborne allergens.

No official validated methods and certified products with a known contamination are available for *Alternaria* metabolites analysis so as no regulatory limits.

There are currently no statutory or guideline limits set for *Alternaria* mycotoxins because surveys to date have shown that their natural occurrence in foods is very low and the possibility for human exposure is very limited. The need for regulation is kept under review as new information becomes available. Indeed it is important to have policies in place that ensure that mycotoxin contamination levels do not pose a significant hazard to human health.

FUTURES

- The development of official validated methods for *Alternaria* metabolites analysis are strongly suggested.
- Surveys to check the occurrence of Alternaria toxins to ensure that contamination level do not pose a significant hazard to human health.

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Chapter 4 - Moniliformin in food and feed

Keywords: Moniliformin, *Fusarium*, Biosinthesys, Maize, Cereals, Methods of analysis, Validation, Toxicity, Mitigation.

INTRODUCTION

Moniliformin (MON) was first reported in 1973 (Cole *et al.*, 1973) from a culture of *F. moniliforme* [*Gibberella fujikuroi*], isolated from naturally infected maize in the USA. ON was isolated and chemically characterized (sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione) from *F. nygamai* Burgess & Trimboli NRRL-6022 (ATCC-12763) isolated from millet in Nigeria (Marasas *et al.*, 1988a; Springer *et al.*, 1974). This strain has been reported to produce large amounts of MON (Steyn *et al.*, 1978). Subsequently, another strain, *F. proliferatum* (Matsushima) Nirenberg NRRL-6322 isolated from raw cotton in North Carolina in the USA. has been used in the production of MON for toxicity studies (Burmeister *et al.*, 1979; Marasas *et al.*, 1988b).

MON is produced at least by thirty *Fusarium* species, isolated from different substrates and geographical areas (Abramson *et al.*, 2001; De Nus *et al.*, 1996; Fotso *et al.*, 2002; Schütt *et al.*, 1998).

In addition to *F. proliferatum*, the *Fusarium* species known to produce significant amounts of MON include *F. avenaceum*, *F. fujikuroi*, *F. nygamai*, *F. pseudonygamai*, *F. subglutinans*, and *F. thapsinum* (Desjardins *et al.*, 1997; Leslie *et al.*, 1996). The literature concerning production of MON by *F. verticillioides* is confounded by misidentification of the strains examined (Burmeister *et al.*, 1979; Leslie *et al.*, 2005; Marasas *et al.*, 1988a,b). Due to taxonomic refinement, it seems clear that the species now recognized as *F. verticillioides* does not generally produce significant levels of MON even though occasionally some strains may produce the mycotoxin when grown on maize (Rabie *et al.*, 1982). The confusion mainly centers on *F. proliferatum* and *F. thapsinum*, which were previously lumped with *F. verticillioides* in the species *F. moniliforme* (Leslie *et al.*, 1996).

MON appears to be a growth regulator in plants and is phytotoxic in corn and tobacco (Vesonder and Golinski, 1989). MON is highly toxic and results in rapid death in chicks and rats. Although MON was discovered more than 3 decades ago (Cole *et al.*, 1973), active investigations on the toxic effects of MON in poultry have been initiated only in recent years. Cardiac injury, with alterations in the cardiac electrical conductance, was shown to be a primary cause of mortality in birds (Nagaraj *et al.*, 1996; Reams *et al.*, 1997). Prolonged feeding of birds with MON has been shown to cause poor growth performance, increased serum pyruvate levels, and cardiopathy (Ledoux *et al.*, 1993; Morris *et al.*, 1997; Reams *et al.*, 1997). Acute mortality and gross lesions, including ascites, hydropericardium, and myocardial pallor, have been observed in broilers, turkeys, and ducklings (Engelhardt *et al.*, 1989)

FUSARIUM AND ITS HOSTS

The genus *Fusarium* includes several species which are important pathogens of maize (*Zea mais*) and other cereals, causing root, stem and ear rot, with severe crop yield reduction of economic relevance, often estimated at between 10 and 30% (Golinski *et al.*, 2002; Logrieco *et al.*, 2002; Uhlig *et al.*, 2007). In addition, certain isolates are also capable of producing mycotoxins which can be accumulated in infected plants or in stored grains (Chelkowski, 1989).

Fusaria are widespread in all cereal-growing areas of the world, but there are some geographical differences in the natural distribution of the *Fusarium* species, as well as of their corresponding mycotoxins, which are influenced primarily by environmental conditions, crop production and storage methods.

Fusarium species are responsible for at least two kinds of maize ear rot, roughly differentiated as red ear rot or red fusariosis, mainly caused by species of the *Discolour* section, and pink ear rot or pink fusariosis mainly caused by representatives of the *Lyseola* section (Chelkowski, 1989). The predominant species causing maize red ear rot are *F. graminearum* (teleomorph *Gibberella zeae*), *F. culmorum*, *F. cerealis* and *F. avenaceum* (teleomorph *G. avenacea*). Red fusariosis is predominant in years and regions characterized by frequent rainfall and low temperature during summer and early fall. Actually, *F. graminearum,* which is not known to produce MON, is increasingly distributed from central to northern European areas, and is occasionally associated with many additional *Fusarium* species depending on the climate conditions:

- *F. avenaceum,* a MON producer, in the north of Europe ;
- *F. culmorum* and *F. cerealis*, which are more common in central European areas;
- *F. subglutinans*, which can predominate in some regions of Europe such as Austria and Poland and has been proved to produce MON (Desjardins *et al.*, 2006).

Pink ear rot prevails in the drier and warmer climates of southern areas of Europe; it is essentially due to the anamorphs of the rather rare *Gibberella fujikuroi*, namely, *F. verticillioides* (syn. *F. moniliforme*), associated with *F. subglutinans*, predominant in the central areas, and to a lesser extent with *F. proliferatum* which is more common in the southern European areas (Logrieco *et al.*, 2002).

Fusarium head blight (FHB) is the most serious fungal disease affecting wheat production in the world (Duveiller *et al.*, 2008; McMullen *et al.*, 1997; Windels, 2000). It is associated to a complex of fungi, mainly *F. graminearum*; the species predominantly found associated with FHB in Europe are *F. culmorum*, *F. avenaceum*, *F. sporotrichiella* (Bottalico, 1998). The

differences in the diffusion of the diverse species are due to temperature requirements, as seen in the previous paragraph.

The *Fusarium* species more frequently associated with MON production are listed in Table 1:

Table 1. *Fusarium* species more frequently associated to MON production.

***Fusarium* species**	**Teleomorph**	**Host of primary concern**	**Endemic geographical regions**	**Other mycotoxins* produced**
F. acuminatum	*Gibberella acuminata*	Small grains	Worldwide	T2
F. avenaceum	*Gibberella avenacea*	Maize, small grains	Worldwide	BEA, ENN, FUS
F. chlamydosporum		Maize, small grains	Worldwide	
F. fujikuroi	*Gibberella fujikuroi*	Rice	Worldwide	GB, BEA, FB
F. napiforme		Millet, sorghum	Africa, Argentina	FB
F. nygamai	*Gibberella nygamai*	Sorghum	Africa, Australia	FB, BEA
F. oxysporum		Maize, small grains, asparagus		BEA
F. proliferatum	*Gibberella intermedia*	Maize, small grains, bananas	Worldwide	FB, BEA, FP
F. pseudonygamai		Millet	Africa	FP
F. subglutinans	*Gibberella subglutinans*	Maize	Worldwide	BEA, FP
F. thapsinum	*Gibberella thapsina*	Sorghum	Worldwide	
F. tricinctum		Maize, small grains	Worldwide	BEA
F. verticillioides	*Gibberella fujikuroi*	Maize, millet, sorghum, bananas	Worldwide	FB, FUS

*BEA: beauvericin; FB: fumonisin B_1, B_2 and B_3; FP: fusaproliferin; FUS: fusarin C; EN: enniantin; GB: gibberellins; small grains= wheat, rye, barley, oat, rice.

Data from: Bottalico, 1998; Bottalico and Perrone 2002; Glenn, 2007; Logrieco *et al.*, 2002; Peralta Sanhueza and Degrossi, 2004.

Although there are several species able to produce MON on a wide variety of hosts, in Europe the problem is strongly linked to the presence of *F. subglutinans* in the maize ear rot complex, and *F. avenaceum* in the *Fusarium* head blight of wheat.

Infection cycle

No detailed information is available on the infection cycle of *F. subglutinans* and *F. avenaceum*, the two principal MON producers in maize and wheat respectively. Nevertheless, some general information about infection cycle of *Fusaria* can be given.

Generally speaking, the optimal environmental conditions for the production and the dispersal of inoculum vary, depending on whether the fungus reproduces sexually and/or asexually. *F. graminearum, F. avenaceum, F. verticillioides, F. proliferatum* and *F. subglutinans* reproduce both sexually and asexually, while only asexual conidial reproduction has been observed in *F. culmorum* and *F. poae*.

Fusaria ear rot pathogens that have sexual reproduction overwinter in crop residues as a mycelium and then form perithecia in spring and early summer as wheat anthesis occurs (Shaner, 2003). High humidity is required for the initial release of ascospores, although dry periods may be required for their forceful discharge into the air from perithecia by wind (Parry *et al.*, 1995). Because of the similarities between the different species of *Fusaria* and the impossibility of describing the characteristics of all the stains above mentioned, we can use *F. gramineaurum* as an example. *F. graminearum* discharge the ascospores usually between 10° and 30°C, with an optimal temperature of approximately 16°C, and this is triggered by a drop in air temperature accompanied by a rise in relative humidity (60-95%), although subsequent dry periods may be required for a powerful discharge from the perithecia (Doohan *et al.*, 2003). Ascospore discharge is more abundant 1-4 days after rainfall exceeding 5 mm. Moisture is required in the form of high relative humidity, but saturation suppresses ascospore discharge (Paulitz, 1996). Other species utilize asexual macroconidia as primary inoculum since their sexually reproduction is not known.

The influence of climatic conditions on the incidence of *Fusarium* species is probably both direct (e.g. an effect on mode of reproduction) and indirect (e.g. an effect of soil and vegetation type). The production and dispersal of inoculum is strongly affected by temperature, humidity, light intensity and wind (Doohan *et al.*, 2003); chlamydospores are produced by some species in response to adverse conditions (Griffiths, 1974). The switch

between the production of ascospores, macroconidia, mesoconidia, microconidia and chlamydospores formation may well be related to both nutritional and environmental factors (Doohan *et al.*, 2003).

As far as MON producers is concerned, *F. avenaceum* rarely produces microconidia and does not produce chlamydospores while *F. subglutinans* produces oval to spindle-shaped microconidia and chlamydospores (Desjardins, 2006).

Generally speaking there are different pathways for *Fusarium* infection:

1. the conidia are air- or splash-dispersed and infect ears through silks or wounds (Koehler, 1942; Kucharek and Kommendahl, 1966, Ooka and Kommendahl, 1977). Macroconidia seem to be more suited for splash rather than wind dispersal (Parry *et al.*, 1995) and on wheat their number progressively increases with rainfall (Rossi *et al.*, 2002);
2. the pathogen systemically develops within the plant. Systemic infection can rise from root, stalk or leaf sheath penetration, or from seed transmission (Comerio *et al.*, 1999; Marin *et al.*, 1996; Munkvold and Carlton, 1997);
3. conidia reach ears via spore-carrying insects, mainly the European corn borer larvae, *Ostrinia nubilalis* (Le Bars *et al.*, 1994), which move inside systemically infected maize stalks or penetrate through bracts.

Wheat, maize and other grain crop residues are generally regarded as the primary source of inoculum for *Fusarium* species (Cotten and Munkvold, 1998). Asexual conidia landing on the silks and growing down to the developing kernels are the primary infection pathways for maize ear rot while ascospores produced from the homothallic reproduction of *G. zeae* and formation of perithecia on the residue of wheat and maize trigger the development of wheat head blight (Shaner, 2003). *F. avenaceum* has a wide distribution in soil and soils debris, mainly in cooler climates.

After the dispersal of inoculum, germination, growth and competition between *Fusaria* are dependent upon the availability of nutrients and environmental factors such as temperature, pH, humidity, aeration and light. In general, warm humid conditions are suitable for good germination and the temperature of growth is different depending on the species considered (Doohan *et al.*, 2003).

Fusarium head blight is a monocyclic disease for which ascospores of *G. zeae* from residue-born perithecia are known to be the only significant source of initial inoculum (Bujold *et al.*, 2001; Dill-Macky, 2008) while the fusariosis of maize can cause secondary infections through infected tassels that produce conidia which can infect ears through silks and wounds.

Ecology

The factors contributing to the development of *Fusarium* head blight and ear rot and subsequent mycotoxin contamination include temperature, relative humidity, amount and timing of rainfall, wind patterns, niche competition (Glenn, 2007). The influence of meteorological factors on *Fusarium* diseases is complicated by the fact that *Fusarium* fungi can cause disease individually or in complex infections (Doohan *et al.*, 1998). In general, optimal conditions for development of *Fusarium* head blight of small grains require moderately warm temperatures and wet weather at anthesis. The same general conditions are necessary for the development of *F. graminearum* ear rot of maize, including high rainfall during maturation (Munkvold, 2003a). Optimal conditions for development of corn ear rot by *F. verticillioides* are warm to hot temperatures and dry conditions at silking and during grain-filling (Doohan *et al.*, 2003; Munkvold, 2003a).

As far as the requirements of *F. subglutinans* are concerned, Castellà *et al.* (1999) found that the optimum conditions for growth occurred in maize cultures between 20° and 25°C and in rice cultures at 15°C. More widely investigated was the incidence and behaviour of *F. subglutinans* on pineapple. In this case, *F. subglutinans* isolated and grown on pineapple plants, exhibited mycelial growth of the fungus in the 10-30°C range, with 25°C being considered the optimal temperature for mycelial development, while sporulation occurred between 15 and 35°C and it was shown that its increase was directly related to temperature increase (Martelleto *et al.*, 1998); the key factor for spore dispersal was a period of rising temperature and declining relative humidity (Matos *et al.*, 1997). Finally, the higher the number of hours of temperatures below 23°C during flowering, and rainfall from flowering to harvest, the higher the disease incidence on fruits. On the other hand, increasing the number of hours of temperatures over 28°C during the inflorescence development decreases the fusariosis incidence on pineapple fruits. The rain effect on the disease incidence may be due to its possible involvement in inoculum production and dissemination; actually, the positive correlation between rainfall intensity and temperature ranging from 23 to 27°C and fusariosis incidence on pineapple indicates that those two environmental factors play an important role in inoculum build up and dissemination before inflorescence reaches its period of high susceptibility to the pathogen (Matos *et al.*, 2000).

As regards *F. avenaceum*, it has been isolated from diseased grain ears over a range of climatic zones, but it is usually predominant in the colder areas of Northern and Central Europe and in Canada (Uhlig *et al.*, 2007). As already said, *F. avenaceum* can reproduce both sexually and asexually (Parry *et al.*, 1995), but high daytime temperatures (30° vs 20°C) caused the formation of a greater proportion of macroconidia, and lowered the abundance of

mesoconidia in *in vitro* cultures (Winder, 1999). The minimum temperature reported for infection of wheat kernels by *F. avenaceum* was 14°C, the optimum 28°C whereas the maximum was 35.5°C. The influence of relative humidity (RH) on *F. avenaceum* infection was irregular: from the 64.8% of glumes infected at 100%, dropping to traces between 95% and 75% RH, and concerning 6.7% of glumes at 65% (Rossi *et al.*, 2001).

For both *F. subglutinans* and *F. avenceum* growing on wheat, rye, barley, oats, maize the optimum condition for MON production are warm temperatures (25-30°C) (Kostecki *et al.*, 1999; Schutt, 2001). MON production by *F. subglutinans* isolates from maize was higher at 30° than 20° or 25°C and on rice rather than on wheat, rye, barley, oat or maize grains (Kostecki *et al.*, 1999). The production of MON by *F. avenceum* on wheat is more influenced by temperature, with higher levels of toxin produced under Mediterranean rather than temperate conditions (Schutt, 2001).

No specific information about the water activity (a_w) requirements of these two species are available, but generally *Fusaria* have a minimum of 0.90 a_w for growth and a higher request for mycotoxin production.

In Table 2 the thermal requirements of these two species are summarized.

Table 2. *F. avenaceum* and *F. subglutinans* thermal requirements.

	F. avenaceum	*F. subglutinans*
Growth	15-35°C	10-30°C
Sporulation	14-35.5°C	15-35°C
Toxin production	25-30°C	25-30°C

Plant-pathogen interaction

Plants have a variety of defensive mechanisms exhibited in response to challenges by plant-pathogenic fungi. One strategy is to release low molecular weight compounds that either kill or inhibit the growth of invading fungi (Osbourn, 1999; Van Etten *et al.*, 1994). Maize, wheat and rye produce a class of antimicrobial compound, benzoxazinoids, as part of their normal developmental routine (Niemeyer, 1988). These are cyclic hydroxamic acids, named DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin3-one) and DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin3-one), that are highly reactive (half-life $\leq$ 24h) and spontaneously degrade to the corresponding benzoxazolinones MBOA (6-methoxy-2-benzoxazolinone) and BOA (2-benzoxazolinone). Many insects, fungi and bacteria are deterred or inhibited by these compounds, resulting in increased plant resistance; yet, *F. verticillioides* detoxifies MBOA and BOA within 24 h by their active metabolisation (Glenn *et al.*, 2001, 2003). As *F.*

verticillioides, in decreasing order, also *F. subglutinans* and *F. graminearum* are able to metabolise MBOA and BOA (Glenn *et al.*, 2001). Data from different studies suggest that this ability in detoxification makes *Fusarium* spp. able to gain advantage over other pathogens of maize, increasing their niche colonization success (Glenn *et al.*, 2003; Fomsgaard *et al.*, 2006; Fritz and Braun, 2006). In this way and by the production of chemical compounds attractive to insects and pests, *F. verticillioides* may promote infestation of the plant by lepidoptera and coleoptera pests (Bartelt and Wicklow, 1999; Cardwell *et al.*, 2000; Schulthess *et al.*, 2002). Once attracted, insects can damage the ears, thus providing *F. verticillioides* and other *Fusaria* with carbohydrates for saprophytic growth and insects becoming also vectors of spores. Additionally, fumonisin production by *Fusarium* species increased the virulence of F. verticillioides against maize (Desjardins *et al*., 1995)

Although the mechanism of interaction between MON and its hosts, at present, is not known, Kostecki *et al*. (1997a) found that the accumulation of MON was higher in cultivars of maize susceptible to fusariosis than in resistant ones.

PHYSICO-CHEMICAL CHARACTERISTICS

In nature, MON occurs as a sodium or potassium salt of the semisquaric acid (Figure 1). The UV-absorbance of MON has a maximum at 227 nm and a shoulder at 258 nm (Sydenham *et al.* 1996). Lansden *et al.* (1974) have suggested that in aqueous solutions MON exists as a dimer. Some chemical properties of MON are summarized in Table 3.

R = H (free acid), Na (sodium salt) or K (potassium salt)

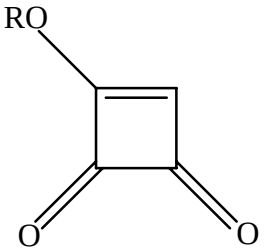

Figure 1. The chemical structure of moniliformin.

Table 3. Some chemical properties of moniliformin.

Property	Value	Reference(s)
Empiric formula (free acid)	$C_4H_2O_3$	Betina, 1989
molecular weight	98.0081 g/mol	Betina, 1989
extinction coefficient (ε)	19 900 $M^{-1}cm^{-1}$ at 227 nm, 5400 $M^{-1}cm^{-1}$ at 258 nm (in water)	Sydenham *et al.*,1996
Melting point (free acid)	158 ºC	Cole and Cox, 1981
pKa-value	0.0–1.72	Bellus *et al.*, 1978; Scharf *et al.*, 1978; Steyn *et al.*, 1978

BIOSYNTHESIS

Moniliformin originates in the acetate pathway. In particular, two malonyl-CoA units are condensated to 1,3 cyclobutanedione. This first intermediate undergoes to enzymatic oxidation, forming three tautomeric derivatives, which finally are reduced to MON. The biosynthetic pathway is reported in Figure 2.

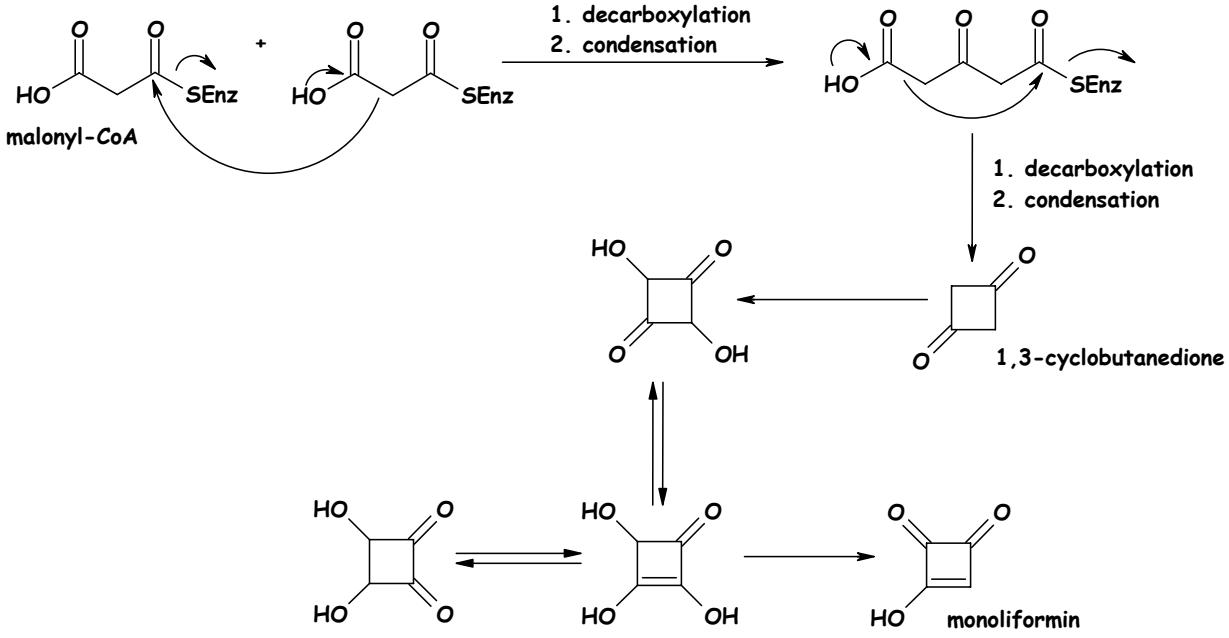

Figure 2. Biosynthesis of moniliformin.

The biosynthetic pathway was investigated by radioactive precursor incorporation experiments (Franck and Breipohl, 1984a). This is the only complete biosynthetic study

reported in the literature to now. More recent biosynthetic studies and genetic information aren't available yet.

CHEMICAL ANALYSES

Source of standards

No data are available about MON standard manipulation.

Filek and Lindner (1996) reported that stock solutions in water, stored in a refrigerator (4°C), were stable for at least three months.

Moniliformin standards are commercially available as sodium salt (Table 4).

Table 4. Sources of Moniliformin standards.

Compound	Concentration or Purity (µg/ml) %	Quantity	Supplier
Solid standard			
Moniliformin sodium salt from *Fusarium proliferatum*	-	1 mg	Sigma-Aldrich
Moniliformin sodium salt from *Fusarium moniliforme*	-	1 mg/5mg	Apollo Scientific Ltd (UK)
Moniliformin sodium salt	Min 95%	1 mg	AppliChem GmbH (Darmstadt, Germany)
Standard solution			
Moniliformin	100 in acetonitrile/water	1 ml	Romer Labs Diagnostic (Tulln, Austria)

Sampling

No specific research has been undertaken on sampling plans for MON in food and feed, a topic which should be faced in the near future.

Extraction and clean- up

MON is extremely soluble in water due to its high polarity. However, water is not a good extraction solvent since it would extract also many undesired impurities from the sample matrix and extensive sample purification steps are then needed. Therefore, MON is usually extracted with 50-96% acetonitrile and/or methanol in water (Jestoi *et al.*, 2003; Rabie *et al.*, 1978; Scott and Lawrence, 1987; Kostecki *et al.*, 1997a; Mubantanhema *et al.*, 1999). Parich *et al.* (2003) tested the extraction efficiency of different acetonitrile/water ratios, and found that 84% acetonitrile in water was the most effective extraction solvent for MON from maize matrix. MON was extracted using ion-pairing reagent tetra-*n*-butyl ammoniumhydroxide (TBAH) (Shepherd and Gilbert, 1986). The method was improved by using tetra-*n*-butyl

ammoniumhydrogen sulfate (TBAHS) as an ion-pairing reagent, achieving better extraction selectivity than using TBAH (Munimbazi and Bullerman, 1998). Extrusion cooking, which is used to manufacture various types of corn-based snack foods and breakfast cereals, subjects these matrices to high temperatures, high pressures, and severe mechanical shear. These forces may cause binding of MON to the food matrix, resulting in low extraction efficiency and poor recovery. Chung *et al.* (2005) developed and optimized an extraction procedure using α-amylase for the detection and quantification of MON in extruded corn grits.

The sample purification steps of the published methods for MON include defattening with hexane, dichloromethane or chloroform (Rabie *et al.*, 1978; Lew *et al.*, 1993; Kostecki *et al.*, 1997b) and/or the use of different purification columns (Florisil, Extrelut, alumina, ion-exchanger resins, Sephadex) (Rabie *et al.*, 1978; Kostecki *et al.*, 1997b; Lew *et al.*, 1993; Scott and Lawrence, 1987; Thiel *et al.*, 1982). Munimbazi and Bullerman (1998) introduced the technique of liquid-liquid partitioning of MON as an ion-paired form with TBAHS to chloroform.

Commercial Solid Phase Extraction (SPE)-columns used for the purification of MON extracts include strong anion exchanger (SAX) columns (Munimbazi and Bullerman, 1998; Caputo and Munimbazi, 2002; Parich *et al.*, 2003; Maragos, 2004; Sorensen *et al.*, 2007), nonpolar C18-columns (Shepherd and Gilbert, 1986; Sewram *et al.*, 1999, Kandler *et al.*, 2002) or the combination of these two (Sharman *et al.*, 1991). However, C18-SPE purification was not recommended by Munimbazi and Bullerman (1998), as they found irreversible adsorption of free acid and ion-paired forms of MON to the sorbent.

In the case of MON, anti-MON antibodies have not been reported, possibly due to the small size of MON resulting in a limited arrangement of functional groups of MON for interactions with an antibody binding site and hence IA columns have not been developed. One alternative to antibodies for selective binding materials is molecularly imprinted polymers (MIPs). Imprinted polymers possess binding sites with functional groups arranged in geometries capable of binding a substrate (Appel *et al.*, 2007). Use of moleculary imprinted solid phase extraction (MISPE) as a clean-up method for mycotoxin determination is increasing because of its efficiency in very complex matrices and because of MIP stability, e.g. in apolar solvents, especially when compared with immunoaffinity (IA) materials (Cigic and Prosen, 2009).

Analytical methods

Determination of MON is very different from other mycotoxins since it is a small, highly polar, acidic molecule with a pKa value of 0.5 and selective determination is rather complicated. Therefore several approaches have been developed for selective extraction, sample purification and the chromatographic separation of MON.

High-performance liquid chromatography (HPLC) has been the most popular chromatographic technique used in the analysis of MON, with a C18-column being the most common stationary phase used. MON is a water-soluble compound and cannot be retained effectively in a reversed-phase column with conventional mobile phases. Therefore ion-pairing mobile phases using TBAH, TBAHS or tetra-*n*-butylammonium dihydrogen phosphate (TBAHP) together with mixtures of water and organic solvents are used (Shepherd and Gilbert, 1986; Scott and Lawrence, 1987; Pineda Valdes and Bullerman, 2000). HPLC is combined with UV-detection at 219-260 nm (Langseth *et al.*, 1999, Uhligh *et al.*, 2004), with 229 nm being the most common detection wavelength used. Triethylamine (TEA) was used as the ion-pair reagent in the liquid chromatography-mass spectrometry (LC-MS) method of Sewram *et al.*(1999). In addition to the ion-pairing HPLC-chromatography, ion-exchange (strong anion exchanger, SAX) columns have been applied in the analysis of MON (Thiel *et al.*, 1982; Thiel, 1990; Kandler *et al.*, 2002).

Filek and Lindner (1996) used HPLC with fluorescence detection (λ_{Ex}=330 nm, λ_{Em}=440 nm) of MON and acetonitrile-ammonium acetate (50 mM) (pH=7) as the mobile phase. Before analysis, MON was derivatised for two hours with 1,2-diamino-4,5-dichlorobenzene (DDB).

One study reported the use of gas chromatography-mass spectrometry (GC-MS) in the analysis of MON (Gilbert *et al.*, 1986). MON was derivatised with N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) containing *tert*-butyldimethylchlorosilane (TBDMCS) to obtain a volatile derivative. Excess derivatisation reagent was removed by size-exclusion chromatography (SEC) prior to the gas chromatographic measurement. The last two methods were sensitive for MON, but they were not applied to the analysis of MON from field samples.

In addition to HPLC and GC, also thin layer chromatography (TLC) with colorimetric or fluorescence quenching for visualisation has been applied in the analysis of MON. MON was developed on silica-plates with different solvents or solvent mixtures and visualised after reaction with 3-methyl-2-benzothiazolinonehydrochloride (MBTH) or 2,4-dinitrophenylhydrazine (2,4-DNP) (Rabie *et al.*, 1978; Scott *et al.*, 1987; Kostecki *et al.*, 1997b; Schutt *et al.*, 1998; Kamimura *et al.*, 1981;) or ninhydrin (Cole *et al.*, 1973).

The charged nature of MON enables its determination using capillary electrophoresis (CE). Three studies reported the use of this separation technique combined with UV-detection at 229 nm (Bohs *et al.*, 1995; Maragos 1998, 2004). A limit of detection of 50 µg/kg in maize matrix was reported for MON using a capillary zone electrophoresis-diode array detector (CZE-DAD) (Maragos 2004).

MON can also be analyzed by LC-MS techniques.

The method by Filek and Lindner (1996) with precolumn derivatisation was applied to MS/MS detection with the advantage that derivatisation increased the molecular mass to such an extent that the protonated molecule was moved out of the noisy low mass range of the mass spectrum. Unfortunately, this study was focused on structural elucidation of the derivatisation product and did not provide validation data or further quantitative analytical results (Zollner *et al.*, 2003). Underivatised MON can be efficiently ionised with electrospray ionization (ESI) (Jestoi *et al.*, 2004a; Jestoi *et al.*, 2004b) or atmospheric pressure chemical ionization (APCI) (Sewram at al., 1999). Due to its acidic properties and low chemical background noise, the negative ion mode provides an abundant deprotonated molecule $[M-H]^-$ and is always preferred. The product ion spectrum of the deprotonated molecule $[M-H]^-$ is dominated by losses of carbon monoxide (Jestoi *et al.*, 2003), and single reaction monitoring (SRM) detection seems not to be more sensitive than single ion monitoring (SIM). Both monitoring techniques afford levels of detection (LODs) of 10 µg/kg and levels of quantification (LOQs) of 20–30 µg/kg, which are well below the detection limits of UV and fluorescence detectors (Sewram at al., 1999; Jestoi *et al.*, 2003). Sewram *et al.* compared quantitative LC/MS and LC–UV data and found a good correlation between both data sets, even if mass spectral data can prove more credible that UV data, more susceptible to the matrix effect (Sewram at al., 1999). To achieve sufficient RP chromatographic retention/separation prior to MS detection, triethylamine was added as an ion pairing reagent to the mobile phase which in addition supported the formation of the deprotonated molecule $[M-H]^-$ in the negative ion mode (Jestoi *et al.*, 2004a; Jestoi *et al.*, 2004b; Sewram at al., 1999; Jestoi *et al.*, 2003). Due to high MS selectivity, a sample clean up of grain extracts was either omitted (Jestoi *et al.*, 2003) or reduced to one SPE step with RP absorbent material (Jestoi *et al.*, 2004a; Jestoi *et al.*, 2004b; Sewram at al., 1999). Consequently, matrix effects have repeatedly been reported especially when a sample clean up was completely omitted (Jestoi *et al.*, 2004b; Jestoi *et al.*, 2003). In this context Jestoi *et al.* (2003, 2004b) reported differences between the standard and matrix matched calibration curves and also found varying recovery values depending on the matrix under investigation.

Other methods for MON in maize and wheat using ion pair-HPLC separation and UV detection have been optimized (Parich *et al.*, 2003) and employed in surveys of Norwegian grain (Uhlig *et al.*, 2004). For maize samples, the method had a LOD of 39 μg/kg and the recovery was 76±9%, while for wheat the LOD was 30 μg/kg with recovery of 87±5%. Recovery as high as 97±3.5% can be achieved by a developed method for the determination of MON in maize by ion chromatography with a LOD of 120 μg/kg (Kandler *et al.*, 2002). In contrast to two previously published ion chromatographic methods (Thiel *et al.*, 1982; Thiel, 1990), the MON peak is base line separated. Additional advantages of this method are the use of ultrapure water as extraction solvent, and the simple clean-up using cation-exchange resins and C-18 cartridges. Nadubinsk´a *et al.* (2002) successfully employed this method for evaluating the contents of MON in maize ears from Slovakia.

MON can also be determined in wheat and maize by an LC-MS/MS multi-method, without the need for any clean-up (Sulyok *et al.*, 2006). A LOD of 20 μg/kg, for both maize and wheat, was obtained, with a recovery of 75% and 81%, respectively.

Sorensen *et al.* (2007) combined their HPLC- hydrophilic interaction chromatography (HILIC)-UV method with MS detection for confirmation of positive samples. Using this procedure, they obtained a recovery rate of 57–74% MON with a LOD of 48 μg/kg and a limit of quantification of 96 μg/kg using UV detection at 229 nm, which was comparable to current methods used. Limit of detection and quantification using ESI--MS detection was 1 and 12 μg/kg, respectively. They concluded that HILIC was not as sensitive as RP when using UV detection due to broader peaks. However, when using HILIC-MS, the higher concentration of organic solvent at the point of elution gave a significantly better spray and thus compensated for the wider peaks compared to HPLC-RP-MS.

Validation and outlook

None of the mentioned methods, including LC-methods, have been validated by interlaboratory studies and there are no certified reference materials or proficiency studies available for the determination of MON. In the near future, certainly, interlaboratory validated analytical methods for the quantification of MON are needed as a prerequisite to have reliable and comparable data on their occurrence in food.

OCCURRENCE DATA

MON was first reported in 1982 in naturally contaminated maize obtained from Transkei-South Africa (Thiel *et al.*, 1982). Since then, MON has been reported as a natural contaminant in maize and other cereals (rice, oats, rye, wheat, barley and triticale) in

different parts of the world (Table 5). MON contamination is higher in maize than in other substrates and in visibly infected samples. The maximum level of MON recorded in maize (530000 μg/kg) was detected in *Fusarium*-damaged maize in Poland (Chelkowski *et al.*, 1987; Chelkowski , 1989).

Recently, MON occurrence has been reported in asparagus spears (Knaflewsky *et al.*, 2008) and in apples showing symptoms of wet apple core rot (Sorensen *et al.*, 2009).

Although the low levels of exposure of the British population do not give particular cause for concern, the Ministry of Agriculture, Fisheries and Food (MAFF) of the U.K.(1998) recommended a continued monitoring program to determine the trend in MON levels. Likewise, as is detailed in Table 5, the co-occurrence of MON with other mycotoxins such as fumonisins, beauvericin, fusarin C, zearalenone, and trichothecenes has been reported.

Since MON has been suggested as one of the etiological factors of Keshan Disease (KSD), Yu *et al.*, (1995) used TLC and HPLC methods for the determination of MON in rice and corn samples collected from KSD areas and non-KSD areas. The results showed that the contamination of MON in grains was significantly different between rice and corn, but not between the grains from the KSD areas and non-KSD areas, thus casting doubt on the role of MON as an etiological factor of KSD.

Severe infections of *F. avenaceum*, *F. tricinctum*, and to a lesser extent *F. subglutinans*, from central to north-east European countries, were usually responsible for MON occurring in scabby grains (Kostechi *et al.*, 1995). In particular, in Poland, significant levels of MON were found in scabby kernels obtained in 1988 from ears of wheat highly infected by *F. avenaceum* (Lew *et al.*, 1993). MON was also reported in freshly harvested durum wheat in Austria (Adler *et al.*, 1995). In all these surveys, the MON content in kernels was well correlated with the presence of *F. avenaceum* (up to 38%) (Kostechi *et al.*, 1995; Chełkowski *et al.*, 2000).

The MON concentrations in grain in surveys carried out in Norway and Finland were generally low with respect to the amounts that are necessary to cause adverse effects *in vivo* (Jestoi *et al.*, 2004a; Uhlig *et al.*, 2004, 2007). Maximum concentrations of MON were 950 μg/kg and 810 μg/kg in wheat samples from Norway and Finland, respectively.

Moniliformin levels found by Uhlig *et al.*, (2004) were statistically related to the level of infection with F. *avenaceum/arthrosporioides* and *F. culmorum*, but not to the level of infection with *F. tricinctum*.

However, under different climatic conditions, both *F. avenaceum* and other *Fusarium* spp. may have a greater potential to produce the toxin in field. In maize, especially, high contamination levels have been reported. More than 300000 μg/kg were found in hand-

selected, visibly infected kernels from Poland, and up to 25000 µg/kg in hand-selected kernels from South Africa (Lew *et al.*, 1996; Sharman *et al.*,1991; Thiel *et al.*, 1982). In all these cases, the high contamination could be related to *F. subglutinans*. When visibly *F. avenaceum*-infected kernels of small-grain cereals were investigated for their MON contamination, maximum concentrations were about 1/10 of the concentrations in maize (Lew *et al.*, 1993; Sharman *et al.*, 1991).

The highest concentration of MON in small-grain cereals was 38300 µg/kg in a hand-selected oat sample from Poland (Sharman *et al.*, 1991). It has to be noted, however, that the reported high concentrations were from hand-selected samples with a high degree of infection, and are therefore not directly comparable to the Norwegian and Finnish surveys where randomly selected samples were analysed.

Scudamore *et al.* (1998a) reported a particularly interesting result. Their analysis of maize gluten and milled maize products, destined for incorporation into animal feed stuffs in the U.K., showed 60% of samples contaminated with concentrations up to 4600 µg/kg of MON. Moreover, in addition to MON, in maize screenings and maize meal, eleven other *Fusarium* toxins (fumonisins, zearalenone, deoxynivalenol, nivalenol, HT-2 toxin, T-2 toxin, fusarenon-X, monoacetoxyscirpenol, 5-acetyl deoxynivalenol, 3-acetyl deoxynivalenol, diacetoxyscirpenol) with a total loading of ca 45000 µg/kg were found.

MON was detected in 52% of 50 poultry feed samples from Slovakia, in concentrations from 42 to 1214 µg/kg (Labuda *et al.*, 2005). The outcomes from this study have clearly shown comparatively lower levels than the 50000 µg/kg proposed as a maximum level in the diet of broiler chickens (Ledoux *et al.*, 1995).

Table 5. Results of surveys for moniliformin showing concentrations and distribution of contamination in food and feed commodities.

Country	Commodity	Year	Co-occurring mycotoxins	N°. of samples	LOQ (μg/kg)	*n* > LOQ	Mean (μg/kg)	Min/Max (μg/kg)	References	Sampling procedure
Food										
South-Africa	Maize	1978	DON, ZEN	2	na	2	na	16000/25000	Thiel *et al.*, 1982	Obtained from a farm
Germany	Maize	na	ZEN, HT-2, T-2, T-2-triol. DAS, DON	58	na	25	na	na/> 650	Thalmann *et al.*, 1985	na
Canada	Wheat	na	DON	24	0.02[a]	0	-	-	Scott and Lawrence, 1987	na
	Rye	na	na	4	na	0	-	-		
	Maize	na	DON	12	0.01[a]	2		<0.01/200		
Poland	Maize	na	na	na	na	na	na	na/530000	Chelkowski *et al.*, 1987	na
Poland	Maize	na	na	na	na	na	na	na/530000	Chelkowski, 1989	na
South Africa	Maize foods	1985	DON, NIV, ZEN, FB_1, FB_2	na		All positives	na	350/11570	Sydenham *et al.*, 1990	Samples intended for human consumption and for beer brewing
Austria	Maize	1988-1989	na	67		21	na	na/20000	Lew *et al.*, 1991	na
UK, USA, France	Maize and maize products	1990	na	36	50[a]	33	na	<50/250	Sharman *et al.*, 1991	– Purchased from retail markets
Worldwide	Maize	1985-1989	na	64	50[a]	27	na	<50/3160		– Maize kernels intended for human consumption
Poland	Wheat	na	na	6	50[a]	6	na	500/17100		– Deliberately selected by firstly field-inspection as being visibly *Fusarium* damaged, and subsequently hand- selected as those cereal grains showing fusariosis symptoms
Poland	Rye	na	na	3	50[a]	3	na	6100/12300		
Poland	Triticale	na	na	3	50[a]	3	na	2600/15700		
Poland	Oats	na	na	3	50[a]	3	na	15700/38300		
Poland	Maize	na	na	20	50[a]	20	na	4200/399300		
Poland	Maize	1990-1991	BEA	8	na	8	na	17000/425000	Logrieco *et al.*, 1993	Corn ears visibly damaged collected at random
Poland	Wheat (healty)	1985-1989	na	5	na	5	na	250/700	Lew *et al.*, 1993	Head with *Fusarium* head blight symptoms collected from about 100 fields
Poland	Wheat (damaged)			5	na	5	na	7200/25200		
Poland	Triticale (healty)			3	na	1	na	na/250		
Poland	Triticale (damaged)			3	na	3	na	2400/5100		
South Africa	Maize	1989	na	10	na	2	na	320/390	Rheeder *et al.*, 1994	– Collected before export. Taken by scooping from the conveyor belt to obtain 1 kg sample
Taiwan	Maize	1989	na	10	na	0	-	-		– Collected after export. Obtained by scooping or probing at 3 different positions per truck
Italy	Maize	1992-1993	na	4	na	1	na	200000	Logrieco *et al.*, 1995	na
Poland	Maize	na	BEA	12	na	6	na	na/8530	Kostecki *et al.*, 1995	na
China	Rice	na	-	123	na	8	na	na/265.3	Yu *et al.*, 1995	
China	Maize	na	-	104	na	47	na	na/1116		

contd

Country	Commodity	Year	Co-occurring mycotoxins	N°. of samples	LOQ (μg/kg)	*n* > LOQ	Mean (μg/kg)	Min/Max (μg/kg)	References	Sampling procedure
Food (contd)										
Austria	Wheat	1991-1992	DON, ZEN	48	10[a]	29	na	<10/880	Adler *et al.*, 1995	Collected from 5 locations in Austria
Poland	Maize	1990-1991	na	20	na	20	na	16800/425000	Lew *et al.*, 1996	na
UK	Maize-based foods	1997-1998	na	238	15[b]	36	na	<10/135	MAFF, 1998	na
	Maize and maize flour		na	31	15[b]	31	na	<10/522		
South Africa	Maize	1997	na	4	5[a]	2	na	<5/17	Sewram *et al.*, 1999	na
Switzerland	Maize based products	1999-2000	FB_1, FB_2, BEA, ZEN	99	na	54	na	na/1350	Noser *et al.*, 2001	Obtained from Swiss retail market
USA	Maize	1998	FB_1, FB_2	100	25[a]	83	na	<25/774	Gutema *et al.*, 2000	Food-grade commercial maize samples obtained from 2 maize processing companies at 5 different locations. Ground to a fine meal
USA	Maize based products	1997	FB_1, FB_2	34	25[a]	26	na	<25/858		Maize based products collected from supermarkets
Austria	Maize crops	1996	DON, FBs, ZEN	46	50[a]	7	na	na/290	Lew *et al.*, 2001	na
		1997		58	50[a]	8	na	na/390		
		1998		48	50[a]	8	na	na/800		
Poland	Wheat	na	DON, NIV, OTA	10	na	4	na	nd/200	Krysinska-Traczyk *et al.*, 2001	Collected from 10 private farms, taken during threshing
USA	Corn tortillas	na	na	14	na	12	na	Na/100	Caputo and Munimbazi, 2002	na
Poland	Wheat with scab symptoms	1998-1999	DON, NIV	129	10[a]	83	na	<10/1720	Tomczak *et al.*, 2002	na
Brazil	Maize	na	na	22	300[a]	0	-	-	Leoni *et al.*, 2003	The bulk sample from silos mixed and reduced by quartering. A 500 g sample destined to MON determination
	Maize products	na	na	68	300[a]	0	-	-		Acquired at retail points in amounts of at least 500 g
Norway	Oats	2000-2001	na	73	130	38	na	<130/210	Uhlig *et al.*, 2004	Sampling carried out by the Norwegian Agricultural Inspection Service regulations
	Barley		na	75	130	53	na	<130/380		
	Wheat		na	83	130	76	na	<130/950		
Finland	Wheat	2001-2002	BEA, ENNs	14	20	10	na	<20/810	Jestoi *et al.*, 2004a	collected from the fields of the most significant cultivation areas in Southern and Central Finland
	Barley			22	20	22	na	<20/750		
	Oats			1	20	1	na	84		
	Rye			1	20	0	-	-		

contd

Country	Commodity	Year	Co-occurring mycotoxins	N°. of samples	LOQ (μg/kg)	*n* > LOQ	Mean (μg/kg)	Min/Max (μg/kg)	References	Sampling procedure
Food (contd)										
Finland	Grain-based products	2002	BEA, ENNs, DON, HT-2	18	20	4	na	<20/42	Jestoi *et al.*, 2004b	Randomly selected grain-based products purchased from local markets. Ground with a laboratory mill, if necessary
Italy	Grain-based products	2002		12	20	1	-	<20		
Finland	Wheat		BEA, ENNs, DON, 3-Ac-DON, NIV, HT-2, T-2, ZEN	9	na	1	-	450	Klemsdal *et al.*, (submitted)	na
Finland	Barley			29	na	2	na	<20		
Poland	Wheat		Antibiotic Y	24	300	11	na	nd/<300	Kulik, 2008	na
Poland	Asparagus spears	2002-2003	FB_1	na	25[a]	na	na	na/1350	Knaflewski *et al.*, 2008	na
	apple		ENNs, aurofusarin, antibiotic Y	20	50	14	na	<50/2870	Sorensen *et al.*, 2009	na
Feed										
USA	maize	1985	Fusarin C	1	na	1	na	2820	Thiel *et al.*, 1986	1 kg sample of corn screenings from a farm
UK	Maize gluten	na	FB_1, FB_2, ZEN, NIV, DON, 15-Ac-DON, T-2, F-X	40	50[a]	17	na	<50/320	Scudamore *et al.*, 1998a	na
	Maize products	na		27	50[a]	24	na	<50/4600		
UK	Rice	na	OTA	40	50[a]	1	na	70 (not confirmed)	Scudamore *et al.*, 1998b	na
Slovakia	Poultry feed	2003-2004	FB_1, FB_2	50	39[a]	26	na	<39/1214	Labuda *et al.*, 2005	Randomly collected

[a] : limit of detection (The minimum concentration of a substance being analyzed that has a 99 percent of probability to be detected
[b] : limit of determination (The lowest concentration of a contaminant that can be routinely identified and quantitatively measured)
na : not available
nd : not determinated
- : not computable
BEA (beauvericin), DAS (diacetoxyscirpenol), DON (deoxynivalenol), 3-Ac-DON (3-acetyl deoxynivalenol), 15-Ac-DON (15-acetyl deoxynivalenol), ENNs (enniantins), FBs (fumonisins), FB_1 (fumonisin B_1), FB_2 (fumonisin B_2), FX (fusarenon X), HT-2 (toxin HT-2), MAS (monoacetoxyscirpenol), NIV (nivalenol), OA (ochratoxin A), T-2 (toxin T-2), ZEN (zearalenone)

MITIGATION OF MONILIFORMIN

Fungal diseases are common problems in crop agriculture. In general, infectious organisms can be controlled through:

- the use of preventing breeding techniques or agents that are selectively biocidal or competitors for the pathogens;
- interference with the mechanism by which the pathogen invades and causes injuries to the host crop plant;
- interference with toxin production, storage or activity (Duvick and Rood, 1999).

Agricultural practices such as crop rotation and soil tillage are recommended to control plant contamination with *Fusarium* spp. even if these techniques are not always recognized as efficient. Removal, burning or burial of crop residues is likely to reduce *Fusarium* inoculum for the following crop (Jouany, 2007). Obst *et al.* (1997) noted that minimum tillage instead of ploughing resulted in an increase of deoxynivalenol (DON) content in the following wheat crop, while Steinkellner and Langer (2004) reported that the deeper the tillage the lower was the concentration of *Fusarium* spp. in the soil. The cropping management seems to be efficient also in lowering the level of mycotoxins as regards *Fusarium* head blight, but not so much as far as maize ear rot is concerned. The different efficacies of crop rotation between maize and wheat depends on the type of primary spore inoculum: asexual conidia landing on the silks and growing down to the developing kernels are the primary infection pathways for maize ear rot while ascospores produced from the homothallic reproduction of *G. zeae* and formation of perithecia on the residue of wheat and maize cause the development of wheat head blight (Shaner, 2003). Thus tilling the soil causes the asexual conidia to be wind dispersed and therefore the operation is not efficient in lowering the level of contamination.

As the contamination by *Fusaria* is most likely when the crop flowering stage occurs at the time of spore release, in maize, earlier planting dates in temperate areas will often result in a lower contamination level (mainly because of reduced ECB incidence and earlier harvest time), even if annual weather changes can challenge this potential advantage (Munkvold, 2003b; Blandino *et al.*, 2009). In wheat and barley, winter varieties develop and mature earlier than spring varieties and consequently they have a reduced risk of *Fusarium* infection (Jouani, 2007).

The harvest and post-harvest control of pathogens is linked to the timing of harvest because, generally, earlier harvest results in lower concentrations of mycotoxins (Jones *et al.*, 1981). The cutting height is another important factor in preventing post-harvest contamination to

avoid the contact of healthy grain with soil, the primary source of *Fusarium* spores (Jouani, 2007).

Post-harvest, damaged grains should be eliminated and the humidity level of the kernels must be lowered in order to reduce the possibilities of infection and the production of toxins by fungi. A water activity lower than 0.65 and a humidity level under 14% in cereals are usually considered limiting factors for fungal growth; effectively, *Fusarium* spp. need 17-19% humidity to grow.

The temperature of storage has an effect on fungal growth too and, especially in silo storage its control is relevant: combined cooling and drying operations associated with ventilation systems are necessary to avoid the worsening of contamination during storage (Jouany, 2007).

The use of resistant cultivars

One of the strategies for the control of toxin production is linked to the possibility of limiting the infection by using varieties that have proved to be more resistant to *Fusarium* spp. and, in the case of maize, to European corn borer (ECB) injuries. ECB larvae, actually, cause physical injuries to maize stalk and ears through which *Fusaria* can infect the host plant; in addition, ECB larvae can carry fungal inoculum from the plant surface into the kernels, favouring plant infection. On ears damaged by corn-borers there were many more species belonging to the *Lyseola* section (e.g. *F. subglutinans, F. verticillioides*) than the *Discolor* section (e.g. *F. graminearum, F. culmorum*), respectively with up to 80% for the first and less than 15% for the second (Logrieco *et al.*, 2002). Magg *et al.* (2003) found that ECB management, through the use of transgenic cultivars expressing the *Bacillus thuringiensis* (Bt) toxin, can lead to a significant ($P<0.05$) increase in grain yield compared to the commercial hybrids and to a reduction of MON levels. In effect, MON concentrations were found to be significantly ($P<0.01$) and positively associated to all ECB resistant traits and negatively with grain yield under infestation (Magg *et al.*, 2003). The same findings were evidenced by Papst *et al.* (2005) that found significantly ($P<0.01$) lower stalk damage ratings and percentages of damaged ears for the Bt hybrids in contrast to their isogenic non-Bt counterparts and the commercial cultivars.

Transgene-enhanced disease resistance has not yet achieved the same level of success as insect resistance in crop plants. There have been several attempts to improve ear mould resistance in maize, such as the use of antifungal proteins or secondary metabolites and the induction of defence pathways. The study of the host plant-pathogen interaction and a deeper knowledge of the basic biology of interactions is fundamental in order to achieve

better results. More studies are needed also in the field of transgenic strategies aimed at reducing mycotoxins such as the modification of mycotoxin catabolic pathways or the possibility of detoxifing mycotoxins *in planta* (Duvick, 2001).
No specific information is available regarding wheat resistant cultivars.

Chemical and biological control

Chemical control of the pathogen is difficult because, to be efficient, the fungicides must be totally lethal to *Fusarium* spp.; if not, they stimulate mycotoxin production *in vitro* (D'Mello *et al.*, 1998).
The triazole fungicides metconazole and tebuconazole are efficient against *Fusarium* spp. in wheat while strobilurin azoxystrobin resulted in a reduction of *Fusarium* head blight (FHB), but the level of DON contamination sometimes increased, probably due to the elimination of competitive microrganisms located in the ear environment (Pirgozliev *et al.*, 2003). In a trial carried out in the north of Italy using three different commercial fungicides, Haidukowski *et al.* (2005) observed that cyproconazole plus prochloraz and a mixture of tebuconazole plus azoxystrobin significantly reduced FHB severity, respectively by 25% and 77%, in grains of five different cultivars of soft and durum wheat.
Treikale *et al.* (2006) managed field trials in Latvia and noticed that effective control of FHB on winter wheat was obtained through the application of new fungicides with different active ingredients. At a high level of development of the disease the application of tebuconazole (125 g) plus prothioconazole (125 g/L) at 1.0 L/ha gave good control of *Fusarium* spp. on ears of winter wheat, reducing FHB by 86.3% while the use of spiroxamine (300 g) plus prothioconazole (160 g/L) resulted in a 76.7% reduction of the disease. The same results in the control of the disease were achieved using different doses of tebuconazole (250 g/L) at 1 L/ha, tebuconazole (167 g) plus triamenol (43 g) plus spiroxamine (250 g/L) at 0.8 L/ha or metconazole (60 g/L) at 1 L/ha. Differences in efficacy were be noted for applications at different wheat growth stages and in different years, as a result of diverse atmospheric conditions.
As far as maize is concerned, Folcher *et al*. (2009) showed that the use of fungicide tebuconazole (250 g/ha) in association with the insecticide deltamethrine (20 g/ha) is not effective in the reduction of *Fusarium* spp. and there is no synergy effect between fungicide and insecticide, but there is still a reduction of the mycotoxins level because of the control of insects. Blandino *et al.* (2008) found that lambda-cyhalothrin significantly reduced ECB infestation and, consequently, *Fusarium* ear rot severity (up to 29%) and fumonisin concentration (up to 67%) when associated to earlier sowing of date maize.

All the chemicals reported above are included in annex 1 after the European revision of pesticides.

Biological control with microbial antagonists or competitors to *Fusarium* spp can be integrated in contamination control strategies by spraying it on plants at flowering stage to eradicate or limit the growth of toxin producers (Jouany, 2007). Some biological agents, such as some strains of *Bacillus subtilis, Bacillus thuringensis, Candida, Pseudomonas or Trichoderma* spp., are already included in the pesticide database of the European Union while a specific dossier has to be prepared for the registration of new bioagents. In particular, on wheat a recent study (Xue *et al.*, 2009) investigated the efficiency of 20 potential bioagents, including 5 fungal and 15 bacterial strains all isolated from maize and soybean roots in a long-term rotation study at the Central Experimental Farm in Ottawa, Ontario, except for one (ACM941 *Clonostachy rosea*) isolated in 1994 from peas in Morden, Manitoba, in 2007 and 2008. As already said, *Fusarium* head blight is a monocyclic disease and control of the initial inoculum (perithecia) should have a significant impact on successful management of FHB by diminishing infection pressure during the growing season when weather conditions favour disease epidemics. When applied in field, all of the 20 bioagents significantly reduced perithecium production compared to the untreated control. In 2007, *C. rosea* strains ACM941, CR24, and CR25 were the most effective treatments, reducing the production of perithecia by 63.7%, 62.9%, and 59.7% respectively, while other bioagents reduced perithecia by 18.1-41.7% which was similar to the effect of tebuconazole (30.4%). In 2008 only six bioagents were tested, chosen from the best of the previous trial, and *C. rosea* ACM941 was the most effective treatment, reducing the production of perithecia by 67.5%, which was significantly better than other bioagents (17.8-25.4%) and tebuconazole (20.5%). When sprayed on to wheat heads, seven of the 20 bioagents tested significantly reduced the FHB index, one reduced *Fusarium* damaged kernels (FDK), and six reduced DON content in grains. None of the bioagents increased the disease severity and DON contamination except for one strain of *Bacillus subtilis* (CH15) that significantly increased FDK. Among the effective bioagents, *C. rosea* ACM941 was the only treatment that significantly reduced the FHB index, FDK and DON, by 46.4%, 29.0%, and 28.7%, respectively. Tebuconazole was more effective than any bioagents, reducing the FHB index by 99.1%, FDK by 73.1% and DON by 79.3%. As with chemical fungicides, timing and deposition of bioagents is critical; an early application of the bioagents gives them a strong competitiveness giving the opportunity to occupy the plant tissue before the establishment of the pathogen. Consequently, the efficacy of these bioagents in suppressing the perithecium production would be greater if they were applied to the plant tissues before the pathogen.

Attempts at biological control of *F. moniliforme* in maize were made with *Bacillus subtilis* RRC101 and *Trichoderma* spp (Bacon *et al.*, 2001).

The bacterium was shown to have highly desirable plant-enhancing characteristics as well as being an endophyte able to protect maize from pathogenic organisms; i*n vitro* studies demonstrate that the bacterium completely inhibited the growth of all isolates of *F. moniliforme* tested (Hinton and Bacon, 1995). I*n planta*, the bacterium was distributed in all organs at concentrations that varied (10^1-10^4 colony forming unit (cfu)/g wet weight) during the entire season. *B. subtilis* RRC101 grows continuously without affecting the growth of maize, showing a protective action on maize seedlings when planted in soils infested with *F. moniliforme*, and increasing root and shoot growth. Bacteria-infected maize showed no evidence of disease in seedlings during a 6-week observation period under gnotobiotic conditions nor during the entire maize growth cycle under field conditions of approximately 120 days (Bacon *et al.*, 2001).

Species of *Trichoderma* are very effective as biocontrol agents against various plant pathogens because their powerful extracellular lytic enzymes produce necrotrophic action on fungi through the lysis of cell walls; they are applied mainly to the soil for biocontrols, and only a few studies deal with their application in the management of post-harvest cereal and foliage diseases (Kubicek and Harman, 1998). Yates *et al.* (1999) demonstrate that, *in vitro*, the isolate of *Trichoderma* spp. suppressed growth of *F. moniliforme* colonies over time, increasing from the 46% suppression observed on day 6 to a maximum of 91% by day 14 and Bacon *et al.* (2001) evidenced the ability of *Trichoderma* to reduce fumonisin accumulation in maize kernels up to 85% when inoculated simultaneously with *F. moniliforme* PATgus. The use of *Trichoderma* as biocontrol is expected to have an application for maize kernels, especially for animal feed.

Decontamination

There are different possibilities for the post-harvest decontamination strategies still at the study level, with a biologic approach or applying physical or chemical methods.

Duvick and Rood (1999) discovered some bacteria with the ability to degrade MON. These bacteria, *Ochrobactrum* spp., isolated from field-growing maize kernels, have the ability to use MON as a sole carbon source, degrading it partially or completely in the process. The treatment can be done in field, topically applying a suspension of bacteria onto maize plants so that MON, including any derivatives or analogues, is partially or completely degraded, or on contaminated corn post-harvest, with inoculation of 10^6 cfu/g of log-phase cells of bacteria.

Another approach, in the area of controlling mycotoxins in animals, is that of supplementing drinking water with a *Lactobacillus acidophilus* fermentation liquid formula. Its use seems to reduce mortality and improve weight gain when poultry are fed with *F. proliferatum* contaminated feed (Wu, 1997). Nevertheless, the mechanism of action of this formula is unknown.

As for many other toxins, ozonization is efficacious in detoxifying MON because of the disappearance of the double bond and the opening of the 4-C structure (Zhang and Li, 1994). Chlorinated lime, heating, activated carbon, ozone, microwave, and UV were also tested for their ability to reduce MON content in water. The most effective procedure is chlorinated lime: to detoxify 1 mg of MON in water, 1.5 mg of effective chlorine in chlorinated lime is needed (Zhang and Li, 1997). On maize grains water wash, fumigation with gaseous disinfectants, radiation with –rays and spray treatments were tested. Using oxygenated water was the most effective method to achieve detoxification. Both methods, chlorinated lime and oxygenated water, are easy to use, inexpensive and free from secondary pollution (Zhang and Li, 1997).

No studies are available regarding the fate of MON during hot drying of grain.

Fate of moniliformin during food processing

The thermal stability of MON has been studied. From experiments conducted in ground maize and wheat spiked with MON at levels of 1000 μg/kg and heated at 50, 100, and 150ºC for 0.5-2 h, moderate decomposition was observed, e.g. 55% remained in corn after 0.5 h at 100ºC (Scott and Lawrence, 1987). Freeze-drying did not affect the stability of MON (Abramson *et al.*, 2001).

The instability of MON was also reported if it was evaporated from aqueous solvents to dryness (Shepherd and Gilbert, 1986; Munimbazi and Bullerman, 1998). In an oxidative environment, MON decomposes, forming carbon dioxide and acetic acid (Franck and Breipohl, 1984b).

More recently, Pineda Valdes *et al.* (2000) established that the stability of MON depends on temperature, pH, and time, and that the percentage of reduction was related to an increase in the value of these parameters. MON has been reported to be almost completely destroyed by heating at pH 10 for 1 h at 175ºC. Since the study was done in aqueous solutions, with a MON concentration of 2000 g/kg, the reduction measured was most likely genuine due to the destruction of MON rather than its reaction and binding with the components of the matrix. The mechanism of destruction and whether the heat treatment reduces the toxicity of MON or not still remain to be established.

There is only limited information on MON degradation during processing. Complete (100%) reduction of MON was observed by Pineda Valdes *et al.*, (2002) when a naturally contaminated maize sample containing 1400 μg of MON/kg of corn was used in a pilot-scale alkaline cooking and tortilla manufacturing process. In a companion laboratory-scale study, using a cultured maize sample containing 17600 □g of MON/kg of maize, a 71% reduction of the toxin was observed during the process. Alkaline cooking appeared to be an effective method for the reduction of MON in maize. From Pineda's results, MON would be expected to be stable in food processes that occur under neutral (baking of maize bread) or acidic (wet milling of maize) conditions. In effect, Caputo and Munimbazi (2002) found that alkaline cooking of maize decreased MON levels by about 70% when producing tortillas. These are promising results because the same procedure partially destroys ZEA, DON, and aflatoxins; although maize tortillas manufacture reduces MON content significantly in finished products, it is still possible to detect it in commercially available products (Table 5). Nevertheless, Adler *et al.* (1995) considered that, even if MON is leached out from spaghetti into boiling water to a large degree, because of its strong polarity, a risk for human health due to MON carry over may still remain, as durum wheat can be highly contaminated.

Pineda Valdes *et al.*, (2003) also investigated the effects of autoclaving, baking, extrusion, frying, and roasting on the stability of MON in spiked (5000 μg/kgof MON) maize-based food products. Roasting maize meal at 218°C for 15 min had the most significant effect ($p<=0.05$) on the reduction of MON (44.6%). Autoclaving creamed maize at 121 °C for 65 min resulted in only 10% reduction of MON. Reductions of MON ranging from 5.4 to 28.9% were observed when maize chips were prepared from spiked masa. MON was reduced by 42.2% when maize muffins were baked and by 26.7% when maize grits were extruded. Overall, MON showed heat stability similar to or greater than other *Fusarium* mycotoxins such as deoxynivalenol and fumonisin B_1.

The effects of milling on MON concentrations were investigated (MAFF, 1998) by analysing five batches of raw maize and taking subsequent fractions from various milling stages (steep liquor, gluten, starch, glucose syrup and pellets), but the work was inconclusive.

In conclusion, MON stability and fate during processing has been little studied in various products. This makes it difficult to adequately assess the degree of consumer exposure.

PHARMACOKINETICS

Absorption, Distribution, Metabolism and Excretion

Since this mycotoxin has only been recently discovered, very limited information is available on its toxicity both *in vitro* and *in vivo*. There are no reports on mycotoxicoses, althought

some published studies do highlight the possible risks associated with the ingestion of MON . Further studies are needed to better assess the risk of these compounds to human and animal nutrition; for example nothing is known about its toxicokinetics (absorption, distribution, metabolism, or excretion).

Carry over

There are no available studies about MON carryover from contaminated feed and its effects on humans in available published papers.

TOXICITY

Acute toxicity

The main symptoms of acute MON toxicity are muscular weakness, respiratory stress, myocardial degeneration, as well as some evidence of histopathological changes in organs such as the kidneys, the lungs and the pancreas followed by coma and death as reported by Kriek *et al.* (1977) after exposure of ducklings to 0,5-10 mg/Kg or 25-100 mg/kg for rats. Similar results were obtained by Ledoux *et al.* (2003) with broiler chickens exposed to 100-200 mg/Kg and by Burka *et al.* (1982) in mice treated intraperitoneally with MON 0,13-0,22 mmol/Kg bw. The high susceptibility of cardiomyocytes to MON correlates well with both the symptoms occurring in acutely intoxicated animals and its biochemical mode of action (Wu *et al.*, 1995).

The naturally mycotoxin-contaminated feed may be more toxic to animals than artificially contaminated feed (Engelhardt *et al.*, 1989). Since unidentified secondary metabolites may be present in naturally contaminated feed, the effects of MON *in vivo*, reported in Table 6, include only studies using purified toxin. However, the symptoms and effects of MON in studies reporting the use of naturally MON-contaminated feed (> 50 mg/kg) in hens and turkeys are in line with the results presented in Table 6. Typical symptoms are reduced body weight, increased heart and kidney weight, myocardial lesions, lowered egg production and egg weight, lymphoid depletion, reduced amounts of blood cells and haemoglobin, and even death (Vesonder and Wu, 1998; Kubena *et al.*, 1999; Morris *et al.*, 1999; Harvey *et al.*, 2002).

In vivo toxicity data of MON reveal higher toxicity in animals after intraperitoneal or intravenous administration compared to oral treatment, which may be due to hepatic detoxification.

Table 6. Results of the in vivo-studies on moniliformin (Jestoi, 2008).

Animal	Dosage/route	Symptons/special remarks	Reference
cockerel	62.5 μg - 1.6 mg/oral	LD_{50} 4.0 mg/kg; ascites, hemorrhages of intestinal and skin	(Cole *et al.*, 1973)
duckling, rat	0.5 -10.0 mg/kg bw.; 25.0 - 100 mg/kg bw/intubation	LD_{50} 3.68 mg/kg (duckling), 41.57 mg/kg (female rat), 50.00 mg/kg (male rat)	(Kriek *et al.*, 1977)
chicken, mouse, chicken embryo	0-16 mg/kg bw; 0-35 mg/kg bw/intubation, intraperitoneal	LD_{50} 5.4 mg/kg (chicken), 20.9 mg/kg (female mouse), 29.1 mg/kg (male mouse), 2.8 μg (chicken embryo)	(Burmeister *et al.*, 1979)
mouse	10-80 mg/kg bw./intubation	LD_{50} 47.6 mg/kg	(Burmeister *et al.*, 1980)
broiler chicken	0-64 mg/kg feed; 2 mg/ml/oral, intraperitoneal	LD_{50} 1.38 mg/kg bw (i.p.); reduced body weight, mortality (64 mg/kg feed)	(Allen *et al.*, 1981)
mouse	0.13-0.22 mmol/kg bw./intraperitoneal	LD_{50} 20±2 mg/kg bw./ muscular weakness, respiratory distress, coma, death	(Burka *et al.*, 1982)
rat	0.25-8 mg/g feed; 2.5-199 mg/kg bw./oral, intubation	haemorrhaging in the intestine, death (> 1 mg/kg feed or >20 mg/kg bw.)	(Abbas *et al.*, 1990)
broiler chicken	27-154 mg/kg feed/ *ad libitum* (14 d)	reduced body weight, mortality	(Javed *et al.*, 1993)
broiler chicken	1 mg/kg bw./intravenously	decreased heart rate, mortality	(Nagaraj *et al.*, 1996)
broiler chicken	100 - 200 mg/kg	increased heart, liver and kidney weights, large pleomorphic cardiomyocyte nuclei, loss of cardiomyocytes, renal mineralization, mortality	(Ledoux *et al.*, 2003)
mink	0.6-2.8 mg/kg bw.(acute), 0.6-2.2 mg/kg bw. (subacute)/ intraperitoneal	LD_{50} 2.2-2.8 mg/kg bw./ e.g. vomiting, lethargy, heart dilatation and degeneration, mortality	(Morgan *et al.*, 1999)

Chronic toxicity

The chronic effects of MON were evaluated with broiler chickens from 1 to 7 weeks of age and with turkeys from 1 to 14 weeks of age. *Fusarium fujikuroi* (M-1214) culture material was added to typical corn-soybean diets to supply a 0, 25, or 50 mg/kg diet (broilers) or a 0, 12.5, 25, 37.5, or 50 mg/kg diet (turkeys). Compared with the controls, chicks fed diets containing 50 mg/kg consumed more feed, had a lower body weight and had increased relative heart and proventriculus weights. Chicks fed the diet containing 50 mg of MON/kg also had significantly higher mortality and decreased mean corpuscular volumes compared to the controls. Broilers fed 25 and 50 mg MON/kg feed also had increased serum gamma glutamyltransferase activities. Feed intake and body weight gain of turkeys fed dietary MON were not affected. At 6 and 14 weeks, turkeys fed 25, 37.5, or 50 mg of MON/kg diet increased ($p < 0.05$) their relative heart weights. The results indicate that a ≥ 37.5 mg MON/kg feed is hepatotoxic and a ≥ 25 mg MON/kg feed is cardiotoxic to turkeys and a 50 mg MON/kg diet is toxic to broiler chicken.

Another relevant study performed by Javed *et al.* (2005) reports many pathological effects in 228 male broiler chicks exposed to MON (66, 193 and 367 mg MON/kg feed) from day 1, 7 or 21 for 7-28 days. The age of birds when the diet started influenced the nature and severity of the pathological changes. Birds started on day 1 had ascites (as a sequel to liver and kidney damage), while birds started on day 7 had hydropericardium (results from cardiac damage). Birds started on day 21 had neither ascites nor hydropericardium. These data evidenced that liver and kidney damage occurs before cardiac damage in the MON exposure.

In a study by Kubena *et al.* (1999) the effects of long term feeding of culture material *F. fujikuroi* containing MON were evaluated on laying hens. The animals (3 weeks old) were exposed to 50 or 100 mg MON/kg feed for 420 days. After 112 days liver weights were significantly reduced in hens fed the 50- and 100- mg MON/kg diet, while only in the 100 mg MON/kg feed group they observed a reduction of kidney weights and an increase in heart size. Total serum protein, triglycerides and uric acid concentrations were significantly reduced in hens fed a 100 mg MON/kg diet. In the same group there was an increase in the levels of other markers: serum creatinine concentration, alanine aminotransferase activity, aspartate aminotransferase activity and creatine kinase activity, suggesting disturbances in liver function. Another relevant altered parameter is egg production: measurements every 28 days until the end of the treatment revealed a significant decrease in egg production in laying hens fed 100 mg of MON/kg feed. An interesting aspect is that hens fed the 100 mg of MON/kg feed were able to recover when returned to the control diet.

In summary, the results indicate that the target organs of MON are the liver, kidney and heart. Laying hens seem to be able to tolerate relatively high concentrations of MON for long periods of time without an adverse effect on their health and performance.

Cytotoxicity

The molecular mechanism of the toxic action of MON is thought to be the inhibition of the incorporation of pyruvate into the tricarboxylic acid cycle (TCA). In addition, MON inhibits the oxidation of the TCA-intermediate – *α*-ketoglutarate (Thiel, 1978). The suggested mechanism was confirmed by Burka *et al.* (1982) and Gathercole *et al.* (1986), who found that MON reversibly inhibited pyruvate dehydrogenase. Further studies revealed that MON, competing with pyruvate for the same binding sites, inhibited the site of *α*-ketoglutarate dehydrogenase, pyruvate decarboxylase and acetohydroxyacid synthetase (Pirrung *et al.*, 1996). MON (10^{-9} M) has also been shown to interfere with carbohydrate metabolism by inhibiting transketolase (Burka *et al.*, 1982) and aldose reductase (Deruiter *et al.*, 1992). MON (0,03-0,3 µg/µl) can induce oxidative damage in myoblasts (Reams *et al.*, 1996a), possibly by inhibiting glutathione peroxidase and glutathione reductase (Chen *et al.*, 1990). The reduced viability of blood lymphocytes of broiler chickens fed with MON-contaminated feed (66 - 367 ppm) has been reported (Dombrink-Kurtzman *et al.*, 1993), an indication that MON may have some immunosuppressive effects. This is also suggested in some studies using broiler chickens and turkey poultry fed with MON-contaminated culture material, 75 mg/Kg and 100 mg/Kg respectively (Li *et al.*, 2000a,2000b).

Other studies have suggested that MON could be a cardiotoxic mycotoxin, causing organ-specific lesions, but its action and mechanism remain controversial (Reams *et al.*, 1996a; Reams *et al.*, 1996b; Reams *et al.*, 1997; Gathercole *et al.*, 1986; Chen *et al.*, 1990; Engelhardt *et al.*, 1989; Fan *et al.*, 1991; Zhang and Li, 1989; Nagaraj *et al.*, 1996). Kamyar *et al.* (2006), using isolated terminal ilea of guinea pigs and Caco-2 cells, evidenced that MON (10-30-100 and 300 µM) is not a specific cardiotoxic secondary metabolite, affects contractility only at high concentrations, exerts no electrophysiological effects anddoes not influence intracellular ion concentrations, ATP and pH.

Other phytotoxic and cytotoxic effects of MON are summarized in Table 7.

The effective concentration levels *in vivo* suggest that it has a much more severe toxic action at the cellular level than is observed in *in vitro* studies. This may partially be due to the difficulties of the water-soluble “pure” standard to penetrate through the cell membrane.

Table 7. Different toxic action reported for moniliformin in *in vitro* -tests (Jestoi, 2008).

Property	Organism/cell line	Observed effect(s)/special remark	Effective concentration/dose	Reference
PHYTOTOXICITY	wheat (*Triticum aestivum*), tobacco (*Nicotiana tabacum*), corn (*Zea mays*)	growth regulation, necrosis, chlorosis, distortion of leaf shape	20-200 ppm; 20-2000 μg; 2-200 μg	(Cole *et al.*, 1973)
	duckweed (*Lemna minor*)	reduced growth and leaf chlorophyll content	66.7 μg/ml	(Vesonder *et al.*, 1992)
CYTOTOXICITY	Hep-II (human epithelial), Chang (human liver epithelial)	no cytotoxicity observed	10mM	(Robb and Norval, 1983)
	Human lymphocyte, K562 (human erythroleukemia), MIN-GL1 (human lymphoid)	viability	IC_{50} 22μM (lymphocyte), >100 μM (K-562, MIN-GL1)	(Visconti *et al.*, 1991)
	BHK-21 (baby hamster kidney), MM (McCoy mouse), RH (rat hepoma), MDCK (dog kidney), CHO-K1 (chinese hamster ovary)	viability, MON cytotoxic to MM, RH MDCK and CHO	5-75 μg/ml	(Vesonder *et al.*, 1993)
	SK (swine kidney)	viability	IC_{50} > 50 μM	(Hanelt *et al.*, 1994)
	hepatocytes, cardiomyocytes, skeletomyocytes, splenocytes, chondrocytes (chicken)	viability, MON cytotoxic to hepatocytes, cardiomyocytes, skeletomyocytes, splenocytes	IC_{50} 42 μM (skeletomyocytes), 95 μM (cardiomyocytes), > 200 μM (splenocytes)	(Wu *et al.*, 1995)
	L6 myoblast (rat skeletal muscle)	morphologic alterations, viability; depletion of creatine phosphokinase, no inhibition of protein synthesis	0.03-0.3 μg/ml	(Reams *et al.*, 1996a)
	rat hepatocytes	inhibition of cell division; chromosome breakage	0.01-100 μg/ml	(Knasmuller *et al.*, 1997)
	PK-15 (porcine kidney)	decrease in metabolic activity	IC_{50} > 40 μM	(Uhlig *et al.*, 2004)

Reproductive toxicity

Female mink exposed to 8.1 or 17.0 mg MON/kg feed (wet weight) MON provided by *Fusarium fujikuroi* culture material (M-1214) from 2 weeks prior to the breeding season until their offspring were 8 weeks old. Consumption of the high-dose (17 mg MON/kg feed) diet resulted in significant neonatal mortality and reduced body weights at birth and at 8 weeks of age. Necropsy of 8-week-old pups from the control and MON exposed groups revealed no histological alterations in liver, lung or heart tissues. These results indicate that long-term (105–135 days) exposure to *Fusarium fujikuroi* culture material containing 17 mg MON/kg feed is not lethal to adult female mink, but can have adverse effects on neonatal mink (Morgan *et al.*, 1998).

Developmental toxicity

As reported by Hood and Szczech (1983) MON does not seem to have any teratogenic effects in experimental laboratory animals (dose is not defined).

Genotoxicity

At low doses (1.0 μg/ml), chromosonal aberrations in primary cultures of rat hepatocytes have been shown (Knasmuller *et al.*, 1997), although Norred *et al.* (1992) could not verify these findings.

In contrast, MON was inactive in bacterial genotoxicity assays; MON is not mutagenic in the Ames test indicating that, probably, it is not a genotoxic carcinogen (Knasmuller *et al.*, 1997; Wehner *et al.*, 1978).

Carcinogenicity

No studies were found on the carcinogenity of MON.

Human studies

In humans, MON is suspected to be associated with an endemic disease in China called Keshan disease (Chen *et al.*, 1990; Zhu *et al.*, 1982) even if a clear correlation is lacking.

LEGISLATION ON MONILIFORMIN

Although MON is a highly toxic metabolite and reported to occur worldwide in cereals, no regulatory limits and trade specifications have been established so far for this mycotoxin.

Only one study proposes a tolerance limit for MON in poultry of a 50 mg/kg diet (Ledoux *et al.*, 1995). Compilation of data from literature for a particular mycotoxin is fraught with

difficulties arising from expected variations between studies and from the criteria used to develop estimates of tolerance. Dose in terms of intake is more important in determining toxicity than dietary levels. In addition, rates of detoxification of absorbed mycotoxins will have an impact on the eventual outcome. It should also be recognized that no allowance has been made for additive or synergistic effects arising from co-occurring mycotoxins. The choice of performance and biochemical criteria may also influence the selection of tolerance values (D'Mello *et al.*, 1999). According to Ledoux *et al.* (1995), significant increases in mortality of broiler chicks are not observed until dietary levels of MON exceed 200 mg/kg. If feed intake depression is to be avoided, then levels must not exceed 100 mg/kg, and if normal heart weight is the selected criterion, levels of MON should remain below 50 mg/kg diet.

More data about toxicity, occurrence and validated methods are needed to set regulatory limits for MON in food and feed.

CONCLUSIONS

MON is produced by at least thirty *Fusarium* species, isolated from different substrates and geographical areas, but in Europe the problem is strongly linked to the presence of *F. subglutinans* in the maize ear rot complex and *F. avenaceum* in the *Fusarium* head blight of wheat.

Moniliformin is a toxin of possible concern in animal feeding stuffs (especially maize-based), but the lack of data on occurrence and its carryover into animal products make it impossible to evaluate its significance to animal and human health. Further studies on MON are needed to allow a more detailed risk assessment. Besides, some of the published data in which maize containing MON has been fed to animals may need re-evaluation on the basis that contaminated maize may contain a cocktail of toxic *Fusarium*-derived residues such as fumonisins, zearalenone and trichothecenes.

Knowledge on mitigation is still poor and mainly based on the actions suggested for other fuasaria.

No specific research has been undertaken about sampling plans for MON in food and feed, which is a topic which should be faced in the near future.

Analytical methods available have not been validated by interlaboratory studies and there are no certified reference materials or proficiency studies available for the determination of MON.

MON stability and fate during processing has been poorly studied in various products, but it showed heat stability similar to or greater than other *Fusarium* mycotoxins such as deoxynivalenol and fumonisin B_1.

It is difficult to adequately assess the degree of consumer exposure and more data about toxicity, occurrence and validated methods are needed to set regulatory limits for MON in food and feed.

FUTURES

- Studies on ecology of main fungi involved in MON production are suggested.
- Validated analytical methods for the quantification of MON are needed.
- Surveys on MON contamination in cereals and co-occurrence with other mycotoxins are necessary.
- Detailed studies on toxicity are mandatory for any approach of risk assessment.

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Chapter 5 - Nivalenol in feed

Keywords: Nivalenol, *Fusarium*, Trichothecene, Biosynthesis, Analytical methods, Maize, Wheat, Barley, Feedstuffs, Mitigation, Metabolism, Toxicity.

INTRODUCTION

Nivalenol (12,13-epoxy-3,4,7,15-tetrahydroxytrichothec-9-en-8-one; NIV) is a type B trichothecene primarily produced by *Fusarium cerealis* (*F. crookwellence*), *F. poae* and *F. nivale* and to a lesser extent also by *F. culmorum* and *F. graminearum* (Eriksen, 2003). NIV was first isolated from *F. nivale* Fn2B, an atypical strain of *F. sporotrichioides* (IARC, 1993; Weidenbőrner, 2001).

NIV occurs more frequently in years with dry and warm growing seasons (Pettersson, 1995); it is more common in Europe, Australia and Asia than in America, where the occurrence of NIV is limited. NIV may occur together with fusarenon X (FusX), the C-4 acetylated derivative of NIV, and other toxins produced by *Fusari*a.

Trichothecenes are secondary fungal metabolites harmful to human and animal health, causing a range of acute and chronic symptoms (D'Mello and Macdonald, 1997; D'Mello *et al*., 1999). This class of mycotoxins comprises a very large family of related chemicals produced by a number of taxonomically unrelated fungal genera, including *Fusarium*, *Mycothecium*, *Trichoderma*, *Trichothecium*, *Stachybotrys*, *Verticimonosporium* and *Cephalosporium* (Ueno, 1985).

Fusaria produce type A and B trichothecenes. Types A and B are distinguished by the presence of an oxygen or carbonyl functional group at the C-8 position, respectively. The lack of the carbonyl group tends to make type A trichothecenes more toxic (Smith and Solomons, 1994). The type A trichothecenes include T-2 toxin (T-2), HT-2 toxin (HT-2), neosolaniol (NEO), diacetoxyscirpenol (DAS) and monoacetoxyscirpenol (MAS), while type B trichothecenes include deoxynivalenol (DON, also known as vomitoxin) and its 3-acetyl and 15-acetyl derivatives (3AcDON and 15AcDON, respectively), nivalenol (NIV) and fusarenon X (FusX) (Krska *et al*., 2007b). The synthesis of the two types of trichothecenes appears to be characteristic of a particular *Fusarium* species. For example, production of type A trichothecenes predominates in *F. sporotrichioides* and possibly also *F. poae*, whereas synthesis of type B trichothecenes occurs principally in *F. culmorum* and *F. graminearum* (Placinta *et al*., 1999). In addition, within-species chemotypes are discernable. Thus, Perkowski *et al*., (1997) identified two chemotypes of *F. graminearum*, one producing NIV, DON and FusX and the other elaborating a mixture of DON and both its acetylated derivatives.

Contamination of cereals and related products with *Fusarium* toxins causes feed-borne intoxication especially in farm animals (Fink-Gremmels and Malekinejad, 2007). Common symptoms are feed refusal, vomiting, reduced weight gain, growth retardation, diarrhoea, haemorragic lesions, skin toxicity, immunosuppression, reduced ovarian function and

reproductive disorders (Rocha *et al*., 2005). The toxicity of trichothecenes is largely due to their ability to inhibit protein as well as RNA and DNA synthesis (Klotzel *et al*., 2006).

The trichothecenes have been shown to reduce performance, affect reproductive systems, impair immune function in livestock and aquatic species, and can pose a severe threat to humans and animals consuming contaminated cereal products (Woodward *et al*., 1983; Arukwe *et al*., 1999; Eriksen *et al*., 2004; Diaz *et al*., 2005; Morgavi and Riley, 2007; Zhou *et al*., 2008).

In addition to health problems and/or economic losses, trichothecenes, when present in raw materials, can have negative effects on some technological processes such as malting, brewing, fermentation and baking (Lancova *et al*., 2008).

Trichothecenes are in general very stable compounds, both during storage/milling and the processing/cooking of food, and they do not degrade at high temperatures (Eriksen and Alexander, 1998).

FUSARIUM AND ITS HOSTS

The genus *Fusarium* was established by Link (1809), nearly 200 years ago and currently contains over 20 species (De Hoog *et al*., 2000); some of them are the most important toxigenic plant pathogenic fungi. They infect important crops such as soft and durum wheat, barley, oats, rice, maize, potato, asparagus, mango, grasses, and other food and feed grains (Nelson, 2002).

Fusarium species produce long, multicellular, canoe-shaped or banana-shaped macroconidia. These large asexual conidia are the defining morphological characteristic of the genus. Many species will also produce small, generally single-celled microconidia that range in shape from fusiform to oval to spherical. Additionally, some species produce thick-walled resistant chlamydospores important for long-term survival. Microconidia and macroconidia are important for wind and splash dispersal of the fungi. The conidia are also generally the propagules that result in infection of host plants.

Fusarium species are diverse in their host-associations and mycotoxin profiles, clearly identifying one species from another based on a range of morphological, molecular, and metabolic data.

Many trichothecene-producing *Fusarium* species are common causal organisms of *Fusarium* head blight, also known as ‘scab’ or ‘head blight’, foot rot, root rot, stem and ear rot disease of cereals, including the NIV producers *F. poae, F. culmorum* and *F. graminearum*). Consequently, infection and colonization of cereal heads by such fungi not only affects yield, but can also result in trichothecene contamination of grain (Rocha *et al*., 2005). The

incidence of these mycotoxins in feed and food crops can vary considerably from year to year depending on many factors, weather conditions and agricultural practices being the most significant (Edwards, 2004).

Infection cycle

No specific studies have been conducted on the infection cycle of NIV producers fusaria, but it is known that in general fusaria can grow successfully on a variety of substrates, can tolerate diverse environmental conditions, and have high levels of intraspecific genetic and genotypic diversity (Miedaner and Schilling, 1996; Kerényi *et al*., 1997).

Meiotic recombination can generate and maintain genotypic variation and result in the reassortment of genes that govern traits such as virulence or toxin production (Cumagun *et al*., 2002). The sexual spores (ascospores) produced by some *Fusarium* species may also function as infectious propagules (Glass and Kuldau, 1992). Although several *Fusarium* species have a known sexual cycle, i.e., they mate in either a homothallic or heterothallic manner followed by subsequent meiosis and the production of ascospores. The sexual stages of important pathogenic species, including *Fusarium avenaceum*, *Fusarium cerealis*, *Fusarium culmorum*, *Fusarium equiseti*, *Fusarium poae*, and *Fusarium sporotrichioides*, are not known (Kerényi *et al*., 2004).

Fusarium is a soil-borne pathogen. The most important sources of inoculum are seeds and crop debris. A warm dry spring induces spore production and they are rain dispersed. The spores land on spikes and colonize the exterior of the plant. During anthesis, the mycelium grows into the flowers and infects through weak points, such as stomata and anthers. Following the initial colonization, the mycelium easily spreads to the epidermis and parenchyma of the flower, stigmas and anthers. In some cases, the mycelium also resides between the kernel epidermis cell wall and cuticle (Bushnell *et al*., 2003). This growth pattern is believed to serve as a mechanism for the dispersal of the fungi (Goswamin and Kistler, 2004). The mycelium penetration is thought to be aided by excretion of hydrolytic enzymes (cutinases, celluloses, amylases and pectinases) (Jenczionka *et al*. 2003). Heavy rainfall in June splashes spores onto ears and high rainfall/humidity through summer allows infection to spread and can result in very high levels of seed-borne infection.

Ecology

Fusarium species are common in tropical and temperate regions and are also found in desert, alpine, and arctic areas, where harsh climatic conditions prevail (Booth, 1971); they are abundant in fertile cultivated soils (Burgess, 1981).

The widespread distribution of *Fusarium* species may be attributed to the ability of these fungi to grow on a wide range of substrates and their efficient mechanisms for dispersal (Burgess, 1981).

Fusarium poae is widely distributed in soil in temperate climates and it is a major component of the head blight complex of barley, wheat and oat in Europe (Bottalico and Perrone, 2002; Parry *et al*., 1995).

Ecological needs of *F. poae* and *F. cerealis*, the main responsible for NIV contamination, are not described in literature.

Plant-pathogen interactions

Fusarium head/ear blight (FHB, FEB) or scab of small grain cereals affects the ears (heads) of the host, i.e. wheat, barley, oats, rye and triticale. FHB has been linked with up to 17 causal organisms, although most records of the disease are associated with five species: *Fusarium culmorum*; *F. avenaceum* (*Gibberella avenacea*); *F. graminearum* (*G. zeae*); *F. poae* and *Microdochium nivale*. The disease occurs in most areas of the world where small grain cereals are grown.

F. graminearum is considered the main causal agent of scab disease that is distributed worldwide on wheat and oats. It infects barley and rye in temperate regions and maize and rice in the tropics. On maize, the disease is prevalent in the cooler moist regions of cultivation and in cool seasons. The fungus can cause shrivelling of grains, the ears often being covered with a pinkish mat or mycelium. The ears may be infected at any time, but the crucial stage is around flowering when the stigma surface is available and receptive to spores which may arrive by wind, rain splash or by plants rubbing together. The spores penetrate the ovary wall and inhibit its development. At first, only conidia are produced from the mycelium coating the ear but perithecia may occur later giving the typical “scabby” symptoms (Jones and Clifford, 1983). This pathogen is responsible for billions of dollars in economic losses worldwide each year (De Wolf *et al*., 2003; O'Donnell *et al*., 2004). Infection causes contamination with mycotoxins, mainly deoxynivalenol (DON) and zearalenone (ZEA). NIV was less surveyed, but many reports are available regarding cereal contamination. It is probably due to its production by *F. graminearum* or possibly by the other producers even if less/never cited as associated to FHB.

PHYSICO-CHEMICAL CHARACTERISTICS

Nivalenol (3α,4β,7α,15-Tetrahydroxy-12,13-epoxytrichotec-9-en-8-one; $C_{15}H_{20}0_7$, MW 312.3 g/mol) belongs to type B trichothecenes, which are characterized by the presence of a

carbonyl group at C-8 position. Nivalenol is a white crystalline powder with a melting point at 138-140°C.

It is soluble in common polar organic solvents suchas acetonitrile, methanol and ethyl acetate, and slightly soluble in water and chloroform. It shows a UV absorption at 220 nm, with an extinction coefficient ε in acetonitrile of 6900 (concentration ~ 10 mg/L) (Krska *et al*., 2007a).

Acetonitrile is recommended as a suitable solvent for NIV since it is water-miscible and UV-transparent at $\lambda \leq 220$ nm, namely the UV range where nivalenol shows its characteristic absorption. Solutions of NIV in acetonitrile are stable at temperatures up to 25°C for at least 24 months, whilst long-term storage of NIV in ethyl acetate or as thin film at temperatures above freezing should be avoided (Widestrand and Petterson, 2001).

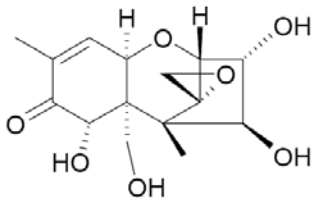

Figure 1. Chemical structure of the type B trichothence nivalenol.

BIOSYNTHESIS

Classified into the chemical group sesquiterpene, trichothecenes have a skeleton derived from farnesyl pyrophosphate (FPP), which is synthesised by the condensation of isopentenyl pyrophosphate (IPP), an isoprene unit, with its isomer dimethylallylpyrophosphate, followed by the repeated condensation of IPP with the resulting prenyl pyrophosphate called geranylpyrophosphate.

FPP is a common intermediate in protein isoprenylation and in the biosynthesis of secondary metabolites (Figure 2).

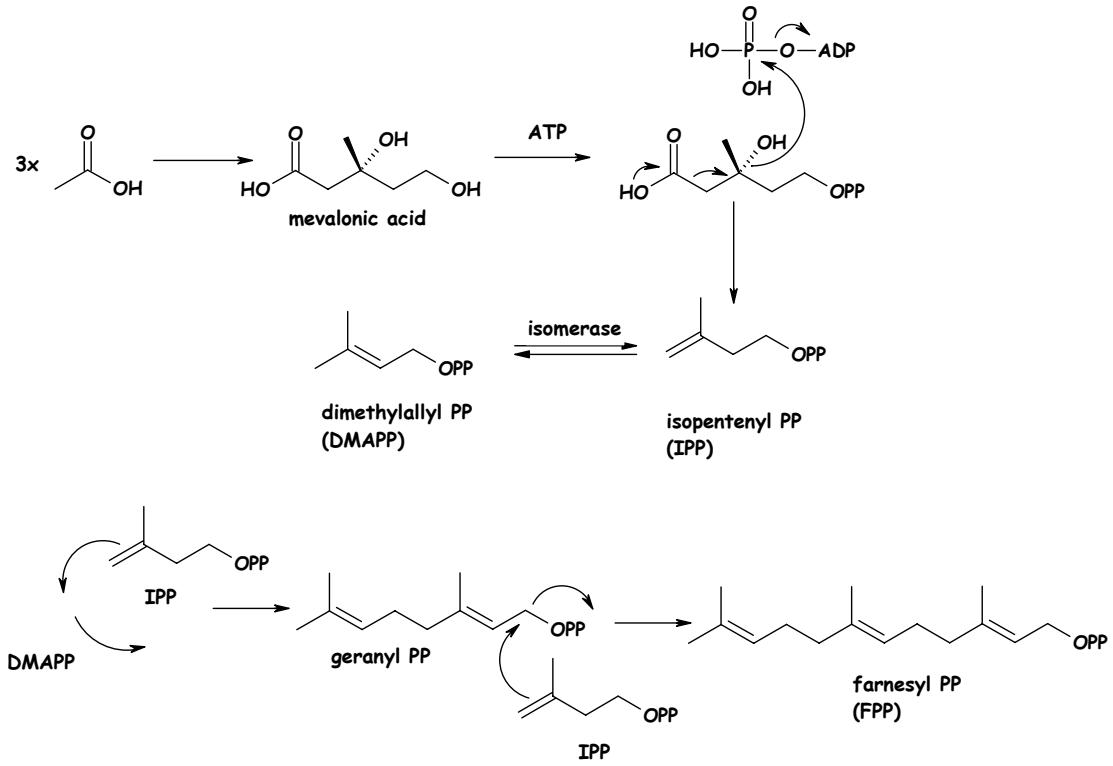

Figure 2. Farnesyl pyrophosphate formation.

In the pathway producing trichothecenes and related mycotoxins the first identified intermediate was trichodiene (TDN), which was proved to originate from mevalonate via all-*trans*-FPP and nerodyl pyrophosphate, as reported in Figure 3. (Blackwell *et al*., 1985; Zamir *et al*., 1989).

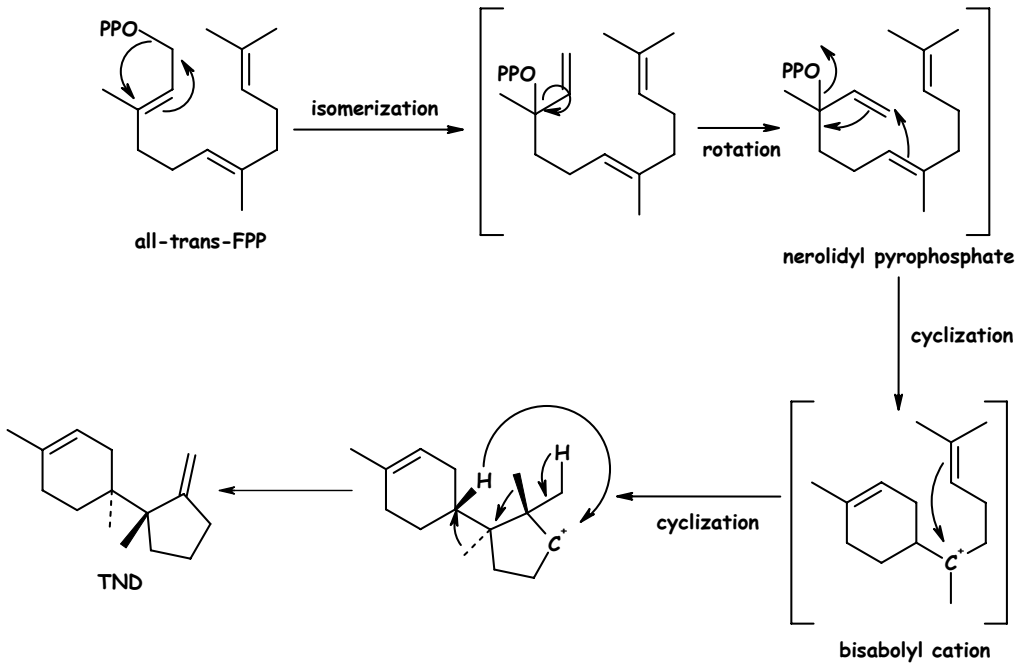

Figure 3. TND biosynthetic pathway.

The following oxygenation reactions, which are mediated by cytochrome P450 monoxygenases (CYPs), give rise to the addition of hydroxyl groups at C-3, C-4, C-8 and C-15 and to the formation of an epoxy group at position 12,13. The oxygenation steps after TND are sequential and not random, following the sequence reported in Figure 4. The last oxygenation step giving isotrichotriol seems to be the rate-limiting step of the four consecutive steps of oxygenation of TND in the early biosynthetic pathway (Hesketh *et al*., 1991; Hesketh *et al*., 1992; Zamir *et al*., 1999).

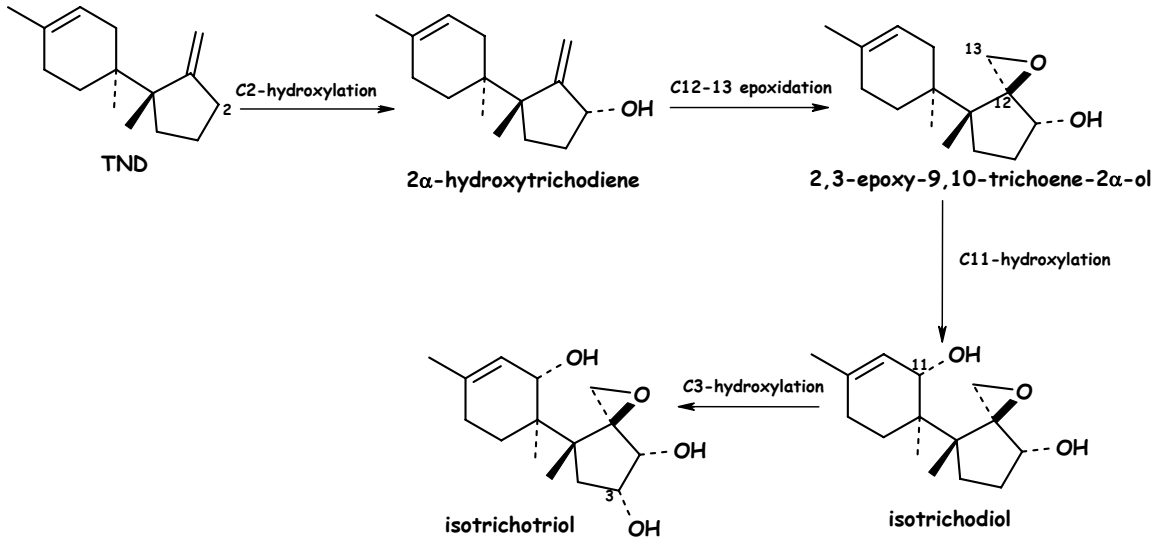

Figure 4. TND conversion to isotrichotriol.

The conversion of isotrichotriol to isotrichodermol was shown to proceed non-enzymatically in acidic conditions, although the reaction was not rapid enough to feature in a biosynthetic pathway. Since the transient formation of trichotriol was observed during the acidic-catalyzed chemical conversion, an intermediate role for trichotriol was proposed in the second cyclization. Hesketh's group also identified the natural occurrence of isotrichotriol, trichotriol and 9α-epimer of trichotriol in *F. culmorum*. Considering the co-occurrence of the three tetraoxygenated TDN isomers, the proposed transient carbocation intermediate appears to be a common pathway intermediate. The unstable compound undergoes either irreversible cyclization to isotrichodermol by intramolecular attack of the C-2 hydroxyl to C-11 or reversible epimerization to trichotriol and its 9α-epimer by regiospecific attack of a water nucleophile at C-9 (Figure 5) (Hesketh *et al*., 1992).

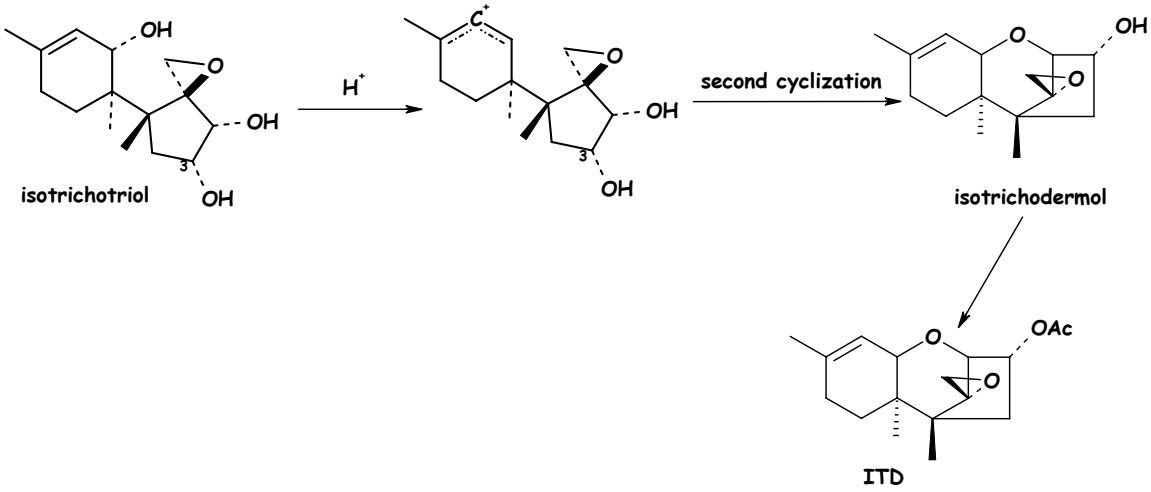

Figure 5. Second cyclization scheme.

Since the *Fusarium* culture is not acidic enough to rapidly promote the cyclization of isotrichotriol to isotrichodermol, the major biosynthetic route may involve a direct enzymatic cyclization. However, a gene for this second cyclization step is not present in the cluster of genes involved in the biosynthesis of trichothecenes in *Fusarium* species. It is possible that a locally high concentration of isotrichotriol on the endoplasmatic reticulum membrane greatly enhances progression of the forward chemical reaction even under mildly acidic conditions.

All such trichothecene-related compounds had a trichothecene structure with a hydroxyl or acetyl at C-3: these included all the species reported in Figure 6, generated by the random oxygenation at C-7, C-8 and C-15 followed by an acetylation step (Zamir *et al*., 1991).

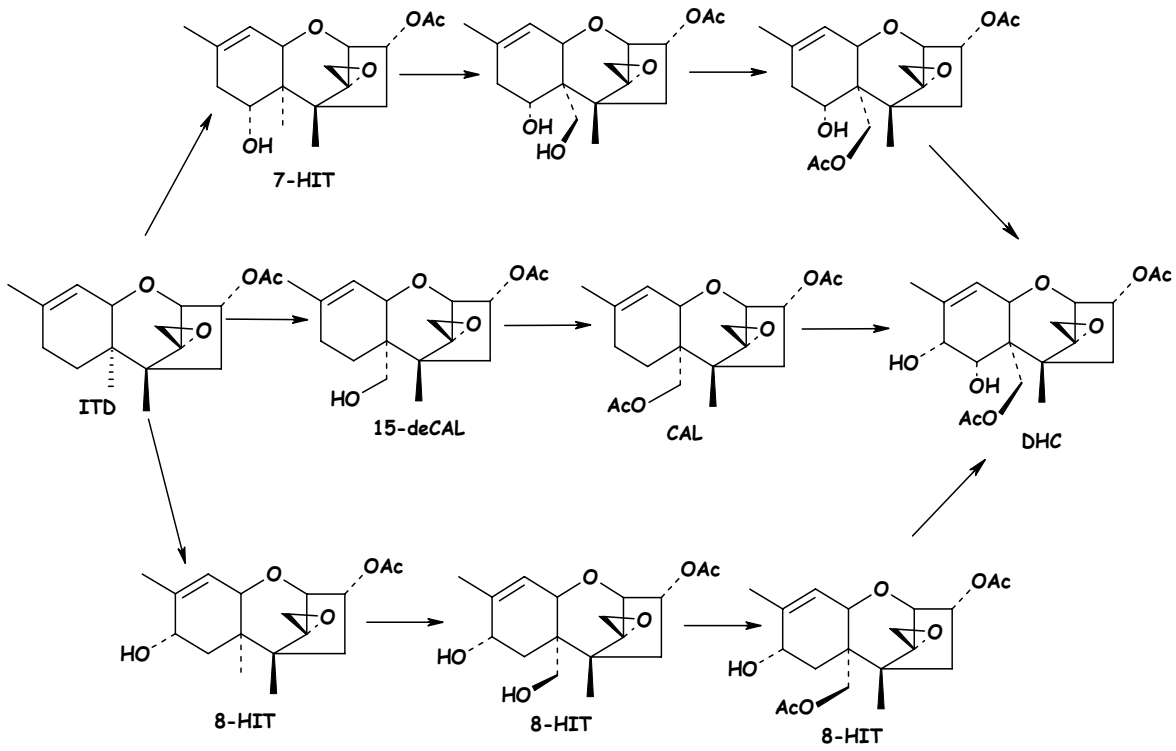

Figure 6. Trichothecenes differentiation pathway.

After the formation of calonectrin (CAL), a diversity of trichothecene structures arises. In particular, the substrate specificity of certain late biosynthetic enzymes differs between type A and type B trichothecene producers (Hesketh *et al*., 1991). Partly due to the difficulties of prepararing labelled derivatives for feeding experiments, chemical procedures were not used to determine the biosynthetic grids. Instead, the availability of gene manipulation techniques and the discovery of the trichothecene pathway genes have opened alternative ways to study the late steps in the biosynthesis of trichothecenes.

However, by now, the formation pathway of NIV has only been proposed. In particular, the tentative NIV formation pathway has been proposed on the basis of several experimental results using the targeted gene disruption mutants of *F.graminearum*, as reported below (Kimura *et al*., 2007) (Figure 7).

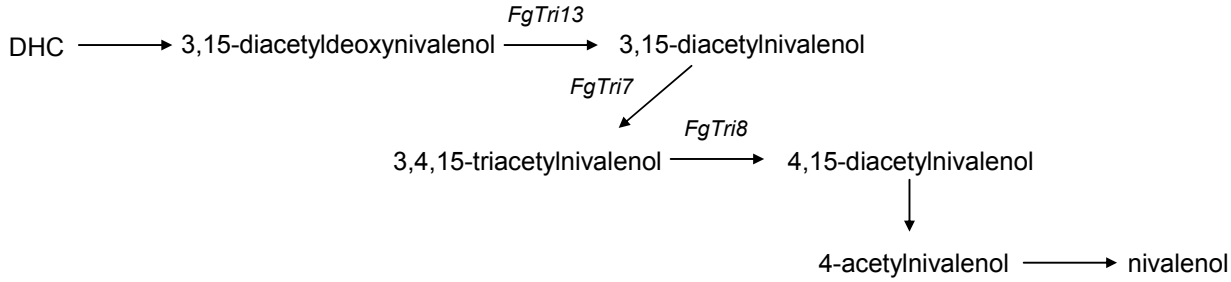

Figure 7. Biosynthetic pathway proposed for nivalenol.

Although the trichothecenes pathway has been elucidated, NIV biosynthesis is still to be defined in the very last steps. Moreover, to date very few data on genes involved in the biosynthesis are available.

CHEMICAL ANALYSES

Sampling

In general, *Fusarium* toxins are heterogeneously distributed in the commodities to be inspected, which makes it difficult to obtain a representative sample. To reduce variance, clearly defined sampling plans are required, generally including large sample sizes. To prepare a representative test portion for analysis, the sample is ground and thoroughly mixed (Pronk *et al*., 2002).

A specific sampling method for commodities containing NIV does not exist, but we can refer to Commission Regulation (EC) No. 401/2006 of 23 February 2006, "laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs".

Extraction and Clean-up

The analytical procedures reported in literature are usually focused on the simultaneous determination of the main compounds belonging to the same group. Thus, extraction procedures as well as analytical methods are usually developed trying to find the best compromise in order to get reliable results for all the analytes considered.

Since type B trichothecenes, including NIV, are mainly contaminants of grains, the majority of analytical methods have been developed for cereals and grains, without any distinction between food and feed. More specific methods have then been developed only for food, in order to achieve lower detection limits and to obtain data for risk assessment (Langseth and Rundberget, 1998).

Samples are usually extracted with acetonitrile/water mixtures in different ratios, sometimes also applying pressure in order to speed up and improve the extraction process. Typically, extracts are afterwards cleaned with MycoSep columns (mixture of charcoal, ion-exchange resins and other materials) (Krska *et al*., 2001). Alternatively, solid phase extraction (SPE) has been described for sample extract clean up applying either carbograph-4 material (Cavaliere *et al*., 2005), polymeric adsorbent materials with Reverse Phase (RP) and anion exchange functionalities (Meky *et al*., 2003; Kloetzel *et al*., 2006; Lattanzio *et al*., 2008), or reversed-phase materials partly in combination with a liquid/liquid extraction step (Sypecka *et al*., 2004). No immunoaffinity material is available for NIV clean up (Josephs *et al*., 2001; Krska *et al*., 2001).

Several authors proposed to omit any kind of sample clean up when mass spectrometry based detection is applied, on account of the high selectivity of this technique. Quantitative data down to the 50 µg/kg level were readily achieved, expecially for NIV and DON, when the crude extract is diluted with water and directly injected into the liquid chromatography coupled with mass spectrometric detector (LC/MS).

Source of standards

Availability of NIV standards is shown in Table 1.

Table 1. Sources of nivalenol standards.

Compound	Concentration (µg/ml)	Quantity	Supplier
Standard Solution			
Nivalenol solution (OEKANAL)	100 in acetonitrile	2 ml	Fluka. Sigma-Aldrich (Buchs - Switzerland)
Nivalenol solution	100 in acetonitrile	1/5 ml	Chiron AS (Trondheim - Norway)
	100 in acetonitrile	1/5 ml	Romer Labs Diagnostic (Tulln - Austria)
Nivalenol BCR (IRMM-316)	18.8 ± 0.9 in acetonitrile	4 ml	Fluka. Sigma-Aldrich (Buchs - Switzerland)
Solid Standard			
Nivalenol hydrate (OEKANAL)		5 mg	Fluka. Sigma-Aldrich (Buchs - Switzerland)
Nivalenol hydrate		5/10 mg	Chiron AS (Trondheim - Norway)
		5/10 mg	Romer Labs Diagnostic (Tulln - Austria)

Analytical methods

Many analytical methods for the determination of type B trichotecenes and, among them, NIV have been published in the last decade. Accurate and reliable detection of these compounds is usually required down to 100 – 500 µg/Kg range in naturally contaminated samples. Suitable analytical methodology, which has been summarised in several reviews

(Josephs *et al*., 2001; Krska *et al*., 2001; Sforza *et al*., 2006; Zoellner and Mayer-Helm, 2006; Lattanzio *et al*., 2009), relies predominantly on gas chromatography coupled with mass spectrometric detector (GC/MS), gas chromatography coupled with electron capture detector (GC/ECD) and LC/MS techniques, on account of the lack of chromophore or fluorophore moieties on the analyte. Major advantages of these methods are their high separation efficiency in mixture and the cost effective and relatively easy way to achieve reliable results. Concerning the analytical determination of *Fusarium* mycotoxins in feed and, among them, NIV, only one updated review was found in literature (Krska *et al*., 2007b).

Gas chromatography analysis is widely employed for the determination of type B trichothecenes, although the conjugation of the carbonyl group with the double bond makes them more suited for HPLC analysis. Gas chromatography determination is mostly based on derivatization of the hydroxyl groups by trimethylsilylation or fluoroacylation to trifluoroacetyl (TF), pentafluoropropionyl (PFP), or heptafluorobutiryl (HFB) derivatives. FID detectors are commonly utilized or alternatively, ECD for the fluoroacyl derivatives. Although several GC/MS methods have been proposed as alternatives to GC/ECD or GC/FID, none seems to allow for a good quantitative determination (Eskola and Rizzo, 2001; Jestoi *et al*., 2004).

The GC/MS approach seems to give the best results for confirmatory analyses and also for obtaining structural information on several derivatives of the main trichothecenes, which is fundamental for toxicological and metabolic studies.

Liquid cromatography based methods without MS detection have been applied less frequently, mainly due to the low sensitivity of trichothecenes towards UV detection, which can be performed only at 220 nm (Sforza *et al*., 2006). For this reason, LC with UV detection of trichothecenes usually require pre- or post-column derivatization assays. Post-column derivatization involving alkaline degradation was reported for type B trichothecenes such as NIV (Langseth and Rundberget 1998; Young *et al*., 2006).

Since LC with fluorescence detection (FLD) generally allows for high sensitivity and good selectivity, several fluorophores have been proposed as hydroxyl derivatizing reagents, such as 1-ethoxy-4-(dichloro-1,3,5-triazinyl) naphtalene, 9-fluorenylmethyl chloroformate, carbonyl-3-coumarin chloride, anthracene-9- carbonyl chloride and 1-anthroylnitrile (Bayliss *et al*., 1988; Dall'Asta *et al*., 2004). The limit of these methods is usually due to low conversion rates recorded for type B trichothecenes and, especially, NIV, on account of the presence of a higher number of hydroxyl groups which gave rise to incomplete or unreproducible derivatization (Mateo *et al*., 2001).

ELISA assays though frequently used for screening purposes, have been found to produce inaccurate quantitative data and to overestimate trichothecenes levels in samples. Moreover,

no specific antibodies have been developed for NIV, whereas several specific immuno-assays (ELISA, lateral-flow devices) have been improved for DON (Josephs *et al*., 2001).

HPLC separation prior to MS detection relies on reversed-phase materials (RP-18) with acetonitrile/methanol/water, methanol/water or acetonitrile/water mixtures as mobile phases in both isocratic or gradient mode (Zoellner and Mayer-Helm, 2006). Methanol was reported to enhance MS sensitivity for type B trichothecenes when compared to acetonitrile (Berthiller *et al*., 2005). Acetic acid and ammonium acetate are commonly applied as additives either to improve chromatographic separation or to force adduct formation for improving MS sensitivity or structural elucidation. Depending on the analytes, matrix and column lengths, run times range between 6 and 30 minutes (Sforza *et al*., 2006; Zoellner and Mayer-Helm, 2006).

A critical point is the NIV separation from disturbing co-eluting matrix compounds, on account of its higher polarity in comparison to other trichothecenes. A possible solution is the increasing of chromatographic retention in order to sufficiently separate interfering compounds from the analyte. On the other hand, a more extensive sample preparation protocol is feasible to avoid matrix interferences (Plattner and Maragos, 2003).

Both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) modes have been successfully applied in the positive as well in the negative ion mode for the analysis of type B trichothecenes, although sensitivity seems to be strongly dependent on the geometry and technical details of the MS instrumentation. For this reason, a better and stable ionisation performance may be achieved and interference contamination may be reduced when the matrix and salt contaminated first portion of the LC effluent is diverted into waste (Sorensen and Elbaek, 2005). Usually, NIV and other type B trichothecens are better detected using the negative ion mode. Very recently, multiresidual methods have been proposed by switching between negative and positive ionisation to achieve similar sensitivity for both types of trichothecenes (Berthiller *et al*., 2005; Sulyok *et al*., 2007).

Several authors added additives, such as ammonium acetate, sodium acetate and acetic acid to the mobile phase, in order to support the ionisation process via adduct formation.

APCI sensitivity was reported to be lower than ESI, on account of its higher in-source fragmentation. Consequently, ESI is mainly preferred in quantitative applications, since it also seems to be more robust than APCI (Sforza *et al*., 2006).

Analyte quantification has been proposed by select ion monitoring (SIM) or, in order to get more accurate quantification, using multi-stage MS applications with triple quadrupole or ion trap instrumentation (Razzazi-Fazeli *et al*., 1999 and 2003; Melchert and Pabel, 2004; Berthiller *et al*., 2005; Sulyok *et al*., 2007).

Validation and outlook

In general, LC/MS provides accurate quantification of trichothecenes down to low µg/kg level. Recovery rates range between 70% and 108% and depend on the polarity of the analytes. Generally, polar trichothecenes such as NIV exhibit the lowest recovery rates, indicating significant losses during sample preparation. Increasing the polarity of the extraction medium has been reported to significantly enhance the recovery rates of NIV although leading to strong ion suppression phenomena due to higher amounts of polar coextracted matrix compounds (Kloetzel *et al*., 2005).

Typically, linearity ranges are achieved with one or two orders of magnitude with good correlation coefficients (Zoellner and Mayer-Helm, 2006). Most authors did not use internal standards to compensate for analyte losses, matrix effects or performance deviations of the MS detector. However, matrix-matched calibration curves and isotope-labelled internal standard addition should be taken into account to cope with matrix related method validation problems. Unfortunately, isotope labelled NIV is still not commercially available, although Asam and Rychlik (2007a and b) proposed the in-house preparation of [$^{13}C_2$]-4-acetyl-nivalenol as an internal standard to compensate for NIV losses, achieving recovery rates of 94-101%.

Several studies about NIV elemental analysis have been performed. The difference in molecular weight between NIV and NIV-monohydrate, which was found to be the main compound in the calibrant solution, was 5.4%. Consequently, 10 mg of NIV-monohydrate contained only 9.46 mg of NIV (Krska *et al*., 2005; Welzig *et al*., 2005). Calibrant with such insufficiently characterized calibrants resulted in systematic errors that could be avoided through a regular check by certified calibrants. Krska *et al*. (2005) reported the urgency of purity assessment of trichothecenes calibrants: intercomparison studies made it possible to state that purity assessment and the use of common calibrants improved the partecipants overall performace and led to a better comparability of measurement results at a EU level.

Since NIV is a very polar compound, in order to avoid analyte losses and matrix related errors during quantification, matrix-matched calibration and the use of internal standards should be recommended for NIV analysis. Moreover, the development of specific immunoaffinity devices as well as isotope-labelled internal standards should be encouraged. No specific validation has been reported to date for NIV occurrence in feed, which is usually a very complex matrix.

OCCURRENCE DATA

Nivalenol occurs throughout the world in various cereal crops (e.g. wheat, maize, barley, oats and rye) and processed grains. Surveys on the occurrence of NIV in grains show that the degree of contamination differs significantly in different parts of the world, climatic zones and years. NIV was frequently found in cereal grains and animal feeds, often together with DON and zearalenone (ZEN), but the occurrence of NIV is generally lower than for DON. DON, followed by NIV, T-2 and HT-2 toxins have been the most common trichothecenes found in grains in Europe (Petterson, 1995b), but there are currently data from other countries on the degree of *Fusarium* infection and trichothecene contamination.

In the final report SCOOP task 3.2.10 (2003), 16% of NIV-positive samples were described in 4166 cereal samples from Europe with mean contamination levels of 340, 351, 440, and 1860 µg/kg, for maize, barley, wheat and oats, respectively.

Placinta *et al*. (1999), provide an exhaustive review of the worldwide trichothecene contamination of cereal grains and animal feed. DON and NIV are the most commonly found trichothecenes in the world.

Maize

Nivalenol has often been reported in maize red ear rot throughout the European maize growing areas (Logrieco *et al*., 2002). Its formation in infected ears may be due to NIV-chemotypes of *F. graminearum*, especially when found together with DON and ZEN, and in the absence or scant presence of *F. cerealis*, as reported for Romania (Moldavia) (Ciudin and Bazgan, 1991), Italy (Logrieco *et al*., 1992), and Hungary (Szécsi and Bartok, 1995). But, the occurrence of NIV in European areas appeared to be related more to the spread of *F. cerealis* than that of *F. graminearum*. In fact, strains of *F. cerealis* from red ear rot of maize were essentially able to produce NIV and FusX associated with ZEN, but not DON (Sydenham *et al*.,1991), and this capability was confirmed for several strains collected from Finland, Germany, Yugoslavia, Italy, Austria and Poland (Golinski *et al*., 1988; Bottalico *et al*., 1990).

In 1988–89 surveys of Austrian *Fusarium*-infected ears, in spite of the widespread presence of *F. graminearum*, Lew *et al*. (1991) ascribed the occurrence of NIV to the presence of *F. cerealis*. Moreover, in the 1988 Polish survey of *Fusarium* ear rot (or *Gibberella* ear rot), a total of 1350 corn ears were collected randomly from a corn field at the Radzikow Plant Breeding Station, 30 km west of Warsaw. Kernels from visibly identifiable zones of such rotted ears as well as underlying portions of the cobs were ground separately and analyzed for trichothecenes. Almost all samples predominantly colonized by *F. cerealis* were found to

be highly contaminated by NIV (33-56 mg/kg) and FusX (0.6-20 mg/kg) (Visconti *et al*., 1990).

In another Polish survey, high concentrations of NIV (0.6-42.2 mg/kg), FusX (0.1-0.8 mg/kg) and ZEN (0.5-99.6 mg/g), were found in ears predominantly infected by *F. cerealis* (Grabarkiewicz-Szczesna *et al*., 1996).

Finally, in Polish samples of maize ears affected by pink ear rot caused by *F. poae,* NIV was found, associated with FusX, both in grains and in cobs (Chelkowski *et al*., 1994). *Fusarium poae*, one of the pathogens responsible for scab of cereals, is very common in Poland and in other countries of Northern and Eastern Europe. Its potential to biosynthesis of very toxic NIV was a motivation for Grabarkiewicz-Szczesna *et al*. (1999) to start investigation on scab of corn cobs caused by the pathogen and trichothecene accumulation in infested kernels. Corn cultivars, commonly used for feeding purposes, were inoculated by a toothpick overgrown with *F. poae*. During 1994, 9 hybrids and during 1995 6 hybrids were tested. Fraction of kernels with scab symptoms and *Fusarium* damaged kernels (FDK) (average of 10 cobs per genotype), were analysed for NIV presence in two replicates for each sample. Fraction of healthy kernels (HLK) was free of detectable amounts of trichothecenes while scabby kernels were contaminated with NIV, FusX and trace amounts of DON (only few of genotypes). The results of chemical analysis of FDK fraction collected during 1994 and 1995 experiments are presented in Table 2. In 1994 the average percentage of FDK fraction was estimated at 8.2% with a mean concentration level of NIV equal to 2200 µg/kg. The average concentration of NIV was 28 fold lower in 1995 (78 µg/kg), when compared to 1994. No relation between scab severity and the toxin amounts in FDK fraction was observed. FDK fraction of Smolimag exhibited, during both seasons 1994 and 1995, the highest (among all tested cultivars) concentration level of NIV, that indicates very high susceptibility of the cultivar to toxin biosynthesis as well as to infection with the pathogen and disease development.

Wheat

Wheat, too, is commonly cultivated and is a very important crop in Poland. *F. graminearum* is the principal fungus identified as the cause of the wheat disease *Fusarium* head blight (FHB) or scab. FHB occurs worldwide in most small grain cereal growing areas, particularly of wheat and barley, and has caused devastating losses over the past decades. In addition to losses in crop yield and lower grain quality, the production of trichothecenes in scabby cereals is an important concern to both livestock and human health (Plattner and Maragos, 2003). Suitable conditions like rainy weather coinciding with a significant inoculum at anthesis play a crucial role in the disease's severity.

Samples of kernels of 25 winter wheat cultivars, very common in Poland, were collected during the harvest of 1993 in the south-east of the country, near Lublin (Grabarkiewicz-Szczesna *et al*., 2001). In morphological analysis of the samples, species of the genus *Fusarium* represented 81% of all infesting isolates. *F. graminearum* was found as the dominating species, present in 42% of tested cultivars, while *F. nivale*, *F. poae*, *F. avenaceum* and *F. culmorum* were found in 35, 35, 31 and 12% of the samples, respectively. Chemical analysis revealed that DON was most frequently present in the samples (96%) and mean value (104 µg/kg), was the highest measured. NIV was found not as frequently as DON in 76% of positive samples with an average of 97 µg/kg (results in Table 2). Contamination with DON was observed to be higher when wet weather during anthesis followed dry spring, while NIV is a typical metabolite after dry and hot summers and harvest is performed earlier than usual (Pettersson, 1996). The dry weather conditions during the 1993 vegetation season, including anthesis, explains the relatively high incidence ratio and average concentration level of NIV in wheat samples collected in that period.

In the same years Muller *et al*., (2001) collected wheat samples randomly, during the period 1989-1993 from farms in an area of Southwest Germany, for analysis of trichothecenes. Based on incidence and level, DON was the predominant toxin in each of the years surveyed. DON was detected in 77 to 93% of samples, whereas the overall frequency of NIV was only 38%. Data are shown in Table 2.

Scab epidemics of wheat occurred in Żuławy (Northern Poland) in 1998 and in Wielkopolska (West) in 1999. Four species were identified in wheat heads with scab symptoms: *F. culmorum*, *F. graminearum*, *F. avenaceum* and *F. nivale*. A significant increase in the frequency of *F. graminearum,* between 26% and 38%, was observed in the two regions, compared to about 10% during the previous decade (Tomczak *et al*., 2002). Fields of wheat were inspected in July and August in 1998–2000. A short time before harvest, samples of wheat heads were collected: 61 in 1998, 68 in 1999 and 45 in 2000 (for results look at Table 2). Taking into consideration frequency and concentration, the most important mycotoxin detected in field samples of wheat was DON, occurring in 60–65% of samples in 1998 and in 89–95% of samples in 1999. NIV was detected at high concentrations, up to 1420 µg/kg only in 1998, when this metabolite was present in 80–85% of samples. Simultaneous co-occurrence of the three mycotoxins DON, NIV and MON (moniliformin), was found in a high percentage of positive samples, 33% and 35%, during 1998 and 1999, respectively. In 2000 *Fusarium* scab was not observed in the fields because of very dry weather conditions.

Barley

Every year barley heads are also usually infected with *Fusarium* species, and this infestation results in a reduced number of kernels per head and/or in an increased number of small kernels. Grain samples of 15 naturally contaminated barley cultivars, collected after harvest of 1997 in south-east Poland, were analysed for *Fusarium* trichothecenes (Perkowski *et al*., 2003). Before chemical analysis, all grain samples were dried, manually threshed and separated into two groups: kernels >2.5mm and kernels <2.5mm. On average, kernels >2.5mm accounted for 87.2% of sample weight, and kernels <2.5mm accounted for the remaining 12.8%, but fraction <2.5mm accumulated very higher toxin concentrations than fraction >2.5mm and contained, for example, up to 94% of NIV of the total toxin content of the whole sample.

Mean concentrations of NIV are shown in Table 2, with an average for the 15 cultivars of 61 µg/kg. Analyses revealed considerable differences in mean toxin concentrations between the analysed cultivars. General concentrations of the toxins varied greatly, but were generally higher than in barley samples analysed in Poland in 1994 and 1995, (NIV detected in 20% of samples and mean concentration of 18 µg/kg) (Perkowski *et al*., 1997) and in Germany (Muller *et al*., 1997). This may be due to the unusually high precipitation in the summer of 1997.

In the study of Muller *et al*. (1997), barley samples for feed use were collected randomly after the 1987, 1989, 1990, 1991 and 1992 crops, respectively, from farms located in an area of south-west Germany. During the whole survey DON was the major toxin based on incidence and levels. The percentage of samples positive for this toxin ranged between 71 and 98%. It is interesting to note that the frequency of NIV increased from 1987 through 1992 from 11 to 41%, together with a significant sevenfold increase of the mean concentration (Table 2). This may have resulted from an increase of growth and/or of toxin production by NIV producing *Fusarium* strains due to the dry climate in 1989–1992.

Different cereals and feedstuffs

During recent decades, NIV has been found in cereals from Sweden, North Europe and other parts of the world. Concentrations up to 3400 µg/kg have been found in Swedish oats (Pettersson, 1993) and 6600 µg/kg in wheat from China (Ueno *et al*., 1986).

Samples of oats, barley and wheat from different Swedish plant production trials were collected every year from 1987 to 1990. They were analysed for NIV and other trichothecenes by capillary gas-chromatography with EC-detection. The yearly occurrences of NIV in the different cereals are given in Table 2. The occurrences varied greatly in different

years and cereals. Between 0 and 63% were contaminated with more than 50 µg/kg. The levels were normally between 50 and 30 µg/kg. It was found most often and at highest concentrations in oats. Oats containing NIV were often also contaminated with other trichothecenes, HT-2, T-2, and DON. Wheat was less contaminated. Both spring and winter wheat were analysed, but the toxin occurred only in winter wheat. NIV has been reported to be more common in wheat than in other cereals in Europe (Gareis *et al*., 1989), but these results from Sweden indicate the opposite. *Fusarium* sp. have been isolated from NIV-contaminated samples. *F. poae* was the most common, followed by *F. culmorum* and *F. avenaceum*.

A total of 449 grain samples of barley, wheat and oats were collected from different regions of Norway from 1996-1998 crops, mainly from grain loads and silos. The samples were analysed for type A and B trichothecenes, by gas chromatography with mass spectrometric detection (GC-MS) (Langseth and Rundberget, 1999). DON and HT-2 toxin were found both in highest incidence (31 and 33%, respectively in all samples) and concentrations (102 µg/kg and 107 µg/kg, respectively), followed by T-2 toxin and NIV. Oats were the grain species most contaminated by all the four trichothecenes, followed by barley and wheat, when calculated on the basis of concentration and incidence. Both the present and an earlier study (Langseth and Elen, 1996), indicate that the NIV level in Norwegian cereals is low (34 µg/kg as mean value), and lower than in the neighbouring countries. The colder and more humid climate in the main growing areas of Norway may explain the difference. In Sweden the occurrence of NIV has been found to be more prevalent in years with dry and warm summers (Pettersson, 1995), which also explain the very low level found in the present study.

The major grain species grown in Lithuania are summer barley, winter rye and winter wheat (85%). The remaining 15% are spring wheat, winter barley, oats, legumes, buckwheat and mixed grain (barley and legumes for feed production only). Grain produced for the commercial market in Lithuania is mainly for human consumption or pig or poultry feed (Keblys *et al*., 2000). The problems with grain quality, especially fungal infections and mycotoxins, occurr mainly in small holdings, which produce about 75% of grain in Lithuania. So, to represent well the year 1999 crop, Garaleviciene *et al*. (2002a) purchased barley, wheat and oat samples at a joint stock company, just after harvest in July for analysis of different mycotoxins. Also samples of mixed feeds for swine and poultry, made from cereals stored for at least 5 months, were taken in summer 1999 at the same factory. Wheat samples were found to be positive for DON and NIV; around 65% of analysed samples contained low amounts of DON and NIV (data in Table 2). On the other hand, 10 of 12 analysed barley samples contained small amounts of DON but high concentrations of NIV

(138 µg/kg as mean value, and 303 µg/kg as max value). Mixed feed samples also showed the presence of NIV.

Also in a previous study (Keblys *et al*., 2000), barley was the most frequently contaminated grain species by NIV. It was present in 62% of barley samples, but was found in only one rye and in no wheat samples. Totally, NIV was the toxin detected in highest concentration, but was found in only 13% of the samples.

In Table 2 there are also presented data concerning the analysis of commodities including cereals, cereal by products, corn plants and corn silage, as well as non-grain based products such as hay, legumes and oilseed by products which serve as feedstuffs or mixed feed components in Germany (Schollenberger *et al*., 2006). NIV co-occurred in these commodities with other trichothecenes.

Otherwise NIV was not detected in a total of 50 samples of poultry feed mixtures of Slovakian origin, analysed for *Fusarium* mycotoxins (Labuda *et al*., 2005).

Table 2. Results of surveys for nivalenol, showing concentrations and distribution of contamination in cereals and feed commodities.

Country	Commodity	Year	Co-occurring mycotoxins	N° of samples	LOQ (µg/kg)	*n* > LOQ	Mean (µg/kg)	Min/Max (µg/kg)	References	Sampling procedure
Maize										
Poland	Milpa hybrid corn	1994	FusX	1	NA	-	2250	-	Grabarkiewicz-Szczesna *et al*., 1999	Corn cultivars, commonly used for feeding purposes
	KLG 2210 hybrid corn		FusX	1		-	2150	-		
	Buran hybrid corn		-	1		-	130	-		
	Mona hybrid corn		-	1		-	ND	-		
	Anna hybrid corn		-	1		-	ND	-		
	Smolimag hybrid corn		FusX	1		-	5800	-		
	SMH 4792 hybrid corn		FusX	1		-	620	-		
	Ela hybrid corn		FusX	1		-	3200	-		
	Zenit hybrid corn		FusX	1		-	5500	-		
	Mona hybrid corn	1995	FusX	1		-	123	-		
	Betulisa hybrid corn		FusX	1		-	24	-		
	KLG 2210 hybrid corn		-	1		-	ND	-		
	Ruten hybrid corn		FusX	1		-	40	-		
	Smolimag hybrid corn		FusX	1		-	249	-		
	RAHBE 921573 hybrid corn		FusX	1		-	32	-		
Wheat										
Poland	Winter wheat cultivars	1993	DON-MON	25	NA	NA	97	8/453	Grabarkiewicz-Szczesna *et al*., 2001	Cultivars most common in Poland, collected during harvest
Germany	Wheat samples	1989	DON-T2-HT2-FusX-3AcDON-15AcDON	53	LOD range:1-5	49 pos.	19.1	4/80	Muller *et al*., 2001	Randomly collection after harvest
		1990	"	54		19 pos.	59.8	5/145		
		1991	"	57		42 pos.	13.2	2/74		
		1992	"	72		67 pos.	68.1	3/341		
		1993	"	60		15 pos.	21.3	10/42		
Poland	Żulawy region wheat	1998	DON-MON	20	NA	NA	180	10/630	Tomczak *et al*., 2002	Wheat head samples collected a short time before harvest
	Wielkopolska region wheat	1998	"	23		NA	1080	20/1420		
	Żulawy region wheat	1999	"	26		NA	150	40/320		
	Wielkopolska region wheat	1999	"	38		NA	80	20/210		
Barley										
Poland	Ars barley cultivar	1997	DON-T2-HT2-DAS	1	LOD:10	-	80	-	Perkowski *et al*., 2003	Samples collected manually from mature barley cultivars
	Agat barley cultivar		DON-T2-HT2-DAS	1		-	70	-		
	Bielik barley cultivar		DON-T2-HT2	1		-	80	-		
	Dema barley cultivar		DON-T2-HT2	1		-	70	-		

	Diva barley cultivar		DON-T2-HT2	1		-	40	-		
	Grosso barley cultivar		DON-T2-HT2-DAS	1		-	120	-		
	Hockey barley cultivar		DON-T2-HT2	1		-	50	-		
	Klimek barley cultivar		DON-T2-HT2-DAS	1		-	20	-		
	Lot barley cultivar		DON-T2-DAS	1		-	40	-		
	Lotus barley cultivar		DON-T2-HT2-DAS	1		-	130	-		
	Nadgar barley cultivar		DON-T2-HT2-DAS	1		-	10	-		
	Roland barley cultivar		DON-T2-HT2-DAS	1		-	20	-		
	Rudzik barley cultivar		DON-T2-HT2-DAS	1		-	50	-		
	MOB-487 barley cultivar		DON-T2-HT2-DAS	1		-	60	-		
	NAD-687 barley cultivar		DON-T2-HT2-DAS	1		-	70	-		
Germany	Barley samples	1987	DON-T2-HT2-FusX-3AcDON-15AcDON	44	LOD range:1-5	11 pos.	5.2	3/10	Muller *et al*., 1997	Randomly selection 1-4 weeks after harvest
		1989	"	40		15 pos.	18.2	3/45		
		1990	"	47		11 pos.	17.4	4/38		
		1991	"	51		24 pos.	30.4	2/196		
		1992	"	58		41 pos.	34.4	2/333		
Different Cereals and feedstuffs										
Sweden	Barley	1987	-	45	NA	NA	61	NA/80	Petterson *et al*., 1995	Samples from different plant production trials
	Barley	1988	-	32		NA	700	NA/700		
	Barley	1989	-	67		NA	78	NA/130		
	Barley	1990	-	39		NA	169	NA/215		
	Oats	1986	DON-T2-HT2	18		NA	194	NA/300		
	Oats	1988	DON-T2-HT2	52		NA	50	NA/150		
	Oats	1989	DON-T2-HT2	46		NA	1094	NA/4700		
	Oats	1990	DON-T2-HT2	71		NA	147	NA/360		
	Wheat	1987	-	42		NA	85	NA/140		
	Wheat	1988	-	42		NA	60	NA/60		
	Wheat	1989	-	91		NA	ND	ND		
	Wheat	1990	-	96		NA	75	NA/75		
Norway	Barley	1996/98	DON-HT2-T2	102	LOD:20	NA	30	NA/50	Langseth and Rundberget, 1999	Sampling with automatic equipment from loads and silos
	Oats		"	178		NA	56	NA/211		
	Wheat		"	169		NA	20	NA/10		
Lithuania	Wheat	1999	DON	23	LOD:10	NA	22	NA/41	Garaleviciene *et al*., 2002a	Sampling after harvest at the stock company "Kauno Grudai"
	Barley		DON-HT2	12		10	138	NA/303		
	Oats		DON-HT2-T2	5		NA	112	NA/191		
	Mixed feed for pigs		DON-FusX- HT2-T2-ZEN-OA	25		NA	86	NA/235		
	Mixed feed for poultry		DON-FusX- HT2-T2-ZEN-OTA	27		NA	104	NA/221		

Lithuania	Barley	1998	DON-HT2-T2	29	LOD:10	NA	101	NA/571	Keblys *et al*., 2000	Samples collected in different regions after harvest
	Rye		"	46		1	20	NA/20		
	Wheat		"	84		ND	ND	ND		
Germany	Wheat	2000/01	DON-T2-HT2-ZEN	41	LOD:14	NA	33	NA	Schollenberger *et al*., 2006	Collection randomly
	Oats		DON-T2-HT2-FusX-ZEN	17		NA	155	NA/162		
	Corn		DON-T2-HT2-FusX-ZEN	41		NA	291	NA/1388		
	Corn byproducts		DON-T2-HT2-FusX-ZEN	13		NA	694	NA/2050		
	Corn plants		DON-T2-HT2-ZEN	8		NA	1312	NA/6640		
	Corn silage		DON-T2-ZEN	5		NA	1612	NA/2809		
	Hay		DON-ZEN	28		NA	131	NA/222		
	Lupines		T2-HT2	9		NA	23	NA/23		
	Peas		DON	25		NA	ND	ND		
	Soya meal		DON-ZEN	13		NA	ND	ND		
	Rapeseed meal		DON	12		NA	ND	ND		
	Oilseed byproducts (sunflower, palmakernel)		DON-HT2-ZEN	8		NA	ND	ND		

-: Not calculated
n: Number of samples
NA: Not available
ND: Not detected
DON: Deoxynivalenol
FusX: Fusarenon X
MON: Moniliformin
T2: T-2 toxin
HT2 : HT-2 toxin
3AcDON : 3-acetyl-deoxynivalenol
15AcDON: 15-acetyl-deoxynivalenol
DAS: Diacetoxyscirpenol
ZEN: Zearalenone
OTA: Ochratoxin A

MITIGATION OF NIVALENOL

In order to reduce the risk of an epidemic of FHB, measures must be taken to reduce the quantity of inoculum available for dispersal, to prevent the dispersal of inoculum and to prevent the infection of spikelets. In order to achieve these objectives, several preventive measures may be employed, including the optimisation of the cropping system, the growing of resistant cultivars and the use of fungicides or biological control agents, because under field conditions, factors such as agricultural practices, plant varieties, geographical differences may influence mycotoxin production (Stenglein, 2009).

Therefore, early detection and control of trichothecene-producing *Fusarium* spp. is critical to prevent toxins entering the food chain (Wagacha and Muthomi, 2007).

Cropping system

Fusarium species that infect cereals are capable of surviving saprophytically on crop debris (Parry *et al*., 1995; Jones, 2000). Management practices, such as tillage systems and crop rotation, are important factors influencing contamination with fusaria. The use of reduced tillage practices increases the incidence and severity of FHB (Pereyra and Dill-Macky, 2004). Ploughing to bury crop debris removes the source of inoculum from the soil surface, which could be available for dispersal to ears. The effect of soil preparation (tillage), nitrogen fertilization levels, crop rotation, previous crop, intensity of cultivation and weed control play an important role in FHB infection (Oerke *et al*., 2002). The role of weeds in the development of FHB is unclear, and the efficacy of weed control in reducing FHB is debatable (Parry *et al*., 1995). It is critical to avoid planting wheat adjacent to fields with large amounts of small grain or corn residue remaining on the soil surface. Rotation to a legume crop between corn and small grain crops provides time for the residues to break down and the pathogen population declines (Champeil *et al*., 2004).

The avoidance of water stress by the use of irrigation and well-drained soils has been shown to reduce the severity of *Fusarium* foot rot in wheat (Cook, 1980), and could therefore reduce available inoculum for the development of FHB. However, the use of overhead irrigation has also been shown to increase the severity of FHB (Tusa *et al*, 1981; Strausbaugh and Maloy, 1986; Teich, 1987).

According to Cook (1980), any cultural practice that increases plant water stress, including the application of high levels of nitrogen fertilizer, increases the incidence and severity of *Fusarium* foot rot. Martin *et al*. (1991) observed that increasing nitrogen from 70 kg N/ha to 170 kg N/ha significantly increased the incidence of *Fusarium* infected grain in wheat, barley

and triticale (Parry *et al*., 1995). A well balanced nitrogen fertilisation is a good preventive action.

The best approach in managing FHB in general should focus on an integrated approach. Proper timing and application of fungicides and/or biocontrol products is critical. Fungicides with no harmful effects on biological antagonists against fusaria should be used. In the medium term, good agricultural practices, such as crop rotation and proper tillage should be considered to maintain low inoculum levels. Breeding for resistance is necessary as a long-term strategy (Wagacha and Muthomi, 2007).

Chemical and biological control

Seed-borne infection is not easily controlled by surface-acting fungicides because the fungus is present within the seed coat, rather than as a contaminant on the seed surface. While seed treatment greatly reduces the risks of seedling blight, it does not guarantee adequate protection against later invasion by soilborne *Fusarium* species. Fungicides such as prothioconazole and boscalid both registered in the European Union, applied for the control of other diseases can give good control of stem base *Fusarium* infection.

The use of fungicides in the management of FHB has been shown to be at most 77% and 89% effective in the reduction of disease severity and mycotoxin content, respectively (Haidukowski *et al*., 2004), but research was focused on DON. . The fungicides registered in the European Union used in the control of FHB include prochloraz, propiconazole, tebuconazole, cyproconazole and azoxystrobin (Hutcheon and Jordan, 1992; Matthies and Buchenauer, 2000; Haidukowski *et al*., 2004). Ramirez *et al*. (2004) reported the effectiveness of fungicides against *F. graminearum* to be influenced by complex interactions between water, temperature, fungicide concentration and the time of inoculation. Although some of the fungicides are ineffective against FHB, some have been shown to stimulate DON and NIV production particularly in sub-optimal fungal growth conditions and low fungicide dosage (D'Mello *et al*., 1999; Jennings *et al*., 2000; Magan *et al*., 2002; Ramirez *et al*., 2004). In addition, food safety concerns limit the chemical management option due to fungicide residues in grain and wheat products (Jones, 2000).

However, chemicals applied to wheat at the flowering stage are used to reduce yield losses associated with FHB and trichothecene contamination of infected grains.

Treatments that included metconazole or tebuconazole significantly decreased *F. graminearum* or *F. culmorum* infection levels, but the effect was variable across the years and type of fungicide applied. In 2002, DON concentration in wheat treated with tebuconazole and metconazole was reduced by 46% and 48%, respectively, while NIV

concentration was unaffected. Yet, in 2000 and 2001, significant reductions in *F. graminearum* or *F. culmorum* infection levels were not always associated with significant reductions in DON and NIV mycotoxins. The fungicides applied in naturally infected fields did not always reduce mycotoxin producers or alternatively the trichothecenes produced (Ioos *et al*., 2005).

There have been efforts to identify biological antagonists, which could be used in integrated pest management (IPM) strategies, even if till now results have been limited to research application and no data are available regarding field scale application. The short time period during anthesis, when wheat ears are most susceptible to FHB, could offer an ideal opportunity for biological control, thus avoiding the hazards associated with late fungicide application (Parry *et al*., 1995). Isolates of *Clonostachys rosea* have been shown to consistently suppress sporulation of *F. culmorum* and *F. graminearum* on wheat straw and of *F. culmorum*, *F. graminearum*, *F. proliferatum* and *F. verticillioides* on maize stalks (Luongo *et al*., 2005). A strain of *F. equiseti* has been shown to consistently decrease DON (70%) on wheat inoculated with *F. culmorum* with similar performance to a standard fungicide tebuconazole (Dawson *et al*., 2004). Diamond and Cooke (2003) reported a 60% reduction in FHB symptoms compared to the control after 25 days on ears pre-inoculated with *Phoma betae* and challenged with *F. culmorum*; they further reported a significant increase in number of grains per ear. Two strains of *Pseudomonas fluorescens* have been reported to inhibit the growth of *F. culmorum* both *in vivo* and *in vitro* (Kurek *et al*., 2003).

No data are available on the biological control *of F. poae, F. cerealis and F. nivale*, the most relevant species for NIV production.

Resistant cultivars

Growing of wheat cultivars resistant to *Fusarium* spp. should be the most economic, environment-friendly and effective method of disease control. Two types of resistance to FHB of wheat have been reported; resistance to primary or secondary infection (Schroeder and Christensen, 1963). Kang and Buchenauer (2000) reported that FHB resistant cultivars were able to develop active defence reactions during infection and spreading of *F. culmorum* in the host tissues. The researchers also reported lower accumulations of DON in the tissues of infected ears of resistant wheat cultivars. Miller *et al*. (1985) found that resistant cereal lines inoculated with *F. graminearum* contained lower concentrations of DON in the grain than susceptible cultivars. This suggests a resistance mechanism that neutralizes DON production and DON may play a role in FHB pathogenesis (Snijders and Krechting, 1992). It is now generally agreed that FHB resistance is controlled by a polygenic system. Effects of

dominance of genes probably influence FHB resistance, but additive effects appear to be important, and resistance genes can be cumulated (Snijders, 1990; Bai *et al*., 1999).

Sources of resistance to FHB have been found in China, South America and the Czech Republic (Snijders, 1990; Mesterhazy, 1995; Mesterhazy *et al*., 1999). Currently, there are no wheat cultivars with a high level of resistance to FHB although some cultivars have useable levels of partial resistance that limit yield loss and mycotoxin accumulation (Pereyra and Dill-Macky, 2004). Wisniewska and Kowalczyk (2005) reported a breeding line with useable resistance to *F. culmorum* and other *Fusarium* spp. They found a good candidate for deriving new lines and cultivars with improved resistance to three fungal diseases: *Fusarium* head blight, leaf rust and powdery mildew. One of the wheat accessions was less susceptible to both pathogens, *F. culmorum* and powdery mildew, during the 5 years of experiments..

Decontamination

It is essential for grain producers to decontaminate mycotoxin-contaminated grain. From a practical point of view, one of the best methods is to separate grain into size fractions and to exclude the smaller kernels (Perkowski and Miedaner, 1994). This is not a final solution, but it helps in reducing the risk of high contamination.

Mycotoxin-transforming microorganisms have been investigated with some success for their potential applications in detoxifying mycotoxins in contaminated food and feed (Karlovsky, 1999; Volkl *et al*., 2004; Zhou *et al*., 2008). Typically, microorganisms obtained from different sources are only active under specific conditions that are similar to those of the environment where they have been isolated. These conditions often include nutrition, the presence or absence of oxygen (aerobic vs. anaerobic), pH and temperature. Microorganisms originating from aquatic species should have significant advantages when used to detoxify mycotoxins in the aquaculture industry, as compared with the use of microorganisms obtained from other sources. Microbial populations in fish digestive tracts have been well studied (Trust and Sparrow, 1974; Lindsay and Harris, 1980; Lesel *et al*., 1986). These populations grow mainly upon the food consumed by the host animal and digestive secretions (Lesel, 1993). Fish gut bacterial flora has a diversified enzymatic potential, and it is logical to propose that the bacteria themselves or enzymatic metabolites might interfere with the feedstuff consumed by the host animal. The possibility of finding microbes in fish guts capable of transforming trichothecenes into less toxic compounds was studied by Guan *et al*. (2009). This study found the presence of microorganisms capable of transforming trichothecene mycotoxins from the digesta of brown bullhead catfish.

It has been found that fish harbour a large number of microbial populations in their digestive tracts. The identification of the active microbial culture C133 has clearly demonstrated that fish gut microorganisms can be sources of mycotoxin-transforming microorganisms (Guan *et al*., 2009).

A number of studies have reported the transformation of trichothecenes by microorganisms from other animal intestinal systems, such as rumen fluid, chicken, rat, and swine digesta (Kiessling *et al*., 1984; King *et al*., 1984; Prelusky *et al*., 1988; Swanson *et al*., 1988; Beeton and Bull, 1989; Young *et al*., 2006), but no commercial products obtained from these microorganisms are yet available. No other data on nivalenol decontamination from feed processing were found.

PHARMACOKINETICS

Absorption, Distribution and Excretion

Hedman *et al*. (1997a) reported that in pigs, fed a diet containing NIV at the dose of 0.05 mg/kg bw twice per day for three days, NIV was detected in the blood 20 min after ingestion. During the first 7.5 hours, 11-43% of the NIV dose was absorbed, with systemic peak concentrations of 3-6 ng/ml, mostly occurring 2.5-4.5 hours after feeding. Sixteen hours after feeding, NIV was still absorbed from the intestine (1-3 ng/ml systemic concentrations). The authors reported that NIV was excreted in the feces (up to 3.2 mg/kg). Poapolathep *et al*. (2003) demonstrated, with experiments in 4-week-old ICR female mice, that a large proportion of oral ^{3}H-NIV passed through the gastrointestinal lumen without being absorbed, in contrast to fusarenon X (the precursor of NIV), which was absorbed from this tract more rapidily and efficiently than NIV. The same authors calculated (Table 3) the pharmacokinetics parameters of NIV after a single oral administration of ^{3}H-NIV (20 µg/kg).

Five male Wistar rats, orally administered NIV (5 mg/kg bw), showed an excretion of NIV and its metabolites (de-epoxynivalenol) mainly in feces (87%), instead of urine (1%) (Onji *et al*., 1989). These data were confirmed also by Poapolathep *et al*. (2003), who gave ^{3}H-NIV p.o. to 4-week-old ICR female mice. They observed that NIV was mostly excreted unchanged in the feces, except for an unknown metabolite identified in the HPLC profile.

Recently, Tep *et al*. (2007) have investigated the mechanism involved in NIV transepithelial transfer, showing that the human intestinal Caco-2 cell line, after 6 hours of exposure to 5 µM of NIV, exhibited an absorption rate of 27.8±8.7%. Absorption of NIV was slow, and was not due to intracellular NIV retention, since no time-dependent accumulation could be detected in the cells. Furthermore, transport experiments carried out by the authors suggested the

involvement of an active carrier-mediated efflux of NIV, such as P-gp and MRP2 transporters.

Poapolathep *et al*. (2004) carried out experiments in ICR mice with radiolabeled NIV, and showed that NIV transferred in unchanged form to fetal or suckling mice via placenta or milk, respectively.

Table 3. Pharmacokinetic parameters of NIV after a single oral administration of ^{3}H-NIV (20 µg/kg) as presented in Poapolathep *et al*., 2003.

Pharmacokinetic parameters (units)	**NIV**
C_{max} (ng/ml)	1.035
T_{max} (min)	60
$t_{1/2\alpha}$ (h)	2.53
$t_{1/2\beta}$ (h)	14.34
AUC (ng h/ml)	8.55

The pharmacokinetic parameters of ^{3}H-NIV were determined by a two-compartment model. Values are mean of two mice. C_{max}, maximum concentration; T_{max}, time of peak plasma concentration; $t_{1/2\alpha}$, distribution half-life; $t_{1/2\beta}$, elimination half-life; AUC, area under the curve (Poapolathep *et al*., 2003).

Metabolism

Hedman and Pettersson (1997) evaluated the capacity of the gastrointestinal microflora of the pig, cow and chicken to metabolize NIV; after one week of feeding of 2.5 or 5 mg/kg NIV, nearly all excreted NIV in feces had been de-epoxylated in five of six pigs. After three weeks from the start of feeding also the sixth pig had acquired this ability. In cows, anaerobic incubation of NIV with cow rumen fluid produced the de-epoxide in a high proportion. Finally, in the feces of chickens fed 2.5 or 5 mg/kg NIV for three weeks only an unknown metabolite was found.

In multiple oral administrations of 5 mg NIV/kg bw to male Wistar rats, 80% and 1% of the total dose were excreted as the de-epoxymetabolite in feces and urine, respectively. Much lower levels of the ingested NIV were detected unchanged (7% and 1% of the total dose in feces and urine, respectively) (Onji *et al*., 1989).

In contrast, Hedmann *et al*. (1997a) did not observe any NIV-metabolite in the urine, plasma and feces of pigs fed 0.05 mg/kg bw twice per day for up to one week, indicating a lack of metabolism (Pronk *et al*., 2002). However, when male swine were fed diets containing 2.5 or

5 mg/kg NIV for three weeks, starting at one week, over 90% of the total dose of NIV in feces was the de-epoxy-NIV. In the bile, 32-44% of NIV was also in the de-epoxy form. It is suggested that a few days are necessary to produce the de-epoxy-metabolite by gastrointestinal microflora (Pronk *et al*., 2002).

Regarding liver biotransformation enzymes, after 4 weeks of oral exposure of C57B16 male mice to NIV (three days a week), Gouzé *et al*. (2007) did not observe any significant change in ethoxyresorufin *O*-deethylase (EROD) activity, whereas methoxyresorufin *O*-demethylase (MROD) and penthoxyresorufin *O*-depenthylase (PROD) activities decreased by 38 and 45%, respectively, at the highest dose (8.87 mg/kg). In contrast, the protein expression of P450 1A, 2B, 2C, 3A and 4A subfamilies was unchanged at all doses (0.071 to 8.87 mg/kg bw). Moreover, doses of 0.071 and 0.355 mg/kg of NIV induced a significant increase (34 and 24%, respectively) of hepatic GST, accepting 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, but not accepting 1,2-dichloro-4-nitrobenzene (DCNB) as substrate. The NOAEL (No Observed Adverse Effect Level) for NIV proposed by this study was of 1.774 mg/kg bw, corresponding to an exposure to 5 mg/kg in contaminated food, far higher than the maximal occurrence measured in European cereals (<2 mg/kg). An increased activity of GST enzymes, in particular GST 1-2, was also observed by Yabe *et al*. (1993), who fed rats with diet containing 6-12 mg/kg of NIV for 2 or 4 weeks. They also described an increase of cytochrome P-450 activity in the hepatic microsomes.

In summary, from the available data NIV is rapidly and extensively absorbed, and excretion occurs mainly via *faeces*. After adaptation, the intestinal microflora of swine and rats can de-epoxydate NIV. NIV is not de-epoxydated in chickens (Pronk *et al*., 2002).

Carry-over

Available data on NIV and others *Fusarium* mycotoxins (especially DON, T-2 and DAS) reveal that not only cereal grains, but also animal feed are contaminated by these toxins. They can cause problems to humans when ending up in human foodstuffs, which can be of plant- or animal-origin (residues in meat, milk, eggs of animals given contaminated feed). The majority of confirmed cases are of plant-origin; in contrast there is almost no carry over of trichotecenes into food of animal origin. Data available mostly concern deoxynivalenol (DON) (Pronk *et al*., 2002).

TOXICITY

Acute toxicity

LD50 values (mg/kg) of NIV in 6-week-old male ddY mice were determined as 38.9 (po), 7.4 (ip), 7.2 (sc), and 7.3 (iv) (Ryu *et al*., 1988). Different NIV effects have been reported after acute toxicity studies, such as bone marrow toxicity, erytropenia and slight leucopenia, haemorrhage and congestion in the intestine, and toxicity to lymphoid organs (Ryu *et al*., 1988), diarrhea, damage to epithelial mucous membranes of intestine, in the thymus and testis (Ueno, 1984). In F344 rats the oral and subcutaneous LD50 values for NIV were 19.5 and 0.9 mg/kg bw, respectively, causing sedation, diarrhea and congestion of the lungs and digestive tract (IARC, 1993).

Chronic toxicity

Animals

Ryu *et al*. (1987) performed a short-term feeding trial for 24 days using feed supplemented with rice artificially molded with NIV producing fungus, *Fusarium nivale Fn 2B*, in female C57BL/6CrSlc SPF mice. A significant erythropenia and slight leukopenia were observed in the 30 mg/kg group, but no marked changes were observed in other hematological parameters, feed consumption, body weight gain, or weights of the liver, spleen, and thymus. Ultrastructural studies also revealed polyribosomal breakdowns of the bone marrow cells in the 30 mg/kg group.

Mice (C57BL/6CrSlc SPF mice) were fed pulverised *F. nivale* (claimed by the authors not to produce other trichothecenes on rice) grown on polished rice in doses 0, 6, 12 or 30 mg NIV /kg feed (0, 0.7, 1.4 or 3.5 mg NIV /kg bw) for 12 weeks, with an interim sacrifice of 10 animal/sex/group after 4 weeks. Reduced body weight gain and feed consumption were observed. A significant decrease in relative organ weight was observed in the thymus and spleen in females at the highest dose after 4 weeks, and in the spleen and kidneys in males at the two highest doses. After 12 weeks, a reduction in relative organ weight was only found in the liver of both males and females. No histopathological changes were observed in the thymus, spleen, brain, pituitary gland, stomach, kidneys, adrenal glands, liver, small intestine with or without Peyers patch, mesenterial lymph node, ovaries, sternum or femurs (Yamamura *et al*., 1989).

These result were confirmed recently by Takahashi *et al*. (2008), who carried out subchronic toxicity experiments with male and female F344/DuCrj rats at 5 weeks of age, fed diet containing from 0 to 100 mg/kg concentration of NIV for 90 days. They observed no deaths throughout the feeding period, and in both sexes body weights were decreased at 100 mg/kg

from week 1 of the experiments. These results were also evident at 25 mg/kg from week 6 to the end of the experiments in males, and at week 4 in females. In the same study the histopathological assessment revealed that the treatment-related changes predominantly regarded the hematopietic and immune organs, and the anterior pituitary in both sexes and females reproductive organs at 100 mg/kg (thymic atrophy, hypocellularity in the bone marrow, increase of castration cells in the anterior pituitary and increase of avarian atretic follicles). Based on the hematological data from this study, the NOAEL of NIV was determined to be less than 6.25 mg/kg (0.4 mg/kg body weight/day) for both males and females. A reduction in feed intake was found in pigs given diet containing 5.8 mg NIV/kg feed or more for 29 days, but not in pigs given a diet containing 2.9 mg NIV/kg feed (Williams and Blaney, 1994; Williams *et al*., 1994). The feed in this last feeding trial also contained zearalenone; estrogenic effects in the pigs were observed, and an interaction with zearalenone cannot be excluded. Recently, Gouzé *et al*. (2007) gave oral doses of NIV to 6 week-old C57B16 male mice, three days a week for 4 weeks, in order to evaluate the effect of the toxin on the immune and metabolic defence systems. They observed that at doses of 1.77 and 8.87 mg/kg, NIV caused significant decreases of body weight. The same doses induced a significant increase of plasma levels of alkaline phosphatase, while urea, glucose, cholesterol and triglyceride levels in plasma were decreased when compared to controls, suggesting hapatotoxicity. Hinoschita *et al*. (1997) showed that NIV given in the diet at 0, 6 or 12 mg/kg bw for 4 or 8 weeks to different mice strains (C3H/HeN, C3H/HeJ or BALB/c) induced IgA deposits in the glomerular mesangium and elevated IgA levels which resemble IgA nephropathy, with an association between the degree of these pathological changes and the dose and duration of NIV exposure.

The fact that the immune function is affected by NIV was also confirmed by Kubosaki *et al*. (2008), who observed, in male F344 rats after 90-day dietary exposure, a slight increase of IgM at 6.9 mg/kg, and a dose-dependent decrease of T lymphocyte/B lymphocyte ratio from 1.5 mg NIV/kg, an increase of NK activity and an elevated $CD4^+$ helper/$CD8^+$ cytotoxic T lymphocyte ratio at 6.9 mg NIV/kg. An increased $CD4^+$ helper/$CD8^+$ cytotoxic T lymphocyte ratio was also found by Sugita-Konishi *et al*. (2008), after a subchronic toxicity study (90 days) in F344 female rats, at a dose of 100 mg/kg NIV. The same authors also showed a decreased count of white blood cells, and calculated the lowest observable effect level as 0.4 mg/kg bw/day.

Takahashi *et al*. (2008) carried out subchronic toxicity experiments in male and female F344/DuCrj rats at 5 weeks of age, fed a diet containing from 0 to 100 mg/kg NIV for 90 days. They observed a decrease of the white (WBC) (mainly lymphocytes), red (RBC) and

platelet (Plt) blood cell counts at 100 mg/kg in males, while in females, a significant decrease of WBC was observed from 6.25 mg NIV/kg, and a decrease of platelets was observed at 100 mg NIV/kg.

In a study by Hedman *et al*. (1997b), where young pigs were fed diets with purified NIV for three weeks, no differences in total or differential blood leukocyte counts between control and exposed pigs, were observed in samples collected after 0, 1 or 3 weeks, nor in the number of thymocytes. Spleen cell numbers showed a dose-dependent decrease after 3 weeks of exposure at 5 mg/kg bw. Flow cytometric analysis of lymphocytes revealed a decreased number of the $CD4^+$ and the $CD8^+$ population in the spleen, reflecting the lower numbers of splenocytes. Moreover, exposure to NIV caused a transient decrease of blood $CD4^+$ cells after 1 week of treatment. Finally, the authors showed that a time-dependent tendency of increasing plasma concentrations of IgA and of decreasing concentrations of IgG in the 2.5 mg/kg group, but no differences in Ig levels were described between controls and experimental groups at any time.

NIV, orally administered to C57B16 male mice 6 weeks-old, three days a week for 4 weeks at a dose of 8.87 mg/kg, was also able to induce a 50% increase in IgG plasma levels and the release of IL-4 by splenocytes stimulated with ConA mitogen (Gouze *et al*., 2007).

Choi *et al*. (2000) examined the effect of NIV on antigen-specific IgE production using ovalbumin (OVA)-specific T cell receptor $\alpha\beta$-transgenic mice. The mice produced significant amounts of total and antigen-specific IgE, IgG1, and IgA in serum when given OVA orally. Administration of NIV with OVA significantly suppressed total IgE and OVA-specific IgE, IgG1, and IgA production. Cytokine assay using splenocytes obtained from mice given the OVA plus NIV diet revealed that interleukin 4 (IL-4) production was suppressed and interleukin-2 (IL-2) production was enhanced. These results suggest that the inhibition of IL-4 production and enhancement of IL-2 production induced by NIV suppressed total and antigen-specific IgE production.

Garaleviciene *et al*. (2002b) fed White Leghorn hens, 55 weeks old, diets containing 0, 1, 3 and 5 mg/kg bw of NIV for 50 days. There were effects neither on feed intake, nor on egg production, nor on egg quality.

In summary, major toxic effects in subacute, subchronic and chronic toxicity experiments with NIV in mice were immunotoxicity, hematotoxicity and reduced body weight gain and reduced feed intake, organ weight changes (without histopathology findings). In subacute feeding studies with swine NIV caused mild pathological changes in the gastrointestinal tract, spleen and kidney, body weight gain and food consumption (Pronk *et al*., 2002).

Humans

No human data were retrieved.

Developmental and reproductive toxicity

Only a few studies in peer-reviewed literature have examined the development and reproductive toxicity of NIV, and they have been reviewed by Pronk *et al*. (2002).

Pregnant mice on days 7-15 of gestation were treated with an ip injection of different doses of NIV, and there were evidences of high embryolethality after exposure to NIV 0.5 and 1.5 mg/kg bw. No fetal malformation were observed in the treated groups. A single administration of 3 mg/kg bw on day 7 affected the embryo within 10 h, damaged the placenta within 24 h, and caused stillbirths at 48 h (Ito *et al*., 1986).

In another study (Ito *et al*., 1988), ICR mice were given mouldy rice powder containing NIV at different concentrations in feed on days 7-15 of gestation. The results revealed that also in this case NIV (10 and 30 mg/kg bw) was both embryotoxic and caused maternal weight loss. Moreover intrauterine growth retardation was found, while no significant adverse effects on skeletal and visceral malformation were observed.

Recently, Sugita-Konishi *et al*. (2008) described adverse effects on the female reproductive system of F344 rats fed diets containing 100 mg/kg of NIV for 90 days.

Thus, in mice, NIV is embryotoxic and fetotoxic but not teratogenic (Pronk *et al*., 2002). The LOAEL in reproduction studies with NIV given by oral exposure was stated to be 1.4 mg/kg bw given in the feed throughout gestation and 5 mg/kg bw when given by gavage on days 7-15 (Ito *et al*., 1988). Data on others species are lacking, as well as data on reproductive toxicity *(*Pronk *et al*., 2002).

Genotoxicity and carcinogenicity

Hsia *et al*. (1988) observed an increased frequency of chromosomal aberrations in Chinese hamster V79 cells treated with NIV at very low concentrations (ng levels/ml medium).

In the alkaline single-cell gel electrophoresis (or Comet) assay, NIV resulted genotoxic in cultured Chinese hamster ovary (CHO K1) cells. Nivalenol at 50 and 100 µg/ml damaged DNA of CHO cells in the absence of S9 mix (Tsuda *et al*., 1998). Recently, Bony *et al*. (2007) exposed human enterocyte-like Caco-2 cell-line, both in the dividing (undifferentiated) stage and 10-12 days post-confluently (differentiated cells) to NIV. Genotoxicity of NIV, by means of the Comet assay, was observed in the sub-cytotoxic 0-0.5 µM range (below the IC_{10}), after 24 and 72 h exposure in post confluent Caco-2 cells.

In vivo, in male ICR mice, DNA damage was seen in the bone marrow and in the kidney after 2 h, in the stomach and colon at 2 and 8 h, respectively (both after ip and oral administration), but not in the liver and thymus. Upon histopathological examination, no tissue necrosis was observed in the organs with DNA damage. The available data do not allow an adequate evaluation of the genotoxicity (Tsuda *et al*., 1998). The *in vivo* findings were not secondary to cytoxicity, as no apoptotic cells and histopathological results including necrotic changes were detected in any organ.

Female mice (C57BL/6CrSlc SPF mice) were given feed containing pulverised *F. nivale* (claimed by the authors not to produce other trichothecenes on rice with no detectable fusarenon-X (4-acetyl NIV). The doses were 0, 6, 12 or 30 mg/kg feed (0, 0.7, 1.4 or 3.5 mg NIV/kg bw) for one or two years. Decreased body weight gain and feed consumption were seen in all the treated groups. The absolute weight of the liver was significantly reduced in the group receiving 3.5 mg/kg bw and the kidney weight in the groups receiving 1.4 and 3.5 mg NIV/kg bw. Severe leukopenia was also observed in the treated animals treated for one year also at the lower dose, whereas the white blood cell count was not affected in the two year study. No histopathological changes including tumours were found in the liver, thymus, spleen, kidneys, stomach, adrenal glands, pituitary glands, ovaries, bone marrow, lymph node, brain and small intestines with or without Peyers patches (Ryu *et al*., 1988; Ohtsubo *et al*., 1989). A lowest observable adverse effect level (LOAEL) derived from these studies was 0.7 mg/ kg bw with growth inhibition and leukopenia as effects. A no observable adverse effect level (NOAEL) could not be derived from these studies.

IARC (1993) concluded that there is inadequate evidence of the carcinogenicity of NIV in experimental animals; the overall conclusion was that NIV was not classifiable (Group 3). NIV is a weak inducer of chromosomal aberrations in mammalian cells in vitro and from indicator tests it appears that NIV has the potential to induce DNA-damage. However, the available information is too limited to evaluate the genotoxic potential of NIV (Pronk *et al*., 2002).

Cytotoxicity

In a variety of systems, such as Ehrlich ascites tumour cells and HeLa cells, NIV has been found to possess protein synthesis inhibiting activity, and to also inhibit DNA synthesis, while RNA synthesis was marginally affected (Saito and Ohtsubo, 1974).

Nasri *et al*. (2006) have shown that in Jurkat T-lymphocytes NIV and DON induce citotoxicity (the IC50 value for mitochondrial activity was 0.4 µM), and the mechanism is mainly via apoptosis. In fact, they observed phosphatidylserine externalization, mitochondrial release of

cytochrome *c,* procaspase-3- degradation and Bcl-2 degradation. In contrast, type A trichotecenes (such as T-2 and diacetoxyscirpenol) reduce mitochondrial activity at approximately 1000-fold lower concentrations than NIV and DON, resulting in necrosis. High concentrations of trichotecenes promote apoptosis of many other cell types, such as macrophages (Yang *et al*., 2000). These data were confirmed by Marzocco *et al*. (2009), who have shown that NIV had anti-proliferative effects in murine macrophages (J774A.1 macrophage), and that its cytotoxic effects could be partly ascribed to an acceleration of apoptotic pathways which involved enhanced ERK activation, induction of the pro-apoptotic protein Bax, induction of caspase-3 activation and activation of a DNA repairing enzyme, PARP. Moreover, the authors observed an arrest of cell cycle in G0/G1 phase after treatment with NIV, confirming previous data reviewed by Rocha *et al*. (2005). Minervini *et al*. (2004) described the apoptotic effects of NIV on K562 human erythroleukemia cell line, by means of 100% of debris, obtained by flow-cytometer analysis, at 84 µM and changes concentration-dependent in the distribution of cells in the different cell cycle phases.

Thuvander *et al*. (1999) have shown that in human (from male and female healthy donors) lymphocyte cultures treated with NIV (4×10^{-6}, 2×10^{-6}, 4×10^{-7} and 2×10^{-7} M) for 72 hours, mitogen-induced lymphocyte proliferation was inhibited. The authors also studied the effects of NIV on Ig production, and found that the toxin also inhibited IgA, IgM and IgG production in a concentration-dependent manner with limited variation in sensitivity between individuals.

NIV inhibited blastogenesis in cultured human lymphocytes. The [^{3}H]thymidine uptake was inhibited by 50% in mitogen-stimulated human lymphocytes at the concentration of 72 ng/ml (Forsell and Pestka, 1985).

Nivalenol inhibited in a concentration-dependent manner T and B cell proliferation. Furthermore, NIV also suppressed natural killer activity and inhibited antibody-dependent cellular cytotoxicity reaction (Berek *et al*., 2001). The anti-proliferative potential of NIV was confirmed recently also by Luongo *et al*. (2008) in porcine whole-blood cells.

In summary, NIV could inhibit protein and DNA synthesis, and was found to be cytotoxic and to have anti-proliferative effects on blood cells.

LEGISLATION ON NIVALENOL

Given the worldwide trade in cereals, feeds and foods that are increasingly contaminated with mycotoxins, including trichothecenes, it has now become important to assess the potential health risks of the most commonly occurring trichothecenes and to establish tolerable daily intakes (TDIs) where possible.

In a set of opinions, the Scientific Committee on Food (SCF) of the European Commission evaluated the *Fusarium* toxins and more recently the toxicity of NIV (SCF, 2000). They set a temporary (t-TDI) of 0.7 mg/kg bw/day, based on a LOAEL (Lowest Observed Adverse Effect Level) of 0.7 mg/kg bw/day found in long-term dietary studies with mice (Table 4). For NIV reduced growth, haematotoxicity and immunotoxicity were the most critical effects. The SCF applied a large uncertainty factor of 1000 because of the use of a LOAEL and the limited database. The TDI was made temporary pending a group evaluation of the trichothecenes, and also because of gaps in the database. A need for further studies was expressed. Available data on oral LOAEL values of NIV are summarised in Table 4.

Table 4. Oral LOAEL values of NIV considered for the evaluation of the temporary TDI.

Study	**Critical Effect**	**LOAEL/NOAEL (mg/kg bw/day)**	**Reference**
Mouse, 2 years (in feed)	Growth retardation, leucopenia	0.7 (LOAEL)	Ohtsubo *et al*., 1989
Mouse, 1 year (in feed)	Growth retardation, leucopenia	0.7 (LOAEL)	Ryu *et al*., 1988
Mouse, throughout gestation period (in feed)	Intrauterine growth retardation	1.4 (LOAEL)	Ito *et al*., 1988
Mouse, 7-15 day of gestation (by gavage)	Intrauterine growth retardation	5 (LOAEL)	Ito *et al*., 1988

(SCF, 2000)

In 2001, the SCOOP (Scientific Co-operation on Questions relating to Food) task 3.2.10 “Collection of occurrence data of *Fusarium* toxins in food and assessment of dietary intake by the population of EU Member States” was established. SCOOP data are used by the SCF for its evaluation and advisory work on the risks to public health arising from dietary exposure to certain mycotoxins. The objectives of SCOOP task 3.2.10 were to provide the scientific basis for the evaluation and management of risk to public health arising from dietary exposure to *Fusarium* toxins, taking into account recent available data. Special emphasis was put on the evaluation of the dietary intake of *Fusarium* toxins in each of the Member States and in high-risk sub-groups of the population. In 2002, the SCF concluded that the available data, while limited, did not support the establishment of a group TDI for DON, NIV, T-2 and HT-2. They therefore confirmed the tTDI of 0.7 mg/kg bw/day for NIV and recommended that further studies should fill the data gaps (SCF, 2002). There are only

limited data on intake; however, estimates from the Nordic countries indicate that intake may be well below the t-TDI (Tritscher and Page, 2004).

NIV was also evaluated by the Nordic Working Group (Eriksen and Alexander, 1998), but they did not find the available toxicity data sufficient to set a tTDI for NIV.

More recently, Eriksen and Pettersson (2004) confirmed that the available information on the toxicity of NIV to pigs and poultry is not sufficient to provide any scientific base for any guidelines for NIV in pig and poultry feed. The authors proposed, until more information eventually becomes available, that a temporary limit could be set at the same level as for DON, i.e. 0.3 mg/kg feed for pigs, while for poultry the data available does not permit any limit to be set.

In 2006 the European Commission gave guidance values of some *Fusarium* toxins in feed materials (European Commission, 2006a), and set maximum levels for certain contaminants (including DON, ZEN, T-2, HT-2 and Fumonisins), in foodstuffs (European Commission, 2006b). In 2007, amending Regulation 1881/2006, the Commission set maximum levels for *Fusarium* toxins in maize and maize products. In both cases no limits were established for NIV.

Most countries regulate the maximum tolerable levels of mycotoxins in food by law or codex standards, for the maximum protection of consumers. Such regulations do not exist for feeds or feed ingredients (Rafai *et al*., 2000).

Worldwide regulations for NIV in food or feed have not yet been established, but given the relatively high toxicity of this mycotoxin, NIV might need to be given more regular consideration.

CONCLUSIONS

NIV is a type B trichothecene primarily produced by *Fusarium cerealis* (*F. crookwellence*), *F. poae* and *F. nivale* and to a lesser extent also by *F. culmorum* and *F. graminearum*. Few studies are available on the main NIV producers and all information on fungi and their interaction with the host, and regarding mitigation, results from studies on *F. graminearum* or *F. culmorum*..

Although the trichothecenes pathway has been elucidated, NIV biosynthesis is still to be defined in the very last steps. Moreover, to date very few data on genes involved in the biosynthesis are available.

Regarding the analytical aspects, GC analysis is widely employed for the determination of type B trichothecenes, although the conjugation of the carbonyl group with the double bond makes them more suited for HPLC analysis. The GC/MS approach seems to give the best

results for confirmatory analyses and also for obtaining structural information on several derivatives of the main trichothecenes, which is fundamental for toxicological and metabolic studies. ELISA assays though frequently used for screening purposes, have been found to produce inaccurate quantitative data and to overestimate trichothecene levels in samples. Moreover, no specific antibodies have been developed for NIV. Since NIV is a very polar compound, in order to avoid analyte losses and matrix related errors during quantification, matrix-matched calibration and the use of internal standards should be recommended for NIV analysis. No specific validation has been reported to date for NIV occurrence in feed, which is usually a very complex matrix.

NIV is rapidly and extensively absorbed in animals and excretion occurs mainly via *faeces*. After adaptation, the intestinal microflora of swine and rats can de-epoxydate NIV, while this does not happen in chickens.

The major toxic effects in subacute, subchronic and chronic toxicity experiments with NIV in mice were immunotoxicity, hematotoxicity, reduced body weight gain and reduced feed intake, organ weight changes (without histopathology findings). In subacute feeding studies with swine, NIV caused mild pathological changes in the gastrointestinal tract, spleen and kidney, body weight gain and food consumption. Besides, NIV could inhibit protein and DNA synthesis, and was found to be cytotoxic and to have anti-proliferative effects on blood cells.

The tTDI of 0.7 mg/kg bw/day was defined for NIV and further studies to fill the data gaps were recommended. There are only limited data on intake; however, estimates from the Nordic countries indicate that intake may be well below the t-TDI.

Worldwide regulations for NIV in food or feed have not yet been established, but given the relatively high toxicity of this mycotoxin, NIV might need to be given more regular consideration.

FUTURES

- Specific studies on main NIV pproducers and their interaction with hosts should be planned
- The development of specific immunoaffinity devices as well as isotope-labelled internal standards
- Specific validation for NIV occurrence in feed should be encouraged

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Chapter 6 - Diacetoxyscirpenol in food and feed

Keywords: Diacetoxyscirpenol, *Fusarium*, Trichothecene, Scirpentriol, Biosynthesis, Analytical methods, Cereals, Potato, Mitigation, Metabolism, Toxicity, Animals, Humans.

INTRODUCTION

The genus *Fusarium* was established by Link (1809), nearly 200 years ago and encompasses a diverse array of species of significance because they are devastating plant pathogens that often produce a wide range of secondary metabolites. The association of some of these metabolites with cellular toxicity, effects on growth and development of animals, and cancer in humans and domesticated animals is of particular interest to agriculture and food safety. Fungal secondary metabolites negatively impacting animals are referred to as mycotoxins. The main mycotoxin classes of concern produced by *Fusarium* species include trichothecenes, fumonisins and zearalenone (ZEN). Several trichothecenes are of concern, including diacetoxyscirpenol (DAS), T-2 toxin, deoxynivalenol (DON), and nivalenol (NIV) (Glenn, 2007).

Currently, more than 40 naturally occurring trichothecenes are known from *Fusarium* species (Desjardins, 2006), with a combined total of nearly 150 variants produced by species of *Fusarium*, *Cephalosporium*, *Cylindrocarpon*, *Myriothecium*, *Stachybotrys* and *Trichoderma* (Grove, 1988).

In addition to their animal toxicity, trichothecenes are also phytotoxic to a range of plants and production of the toxins enhances the virulence of some *Fusarium* species on some host plants but not on others (Desjardins, 2006). For example, production of DAS by *F. sambucinum* was shown to enhance virulence toward parsnip roots, causing more rot if the fungus produced the DAS (Desjardins *et al*., 1992). In contrast, DAS production was not necessary for *F. sambucinum* to cause potato tuber dry rot.

On cereals two main diseases are of primary concern: *Fusarium* head blight of small grains, primarily wheat, barley and oats, and *Fusarium* ear rot of maize. Other grains such as rice, pearl millet, and sorghum are involved in the disease as well, because of their importance in some feed markets and geographical regions of the world.

Diacetoxyscirpenol, 3-hydroxy-4,15-diacetoxy-12,13-epoxytrichothec-9-ene, or anguidine, is one of the trichothecene mycotoxins produced by certain species of *Fusarium*, such as *F. poae*, *F. semitectum*, *F. moniliforme*, *F. sporotrichioides*, *F. acuminatum*, *F. culmorum*, *F. crookwellense, F. venenatum*, *F. sambucinum*, *F. graminearum*, *F. equiseti*, *F. solani*, *F. roseum*, *F. tricinctum*, *F. avenaceum*, *F. langsethiae*, *F. compactum* and *F. clamydosporum* (Omurtag *et al*., 2007; Schollenberger *et al*., 2007a).

DAS was discovered in 1961 as a phytotoxic compound from a culture of *F. equiseti* and *Gibberella intricans* and its chemical properties and structure have been characterized. According to chemical classification of trichothecenes, DAS as well as T-2 and HT-2 toxins,

belongs to group A which is characterized by the absence of a ketone on C-8 position and the absence of a macrocyclic ring (Lautraite *et al*., 1997). Particularly, it is included in the scirpentriol subgroup which comprises a family of type A trichothecene toxins: scirpentriol (SCIRP), the parent alcohol and its seven acetylated derivatives such as DAS, monoacetoxyscirpenol (MAS) and triacetoxyscirpenol (TAS) (Schollenberger *et al*., 2007a).
Among trichothecenes produced by *Fusarium* spp., DAS is one of the most toxic.
The presence of DAS in animal feeds and human foods is a possible health threat to humans and animals in some parts of the world, as historically documented by studies on a variety of animal toxicosis, human alimentary toxic aleukia (ATA), Msleni joint disease, and more recently evidenced by studies on human toxicoses and bone and joint disease in China (Busby and Wogan, 1981; Wang *et al*., 1993; Beardall and Miller, 1994; Wang *et al*., 1996).
The toxic effects of DAS in humans and animals are similar and include vomiting, diarrhea, hypotension, and myelosuppression.

FUSARIUM AND ITS HOSTS

The genus *Fusarium* currently contains over 20 species (De Hoog *et al*., 2000).
Fusarium species grow rapidly on Sabouraud dextrose agar at 25°C and produce woolly to cottony, flat, spreading colonies. The only slow-growing species is *F. dimerum*. From the front, the colour of the colony may be white, cream, tan, salmon, cinnamon, yellow, red, violet, pink, or purple. From the reverse, it may be colourless, tan, red, dark purple, or brown. Sclerotia may be observed macroscopically and is usually dark blue in colour. On the other hand, sporodochia, are usually absent in culture. When present, it may be observed in cream to tan or orange colour, except for *F. solani*, which gives rise to blue-green or blue sporodochia (De Hoog *et al.*, 2000).

Hyaline septate hyphae, conidiophores, phialides, macroconidia, and microconidia are observed microscopically. In addition to these basic elements, chlamydospores are also produced by *F. chlamydosporum*, *F. napiforme*, *F. oxysporum*, *F. semitectum*, *F. solani* and *F. sporotrichoides* (De Hoog *et al*., 2000).
Phialides are cylindrical, with a small collarette, solitary or produced as a component of a complex branching system. Monophialides and polyphialides (in heads or in chains) may be observed. Macroconidia (3-8 x 11-70 µm) are produced from phialides on unbranched or branched conidiophores. They are 2- or more celled, thick-walled, smooth, and cylindrical or sickle- (canoe)shaped. Macroconidia have a distinct basal foot cell and pointed distal ends. They tend to accumulate in balls or rafts. Microconidia (2-4 x 4-8 µm), on the other hand, are

formed on long or short simple conidiophores. They are 1-celled (occasionally 2- or 3-celled), smooth, hyaline, ovoid to cylindrical, and arranged in balls (occasionally occurring in chains). Chlamydospores, when present, are sparse, in pairs, clumps or chains. They are thick-walled, hyaline, intercalary or terminal (Larone, 1995; Sutton *et al.*, 1998; De Hoog *et al.*, 2000).

Macroscopic and microscopic features, such as colour of the colony, length and shape of the macroconidia, the number, shape and arrangement of microconidia, and presence or absence of chlamydospores are key features for the differentiation of *Fusarium* species (De Hoog *et al.*, 2000). Molecular methods, such as 28S rRNA gene sequencing, may be used for rapid identification of *Fusarium* strains to species level (Hennequin *et al.*, 1999).

According to the taxonomic system proposed by Nelson *et al*., 1983, there are 4 sections in the *Fusarium* classification: Elegans, Liseola, Sporotrichiella, and Discolor. The Elegans group contain *F. oxysporum* species. The Liseola group of *Fusarium* has teleomorphs, where they are known, in the genus *Gibberella*, which produces very dark, almost black, perithecia, but none of them are known to produce trichothecenes. *Fusarium verticillioides*, which should no longer be referred to by the once popular name *Fusarium moniliforme* (Seifert *et al*., 2003), is a very toxigenic species and is one of the major sources of the fumonisins. The Discolor group contains the most important species, *Fusarium graminearum* and *Fusarium culmorum*, producing deoxynivalenol and nivalenol (Moss and Thrane, 2004).

Finally, *F. sporotrichioides*, of the Sporotrichiella group, is the most important producer of the very toxic and immunosuppressive trichothecene, T-2 toxin, although a single strain of *Fusarium sporotrichioides* has been reported to produce deoxynivalenol and several strains to produce nivalenol. The most widespread and important producer of DAS is *F. sambucinum* Fuckel, which, as a storage rot, is of considerable importance on potato. As a root rot it attacks a wide variety of hosts as flax, lupin, strawberry, tomato and especially cereals (Booth, 1973). Recently, some strains were isolated from wheat in Upper Egypt (Sabir, 2006), even if the presence of DAS in cereals is generally due to other *Fusarium* species, such as *Fusarium sporotrichioides*, *F. acuminatum*, *F. avenaceum*, *F. equiseti, F. graminearum, F. oxysporum, F. poae* and *F. semitectum*.

Macroscopic and microscopic features of *F. sambucinum* are described as follows (Nelson *et al.*, 1983):

Conidia: microconidia are generally absent. Macroconidia are short and stout, distinctly septate, with thick walls and moderate to strongly curved ventral and dorsal surfaces. The

basal cell varies from distinctly foot-shaped to notched to rounded. The apical cell is constricted into a snout, often with a small papilla, and is sometimes strongly curved.
Conidiophores: unbranched and branched monophialides.
Chlamydospores: generally they are formed abundantly and quickly (less than one month) and are single, in chains, or in clumps.
Perfect state: *Gibberella pulicaris* (Fr.) Sacc.
Colony morphology: on PDA growth is rapid, with or without dense aerial mycelium. When aerial mycelium is present it is white, tan, or pink to reddish brown in colour. Dark blue sclerotia may be present. Cream to tan to orange sporodochia or pionnotes may be present or absent. The undersurface may be of various colours; it is frequently carmine red, but also may be tan to brown.

Previous data, reviewed by Marasas *et al.* (1984) and Marasas (1991), showed that *Fusarium sporotrichioides*, *F. acuminatum* and *F. oxysporum* produced DAS, *F. poae* produced DAS and MAS, *F. semitectum* produced DAS, MAS and SCIRP, *F. equiseti* produced DAS, MAS, SCIRP and TAS and *F. sambucinum* produced DAS, MAS and TAS. The toxigenicity of *Fusarium* species on grains and culturea has been reviewed also by Pitt and Hocking (1999) and Leslie and Summerell (2006), as reported in Table 1.

Table 1. Scirpentriol group toxins detected in *Fusarium* cultures and conditions of culturing.

	Scirpentriol toxins detected	Scirpentriol toxins not detected	Analytical method	Substrate	Temp °C	Growth period (days)	References
F. sporotrichioides	DAS		GC/MS	Liquid	22	21	Abramson *et al*., 1993
	DAS		HPLC	Cereals	20,26,33	21	Mateo *et al*., 2002
	MAS; 4,15DAS		HPLC/MS	Solid	15, 25	15–40	Park and Chu, 1993
	SCIRP, DAS		GC/MS	Agar, wheat	25	7–14	McLachlan *et al*., 1992
	SCIRP, MAS, DAS		GC/ECD/MS	Agar, cereals	20,25,28	7-21	Thrane *et al*., 2004
	15-MAS	DAS	GC/MS	Barley	21–25	3 + 14	Salas *et al*., 1999
F. chlamydosporum	MAS, DAS		HPLC/MS	Solid	15, 25	15	Park and Chu, 1993
F. poae	DAS		TLC+GC/MS	Liquid	22	21	Abramson *et al*., 1993
	DAS		GC/MS	Rice	25	10	Sugiura *et al*., 1993
	4,15DAS	SCIRP 15-MAS TAS	GC/FID	Liquid	22-26	14	Lauren *et al*., 1992
	SCIRP, 15-MAS, 4,15-DAS, TAS		GC/MS	Liquid, rice	28(28 + 10)	10(14 + 14)	Liu *et al*., 1998
	SCIRP, 15-MAS		GC/MS	Barley	21-25	3 + 14	Salas *et al*., 1999
	SCIRP, 15-MAS, DAS		GC/ECD/MS	Agar, cereals	20,25,28	7-21	Thrane *et al*., 2004
F. langsethiae	SCIRP, 15-MAS, 4,15-DAS	TAS	GC/MS	Agar	25	14	Torp and Langseth,1999
	SCIRP, 15-MAS, DAS		GC/ECD/MS	Agar, maize	20,25,28	7-21	Thrane *et al*., 2004
F. avenaceum	DAS		GC/MS	Maize	25	28	Hussein *et al*., 1991
F. semitectum	DAS		GC/ECD	Maize	25 + 15	7 + 14	Molto *et al*., 1997
F. equiseti	DAS		GC/MS	Liquid	22	21	Abramson *et al*., 1993
	SCIRP, 4,15-DAS		GC/MS	Rice	25 + 10	14 + 14	Morrison *et al*., 2002
	MAS, DAS		GC/MS	Liquid, rice	14/28	28	Greenhalgh *et al*., 1985
	15-MAS, DAS		GC/MS	Agar	25	14	Kosiak *et al*., 2005
	SCIRP, MAS, DAS		TLC, GC/MS	Rice	25-27,+10	14 + 14	Bosch and Mirocha, 1992
	SCIRP, 15-MAS, DAS		GC/MS	Agar	25	14	Hestbjerg *et al*., 2002
	SCIRP, DAS		GC/MS	Agar, wheat	25	7-14	McLachlan *et al*., 1992
	SCIRP, 4-MAS, 15-MAS, DAS	TAS	GC/MS	Agar	25	14	Nielsen and Thrane, 2001
F. acuminatum	SCIRP, DAS		GC/MS	Agar, wheat	25	7-14	McLachlan *et al*., 1992
	DAS		GC/ECD	Corn	25	21	Rabie *et al*., 1986
	SCIRP derivates		GC/ECD	Liquid, solid	25	14	Wing *et al*., 1993
F. compactum	DAS (main), SCIRP derivatives		GC/ECD	Liquid, solid	25	14	Wing *et al*., 1993
F. sambucinum	DAS		GC/MS	Maize	25	28	Hussein *et al*., 1991
	DAS		LC/HPTLC	Corn	25	28	Mulè *et al*., 1992
	DAS		HPLC	Corn	25	28	Altomare *et al*., 1995
	DAS		LC/MS	Potato	10/20	30/50	Ellner , 2002
	DAS	15-MAS, TAS	TLC	Agar	25	14	Thrane and Hansen, 1995
	DAS		GLC	Liquid	28	7	Beremand *et al*., 1991
	4,15-DAS	15-MAS	GC/FID	Liquid	28	7	Desjardins and Plattner, 1989
	15-MAS, 4,15-DAS		GC/FID	Potato	25	6	Desjardins and Plattner, 1989
	4,15-DAS	SCIRP, 15-MAS, TAS	GC/FID	Liquid	22-26	14	Lauren *et al*., 1992
	15-MAS, 4,15-DAS	SCIRP, 4-MAS	TLC	Liquid, wheat		28	Latus-Zietkiewicz *et al*., 1995
	15-MAS, 4,15-DAS	SCIRP, 4-MAS	TLC	Potato		28	Latus-Zietkiewicz *et al*., 1995
	SCIRP, 4-MAS, 15-MAS, 4,15-DAS		GC/MS	Wheat	25	5	Jelen *et al*., 1995
	SCIRP, 4-MAS, 15-MAS, 4,15-DAS,		MS, NMR	Corn	25	21	Steyn *et al*., 1978

	TAS		GC/MS, NMR	Liquid	29	3 + 6	Richardson *et al*., 1990
	SCIRP, 3-MAS, 4-MAS, 15-MAS	TAS	GC/MS, NMR	Liquid	29	3 + 6	Richardson *et al*., 1990
	3,4-DAS, 3,15-DAS, 4,15-DAS, TAS	TAS	GC/MS	Agar	22-26	14	Nielsen and Thrane, 2001
	SCIRP, 4-MAS, 15-MAS, DAS		TLC, HPTLC	Corn	10	21	Sanson *et al*., 1989
	SCIRP, 4-MAS, 15-MAS, 3,15-DAS						
F. venenatum	DAS	15-MAS, TAS	TLC	Agar	25	14	Thrane and Hansen, 1995
	DAS		HPTLC	Corn	25	28	Altomare *et al*., 1995
	4,15-DAS	SCIRP, 15-MAS, TAS	GC/FID	Liquid	22-26	14	Lauren *et al*., 1992
	SCIRP, 4-MAS, 15-MAS, DAS	TAS	GC/MS	Agar	25	14	Nielsen and Thrane, 2001
F. culmorum	4,15-DAS	SCIRP, 15-MAS, TAS	GC/FID	Liquid	22-26	14	Lauren *et al*., 1992
F. graminearum	DAS		GC/MS	Maize	25	28	Hussein *et al*., 1991
	DAS		G/MS	Liquid	22	21	Abramson *et al*., 1993
	DAS		GC/ECD	Maize	25 + 15	7 + 14	Molto *et al*., 1997
	MAS, 4,15-DAS		HPLC/MS	Solid	15,25	15-40	Park and Chu, 1993
F. crookwellense	4,15-DAS	SCIRP, 15-MAS, TAS	GC/FID	Liquid	22–26	14	Lauren *et al*., 1992
	DAS		TLC+GC/MS	Corn	25	14	Vesonder *et al*., 1991

DAS = Diacetoxyscirpenol
DON = Deoxynivalenol
FUS-X = Fusarenon-X
HT-2 = HT-2 toxin
MAS = Monoacetoxyscirpenol
NIV = Nivalenol
SCIRP = Scirpentriol
TAS = Triacetoxyscirpenol
T-2 = T-2 toxin

Infection cycle

The main producer of DAS is *Gibberella pulicaris* (Fries; Booth, 1973) Sacc. (asexual stage: *F. sambucinum* Fuckel or *F. sulphureum* Schlecht). Generally, *Fusarium* species are ubiquitous in soil. Some can produce specially developed resting spores (chlamydospores). Most also have highly developed competitive saprophytic abilities which enable them to rapidly colonise debris and stubble. Hence *Fusarium* mainly overwinter in the soil or on debris. Weeds, early-sown autumn crops, volunteers and infected seed may also act as overwintering sources of inoculum. From these bases, mycelium may grow directly into plant roots or stem bases, or spores may be produced which are splash-dispersed onto stem bases and the various symptoms then develop. Drought stress makes the disease development more severe. *Fusarium* species may infect the ear at the end of the growing season, and they also remain in or rapidly colonise any stubble or debris remaining after harvest.

Fusarium sambucinum is commonly found on seed tubers but also survives for very long periods in the soil. Seed pieces decay when the pathogen infects cut or injured surfaces or when seed tubers are infected before cutting. Tubers begin to rot either while they are being held after cutting or after they are planted. Precutting seed puts the tubers at risk for *Fusarium* dry rot, particularly if the isolate of the fungus is resistant to benzimidazole fungicides. The pathogen can move into the tuber through the cut surface and quickly rot the seed piece. Unfortunately, the temperature most favourable for healing the cut surface, about 12-13°C, is high enough to allow *Fusarium* to colonize the tissue. Seed decay results in poor stands and stunted plants.

Progeny tubers are not usually infected until harvest because *F. sambucinum* and *F. solani* cannot cause infection unless they penetrate the skin and the potato skin is rarely injured during the growing season. Growth cracks, however, can be infected by *Fusarium* spp. Damage to tubers at harvest provides a multitude of points of entry for spores that are dormant in soil or on the surface of the tuber. If postharvest applications of a fungicide do not inhibit further development of the disease, the fungus begins to grow in the tuber tissue, causing dry rot lesions at the point of injury. The severity of dry rot in storage depends on the magnitude of injury. In storage the progress of the disease is limited primarily by temperature: the colder the temperature, the slower the disease will progress. Young tubers appear to have some resistance to dry rot which slows disease. Dry rot progresses noticeably faster during the last half of the storage season.

If tubers with dry rot are planted, the seed pieces may decay. The fungus is very effectively spread during seed cutting and handling. In addition, the fungus is introduced into the soil,

where it can survive and contaminate progeny tubers. When harvest occurs, the cycle begins again.

Fusarium sambucinum is not reported on cereals, commodities cited in almost all surveys on DAS contamination, and no reports are available regarding the main *Fusarium* species responsible for DAS contamination in cereals. *Fusarium* head blight in small cereals and Fusarium ear rot in maize are considered the origin of DAS contamination; in fact, they are commonly associated to a complex of Fusaria, several of them (*Fusarium sporotrichioides*, *F. acuminatum*, *F. avenaceum*, *F. equiseti, F. graminearum, F. oxysporum, F. poae* and *F. semitectum*) reported as DAS producers.

Ecology

Fusarium species are widely distributed in soil and on subterranean and aerial plant parts, plant debris, and other organic substrates. They are common in tropical and temperate regions and are also found in desert, alpine, and arctic areas, where harsh climatic conditions prevail (Nelson *et al.*, 1994). Many *Fusarium* species are abundant in fertile cultivated and rangeland soils but are relatively uncommon in forest soils (Burgess and Liddell, 1983). *Fusarium* species are often regarded as soilborne fungi because of their abundance in soil and their frequent association with plant roots, as either parasites or saprophytes. However, many have active or passive means of dispersal in the atmosphere and are common colonizers of aerial plant parts, where they may result in diseases of considerable economic importance. Some of these airborne *Fusarium* species are encountered rarely in isolations of cultures from soil or roots. The widespread distribution of *Fusarium* species may be attributed to the ability of these fungi to grow on a wide range of substrates and to their efficient mechanisms for dispersal (Nelson *et al.*, 1994). Natural *Fusarium* infections occur between 6°C and 21°C.

Fusarium sambucinum (Fuckel), a field fungus occurring in moderate climatic zones, is distributed widely on vegetables, fruits, conifer trees, and soil (Nelson *et al.*, 1983). As a plant pathogen it is the main species that causes dry rot of potato tubers in North America and Europe (Boyd, 1972; Seppanen, 1989). It is a ubiquitous species, which is more common in the northern than in the southern hemisphere and has a wide host range, including stored fruits and potatoes (Weidenbőrner, 2001).

Water activity and temperature show a significant effect on the growth of *F. sambucinum*. The results of an investigation by Sempere Ferre *et al*. (2007) showed that the fungus grew most rapidly at water activity of 0.995 and temperature of 25°C.

Another investigation conducted by Lui and Kushalappa (2002) studied the disease dynamics of dry rot development in potato tubers after harvest. After inoculation with a spore suspension of *F. sambucinum* (10^4/mg), potato tubers were incubated in growth chambers at different temperatures (4-20°C) and wetness duration till to 90 days. The amount of infection was limited at the lowest temperatures and increased with an increase in wetness duration and temperature. At a storage temperature of 16°C, lesions expanded rapidly reaching maximum in about 45 days of storage. Two cubic regression models were developed to predict infection potential from incubation temperature and duration of wetness and lesions expansion potential as a function of storage temperature and duration. These models explained 94 and 99% of variation and could be applied to improve potato storage after their validation.

Plant-pathogen interactions

Fusarium species have been important for many years as plant pathogens causing diseases such as crown rot, head blight, and scab on cereal grains; vascular wilts on a wide range of horticultural crops; root rots; cankers; and other diseases such as pokkah-boeng on sugarcane and bakanae disease of rice (Booth, 1971).

Fusarium species infect important crops such as wheat, durum, barley, oats, rice, maize, potato,

Fusarium sambucinum is a major cause worldwide of dry-rot of potato tubers (Boyd, 1972) and this disease may result in significant economic losses during storage (Gould *et al.*, 2008). *Fusarium* dry rot is caused also by *Fusarium culmorum* (W.G. Smith) Sacc. and *Fusarium oxysporum* Schlecht, under field and storage conditions (Boyd, 1972; Schisler and Slininger, 1994). Crop losses attributed to the dry rot have been estimated to average 6%, with losses up to 25% reported (Chelkowski, 1989). In addition to destroying tuber tissues, *Fusarium* spp. can produce toxins that have been implicated in mycotoxicoses of humans and animals (Senter *et al.*, 1991; Schisler *et al.*, 1997).

The ability of field strains of *F. sambucinum* to cause dry-rot is correlated with their ability to detoxify sesquiterpene phytoalexins produced by potato. All highly virulent field strains can detoxify the sesquiterpenes rishitin and lubimin.

Fusarium dry rot is characterized by shrunken, collapsed diseased tissues and an internal light to dark brown or black rot of the potato tuber, that are usually dry. The rot may develop at an injury such as a bruise or cut. The pathogen penetrates the tuber, often rotting out the centre. Extensive rotting causes the tissue to shrink and collapse, usually leaving a dark

sunken area on the outside of the tuber and internal cavities. Yellow, white, or pink mould may be present.

It is an economically important disease worldwide, which affects tubers in storage and may also damage seed pieces, reducing crop stand. *Fusarium* dry rot accounts for losses of up to 25% of potatoes in storage (Slininger *et al.*, 1996 and 1998 and 2004; Lui and Kushalappa, 2002).

Diagnosis of dry rot can be complicated by the presence of soft rot bacteria, which often invade dry rot lesions, particularly if the tubers have been stored at high humidity and condensation has occurred on the surfaces. Soft rot bacteria cause a wet rot that can very quickly encompass the entire tuber and mask the initial dry rot symptoms.

Several other diseases and physiological disorders, including Pythium leak, pink rot, late blight, and suboxygenation (blackheart), also cause brown to black internal discoloration of tubers. Leak and pink rot are wet rots; tubers exude a clear fluid when squeezed. Late blight is a less aggressive rot that generally does not penetrate into the centre of the tuber and causes reddish-brown lesions. Poor air circulation or extremes in temperature that result in low internal oxygen concentrations can cause a smoky gray to black discoloration of tissue, but the tissue is never brown and is very firm (Snowdon, 1990).

Fusarium sambucinum is very common on citrus, conifer seedlings, and the genera *Dahlia, Humulus, Junglans, Lycopersicon, Morus, Musa, Pinus* and *Ricinus* (Beremand *et al*., 1991).

PHYSICO-CHEMICAL CHARACTERISTICS

Diacetoxyscirpenol (DAS; $C_{19}H_{26}O_7$; 12,13-Epoxytrichothec-9-ene-3,4,15-triol-4,15-diacetate; MW: 366.41) is a type A trichothecene. It is a white crystalline powder with a melting point at 160°-164°C (Figure 1).

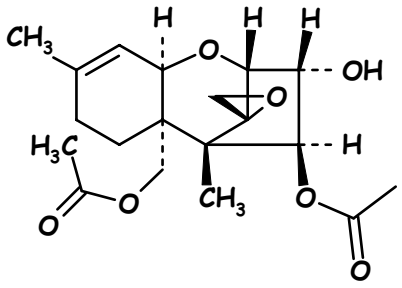

Figure 1. Chemical structure of DAS.

DAS, as well as the other type A trichothecenes, is highly soluble in ethyl acetate, acetone, chloroform, methylene chloride and diethyl ether. It can react with oxidazing agents, acids

and alkalis. When treated with alkali or dilute solutions of potassium carbonate, sodium hydroxide or ammonium hydroxide DAS is hydrolised to scirpentriol.

DAS is chemically stable and can persist for long periods once formed. Prolonged boiling in water or under highly acidic conditions causes a skeletal rearrangement due to opening of the epoxide ring. Primary and secondary hydroxyl groups are easily oxidised to the aldehyde and ketone derivatives by reagents such as a mixture of chromic oxide and sulfuric acid in acetone, a mixture of chromic oxide and pyridine and of chromic oxide and acetic acid.

BIOSYNTHESIS

Classified into the chemical group sesquiterpene, trichothecenes have a skeleton derived from farnesyl pyrophosphate (FPP), which is synthesised by the condensation of isopentenyl pyrophosphate (IPP), an isoprene unit, with its isomer dimethylallylpyrophosphate, followed by the repeated condensation of IPP with the resulting prenyl pyrophosphate called geranylpyrophosphate.

FPP is a common intermediate in protein isoprenylation and in the biosynthesis of secondary metabolites (Figure 2).

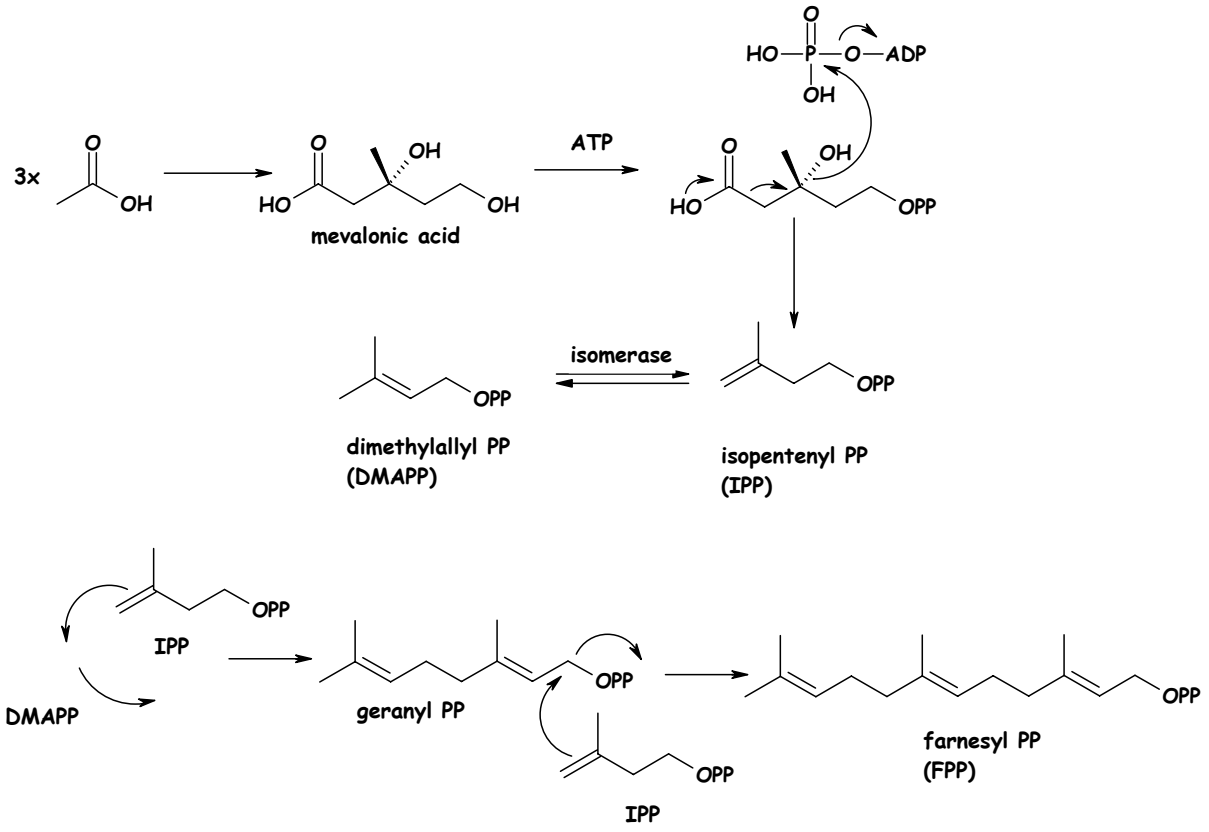

Figure 2. Farnesyl pyrophosphate formation.

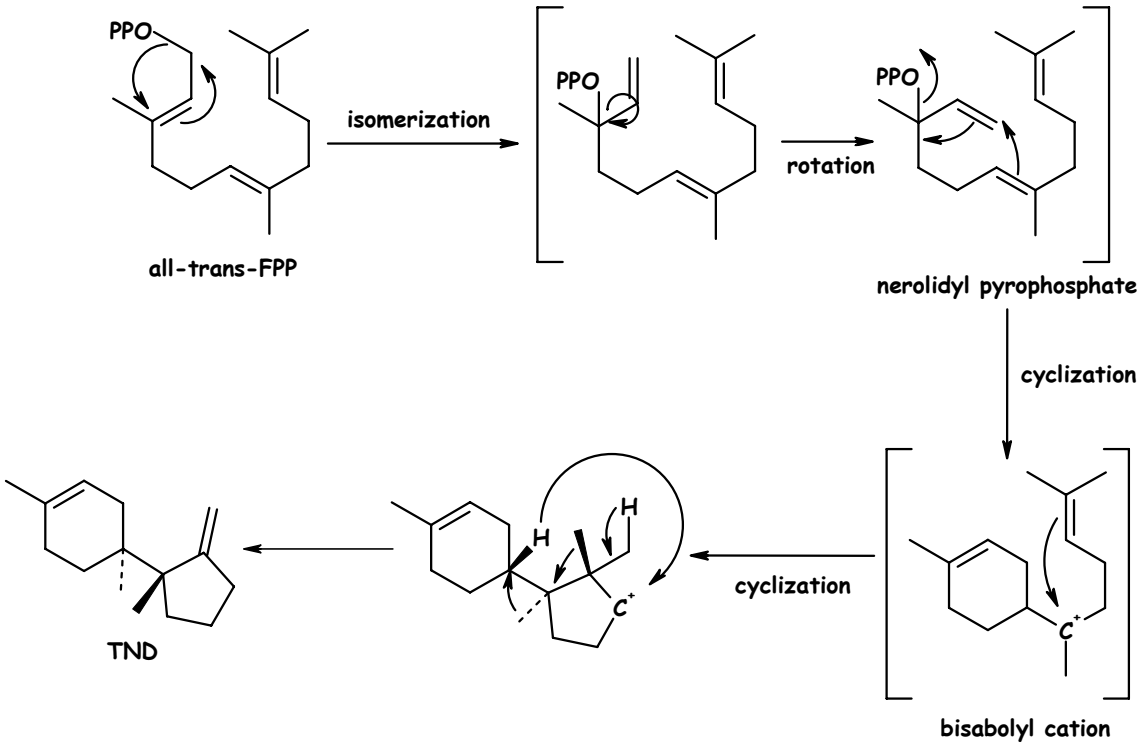

Figure 3. TND biosynthetic pathway.

In the pathway producing trichothecenes and related mycotoxins the first identified intermediate was trichodiene (TDN), which was proved to be originated from mevalonate via all-*trans*-FPP and nerodyl pyrophosphate, as reported in Figure 3 (Blackwell *et al*., 1985; Zamir *et al*., 1989).

The following oxygenation reactions, which are mediated by cytochrome P450 monoxygenases (CYPs), give rise to the addition of hydroxyl groups at C-3, C-4, C-8 and C-15 and to the formation of an epoxy group at position 12,13. The oxygenation steps after TND are sequential and not random, following the sequence reported in Figure 4. The last oxygenation step giving isotrichotriol seems to be the rate-limiting step among the four consecutive steps of oxygenation of TND in the early biosynthetic pathway (Hesketh *et al*., 1991 and 1992; Zamir *et al*., 1999).

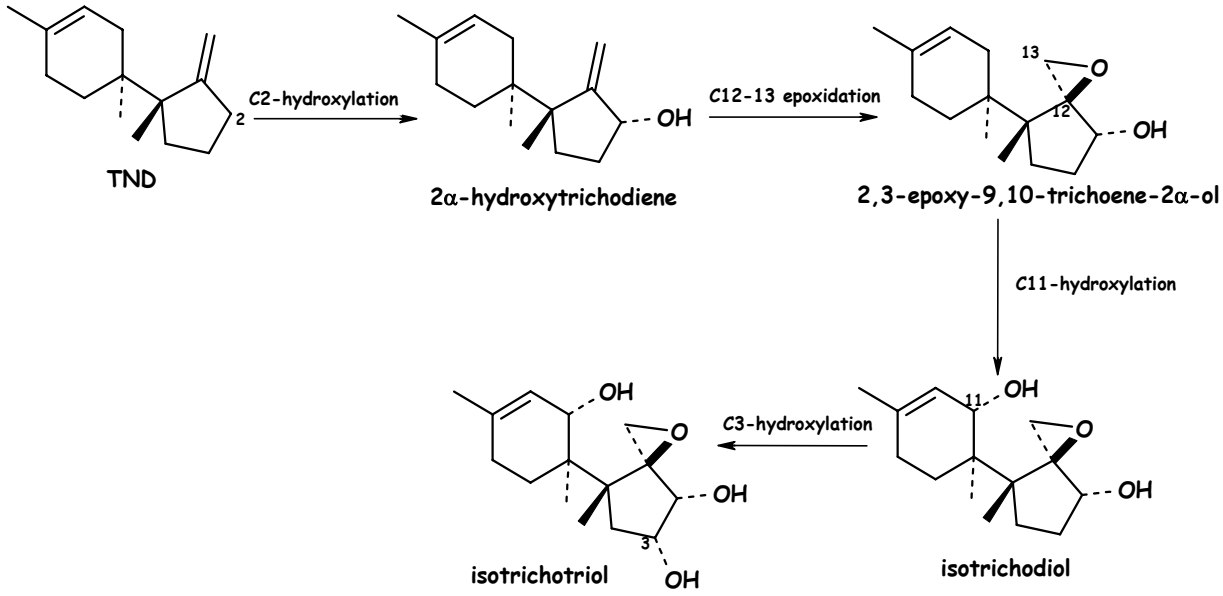

Figure 4. TND conversion to isotrichotriol.

The conversion of isotrichotriol to isotrichodermol was shown to proceed non-enzymatically in acidic conditions, although the reaction was not rapid enough to feature in a biosynthetic pathway. Since the transient formation of trichotriol was observed during the acidic-catalyzed chemical conversion, an intermediate role for trichotriol was proposed in the second cyclization. Hesketh's group also identified the natural occurrence of isotrichotriol, trichotriol and 9α-epimer of trichotriol in *F. culmorum*. Considering the co-occurrence of the three tetraoxygenated TDN isomers, the proposed transient carbocation intermediate appears to be a common pathway intermediate. The unstable compound undergoes either irreversible cyclization to isotrichodermol by intramolecular attack of the C-2 hydroxyl to C-11 or reversible epimerization to trichotriol and its 9α-epimer by regiospecific attack of a water nucleophile at C-9 (Figure 5) (Hesketh *et al*., 1992).

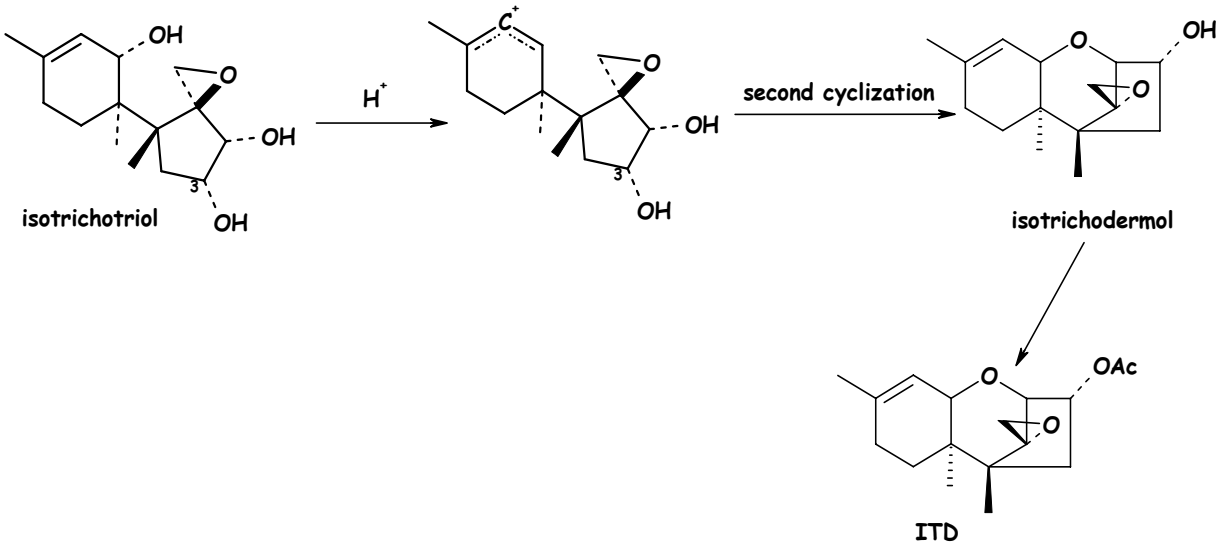

Figure 5. Second cyclization scheme.

Since the *Fusarium* culture is not acidic enough to rapidly promote the cyclization of isotrichotriol to isotrichodermol, the major biosynthetic route may involve a direct enzymatic cyclization. However, a gene for this second cyclization step is not present in the cluster of genes involved in the biosynthesis of trichothecenes in *Fusarium* species. It is possible that a locally high concentration of isotrichotriol on the endoplasmatic reticulum membrane greatly enhances progression of the forward chemical reaction even under mildly acidic conditions.

All such trichothecene-related compounds have a trichothecene structure with a hydroxyl or acetyl at C-3: these include all the species reported in Figure 6, generated by random oxygenation at C-7, C-8 and C-15 followed by an acetylation step (Zamir *et al*., 1990; Zamir *et al*., 1991).

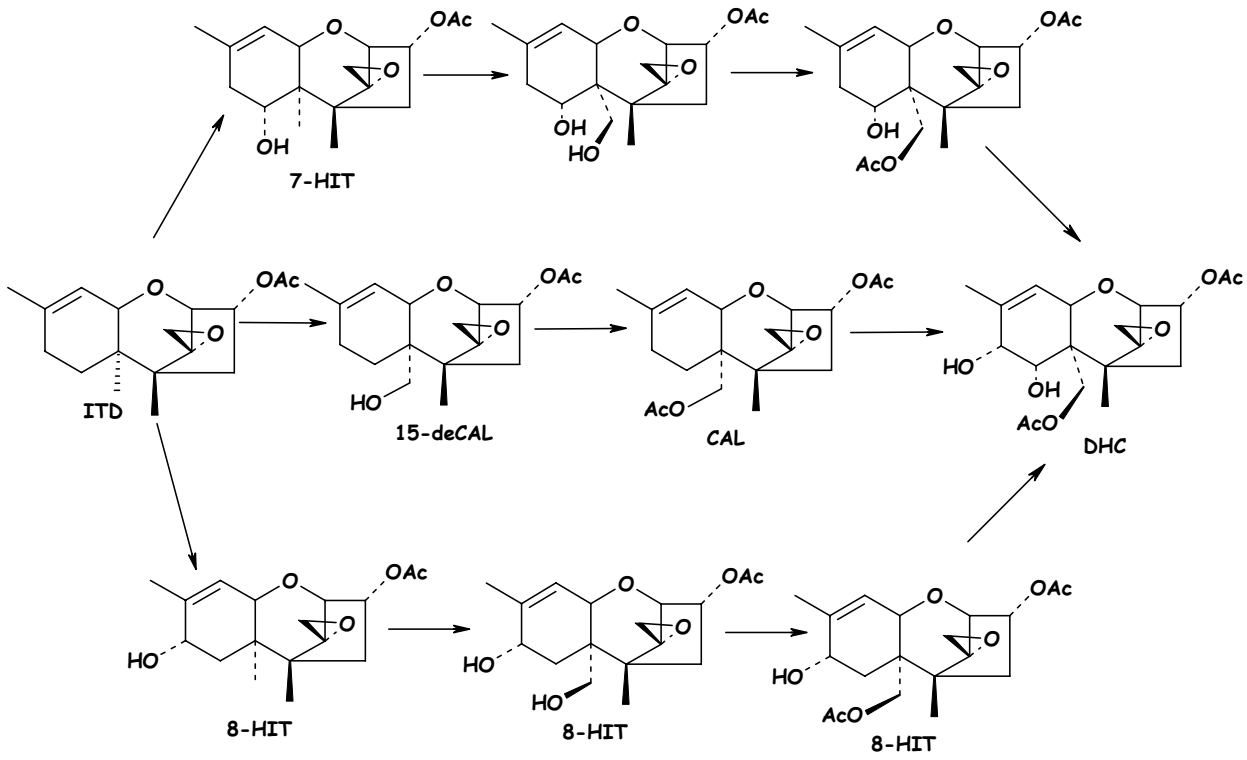

Figure 6. Trichothecenes differentiation pathway.

After the formation of calonectrin (CAL), a diversity of trichothecene structures arises. In particular, the substrate specificity of certain late biosynthetic enzymes differs between type A and type B trichothecene producers (Hesketh *et al*., 1991). Partly due to the difficulties of prepararing labelled derivatives for feeding experiments, chemical procedures were not used to determine the biosynthetic grids. Instead, the availability of gene manipulation techniques and the discovery of the trichothecene pathway genes have opened alternative ways to study the late steps in the biosynthesis of trichothecenes.

However, by now, the formation pathway of DAS has only been proposed. In particular, the tentative DAS formation pathway has been proposed on the basis of several experimental results using the targeted gene disruption mutants of *F. sporotrichioides*, as reported below (Kimura *et al*., 2007).

CAL —*FsTri13*→ 3,15-diacetoxyscirpenol —*FsTri7*→ 3,4,15-triacetoxyscirpenol —*FsTri8*→ 4,15-diacetoxyscirpenol

In summary, although trichothecenes pathway has been elucidated, DAS biosynthesis is still to be defined in the very last steps. Moreover, to date very few data on genes involved are available.

CHEMICAL ANALYSES

Sampling

A specific sampling method for commodities containing DAS does not exist, but since mycotoxins can be distributed very heterogeneously in a lot, a representative sampling is highly important.

So in sampling, we can refer to Commission Regulation (EC) No. 401/2006 of 23 February 2006, "laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs".

Extraction and Clean-up

The state-of-the-art in the analysis of type A trichothecenes such as DAS has been reviewed by different authors (Krska *et al*., 2001). Various combinations of solvents have been used to extract DAS from grain, food and feeds. Mostly acetonitrile and water (Langseth and Rundberget, 1998; Schollenberger *et al*., 1998) were described as extractants, although also ethylacetate was in use (Desjardins and Plattner, 1989). Extraction has been performed mainly by high-speed blending or mechanical shaking.

Most clean-up procedures are based on solid-phase extraction. They involve charcoal–alumina columns or modified charcoal alumina columns including charcoal/celite and ion-exchange resin, purification on silica, or defatting with hexane followed by purification on Florisil (Schollenberger *et al*., 2007a). These methods may be combined with each other or with further clean-up steps based on other chromatographic principles e.g., ion-exchange resin or reversed-phase cartridge (Schollenberger *et al*., 2007a). The most common clean up method for type A trichotechenes is based on Mycosep columns, which allow for good recovery and multiresidual analysis (Weingaertner *et al*., 1997) Immunoaffinity columns, which are successfully applied for sample clean-up of several mycotoxins, so far have not been used for DAS because of a lack of commercially available columns.

Source of standards

Availability of DAS standards is shown in Table 2. Certified reference materials for method development and validation are not commercially available.

Table 2. Sources of DAS standards.

Compound	Concentration (µg/ml)	Quantity	Supplier
Standard Solution			
Diacetoxyscirpenol solution (OEKANAL)	100 in acetonitrile	2 ml	Fluka. Sigma-Aldrich (Buchs - Switzerland)
Diacetoxyscirpenol solution	100 in acetonitrile	1/5 ml	Chiron AS (Trondheim - Norway)
	100 in acetonitrile	1/5 ml	Romer Labs Diagnostic (Tulln - Austria)
Solid Standard			
Diacetoxyscirpenol from *F. sambucinum*		1/5/100 mg	Sigma-Aldrich
Diacetoxyscirpenol		-	Apollo Scientific Ltd. (Cheshire - UK)
		-	Carbone Scientific Co. Ltd. (London - UK)
		-	UK Green Scientific Co. Ltd. (UK)

Analytical methods

The analytical determination of DAS as well as other type A trichothecenes has been extensively reviewed (Krska *et al*., 2001; Sforza *et al*., 2006; Zoellner and Mayer-Helm, 2006; Schollenberger *et al*., 2007a). Extraction and clean-up formerly were followed by thin-layer chromatography (TLC) as already reviewed (Snyder, 1986), but gas chromatography (GC) and high-performance liquid chromatography (HPLC) have become increasingly popular. These methods generally are more sensitive, selective, precise and accurate compared to TLC (Krska *et al*., 2001). Spectroscopic methods have not been applied for the detection of A type trichothecenes due chiefly to the lack of chromophores in their molecule. Different immunochemical methods including enzyme-linked immunosorbent (ELISA) and radio-immuno (RIA) assay systems have been established for determination of trichothecenes (Candlish *et al*., 1989), and these methods in particular were used for DAS analysis (Klaffer *et al*., 1988). Due to the low interest in the routine analysis of this mycotoxin commercially available kits were so far not brought to market.

Gas chromatography methods have been based on flame ionisation detection (FID) (Schothorst and Jekel, 2001 and 2003), electroncapture (ECD) (Molto *et al*., 2000) or mass spectrometric (MS) detection (Schollenberger *et al*., 2005). Mostly a derivatization step of the hydroxyl groups is included in order to increase volatility and sensitivity. Derivatives are trimethylsilyl (TMS) ether, heptafluorobutyryl (HFB) ester and the fluoroacyl (trifluoracetyl, pentafluoropropionyl, heptafluorobutyryl) esters (Sforza *et al*., 2006; Zoellner and Mayer-Helm, 2006).

HPLC-UV detection is not suitable for A type trichothecenes because of their very low wavelength absorption (below 205 nm) (Samar and Resnik, 2002). Therefore precolumn derivatisation methods have been developed including those involving UV detection of the p-nitrobenzoate (Maycock and Utley, 1985) or diphenylindenone sulphonyl esters (Yagen *et*

al., 1986). Fluorescence measurement of the coumarin- 3-carbonyl chloride derivatives has also been applied (Jimenez *et al*., 2000; Dall'Asta *et al*., 2004a).

HPLC-MS has great potential for the simultaneous detection of various mycotoxins and their degradation products (Sforza *et al*., 2006). Instruments using electrospray (ESI) or atmospheric pressure (APCI) chemical ionisation have been employed for the determination and identification of A type trichothecenes including DAS at trace levels as recently reviewed by Zoellner and Mayer-Helm (2006). Since ESI interface is usually more affected by matrix effects, LC-MS based methods for type A trichothecenes commonly use an APCI interface.

Mass spectrometry based methods are usually developed for multiresidual analysis (Razzazi-Fazeli *et al*., 2002; Dall'Asta *et al*., 2004b; Berthiller *et al*., 2005; Sulyok *et al*., 2006) and can be performed using single stage (MS) or multiple stage (MS/MS, MS^n) modes. For the latter, ion trap or triple quadrupole techniques have been used (Berger *et al*., 1999; Sulyok *et al*., 2006). Quantification is usually based on internal standards: since isotope-labelled DAS is not commercially available, verrucarol has been successfully proposed (Berger *et al*., 1999). Separation is usually achieved with a C18 column, using water-mathanol or water-acetonitrile mixture as mobile phase and operating in the positive ion mode. Ionization is commonly assisted by adding low amounts of ammonium acetate to the eluent, giving the formation of $[M+NH_4]+$ adducts (Sforza *et al*., 2006; Zoellner and Mayer-Helm, 2006).

Since type A trichothecenes such as DAS may form very stable sodium adducts, this peculiar tendency can be used to enhance their naturally poor response in the ESI interface by adding a very low amount of sodium chloride (Dall'Asta *et al*., 2004a).

Validation and outlook

In general, mass spectrometry detection is essential for the reliable detection of small quantities of DAS (Langseth and Rundberget, 1998): GC-MS in contrast to LC-MS is limited to analytes with sufficient volatility. For trichothecene analysis using GC methodology time-consuming and error-prone derivatisation steps are necessary. On the other hand relatively low separation efficiency of HPLC compared to GC must be taken into consideration when multi-trichothecene analysis, especially of mass identical isomers, is required (Zoellner and Mayer-Helm, 2006). The typical limits of detection for the determination of DAS were in the magnitude of 500 μg/kg by TLC, 100–500 μg/kg by GC-FID and GC-ECD, 3–120 μg/kg by GC-MS and 0.5– 60 μg/kg by HPLC-MS.

LC-MS provides accurate quantification of trichothecenes down to low µg/kg level. Recovery rates range between 70% and 108% and depend on the polarity of the analytes. Generally, low polar trichothecenes such as DAS exhibit good recovery rates.

Typically, linearity ranges are achieved with one or two orders of magnitude with good correlation coefficients (Zoellner and Mayer-Helm, 2006). Most authors have not used internal standards to compensate for analyte losses, matrix effects or performance deviations of the MS detector. However, matrix-matched calibration curves and isotope-labelled internal standard addition should be taken into account to cope with matrix related method validation problems, expecially when LC-MS methods are used. Unfortunately, isotope labelled diacetoxyscirpenol is still not commercially available.

Studies about DAS calibrant have been not performed yet and DAS-containing reference material are not commercially available so far. Moreover, the development of specific immunoaffinity devices as well as isotope-labelled internal standards should be encouraged.

The methods for DAS determination reported in literature are usually proposed for food. Although similar protocols may be applied for feed, no data about accuracy, recovery, repeatability etc. are reported for feed, which is usually more complex, thus suffering from stronger matrix effects.

OCCURRENCE DATA

Data about the occurrence of DAS are available for North and South America as well as Europe, whereas only a few reports exist for Africa, Asia and Australia.

The natural occurrence of DAS in agricultural commodities was first observed by Mirocha *et al*. (1976). It was associated with an idiopathic condition in pigs called “hemorrhagic bowel syndrome”, characterized by internal hemorrhages originating in the ileum. Gas chromatographic analysis indicated the presence of DAS from 380 to 500 µg/kg in feedstuffs samples.

Successively DAS has been regularly detected in agricultural crops. Its occurrence in European commodities described before 1995 has been reviewed by Pettersson (1995); subsequent reports for this region and for other countries are summarized in Table 3. Generally, wheat is the matrix most frequently analysed, followed by oats, barley, corn, corn products, corn plant, corn silage and some other substrates used for food and feed (Schollenberger *et al*., 2007a).

DAS was not detected in any sample of wheat, barley and oats from Germany although 721, 240 and 395 samples, respectively, harvested over 5 or 6 years, were analysed by a GC-MS method, with a low detection limit (LOD) (Müller *et al*., 1997a and 1997b and 1998 and

2001). No DAS was detected in corn plant, corn silage and grain-based food from Germany (Schollenberger *et al*., 2005 and 2006), and also in organic and conventional wheat from the UK (Edwards, 2009). General results on representative samples of barley, wheat and oats from the 2004 and 2005 harvest, suggest that DAS is currently very rare in UK grown grain (Baxter and Müller, 2006).

Also 85 commercially processed cereals and pulses, from various parts of Turkey, analysed for DAS levels by Omurtag *et al*. (2007), were DAS free.

The incidence of DAS was very low (0.3%) in 178 oat samples harvested in Norway, which were analysed by GC-MS (Langseth and Rundberget, 1999), and a detectable amount was found in only one sample out of 30 maize samples, analysed by an immunochemical method in Romania (Curtui *et al*., 1998). Scudamore *et al*. (1998) reported high trichothecene levels in corn screenings, bran and germ among others on the British market, but DAS was found in only two samples, out of 40 samples, of corn gluten. A low degree of contamination was also found for DAS, particularly in corn products, out of a total of 125 samples of wheat, oats, corn byproducts, corn plants and corn silage collected by Schollenberger *et al*. (2006).

A European study (European Commission, Scientific Cooperation, 2003), involving different member states, on the occurrence of *Fusarium* toxins, provided results covering 12 different trichothecenes. A total of 1886 cereal samples were analysed for DAS with 4% of positive samples, which ranged from LOD = 3.3 µg/kg (UK) to 50 µg/kg (France). A similar study was carried out by a Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2001), but no data were available for DAS.

Among cereals, oats are known to be very susceptible to infestation by the *Fusarium* spp. producing group A trichothecenes, so they are frequently contaminated with T-2, HT-2 and DAS (Pettersson and Olvang, 1995). Kiecana and Kocylak (1998) reported that in 1997 in southeastern Poland, *F. sporotrichioides* and *F. poae* were found in 7% of isolates from oat. Similar frequencies of *Fusarium* spp. producing group A trichothecenes were recorded in oat grain in Canada, where *F. sporotrichioides* accounted for 6% and *F. poae* for 10% of all *Fusarium* isolates (Clear *et al*., 1996). This means that although contributions of *F. sporotrichioides* and *F. poae* are usually lower than those of *F. culmorum* and *F. graminearum*, in epidemic years group A trichothecenes, naturally present in grain samples, can give rise to food hygiene problems of primary concern.

Of the 99 samples of oat grain analysed in Poland by Perkowsky and Basiński (2002), 34 contained detectable concentrations of T-2, HT-2 and DAS. In particular, DAS was detected in 12% of samples at an average level of 23 µg/kg.

Seventy representative oat samples of both conventional and organic production were drawn at mills and at wholesale stage and analysed for type A trichothecenes in LC-MS/MS. In this case DAS levels were particularly low (below 1 µg/kg), and without any significant difference between organic and conventional products, but a 100% incidence was found for DAS in conventional samples (Gottschalk *et al*., 2007).

Each year in Poland 0.1-4.0% of barley heads are usually infected with *Fusarium* species. Fifteen barley cultivars, analysed in 1997 by Perkowski *et al*. (2003), were found positive for trichothecenes such as DON, NIV, DAS, T-2 and HT-2. Distribution of these mycotoxins was studied in two fractions of kernels (diameter >2.5 mm and <2.5 mm). The highest concentration of the analysed toxins was in the fraction of small kernels. Kernel fractions <2.5 mm, although accounting for only 12.8% of sample weight, contained high proportions of the total toxin content, of which 80% was DAS. These results indicate that the level of contamination can be reduced by the rejection of small kernels. The concentration of DAS varied greatly (range 10-34 µg/kg), but was generally higher than in barley samples analysed in Poland in 1994 and 1995 (Perkowski *et al*., 1997). This may be due to the unusually high precipitation in the summer of 1997.

Results showed by Dänicke (2002) indicated that concentrations of T-2 toxin and DAS in grains and mixed feeds are commonly low and seldom reach levels higher than 0.5 mg/kg. So, harmful levels of T-2 toxin and DAS, which could cause toxicosis in farm animals, are rare and sporadic.

Pavicic *et al*. (1999) reported *Fusarium* mycotoxin frequency in 2562 samples of mixed poultry feed (n=2379) and pig feed (n=183) during 1989-1998. T-2 toxin and DAS were found in 18% and 50.5% of samples, respectively, tested by thin-layer chromatography (TLC). In particular DAS was more frequent in 1992, 1995 and 1997, with incidences of 75%, 50% and 73%, respectively.

A total of 465 samples of grains and animal feeds were collected during the period from 1998 to 2004 by Sokolović and Šimpraga (2006), to evaluate the natural occurrence of T-2 toxin and DAS, analysed by TLC. These toxins were commonly found in grains and feeds with a rate of positive samples up to 60.7%. The incidence of DAS was permanently slightly higher during the whole survey with the highest incidence in 2004. Generally, contamination with DAS of grains is in correlation with contamination in feed. The majority of animal feed samples were poultry feed. Only a few samples contained DAS over 1 mg/kg. Those samples derived from farms with evidenced poultry health problems with indications of possible trichothecene poisoning.

Also Labuda *et al*. (2005) studied samples of feed mixtures, designed for poultry feeding and found that the most frequently occurring mycotoxin was T-2, being found in a total of 45 samples in relatively low concentrations. In addition to DON, ZEN, T-2 and HT-2, DAS was the fifth co-occurring mycotoxin, though just in trace amounts. These concentrations were too small (range 2-5 µg/kg) to cause any serious health problem to animals.

Finally DAS was not found in a study by Schollenberger *et al*. (2006), out of a total of 95 samples of non grain based feedstuffs such as hay, lupines, peas, soya meal, rapeseed meal, sunflower meal, linseed meal. Previously, with regard to hay, Engels and Krämer (1996) found *Fusaria* in 41-100% of freshly harvested grass samples (*Lolium perenne, Lolium multiflorum*), and isolated a variety of toxigenic strains. These authors found T-2 toxin in 25% of samples (range 40-2780 µg/kg, with 2.8% samples above 1000 µg/kg), and DAS in 21.6% of samples (range 3-60 µg/kg), using an ELISA technique.

Though toxigenic *Fusarium* species are important pathogens and root colonisers of various fruits and vegetables, in general, exposure of plants and foodstuffs to *Fusarium* toxins and their relative toxicological risk have not yet been well studied (Logrieco *et al*., 2003). Therefore, in a survey conducted by Schollenberger *et al*. (2005), a group of samples was analysed which included vegetables, fruits, oilseeds and nuts. Commodities were selected which are part of the European diet (WHO, 1998) and for which the isolation of toxin producing *Fusarium* species or in some cases the natural toxin occurrence in diseased plant materials were described. A further intention was the examination of gluten-free foodstuffs. These dietetic foods, primarily corn and rice, have to be consumed by people suffering from celiac disease. With regard to the frequent contamination of these cereals with *Fusarium* toxins (Schollenberger *et al*., 1999), a contamination also of gluten-free foods can be assumed.

For the group of cereal-based products, gluten-free foodstuffs and oilseeds and nuts, trichothecenes of the B as well as of the A type were detected, but DAS was not found in any samples. For vegetables and fruits DAS was detected in only one sample of potato products with a low concentration (Schollenberger *et al*., 1999).

Information about the natural occurrence of trichothecenes in potatoes and potato derived food products infected with *Fusarium* spp. is very limited. Dry rot of potatoes is usually caused by *Fusarium* spp., particularly *F. solani* and *F. sambucinum*, leading to significant losses during storage (DeNijs *et al*., 1996). In Poland, for example, potato is one of the most important harvest crops; most stored potatoes (65%) are used as a feed component, mainly in swine feeding. During harvest and storage time, about 20% of potato tubers are lost. The

losses caused by *Fusarium* dry rot are about 4-5% of stored potatoes (Latus-Zietkiewicz *et al*., 1995).

Different strains producing trichothecene toxins have been isolated from potatoes. Steyn *et al*. (1978) identified DAS as the dominating compound. Several authors artificially infected potato tubers with toxigenic *Fusarium* strains isolated from potatoes and investigated the progress of infection as well as the distribution of DAS in potato tissue (Latus-Zietkiewicz *et al*., 1995; Ellner, 2002). Significant toxin amounts were found in the rotten tissue, but also healthy looking parts, distant 10-15 mm from the diseased area, were contaminated with DAS in concentrations up to 110 µg/kg. The toxin could also be found in tubers without any disease symptoms (Ellner, 2002). DAS was found in potato tubers with dry rot sampled in France at an incidence of 80% and concentrations up to 140 mg/kg (Lafont *et al*., 1983). The authors found high concentrations in rotted tissue, whereas adjacent not rotted tissue contained DAS between 0.05 and 11 mg/kg. Desjardins and Plattner (1989) examined potato tissue without visible rot injury adjacent to the rotted part of the tuber and found 100 µg/kg of DAS also in this tissue compared to 1890 µg/kg in the rotted part.

The reported results suggest that the usual practice of cutting out rotted tissue may not always be effective to entirely remove trichothecenes from tubers infected with *Fusarium* strains.

Soybeans (*Glycine max*) have been grown in the Far East since early times and became of supreme importance as a source of oil and protein throughout the world during the 20th century (Hepperly, 1985). Soya meal is a main protein source in animal nutrition and other soy products, flour, textured products, tofu, fermented products amongst others, are used as foodstuffs. Such foods are consumed in particular by health-conscious people, represent a significant part of vegetarian diets and frequently are used also for infant food formulas (Divi *et al*., 1997).

Fusarium rot of soybeans is described in the literature and a variety of *Fusarium* species have been isolated from this commodity (Pitt and Hocking, 1999). These strains are known to produce a broad spectrum of toxins including ZEN and trichothecenes of the A and B type (DeNijs *et al*., 1996). According to Schollenberger *et al*. (2006), out of 13 samples of soya meal, only one sample was positive in low concentration for MAS, whereas DAS was not detected in any sample. In another study conducted by Schollenberger *et al*. (2007b), a variety of soya derived foodstuffs were analysed for a total of 16 *Fusarium* toxins including type A trichothecenes. Out of 45 samples analysed by GC/MS, only one sample was positive for DAS, with a maximum concentration of 21 µg/kg.

Overall data available at present about the natural occurrence of trichothecenes in soya beans and potatoes and derived products are not sufficient to evaluate the health risk. Further data are needed including the occurrence of the whole spectrum of scirpentriol toxins.

Little is known about the co-occurrence of DAS and other trichothecenes of the scirpentriol family such as MAS and SCIRP. Langseth and Rundberget (1999) assumed that the content of acetoxyscirpentriols in cereals generally is below that of the deacetylated form, scirpentriol. In fact, based on a total of 3453 of cereal samples, the incidence of DAS, MAS and SCIRP was at 1.1%, 1.5% and 6.3%, respectively. The relative content of acetylated isomers and SCIRP may depend on a variety of genetic and environmental factors, one of these factors being the period between the onset of *Fusarium* growth in infected cereals and their harvest.

For non-grain substrates a ranking of scirpentriol toxins with regard to their occurrence is not possible because of a substantial lack of data.

An expanded pool of occurrence data will be a precondition for complete evaluation of DAS occurrence in grain based and non-grain based commodities. It must be kept in mind that the significance of occurrence data depends on the analytical method applied. Data collected using methods with low detection limit such as GC-MS and HPLC-MS seem essential to evaluate the contamination on foods and feedstuffs.

Table 3. Results of surveys for DAS, showing concentrations and distribution of contamination in different commodities.

Country	Commodity	Year	Co-occurring mycotoxins	N° of samples	LOD (µg/kg)	*% Positive*	Mean (µg/kg)	Min/Max (µg/kg)	References	Sampling procedure
Norway	Barley	1996/1998	DON-NIV-T2-HT2	102	20	0	-	-	Langseth and Rundberget, 1999	Samples from grain loads and silos
	Wheat		DON-NIV-T2-HT2	169		0	-	-		
	Oats		DON-NIV-T2-HT2	178		0.3	NA	Traces		
Romania	Wheat	1997	DON-3AcDON-15AcDON-T2-ZEN	25	2	0	-	-	Curtui *et al*., 1998	Randomly in 3 counties of Romania
	Maize		DON-3AcDON-15AcDON-T2-ZEN	30		3	2.6	-		
UK	Corn gluten	1997	DON-15AcDON	40	10	5	NA	70/200	Scudamore *et al*., 1998	Randomly in mills and markets
	Corn products		DON-15AcDON-NIV	27		0	-	-		
Germany	Wheat	2000/2001	MAS-T2-HT2-DON-3AcDON-15AcDON-NIV	41	14	0	-	-	Schollenberger *et al*., 2006	Randomly from farms, mills and experimental stations
	Oats		MAS-T2-HT2-NEO-DON-3AcDON-NIV-FusX	17		0	-	-		
	Corn		MAS-T2-HT2-FusX-DON-3AcDON-15AcDON-NIV	41		5	49	NA/76		
	Corn byproducts		MAS-T2-HT2-FusX-DON-3AcDON-15AcDON-NIV	13		8	21	NA/21		
	Corn plants		MAS-T2-HT2-DON-3AcDON-15AcDON-NIV	8		0	-	-		
	Corn silage		MAS-HT2-DON-15AcDON-NIV	5		0	-	-		
Poland	Oats (15 cultivars)	1997	T2-HT2	99	10	12	23	10/118	Perkowski and Basiński, 2002	Randomly in different regions of Poland
Germany	Organic oats	2005	T2-HT2-MAS-NEO	35	0.04	67	0.04	NA/0.14	Gottschalk *et al*., 2007	Samples drawn at mills and at wholesale stage
	Conventional oats		T2-HT2-MAS-NEO	35		100	0.11	NA/0.38		
Poland	Barley (15 cultivars)	1997	DON-NIV-T2-HT2-ZEN	15	0.01	73	12	10/34	Perkowski *et al*., 2003	Manually during harvest
Croatia	Grains and feed	1998	T2	79	0.1	20.3	200	100/400	Sokolović and Šimpraga, 2006	Samples collected regularly from manufactures and small farms
		1999	T2	95		33.7	400	100/500		
		2000	T2	73		27.4	150	100/500		
		2001	T2	47		19.1	250	100/400		
		2002	T2	50		22	250	100/1200		
		2003	T2-DON	38		21.1	100	100/500		
		2004	T2-DON	28		60.7	100	100/400		

Slovakia	Poultry feed	2003-2004	DON-ZEN-T2-HT2	50	0.3	20	4	2/5	Labuda *et al*., 2005	Samples obtained from the State Veterinary Institution in Nitra
Germany	Ryegrass	1991	T2-ZEN	832	50	21.6	NA	3/60	Engels and Krämer, 1996	Samples from farm plots
Germany	Cereal based foodstuffs	2000/2001	DON-NIV-HT2-T2-3AcDON-15AcDON-ZEN	76	14	0	-	-	Schollenberger *et al*., 2005	Randomly from food stores, health food stores and dietetic food firms
	Gluten-free foods		DON-NIV-HT2-T2-15AcDON-ZEN	23		0	-	-		
	Vegetables and fruits		MAS-HT2-ZEN	85		1.2	21	-		
	Oilseeds and nuts		T2-HT2-ZEN	35		0	-	-		
Germany	Whole soy beans	NA	-	6	14	0	-	-	Schollenberger *et al*., 2007	Randomly from food stores and health food shops
	Roasted soy nuts		15AcDON	5		20	21	-		
	Soy flour and flakes		MAS-HT2-DON-ZEN	10		0	-	-		
	Crisp soy		MAS-HT2-DON-ZEN	5		0	-	-		
	Textured soy protein		MAS-HT2-DON-ZEN	5		0	-	-		
	Tofu		ZEN	5		0	-	-		
	Soy infant formulas		-	5		0	-	-		
	Soy sauce		DON-3AcDON	4		0	-	-		

-: Not calculated data
NA: Not available
DON: Deoxynivalenol
NIV: Nivalenol
T2: T-2 toxin
HT2 : HT-2 toxin
3AcDON : 3-acetyl-deoxynivalenol
15AcDON: 15-acetyl-deoxynivalenol
ZEN: Zearalenone
MAS: Monoacetoxyscirpenol
FusX: Fusarenon X
NEO: Neosolaniol

MITIGATION OF DIACETOXYSCIRPENOL

Potato tubers are infected by *F. sambucinum* via wounds incurred at harvest or during handling and these tubers manifest disease during storage. *Fusarium* spores can survive in soil for several years and can infect the cut surfaces of seed potatoes in the spring. Curing of wounds via deposition of polyphenolic and polyaliphatic compounds in cell walls at the wound site reduces desiccation and blocks infection by post-harvest pathogens like *Fusarium*. The optimal temperature for curing potato tubers is from 10 to 15°C; however, this temperature is high enough to support the development of *Fusarium*. *Fusarium* dry rot progresses slowly at the lower temperatures of 3-5°C, recommended for long-term storage of seed and table potatoes, but progresses more rapidly at the higher temperatures of 8-10°C, required for storing processed potatoes.

Most techniques for managing dry rot are aimed at preventing injury to the tubers, either seed or the harvested crop. Preventing bruises will greatly aid in avoiding infection. Practising the following procedures will help prevent dry rot:

1. It is critical to purchase seed that has as little dry rot as possible. Seed should be inspected, preferably during the last months of storage. If this is not possible, inspect seed carefully upon receipt.
2. Warm seed tubers to at least 10°C before handling and cutting to minimize injury and promote rapid growth. Cold tubers are very prone to shatter bruising.
3. Clean and disinfect the seed storage area before receiving seed. Knives on the cutter should be sharp to make a smooth cut that heals easily. Disinfect seed cutting and handling equipment often and clean up well between seed lots. Adjust the cutter and sort tubers to provide 2-ounce seed pieces that will provide substantial nutrition to the developing plant, even if some rot develops. Grade out rotted tubers before they reach the cutter.
4. Treating seed pieces with fungicide helps control decay and other diseases caused by seedborne pathogens. Check current recommendations for specific fungicides.
5. Protect the seed from wind and sunlight during planting because dehydration greatly weakens the seed piece. Do not precut seed, it is better to cut only as much as can be planted within 24 hours.
6. Plant seed that has a *Fusarium* problem in warm soil and cover with as little soil as is practical. Coarse-textured soils warm up faster and are better drained than fine-textured soils. This allows the seed piece to suberize quickly and helps the plant to become established.
7. Harvest tubers after skins are set and when pulp temperature is greater than 10°C. Use anti bruise practices when harvesting and piling potatoes.

8. If using a postharvest fungicide, be certain that the coverage is adequate to protect the entire surface of the tuber. Use the volume of water and fungicide rate specified on the label.
9. Allow a period for wounds to heal before lowering the temperature in storage. There should be good air circulation, high humidity (greater than 90%), and a temperature around 12°C.
10. Lower the temperature slowly to prevent condensation on the tubers and store them in a cool place, considering your intended market.
11. Monitor storages often for dry rot. Market tubers with a significant amount of dry rot as soon as possible. Never grade tubers and place them back in storage because this favours disease. Grade out rotted tubers when tubers are removed from storage for marketing.

Resistant cultivars

There are no examples available in literature about the use of DAS resistant cultivars of potatoes. Additive genetic variance is lacking or minimal and therefore little or no genetic gain in resistance will probably be achieved (Burkhart *et al*., 2007).

Chemical and biological control

Potato dry rot, and consequently the production of DAS, can be controlled by a combination of storage technologies, physical methods and chemical applications (Eckert and Ogawa, 1988; Ranganna *et al.*, 1997).

To prevent *Fusarium* spoilage it is a common practice of different countries to dip harvested potatoes in fungicide solutions prior to storage (Kawchuck *et al.*, 1994). Growers have few chemical control options to manage development of *Fusarium* dry rot in stored potatoes. Registered fungicides in EU include thiabendazole (TBZ) and imazalil. TBZ, which has been available since the 1970s, is applied to tubers as they are loaded into storage. Repeated use of this fungicide has led to the selection of TBZ-resistant strains of *F. sambucinum* (Kawchuk *et al*., 1994; Shinners-Carnelley *et al*., 2003). The spread of these resistant strains by seeding with infected potatoes has decreased the efficacy of TBZ, leading to more widespread losses due to *Fusarium* dry rot (Hide *et al*., 1992; Desjardins *et al*., 1993; Kawchuk *et al*., 1994; Holley and Kawchuk, 1996; Schisler *et al*., 1998). An effective control of *Fusarium* dry rot has been obtained with the fungicide fenpiclonil and a mixture of thiabendazole and imizalil (Carnegie *et al.*, 1998).

Alternative forms of post-harvest disease control such as hydrogen peroxide solution (Oxidate, Biosafe Systems Inc., Glastonbury, CT) have been developed as reduced risk

options for control of *Fusarium* dry rot (Mac Phail, 2007). Aluminium chloride ($AlCl_3$) and sodium metabisulfite ($Na_2S_2O_5$) have received increasing attention as antifungal agents for the control of plant diseases. In an effort to understand their toxic action on fungi, ultrastructural changes and membrane damage in *F. sambucinum* (Ascomycota) and *Heterobasidion annosum* (Basidiomycota) in response to salt exposure was investigated using transmission electron microscopy. Conidial membrane damage was quantified using SYTOX Green stain, which only enters altered membranes. The results showed that mortality of the conidia was generally closely associated with SYTOX stain absorption in *F. sambucinum* treated with $Na_2S_2O_5$ and in *H. annosum* treated with $AlCl_3$ or $Na_2S_2O_5$, suggesting that these salts cause membrane alterations. For both fungi, ultrastructural alterations in conidia treated with $AlCl_3$ and $Na_2S_2O_5$ included membrane retraction, undulation, and invagination. At higher concentrations or longer exposure periods to the salts, loss of membrane integrity, cytoplasmic leakage, and cell rupture were observed. Ultrastructural alterations and increased SYTOX stain absorption in salt-treated conidia appear consistent with a mode of action where $AlCl_3$ and $Na_2S_2O_5$ alter membrane integrity and permeability (Avis *et al.*, 2009). The above-mentioned research studies could help to develop new commercial products for the treatment of *Fusarium* dry rot on potatoes in Europe.

The high costs of chemical control, failures in chemical control due to resistance development, and a lack of other effective control measures have, therefore, generated considerable interest in biological control that offers an effective and environmentally friendly alternative to the use of synthetic pesticides.

Intense research efforts have been devoted to the development of antagonistic microorganisms to control postharvest disease. So far, biological controls of dry and soft rots with different biocontrol agents such as fungi, bacteria, and yeasts have been reported as effective under experimental conditions (Schisler and Slininger, 1994; Schisler *et al.*, 1997; Kotan *et al.*, 1999 and 2002; Sadfi *et al.*, 2002). Several microbial antagonists have been identified and shown to reduce Fusarium dry rot on potatoes (Schisler *et al.*, 1997; Sadfi *et al.*, 2001 and 2002). The different efficacy of the biocontrol agents could be due to the influence of several factors that include the efficiency of the type/strain of biocontrol agent, the type or aggressiveness of pathogens, the susceptibility of the host to the pathogen(s) and environment (Francés *et al.*, 2006).

Several genera of soil bacteria including *Pseudomonas, Enterobacter, Pantoea* (Schisler and Slininger, 1994) and *Bacillus* (Sadfi *et al.*, 2002) have been identified as potential biocontrol

agents of *F. sambucinum*. Fungal and bacterial isolates from soil were evaluated for antagonism to *F. sambucinum* on lupin rhizosphere samples (Hassan *et al*., 2002) Culture filtrates of *Trichoderma harzianum* showed the greatest inhibitory effect and *Bacillus subtilis* among bacterial isolates. This is interesting because commercial products based on these microorganisms are available.

Bacterial antagonists have been shown to suppress dry rot formation by *Fusarium* spp. on potato (Kiewnick and Jacobsen, 1997; Schisler *et al*., 2000; Slininger *et al*., 2003). Bacterial metabolites such as phenylacetic acid, indole-3-acetic acid and tyrosol identified in the culture supernatant of *Enterobacter cloacae* S11:T:07 were implicated in providing biofungicidal activity (Slininger *et al*., 2004).

Efficacy studies with potatoes dipped in bacterial suspensions in the laboratory (Schisler and Sliningler, 1994; Kiewnick and Jacobsen, 1997), greenhouse, field, cold storage (Sadfi *et al*., 2002) and commercial storage (Schisler *et al*., 2000) have consistently shown significant reduction in *Fusarium* dry rot of potato equal to or better than the fungicide TBZ, demonstrating the potential of bacterial antagonists for managing this post-harvest disease.

In commercial storage bin trials, Schisler *et al.* (2000) reported that the control of *Fusarium* dry rot by *Pseudomonas fluorescens* P22:Y:05 and *Enterobacter cloacae* S11:T:07 was comparable to that achieved using TBZ fungicide. In this study, *Serratia grimesii* 4-9 and *S. plymuthica* 5-6, both members of the Enterobacteriaceae family, also suppressed *Fusarium* dry rot as effectively as TBZ. The high level of disease suppression demonstrated by these and other bacterial isolates indicates the potential for biological control of *Fusarium* dry rot and should interest the industry for product development (Sliningler *et al*., 2007).

Gould *et al*. (2008) evaluated the potential of rhizosphere bacteria isolated from Canadian soils to suppress dry rot of potato caused by *F. sambucinum*. Two bacterial isolates, *Serratia grimesii* 4-9 and S. *plymuthica* 5-6, with broad spectrum *in vitro* antifungal activity against *Fusarium avenaceum*, *Rhizoctonia solani* CKP7 and *Pythium* spp. p88-p3 (Hynes *et al*., 2008), were selected. *S. grimesii* 4-9 and *S. plymuthica* 5-6 were also identified as potential biofungicides for control of *Fusarium* dry rot of potato. The biocontrol agents had no observable negative effects on tuber quality.

Kotan *et al*. (2009) tested a total of 17 Plant Growth Promoting Rhizobacteria (PGPR) strains consisting of eight different species (*Bacillus subtilis*, *Bacillus pumilus*, *Burkholderia cepacia*, *Pseudomonas putida*, *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus macerans* and *Flavobacter balastinium*), for antifungal activity *in vitro* (on Petri plate) and *in vivo* (on potato tuber) conditions against *F. sambucinum*, *F. oxysporum* and *F. culmorum*. The strongest antagonism was observed in *B. cepacia* strain OSU-7.

Eukaryotic microorganisms have also been studied for biocontrol of *Fusarium* dry rot. Niemira *et al.* (1996) reported biocontrol of *Fusarium* dry rot in potato minitubers by arbuscular mycorrhizal fungi. Several strains of yeasts were examined for biocontrol of *Fusarium* dry rot of potatoes, but bacterial agents proved to be more effective (Schisler *et al*., 1995).

Despite their apparent value, no products based on bacterial antagonists to *Fusarium* dry rot are available to growers. There are several reasons that may account for this including: the potential risk to human health of the putative biocontrol agents, the cost of production, inconsistent efficacy, poor shelf-life and the inability to deliver the biocontrol agent in a user-friendly formulation (Hynes and Boyetchko, 2006).

No studies are reported about the efficacy of chemical and biological practices for the mitigation of diacetoxyscirpenol in cereals.

PHARMACOKINETICS

Absorption, Distribution and Excretion

Wang *et al*. (1990) examined tissue distribution and excretion of DAS in male Fisher rats and CD-1 mice at 90 min, 24 hours and 7 days after a single intragastric dose of [^{3}H]DAS (approximately 0.66 mg/kg bw for mice and 0.55 mg/kg bw for rats). The authors observed no significant signs of toxicity or damage to tissues. Moreover, in both species, the excretion was rapid and completed within the first 24 h, with approximately 90% of the dose excreted in the urine and feces and an approximate ratio 4.5:1 for both rats and mice. Within the first 24 hours there was a decline of radioactivity in the gastrointestinal tract, organs and carcass, in which low (1.4-2.8% and 2.8-4.4% of dose in rats and mice, respectively), but significant levels of radioactivity remained for an additional six days. Tissue distribution of DAS was quantitatively similar in rats and mice, and when expressed as percentage of dose, most radiolabel was in the carcass, skin, small intestine, stomach, liver and kidney. When expressed as specific radioactivity, spleen and cecum were the most important districts where DAS accumulated. Furthermore, with time, radioactivity spread over the lympho-hematopietic system, heart and in mouse testis and brain, and in these target organs radioactivity decreased at a slower rate than in the liver, kidney, carcass and skin. Finally, because of some differences between rats and mice in the distribution of DAS in target organs, and in particular because of the higher levels of the toxin in mice liver, the authors suggested a more efficient capacity of metabolizing and excreting DAS in mice.

The same authors (Wang *et al*., 1996) reported that after a single topical administration of [^{3}H]DAS (0.98 mg to 1.44 cm^2 of skin for rats, 0.28 mg to 0.42 cm^2 for mice) rats and mice

exhibited different patterns of absorptions, excretion and tissue distribution between 90 min and 7 days after the treatment. In particular, during this period rats showed increased absorption and excretion, and decreased retention in tissues; over the 7-day period 57.5% of the dose was absorbed and 70-80% of total excretion occurred in the first 24 h, with more than 90% of the dose excreted within three days after application. Absorbed radioactivity was rapidly and widely distributed in tissues (carcass, skin, liver, kidney, gastrointestinal tract, spleen, testes and femur), but with time the authors observed a shift of localization toward target organs, such as immune and hematopoietic tissues, gastrointestinal tract and testes. In contrast, in mice absorption occurred only during the first 24 h, and 7 days after treatment mice retained more radioactivity in tissues than rats. Over the 7-day period, mice absorbed 13.1% of the dose, and the total excretion was 6-fold lower than in rats (9% vs 56%). In mice DAS was also rapidly and widely distributed, with the difference that with time there was a shift from target tissues and excretory organs to the carcass and skin. In both species, despite rapid absorption and excretion, the level of DAS or its metabolites remained stable in organs and tissues.

DAS was rapidly absorbed and metabolized also in swine. As reported by Bauer *et al*. (1985), an oral administration of 2 mg/kg bw showed a maximum absorption within 1h; DAS was then converted to 15-monoacetoxyscirpenol (MAS) and scirpentriol (SCT), and together with DAS, these metabolites were present in serum for only 24 h. In this study, the excretion occurred mainly through the feces, with only low amounts in urine, while up to 26% of the oral dose was excreted via vomit (Bauer *et al*., 1989).

In conclusion, after oral administration DAS is rapidly and extensively absorbed and widely distributed in tissues and organs, particularly the lympho-haematopoietic organs and intestines. In rats and mice, excretion is mainly via urine, while in swine it is mainly via feces and vomit. After topical administration, rats and mice have different patterns of absorption, tissue distribution and excretion, with rats having a greater absorption and higher concentrations in target and other organs (Pronk *et al*., 2002).

Metabolism

DAS is deacetylated to MAS by rabbit and rat liver carboxylesterases. Otha *et al*. (1978), reported that incubation of 20 mg of DAS with microsomes yielded 7 mg of DAS and 7 mg of purified MAS. Bauer *et al.* (1989) reported that in the feces the main metabolites were SCT and de-epoxy-SCT, and MAS and de-epoxy-MAS, though in smaller amounts. The same authors observed DAS, MAS and SCT levels in the blood serum of pigs at different times after the oral administration of 2 mg/kg bw of DAS. After 2 hours they measured 0.32, 2.6

and 2.4 ng/ml, respectively for DAS, MAS and SCT (Bauer et al. 1985). SCT and MAS were detected also in the urine of male Wistar rats (4.9% and 3.5% of the administered dose, respectively), after oral administration of 2.8 mg/kg bw of DAS, three times at 7-day intervals. On the other hand, feces contained only the de-epoxy forms of SCT and MAS, while DAS was detected neither in urine nor in feces (Sakamoto *et al*., 1986). In the bile obtained from isolate rat liver perfused with DAS (2 mg), the glucuronide conjugates of MAS (340 µg, as the major metabolite) and SCT (10 µg) were identified (Gareis *et al*., 1986).

Under anaerobic conditions, DAS could be biotransformed by rumen microorganisms to MAS, SCT and their corresponding de-epoxydes (Swanson *et al*., 1987a and 1987b). Direct de-epoxydation of DAS was not detected, suggesting that C4 deacetylation occurred prior to de-epoxydation.

In another study, the same authors (Swanson *et al*., 1988) analyzed the biotransformation of DAS upon anaerobic incubation with fecal microflora of rats, cattle, and swine and showed that the toxin was completely metabolized primarily to de-epoxy-MAS and de-epoxy-SCT. On the other hand, fecal microflora of chickens, horses and dogs did not reduce the epoxy group, and resulted only in MAS with small amounts of DAS and SCT. Intestinal microflora of rats completely transformed DAS to MAS and SCT de-epoxy-metabolites. Upon incubation of DAS with rat fecal and intestinal microflora, upon aerobic conditions, de-epoxy metabolites were not detected. Young *et al*. (2007) demonstrated that chicken intestinal microbes prefer monodeacetylation (about 66%) of the diacetylated trichothecenes, like DAS.

In conclusion, metabolism of DAS first involves hydrolysis/deacetylation to MAS and SCT, which is then followed by glucuronidation and de-epoxydation. The latter is carried out by the (anaerobic) intestinal and fecal microflora of rats, cattle and swine. Chickens, horses and dogs lack the necessary microflora for epoxide reduction. In ruminants, the ruminal flora is also able to hydrolyse/deacetylate and de-epoxydate DAS (Pronk *et al*., 2002).

Carry over

Available data on DAS reveal that not only cereal grains, but also soy food, potato products, vegetables, fruits, oilseed and nuts and animal feed are contaminated by these toxins (Schollenberger *et al*., 2007a). DAS can cause problems to humans when ending up in human foodstuffs, which can be of plant- or animal-origin (residues in meat, milk, eggs of animals given contaminated feed). The majority of confirmed cases are of plant-origin, while in contrast there is almost no carry over of trichotecenes into food of animal origin (Pronk *et al*., 2002).

TOXICITY

Acute/Subacute toxicity

When given intraperitoneally (ip) or subcutaneously (sc) to male Swiss mice, the LD50-values for DAS were 15.3 and 19.5 mg/kg bw, respectively (Thompson and Wannemacher, 1986). Ueno *et al.* (1973) reported an ip LD_{50} of 14.5 mg/kg bw in male mice for DAS, with cell injury and karyorrhexis in the small intestine and bone marrow. After intravenous (iv) administration, the LD50 in adult mice was 12 mg/kg bw. In newborn mice, the sc LD_{50} was 0.17 mg/kg bw (Ueno, 1983).

The mean lethal dose of purified DAS given orally to 1-day old broiler chicks for seven days was 3.82 mg/kg bw. Reported clinical effects were asthenia, inappetance, diarrhoea and coma. Sublethal doses decreased body weight gain and feed consumption (Chi and Mirocha, 1978). DAS also produced necrosis of lymphoid tissue and bone marrow followed by rapid cell depletion. However, after 24 h, cell repletion started. In chicks, skin lesions were also found, as well as necrosis in the liver, gall bladder and gut (Hoerr *et al*., 1981).

In swine, the iv LD50 for DAS was 0.376 mg/kg bw. The animals developed emesis, frequent defaecation, lethargy, posterior paresis, staggering gait and prostration by 18 h leading to death. The jejunum and ileum and large intestines, portions of which were blood-filled at necropsy, were affected by severe haemorrhagic necrotizing lesions and mucosal congestion. Lymphoid follicular necrosis was present in lymph nodes and spleen (Weaver *et al*., 1978).

DAS was also tested for acute toxicity in rats (LD_{50}-values of 1.3, 0.75 and 7.3 mg/kg bw for iv, ip and oral administration, respectively), rabbits (iv LD_{50} 1 mg/kg bw) and dogs (iv LD_{50} 1.1 mg/kg bw) (Ueno, 1983). The iv LD_{50} values originated from a study of Stähelin *et al*. (1968), who reported that mortality was seen at the earliest 10-48 hrs after injection. Clinical signs appeared within a few hours and consisted of apathy, bradypnoe, cyanosis, bloody stool, diarrhoea, vomiting, tremor and tachycardia, later followed by asthenia.

In an experiment in male CD-1 mice, the ip LD50 value of DAS was 20 mg/kg bw, and the intragastric LD50 value was 15.5 mg/kg bw. Most deaths occurred within 36 hrs following onset of lethargy, trembling, pasty diarrhoea and cyanosis. Morphological changes in the animals that died included extensive necrosis of the lympho-haematopoietic organs and intestines (often transmural) and degeneration of testes. Sublethal doses caused time- and dose-dependent cell depletion and necrosis in lympho-haematopoietic organs (bone marrow, thymic cortex, splenic red pulp, mesenteric lymph nodes), multifocal necrosis of intestinal epithelium and necrosis of germinal epithelium followed by progressive tubule degeneration in the testes. After sublethal exposure, DAS-sensitive organs rapidly recovered, except for

the testis where decreased weights and abnormal spermatogenesis persisted throughout the 2-week observation period (Conner *et al*., 1986).

Dermal application to male CD-1 mice of a single dose of DAS produced 0, 15, 25, 70 and 95% mortality at 5, 10, 20, 30 and 40 mg/kg bw, respectively (Schiefer *et al*., 1986). In this experiment, DAS resulted less toxic than T-2 (which produced 100% mortality from 20 mg/kg bw onwards), but more toxic than HT-2 (0, 0, 20, 40 and 90% mortality at 5, 10, 20, 30 and 40 mg/kg bw, respectively) and 3AcDON (not lethal at any dose). A combination of doses of trichothecenes (T-2 + DAS, T-2 + 3AcDON, T-2 + DAS + 3AcDON, DAS + 3AcDON), reduced the time necessary to reach 50% mortality, in particular for the combinations T-2 + DAS and T-2 + DAS + 3AcDON, as compared to the compounds given individually. The combinations T-2 + 3AcDON and DAS + 3AcDON caused results similar to those seen with the individual compounds.

Following a single dermal application of 2.625 mg of DAS to 1.44 cm^2 of skin, 75% mortality was observed in male Fischer rats within two days after treatment. In contrast, the dose 0.75 mg to 0.42 cm^2 of skin and higher (1.875 mg to 1 cm^2) doses were not lethal to CD-1 mice mice (no deaths occurred within seven days after treatment). Severe necrosis and depletion of cells in the bone marrow, spleen, thymus, and small intestine were the major histopathological lesions in the rat. Although these lesions in the mouse were less severe or absent, the skin of mice was much more severely damaged than that of rats, with extensive edema, inflammation, epidermal sloughing, and hair follicle necrosis present (Wang *et al*., 1996). The observed interspecies difference in acute toxicity was attributable to different patterns of absorption and tissue distribution; the lower absorption in mice (as compared to rats) may be attributable to DAS-induced skin damage, which may have served as a physical barrier to absorption. The minimal damage to rat skin induced by topically applied DAS (compared with mouse skin) could have resulted in elevated penetration of the toxin, and as a consequence, greater deleterious effects on target tissues.

Swine, treated with a single oral dose of 2 mg/kg bw, showed strong salivation (after approx. 10 min), vomiting (lasting for 30-60 min), and apathy, anorexia and posterior paresis for approx. 12 hrs, but no signs of intoxication after 24 hrs (Bauer *et al*., 1985).

Coppock *et al.* (1985) examined swine up to 8 hrs after iv administration of 0, 0.5 or 1 mg DAS/kg bw, and observed clinical signs, clinical chemistry and histopathology, such as bruxism, emesis, mahogany flushing of the skin, diarrhoea, ataxia, anuria, muscular weakness, depression and sometimes coma and death. Lesions were spread in the gastrointestinal tract, lymphoid tissues, pancreas, adrenal gland, brain, kidneys, liver, bone marrow and salivary glands. The cytotoxicity induced in these proliferative and metabolically

active tissues and cells which function as specialized ionic pumps, pointed to DAS as a potent radiomimetic substance.

Coppock *et al*. (1989) observed in swine, cattle and dogs after a single iv injection with DAS (0.5 or 1 mg/kg bw for swine, 0.5 mg/kg bw for dogs and cattle) clinical signs and effects on hematology and bone marrow. Clinical signs of intoxication included ptyalism, emesis (not in cattle), diarrhoea, ataxia, mahogany flushing of the skin (only in swine), muscular weakness and depression. In the bone marrow, pathological changes were found, which included moderate to severe necrosis of bone marrow hematopoietic elements, a marked left shift in the neutrophil population, metarubricytes and large platelets in blood, and replacement of lymphocytes with immature cells. The order of sensitivity to DAS was swine > dogs >> cattle.

When given 1 mg DAS/kg bw by gastric intubation for 1, 4 or 8 consecutive days, male SD rats showed signs of intoxication from the third administration onwards. Animals that had received eight administrations showed reduced spontaneous movement and peribucal necrosis. Total proteins in plasma were decreased, while aspartate aminotransferase activity was increased (only in 8-day treated rats) (Galtier *et al*., 1989).

Janse van Rensburg *et al.* (1987) carried out experiments on groups of 30 male Wistar rats, who received 0 or 1 mg DAS/kg bw by gastric intubation, three times weekly for a maximum of five weeks. Five rats per group were sacrified on day 0, 7, 14, 21, 28 or 35, and the pathological and hematological effects of DAS were studied. Treatment with DAS did not affect body weight but resulted in roughness and discoloration of the fur. Regarding hematological parameters, erythrocyte counts were mainly affected; in contrast to control rats, in which the erythrocyte counts increased gradually upon maturation, in DAS-treated rats the erythrocyte counts were lower than those of the controls from day 7 onwards. No effects were observed on (differential) leucocyte counts. Atrophy and necrosis of the actively dividing cells of the bone marrow, thymus, spleen, lymph nodes and gastrointestinal tract were the major pathological lesions. They were generally mild and noted mainly after 2-4 weeks of treatment, with some regression of effects in bone marrow, thymus and lymph nodes at week 5. According to the authors this could point to an increased detoxifying capability upon repeated exposure (Janse van Rensburg *et al*., 1987).

More *et al*. (1990) showed that the mucus-producing cells of the fundic glands of rat stomach could be affected by low doses of DAS even following only 2 days of treatment.

In summary, orally DAS is toxic to rats, mice and chickens. Only a few studies with repeated administration of DAS are available. The major toxic effects consisted of hematological changes, cell depletion and necrosis of the lympho-hematopoietic organs and intestines and degeneration of the testes (Pronk *et al*., 2002).

Chronic toxicity

Animals

Experiments with male and female broiler breeders fed 0, 5, 10 or 20 mg DAS/kg diet for 2-3 weeks, were carried out by Brake *et al*. (2000), who observed effects on feed consumption, body weight and oral lesions. The authors found dose-related decreases in body weight (females only) and feed consumption, as well as an increased time to consume the feed and dose-related increases in the extent of oral lesions. After ending the DAS treatment, feed consumption recovered, but not body weight. Feed refusal was more pronounced in females than males. The severity of the cytotoxic effects in the mouth increased with increased exposure duration, and the areas most sensitive to DAS treatment were associated with the salivary glands and the tip of the tongue. Removal of DAS caused the disappearance of the lesions, with the exception of those on the tongue tip which had a longer recovery time or (at the higher doses) resulted in necrosis and loss of the tongue tip.

Weight loss was reported after 34 days of feeding DAS (5 mg/kg of feed) to lambs. Further weight loss also was described at 34 days of feeding DAS in combination with aflatoxin (2.5 mg/kg of feed), suggesting a synergistic effect (Harvey *et al*., 1995).

Stähelin *et al*. (1968) described a series of experiments in which DAS was given iv to rats, dogs and monkeys for a period of 4-6 weeks or 4 months (dogs only). In these studies DAS mainly affected blood parameters (leukopenia and/or anaemia), bone marrow, spleen, liver and testes. Konjevic *et al*. (2004) described the poisoning of two Brahma chickens with T-2 toxin, DAS and DON. Two out of 10 chickens died with loss of appetite. Histopathological analysis showed vacuolar dystrophy of the liver, necrosis and depletion of lymphocyte in the bursa of Fabricius and multiple necroses in the glandular stomach and gut. Though 0.70 mg/kg of T-2 in feed, and 0.50 mg/kg DAS are significantly lower than the median lethal dose for chickens reported in literature (4.97 mg/kg), parasitological, virological and histopathological results indicate trichotecenes as the causative agents of this pathological condition. Sklan *et al*. (2003) demonstrated that feeding DAS at concentrations of up to 1 mg/kg for more than 30 days influenced small intestinal morphology, but did not affect growth or antibody production in young poults. Brake *et al*. (2002) have shown that short-term consumption of DAS at levels that might naturally occur appears to have little effect on broiler breeder egg production. In particular, when feed containing 0, 1.25, 2.5, or 5.0 mg DAS/ kg was fed from 67 to 69 wk of age, followed by a 3 wk recovery period on a slat-litter floor, egg production was not affected by levels of up to 5 mg DAS/kg in the older hens. When individually caged broiler breeder females were studied from 23 to 31 wk of age and the basal diet containing 0, 5, 10, or 20 mg DAS/kg was fed from 25 to 27 wk of age, DAS

decreased egg production only at the 20 mg/kg level. Finally, individually caged broiler breeder hens were studied from 23 to 32 wk of age. DAS was fed at levels of 0 (control), 5, 10, and 20 mg DAS/kg for 2 wk beginning at week 24, followed by the basic breeder diet for 7 wk. In this study, DAS had no significant effect on egg production and quality.

Only a few studies with repeated administration of DAS are available. These (mainly subchronic) studies, examined only a few effects, and some only used one dose level. The major toxic effects observed in the subacute studies with DAS resembled those seen in the acute studies and consisted of haematologic changes (leukopenia and/or anaemia), cell depletion and necrosis of the lympho-haematopoietic organs (bone marrow, thymus, spleen, lymph nodes) and intestines and degeneration of testes. Vomiting was observed in swine, dogs and poultry, and the latter also showed feed refusal, reduced body weight gain and mouth lesions (Pronk *et al*., 2002).

Humans

There are very few data reporting toxicity of DAS in humans. However, DAS has been evaluated as a possible drug in the treatments of cancer. Parent-Massin and Parchment (1998) extrapolated a NOAEL of 7 mg/m^2 (e.g. 0.18 mg/kg bw/d) in a phase I evaluation about myelosuppression. In another phase I clinical trial no signs of drug-related toxicity were found in humans at daily intravenous doses of < 2.4 mg/m^2 (5 consecutive days). At higher doses gastrointestinal symptoms, myelosuppression, and at lower doses extent hypotension and transient neurological symptoms were reported (Murphy *et al*., 1978).

Developmental and reproductive toxicity

In pregnant female ICR mice given a single ip injection of 0, 1, 1.5, 2, 3 or 6 mg DAS/kg bw on one of gestation days 7-11, maternal toxicity was observed at 6 (death and vaginal bleeding) and 3 (death) mg/kg bw, but not at lower doses. There was no effect on the total number of implantations, but resorptions increased with dose and with day of injection (from 7-34% on gestation days 7-11 at 1 mg/kg bw, to 100% on all tested gestation days at 6 mg/kg bw). Body weight of the live fetuses was significantly depressed at all doses, and was more depressed with increasing dose. A variety of fetal malformations were observed, including both external (a.o. exencephaly, omphalocele, hydrocephaly, short snout, protruding tongue and meningoencephalocele) and skeletal (anomalies of the skull, sternebrae, vertebrae, vertebral centra and ribs) abnormalities, especially when DAS was given on day 9 of gestation. On this day external and skeletal malformations were observed at all doses (at 1 mg/kg bw even 16 and 64%, respectively) (Mayura *et al*., 1987).

DAS is teratogenic in mice given single oral doses of 0, 1, 2, 3 or 4 mg/kg bw on gestation days 9 to 11. At 3 mg/kg bw and above, DAS reduced resorptions, and induced gross malformations (exencephaly, protruding tongue, short snout and missing tail), skeletal abnormalities (fused ribs, missing sternum centra, missing skull bones) and soft tissue defects (Gentles *et al*., 1993).

The testes of male Lewis rats are sensitive to treatment with DAS (1x ip 1.7 mg/kg bw), as testicular weight and sperm production were reduced by 30 days after treatment, and the frequency of hypocellular seminiferous tubules was increased, by day 60. The hypocellular tubules had few or no germinal epithelial cells and consisted almost entirely of vacuolated Sertoli cells. Beginning 30 days after treatment testicular changes were observed, and there was no evidence of recovery. The effects reflect injury to germinal cells early in the maturation sequence. Other effects of DAS on the reproductive tract included alterations in epididymal transit times, as reflected by decreased epididymal sperm reserves, by 3 days after DAS administration, occurring prior to sperm reduction (Conner *et al*., 1990).

To examine the effects of DAS on the fertility and hatchability of broiler breeders, naturally mated broiler breeders received 0, 1.25, 2.5 or 5 mg DAS/kg feed for 3 weeks from weeks 67-69 of age. A restricted feeding regimen of 154 g/bird/day was used when feeding DAS, and males and females were fed together. In a second experiment, female and male broiler breeders individually received 0, 5, 10 or 20 mg DAS/kg feed for 3 weeks from weeks 25-27 of age, according to a restricted feeding regimen of 114-125 g/bird/day depending upon age. After DAS treatment, semen was pooled from males within each treatment and used to inseminate females from each treatment.

In the first experiment, fertility was consistently improved at 5 mg/kg feed, and intermittently at 1.25 and 2.5 mg/kg feed, but the effect disappeared upon removal of DAS. The hatchability of fertile eggs was only slightly influenced at wk 68, not at other ages. In the second experiment, female-related fertility was increased at 5 and 10 mg/kg feed, and male-related fertility was decreased at 10 and 20 mg/kg feed (with the greater effect at 10 mg/kg feed). Female-related hatchability was not affected by DAS-treatment, but there was a decrease in hatchability attributed to a male effect at 10 mg/kg feed. Apparently, the quality of semen at 10 mg/kg feed was poorer compared to 20 mg/kg feed, given the greater effect on malerelated fertility at 10 mg/kg feed and the effect on hatchability. Upon necropsy, no differences in relative testes weights were found, but many treated males had small, fluid filled cysts on the testes (Brake *et al*., 1999).

While in mice DAS is fetotoxic and teratogenic, data from other species are lacking, and no information on reproductive toxicity in other laboratory animals is available. In poultry, low

doses of DAS improved female-related fertility, presumably because of enhanced spermatozoal storage within the oviduct. Conversely, higher doses of DAS decreased male-related fertility, presumably by a direct toxic effect on the testes as was shown for rats and mice, where DAS induced testicular changes (degeneration, reduced weight and abnormal spermatogenesis) (Pronk *et al*., 2002).

Genotoxicity and carcinogenicity

DAS was not mutagenic to *Salmonella typhimurium*, with and without metabolic activation (Wehner *et al*., 1978). Through an *in vitro* approach, Cooray (1984) showed that DAS did not induce sister chromatid exchanges in human lymphocytes. After ip injection at 0.5-1 mg/kg bw, in male Swiss mice DAS caused a reduction in mitotic activity in bone marrow and an increase in structural chromosal abnormalities in somatic cells (bone marrow) and in germ cells. In bone marrow, the structural damages included breaks, centromeric attenuation, and endomitosis, and in spermatocytes breaks and X-Yunivalents. Also sperm morphology, by inducing increases in head abnormalities (especially amorphous and small heads) and tail abnormalities (coiled tails) were affected by DAS (Hassanane *et al*., 2000). No studies investigating DAS carcinogenicity were found.

Cytotoxicity

Lafarge-Frayssinet *et al*. (1979), using an *in vitro* test which involved mitogen-stimulated murine splenic and thymic lymphocytes, showed that DAS reversibly inhibited the stimulation of both T and B cells and suppressed their ability to synthesize anti-SRBC (sheep red blood cells) antibodies. While at high concentrations a direct cytostatic action was found, low concentrations had opposite effects. As suggested by Thuvander *et al*. (1999), DAS effectively inhibited proliferation and immunoglobulin production (IgA, IgG and IgM) in mitogen-stimulated human lymphocytes in a dose-dependent manner with limited variation in sensitivity between individuals. However, at low levels DAS ($4x10^{-10}$ M) exposure could also result in enhanced proliferative responses, as well as in elevated immunoglobulin production (especially IgA). DAS was somewhat less effective than T-2 (3-4 times more potent), but more effective than DON and NIV (30-100 times less potent). Combinations of DAS with NIV or DON resulted in additive and antagonistic interactions, respectively, while for the combination of DAS with T-2 a synergistic effect could not be excluded. DAS is highly cytotoxic in cultured human cells, and is a potent inhibitor of protein synthesis. In cultured animal cells (baby hamster kidney cells, mouse fibroblasts, mouse P-815 mastocytoma cells) DAS is also highly cytotoxic (Stähelin *et al*., 1968; Saito and Ohtsubo, 1974). DAS has also

been shown to inhibit DNA synthesis (total inhibition by 8 ng/ml, 50% inhibition by 2.7 ng/ml) in mitogen-stimulated human peripheral blood lymphocytes. When metabolic activation (by isolated rat liver cells) was present, the inhibitory effect of DAS was somewhat reduced (Cooray, 1984), suggesting that the observed effect was due to DAS itself rather than one or more metabolites.

When rats were given 1 mg DAS/kg bw for up to 8 days, a significant decrease in microsomal and cytosolic proteins was found, as also the microsomal cytochrome P-450 content and, consistent with the latter, cytochrome P-450-dependent monooxygenase activities. Hepatic microsomal cytochrome b5 content was not affected, nor were the conjugating enzymes, except for *p*-nitrophenol glucuronyltransferase activity which was increased. DAS-treatment had no effect on enzyme activities in kidneys and lungs (Galtier *et al*., 1989).

Jun *et al*. (2007) demonstrated that exposure of Jurkat cells to DAS in the range 0.01-0.15 μM induced apoptotic DNA fragmentation and caspase-8 activation, bid cleavage, mitochondrial cytochrome C release, activation of caspase-9 and caspase-3, and PARP (Poly (ADP-ribose) polymerase) degradation, without any alteration in the Fas (Apoptosis Stimulating Fragment) or FasL (Apoptosis Stimulating Fragment Ligand) levels, while necrosis was not observed. T-cell toxicity of DAS is therefore due in part to apoptosis initiated by caspase-8 activation and subsequent mitochondrion-dependent or -independent activation of caspase cascades, and in part to the interruption of cell cycle progression initiated by down-regulation of cdk4 (Cyclin-dependent kinase 4) and cyclin B1 proteins. Typical cascades of apoptotic cell death after exposure of Jurkat cells to DAS were also observed by Lee *et al*. (2006). Moreover, the same authors estimated a IC_{50} (50% Inhibition Concentration) value of 16.1 ng/ml looking at viability (MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay) and an externalisation of phosphatidylserine of about 24% at 3h and 32% at 6h at 50 ng/ml of DAS. On the other hand, Nasri *et al*. (2006) showed that T-2 toxin and DAS are much more cytotoxic at low concentrations (the IC_{50} values were 0.003 and 0.008 μM for T-2 and DAS, respectively) than B trichothecenes (the IC_{50} values were 0.04 and 0.8 μM for NIV and DON, respectively) on Jurkat T-lymphocytes. While B trichothecenes prevalently induced apoptosis, type A trichothecenes reduced mitochondrial activity at approximately 1000-fold lower concentrations than the type B trichothecenes, with the presence of necrosis. Viability curves for DAS showed a IC_{50} value of about 5 nM after 24h of exposure and 2 nM after 48h of exposure. The characterization of cytotoxic rice extracts on porcine kidney cells (PK 15) has shown that DAS is implicated in combination

with other trichothecenes (mainly FUCH (fusarochromanone), 15AcNIV, and SCIRP) (Morrison *et al*., 2002).

Qureshi *et al*. (1998) carried out experiments on macrophage from chickens. Monolayers of this cell type were exposed to 0, 12.5 and 25 µg/mL of DAS for 1 h. The authors showed that treatment with DAS resulted in decreased viability of macrophages, causing the suppression in macrophage phagocytic function and therefore altering the first line of immunological defense in chickens.

DAS caused, at low concentrations (0.5-10 nM), cytotoxic effects in megacharyocyte progenitors, with possible induction of thrombocytopenia (Froquet *et al*., 2001).

In summary, DAS is cytotoxic and could inhibit lymphocyte proliferation and immunoglobulin production, mainly at high concentrations, while low concentrations (0.5-10 nM), appear to have opposite effects. DNA damage and pro-apoptotic properties were also observed.

LEGISLATION ON DIACETOXYSCIRPENOL

The European Commission has set legislative limits for *Fusarium* mycotoxins, including DON, ZEN and fumonisins, in cereal grains and cereal-based products intended for human consumption (European Commission, 2006b and 2007). A combined limit for trichothecenes T-2 and HT-2 will be introduced in the near future (Edwards, 2009). The European Commission also set guideline limits in 2006 for *Fusarium* mycotoxins in animal feed (European Commission, 2006a). In both cases no European limits were established for DAS.

Only a few countries introduced internal legislative limits for DAS and/or other type A trichothecenes in feedstuffs. According to the Croatian regulation, the maximum allowed quantity of T-2 toxin and DAS in complete and supplemental feed is 500 µg/kg for young chickens, pigs and calves and 1000 µg/kg for adult poultry and pigs (Sokolović and Šimpraga, 2006). In Israel the maximum DAS limit for cereals in mixed feed is 1000 µg/kg (FAO, 1997). Canada has set for DAS limits of 2000 µg/kg and 1000 µg/kg for feed for pigs and poultry, respectively (FAO, 2004).

In 2001, the Scientific Committee on Food (SCF) proposed a temporary TDI for the total of T-2 and HT-2 toxins of 0.06 µg/kg bw and nothing for DAS (European Commission, SCF, 2001). According to mean intake data provided by a study of the Scientific Cooperation, the ingestion of type A trichothecenes could exceed this tTDI, especially in infants and children by more than 5-fold (European Commission, Scientific Cooperation, 2003). A problem in performing dietary exposure assessments is always the lack of sufficient consumption data, particularly for certain sub-populations like infants or children. This is the consumer group

with the highest risk, because they have an exceptionally high intake in relation to their body weight (Gottschalk *et al*., 2007).

Therefore, it is necessary to develop sensitive methods of analysis, to collect more occurrence data and to carry out further investigations and research into the factors involved in the presence of DAS and other trichothecenes in cereals and cereal products, and maximum levels need to be amended in order to avoid disruption of the market and to maintain a high level of public health protection.

CONCLUSIONS

Diacetoxyscirpenol is one of the trichothecene mycotoxins produced by certain species of *Fusarium;* it was discovered in 1961 as a phytotoxic compound from a culture of *F. equiseti* and *Gibberella intricans* and its chemical properties and structure have been characterized. According to chemical classification of trichothecenes DAS belongs to group A trichothecenes. Among trichothecenes produced by *Fusarium* spp., DAS is one of the most toxic trichothecenes.

The most widespread and important producer of DAS is *F. sambucinum*, which, as a storage rot, is of considerable importance on the potato. As a root rot it attacks a wide variety of hosts such as flax, lupin, strawberry, tomato and especially cereals. Recently, some strains have been isolated from wheat in Upper Egypt, but the presence of DAS in cereals is generally due to other *Fusarium* species.

Trichothecenes pathway has been elucidated, but DAS biosynthesis is still to be defined in the very last steps. Moreover, to date very few data on fungal genes involved in the synthesis are available.

The analytical determination of DAS as well as other type A trichothecenes has been extensively reviewed. Extraction and clean-up formerly were followed by TLC, but GC and HPLC have become increasingly popular. The method for DAS determination reported in literature is usually proposed for food. Although similar protocols may be applied for feed, no data about accuracy, recovery, repeatability etc. are reported for feed, which is usually more complex, thus suffering from stronger matrix effects. DAS-containing reference material are not yet commercially available. Moreover, the development of specific immunoaffinity devices as well as isotope-labelled internal standards should be encouraged.

Data about the occurrence of DAS are available for North and South America as well as Europe, whereas only a few reports exist for Africa, Asia and Australia. DAS has been regularly detected in agricultural crops. Wheat is the matrix most frequently analysed of European commodities, followed by oats, barley, corn, corn products, corn plant, corn silage

and some other substrates used for food and feed. In a recent European study, a total of 1886 cereal samples were analysed for DAS with 4% of positive samples, ranging from LOD = 3.3 µg/kg (UK) to 50 µg/kg (France).

Significant DAS amounts were found in potato rotten tissue, but also healthy looking parts, distant 10-15 mm from the diseased area, were contaminated with DAS in concentrations up to 110 µg/kg. The toxin could also be found in tubers without any disease symptoms.

DAS is a cytotoxic compound and could inhibit lymphocytes proliferation and immunoglobulin production, mainly at high concentrations, while low concentrations appear to have opposite effects. DNA damage and pro-apoptotic properties have also been observed.

There is no European legislation on DAS, but some countries have introduced national legislative limits in feedstuffs with values ranging from 500 and 1000 µg/kg.

FUTURES

- Studies on the ecology of main fungi involved in DAS production are suggested.
- Surveys on fungi associated with DAS contaminated cereals samples would help in understanding the key *Fusarium* species.
- The development of specific immunoaffinity devices as well as isotope-labelled internal standards should be encouraged.
- Development of specific protocols for DAS determination in feed.

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Chapter 7 - Sterigmatocystin in food and feed

KEYWORDS: Sterigmatocystin, *Aspergillus nidulans*, *Aspergillus versicolor,* Biosinthesys, Cereals, Cheese, Methods of analysis, Validation, Toxicity, Mitigation.

INTRODUCTION

Sterigmatocystin (STC; $C_{18}H_{12}O_6$) (3a,12c-dihydro-8-hydroxy-6-methoxy-furo[3',2',4,5]furo[3,2-c]xanthene-7-one) (Weidenbörner, 2001) is a mycotoxin produced by fungi of the genus *Aspergillus* (Bennett and Christensen, 1983; Bennett and Papa, 1988; Cole and Cox, 1981; Hajjar *et al*., 1989; Hitokoto *et al*., 1982). Sterigmatocystin is a mycotoxin structurally closely related to aflatoxins (AFs), and consists of a xanthone nucleus attached to a bifuran structure. STC is a polyketide (Dutton, 1988) precursor proposed as an intermediate in several schemes for AF biosynthesis (Biollaz *et al*., 1970, Barnes *et al*., 1994, Weidenbörner, 2001). Hsieh and colleagues (1973) demonstrated the conversion of STC into AF performed by *A. parasiticus* (Hsieh *et al*., 1973 while Singh and Hsieh (1976) proved the enzymatic conversion of STC into AFB_1 by cell-free extracts of *A. parasiticus.* This mycotoxin is known to contaminate various foods and feeds and to cause serious health and economic problems worldwide (Jelinek *et al*., 1989; Lancaster *et al*., 1961; Northolt *et al*., 1980). Sterigmatocystin is mainly produced by the fungi *Aspergillus nidulans* and *A. versicolor*, but also *A. flavus, A. sydowi, A. nidulans, Bipolaris, Chaetomiun* and *Emiricella* spp. are reported (Davis, 1981; Terao, 1983; Scott, 1994, 2004; Frisvad and Thrane, 2004). It has been reported in mouldy grain, green coffee beans and cheese although information on its occurrence in foods is limited. It appears to occur less frequently than AFs, although analytical methods for its determination have not been very sensitive until recently and it is possible that small concentrations in food commodities may not always have been detected. Its acute toxicity, mutagenity, cytotoxicity and carcinogenity have been reviewed by Terao (1983) and it is classified as a 2B carcinogen by the International Agency for Research on Cancer (IARC, 1987). The structure of the adduct of STC at the N-guanine of oligodeoxynucleotides was determined (Gopalakrishnan *et al*., 1992). A 'no significant risk' level for humans of 8 mg/kg of body weight/day was estimated by the US State of California (Scudamore *et al*., 1996).

ASPERGILLUS AND ITS HOSTS

Aspergillus is a filamentous, cosmopolitan and ubiquitous fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment. *Aspergillus* species are common saprophytic moulds that grow in household dust, soil, and decaying vegetable matter, including stale food. Aspergilli are also found as agents of biodeterioration in human environments where they regularly destroy paper, fabrics and building materials (Bennett, 2009).

The genus *Aspergillus* was originally divided into subgenera and groups (Raper and Fennell, 1965), but the current classification scheme replaces the designation “group” with “section” (Gams *et al.*, 1985) to conform to rules of the International Code of Botanical Nomenclature. Currently, the genus *Aspergillus* is classified into seven subgenera that are in turn sub-divided into several sections comprised of related species (Gams *et al.,* 1985).

Members of the genus *Aspergillus* have been of interest for centuries because of their positive impact as fermentation agents and because of their negative impact as degraders of agricultural products, their toxicity, and their pathogenicity (Klich, 2002).

The genus *Aspergillus* consists of more than 180 recognized species. Although it includes the major filamentous fungal pathogen of humans *A. fumigatus,* most of the members are useful microorganisms able to degradate plant polysaccharides (de Vries *et al*., 2000), and they are important industrial microorganisms for the large-scale production of both homologous and heterologous enzymes.

The aspergilli are more often soil fungi or saprophytes and several are important because they produce mycotoxins. The aspergilli can also cause decay and deterioration in stored products, and may cause disease in plants, insects, poultry and humans (de Vries *et al*., 2000).

A partial list of the mycotoxins includes the AFs (produced by *A. flavus*, *A. parasiticus*, *A. nominus*), citrinin, citreoviridin, cyclopiazonic acid (the main producer is *A. flavus*; Pitt and Hocking, 1997), gliotoxin, ochratoxin (produced by *A. ochraceus* and related species, Sweeney and Dobson, 1998 and by species belonging to section *Niger*: *A. carbonarius*, *A. niger* and *A. tubingensis*); pennicillic acid, STC and xanthomegnin.

A. flavus and *A. parasiticus* are common contaminants of agricultural crops. They produce a family of highly toxic and carcinogenic metabolites called AFs. Aflatoxin B_1 is the most potent natural carcinogen known and the most important mycotoxin. Because *A. flavus* and *A. parasiticus* are weedy moulds that can grow on cereals, legumes, nut crops and spices, the potential for AF contamination is a major threat to human and livestock health. Thousands of studies on AF toxicity have been conducted, mostly concerning laboratory models or agriculturally important species. The AF biosynthetic pathway is a model for studying the biochemistry and molecular biology of fungal secondary metabolism (Keller *et al.,* 2005; Payne and Brown, 1998; Yu *et al.,* 2004).

Sterigmatocystin is mainly produced by the fungi *A. nidulans* and *A. versicolor* (Davis, 1981; Terao, 1983; Diaz, 2005; Weidenbörner, 2001). It was first isolated from *Aspergillus versicolor* (Vuillemin) Tiraboschi (Birkinshaw and Hammady, 1957; Davies *et al*., 1960), and has been shown to be produced also by an isolate of *A. rugulosus* (Ballantine *et al*., 1965).

The list of known producers was extended to *A. nidulans* (Eidam) Winter and a *Bipolaris sorokiniana* (Saccardo in Sorok) Shoem, also known as *Drechslera sorokiniana* (Saccardo) Subram & Jain (Rabie *et al*., 1976). Schroeder and Kelton (1975) reported that a large number of isolates (57) of *A. flavus* Link and 20 isolates of *A. parasiticus* Speare also produce STC. They extended the list of known producers to *A. chevalieri*, *A. ruber*, and *A. amstelodami*.

The species able to produce STC are listed below (Smith and Henderson, 1991):

- *Aspergillus japonicus* Saito
- *Aspergillus cespitosus* Raper et Thom
- *Aspergillus egyptiacus* Moubasher et Moubasher
- *Aspergillus flavus* Link
- *Aspergillus multicolour* Sappa
- *Aspergillus parasiticus* Speare
- *Aspergillus sydowii* (Bain et Sart) Thom et Church
- *Aspergillus ustus* (Bain.) Thom et Church
- *Aspergillus versicolor* (Vuillemin) Tiraboschi
- *Bipolaris sorokiniana* Shoem
- *Emericella aurantiobrunnea* (Atkins, Hindson et Russel) Malloch et Cain
- *Emericella cleistominuta* Mehrothra et Prasad
- *Emericella corrugate* Udagawa et Horie
- *Emericella foveolata* Horie
- *Emericella heterotallica* (Kwon, Fennel et Raper) Malloch et Cain
- *Emericella navahoensis* Christensen et States
- *Emericella nidulans* (Eidam) Winter var *acristata, dentata, echinulata, lata, nidulans*
- *Emericella parvathecia* (Raper et Fennel) Malloch et Cain
- *Emericella quadrilineata* (Thom et Raper) C.R.Benjamin
- *Emericella spectabilis* Christensen et Raper
- *Emericella striata* (Rai, Tewari et (Mukerji))
- *Emericella variecolor* Berk et Br. var. *variecolor*

<u>*Aspergillus nidulans* (Eidam) G. Winter</u>

A. nidulans (also known as *Emericella nidulans*) is a saprophytic ascomycete and it is the only member of the genus *Aspergillus* forming sexual spores through meiosis. *A. nidulans* is also a homothallic fungus.

Colonies on Czapek's solution agar at room temperature (24-26°C) attain diameters up to 5.0 to 6.0 cm in 2 weeks, in some strains dark cress green from abundant colonial heads, others cream-buff to honey yellow from abundant cleistothecia; margins thin, irregular, sectoring occasional; colony reverse and agar show varying shades of purplish red during the growing period, becoming very dark in age (Raper and Fennel, 1965).

A. nidulans has:

- conidial heads short, columnar, ranging from 40 to 80 µm by 25 to 40 µm;
- conidiophores sinuous, with walls smooth in shades of cinnamon brown, ranging from 60 to 130 µm;
- vesicles 8 to 10 µm in diameter;
- sterigmata in two series, primary commonly 5 to 6 µm by 2 to 3 µm, and secondaries 5 to 6 µm by 2.0 to 2.5 µm;
- conidia globose, regulose, 3 to 3.5 µm in diameter, green in mass .

Colonies on malt extract agar at room temperature grow very rapidly, attaining a diameter of 6.0 to 7.0 cm in 1 week, plane, in deep dull yellow-greens conidial strains with limited cleistothecia developing within and upon the conidial layer (Raper and Fennel, 1965).

Aspergillus versicolor (Vuillemin) Tiraboschi

Colonies on Czapek's solution agar grow rather slowly at room temperature (24-26°C), attaining a diameter of 2 to 3 cm in 2 weeks, in some strains compact with abundant conidiophores arising from the medium, in other strains showing a marked development of aerial, closely interwoven hyphae bearing more or less abundant conidiophores as short branches, in or buckled, at first white, passing through shades of yellow, orange-yellow. Tan, to yellowish green shades such as pea green depending upon the strain and the amount of sporulation, occasionally in flesh to pink shades with the green colour completely lacking; exudate absent to abundant, ranging from clear to dark wine red; reverse and substratum colourless or nearly so, in some strains passing through shades of yellow to orange then rose, to red or purple-red (Raper and Fennel, 1965).

A. versicolor has:

- conidial heads roughly hemispherical, radiate, up to 100 to 125 µm in diameter;
- conidiophores colourless or yellowfish in strongly pigmented strains, heavy walled, smooth, up to 500 or even 700 µm by 5 µm or approaching 10 µm near the vesicle;
- vesicles 12 to 16 µm in diameter fertile area hemispherical or semieliptical passing almost imperceptibly into the funnel-like enlarged apex of the conidiophore;

- sterigmata in two series, primary commonly 5.5 to 8.0 µm by 3.0 µm, secondary 5.0 to 7.5 µm by 2.0 to 2.5 µm;
- conidia globose, strongly to delicately echinulate, mostly 2 to 3 µm, usually borne in loosely radiating chains (Raper and Fennel, 1965).

Colonies on malt extract agar grow somewhat more rapidly, 3 to 4 cm in 10 days, usually plane, heavily sporing throughout with conidial heads in a dense strand arising from submerged mycelium, less commonly flocculent and bearing reduced or fragmentary heads as aerial branches. The fungus reverse is uncoloured to orange or greenish drab shades (Raper and Fennel, 1965).

Life cycle

A. nidulans is one of the best characterised eukaryotic and the signalling pathways tend to be conserved among *Aspergillus* species. *A. nidulans* develops a sexual stage with fruiting bodies (cleistothecia), where sexual spores (ascospores) are formed. Both asexual and sexual development is linked to STC production (Hicks *et al*., 1997; Kato *et al*., 2003).

Aspergillus has lifes cycle both sexual and asexual cycle (Todd *et al*., 2007). Vegetative hyphae from two individuals may fuse to form a heterokaryon, and nuclei in a heterokaryon or a homokaryon may fuse to form a diploid. Vegetative hyphae differentiate by asexual development to produce spores (conidia) on conidiophores. Asexual development is initiated by differentiation of a specialised foot cell from which a stalk emerges. A vesicle is formed at the end of the stalk and two layers of uninucleate sterigmata metulae and phialides and uninucleate conidia are produced by successive budding from the vesicle. As growth proceeds, sexual development occurs by the production of nurse cells called Hülle cells and closed sexual fruiting bodies (cleistothecia) containing sexual spores (ascospores) arranged in nonlinear asci. Each cleistothecium contains as many as 10,000 ascospores that are the meiotic progeny of a single ascogenous hypha. Asci arise from croziers, which are formed by simultaneous division of the two nuclei of the terminal cell of an ascogenous hypha. The crozier comprises a uninucleate tip cell, a binucleate penultimate cell and a uninucleate basal cell. The ascus is formed by enlargement of the penultimate cell, which is accompanied by fusion of its two nuclei to form a transient diploid zygote that undergoes meiosis immediately. The four nuclei produced by meiosis divide mitotically to generate eight nuclei, each of which is included in one of the eight dark red ascospores in each ascus. The nucleus in each ascospore undergoes a single mitotic division resulting in mature binucleate ascospores. The tip and basal cells fuse to generate a binucleate cell, which forms a second crozier and the process is repeated. All of the ascospores within a single cleistothecium are of selfed or

hybrid origin, which may arise if all of the dikaryotic ascogenous hyphae (and therefore all of the asci) are generated by conjugated divisions of one pair of parental nuclei.

Ecology

A. nidulans is a cosmopolitan saprophytic fungus which is primarily isolated from soil, household dust, soil, and decaying vegetable matter, including stale food.

A. versicolor is a cosmopolitan fungus, primarily isolated from plant materials and from soil, which is particularly common in temperate and colder areas. It is often found in buildings with problems of humidity and ventilation. *A. versicolor* is very common in damp places including: gypsum boards, carpets, mattresses, upholstered-furniture and damp walls. It is commonly found in soil, hay, cotton, cheese and dairy products.

A. versicolor has been found in grains (Wallace and Sinha, 1963), bread (Reiss, 1976), wheat flour (Graves and Hesseltine, 1966), grape juice (Senser *et al*., 1967), meat, and cheese (Raper and Fennell, 1965; Scott *et al*., 1972; Scott, 1989). Relatively high levels of STC have been formed in bread, cured ham and salami after inoculation with *A. versicolor*. It has also been found in a variety of nuts, fermented and cured meats and biltong, spices and cheese (Pitt and Hocking, 1997).

A. versicolor is able to grow on very nutrient-poor materials such as concrete and plaster. This mould has highly variable culture morphology, but produces a consistent chemical profile on laboratory substrates, usually generating high quantities of the carcinogenic mycotoxin STC (especially on CYA and 2% malt agar), and related compounds (e.g., versicolorins; Frisvad and Gravesen, 1994; Frisvad and Thrane, 2002). On YES agar, *A. versicolor* produces versicolins, but only minute quantities of the STCs (Nielsen *et al.,* 1998). On wallpaper paste, conidia of 50% of the isolates contained STC (Larsen and Frisvad, 1994). Reports of cyclopiazonic acid (CPA) and ochratoxin A (OTA) production are probably due to culture contamination and misidentification (Frisvad, 1989; Frisvad and Thrane, 2002). On water-saturated materials, *A. versicolor* produces 5-methoxy-STC and STC in quantities up to 7 and 20 µg/cm^2 respectively (up to 1% of biomass; Engelhart *et al.,* 2002; Nielsen *et al.,* 1998, 1999; Tuomi *et al.,* 2000), whereas they are not produced when a_w < 0.9 (Nielsen, 2002). Interestingly, non-sporulating red-colored samples of contaminated materials contained the largest quantities of STCs; areas with many conidia contained small quantities (Nielsen *et al*., 1998, 1999). Recently, STC was detected in 20% of household dust samples at levels up to 4 ng/g (Engelhart *et al*., 2002).

Mycotoxin production is influenced by a number of different parameters including nutrient availability and environmental factors, such as water activity, pH (Cotty, 1988**;** Buchanan and

Ayres**,** 1975), temperature (Feng and Leonard, 1998), and availability of an air-surface interface (Guzman-de-Peña and Ruiz-Herrera, 1997).

Aspergilli grow well and sporulate abundantly at temperatures between 23° and 26°C. *A. nidulans* and *A. versicolor* exibit weak or absent growth at 5°C or lower and at 37°C or higher temperature. Northolt *et al*. (1980) reported the occurrence of STC in cheese ripening in warehouses in the Netherlands. However, low temperatures (5–7°C) should prevent the growth of *A. versicolor* and production of STC.

Aspergillus mycotoxin production starts at a_w levels between 0.80-0.83, for example 0.80 a_w is necessary for STC production on bread by *A. versicolor*, and of a_w 0.85 on agar media. (Weidenbörner, 2001). *A. versicolor* is a xerophile which has been reported to grow at 0.76 a_w (ICMSF, 1996). Lactose, glycerol, high humidity and temperature are stimulating factors in STC production by *A. versicolor* on cheese (Veringa *et al*. 1989).

Although AF and STC production appears to be influenced by growth medium pH, studies addressing pH regulation of these mycotoxins have produced complex and sometimes contradictory results. Cotty (1988) determined a link between AF production and sclerotial morphogenesis based on changes of both chemical and morphological differentiation in response to pH. At pH 4.0 or below, sclerotial production is reduced by 50% in *A. flavus* while AF production is maximal. Buchanan and Ayres (1975) concluded from their work that the initial pH of the growth medium was not an important determinant for mycotoxin production. In fact, the effect of pH on AF biosynthesis is dependent on the composition of the growth media.

Keller *et al*. (1997) showed that *A. nidulans* and *A. parasiticus* produced less mycotoxin as the pH of the growth medium increased. In addition, pH-sensing mutants of *A. nidulans* exhibit both sporulation and STC production aberrations (Piñero, 1999). *A. versicolor* is less tolerant to low pH.

Nutritional factors such as carbon and nitrogen sources can also affect both mycotoxin production and morphological differentiation. Additionally, some compounds present in seeds commonly infected by *Aspergillus* species can influence both toxin production and fungal development (Calvo *et al*., 2002).

The availability and type of carbon and nitrogen source affect STC and AF production. Simple sugars such as glucose, fructose, sucrose, and sorbitol as sole carbon sources support high fungal growth, sporulation, and AF production (Buchanan and Stahl**,** 1984; Kacholz and Demain, 1983). In contrast, peptone (Buchanan and Stahl, 1984; Burow *et al*., 1997) and the more-complex sugars like galactose, xylose, mannitol, and lactose (Kacholz and Demain, 1983) do not support AF production well. The choice of nitrogen source used in

the growth medium can have different effects on STC and AF production in different *Aspergillus* species. Nitrate as the nitrogen source has been shown by some groups to repress the synthesis of AF intermediates in *A. parasiticus* (Kacholz and Demain, 1983) but enhance STC production in *A. nidulans* (Feng and Leonard, 1998).

Feng and Leonard (1998) also observed no STC production in ammonium-containing media. Other studies (Keller *et al*., 1997) indicate STC and AF production increases in ammonium-based media and decreases in a nitrate based medium. Nitrogen source influences not only mycotoxin production but also the formation of developmental structures in *Aspergillus* spp. Studies in which *A. flavus* is grown on agar media containing either nitrate or ammonium as the sole nitrogen source have shown that development of sclerotia occurs on nitrate but not on ammonium.

PHYSICO-CHEMICAL CHARACTERISTICS

Sterigmatocystin ((3AR-cis)3a,12c-dihydro-8-hydroxy-6-methoxy-7H-furo(3',2':4,5)furo(2,3-c)xanthen-7-one; $C_{18}H_{12}O_6$; MW: 324.28 g/mol) is a toxic metabolite structurally closely related to AFs and consists of a xanthone nucleus attached to a bifuran structure (Figure 1).

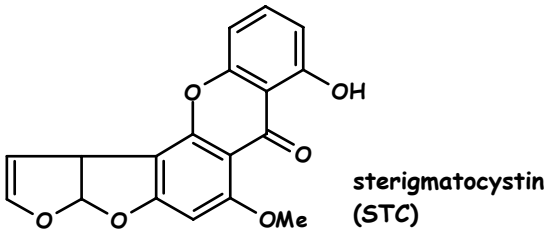

Figure 1. Chemical structure of STC.

Sterigmatocystin crystallises as pale yellow needles and is readily soluble in methanol, ethanol, acetonitrile, benzene and chloroform. It reacts with hot ethanolic potassium hydroxide KOH and is methylated by methyl sulphate and methyl iodide. Methanol or ethanol in acid produces dihydroethoxySTC. There appear to be no reports about the stability of STC, other than in solution. Sterigmatocystin is fairly resistant to degradation. Several parameters such as pH and temperature may affect its stability in solution.

Its most important derivative is O-methyl-STC, which can be found in commodities together with its precursor.

Sterigmatocystin is naturally fluorescent and undergoes phototautomerism under acidic conditions, as reported in Figure 2.

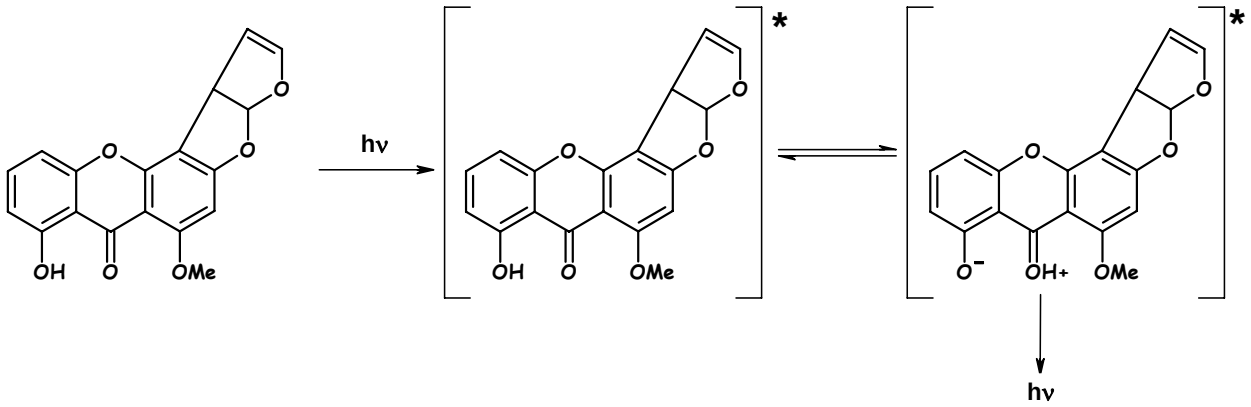

Figure 2. Phototautomerism of STC under acidic conditions.

Both tautomers are emitting species and their fluorescence may be affected by environmental conditions, as reported in Table 1 (Maness *et al*., 1976):

Table 1. Fluorescence of sterigmatocystin (STC) and O-methyl-STC in different environmental conditions.

	60% H_2SO_4		0.1 N HCl		pH 7		2 N NaOH	
	λa (nm)	λf (nm)	λa (nm)	λf (nm)	λa (nm)	λf (nm)	λa (nm)	λf (nm)
STC	377	520	340	570	340	570	380	520
O-methyl-STC	374	510	318	450	318	445	318	445

BIOSYNTHESIS

Sterygmatocystin is an intermediate of the AF biosynthetic pathway, so therefore its biosynthesis has been extensively studied using both radioactive incorporation and genetic approaches (Keller and Adams, 1997; Hicks *et al*. 2002; Yu *et al*. 2004).

Sterygmatocystin is a polyketide-derived furanocoumarin. AF biosynthesis has been proposed to involve at least 23 enzymatic reactions. Thus far, at least 15 structurally well-defined AF intermediates have been identified in the AF biosynthesis pathway. It has been demonstrated that 25 identified genes clustered within a 70-kb DNA region in the chromosome are involved in AF biosynthesis. Sterygmatocystin and dihydrosterigmatocystin (DHSTC), produced by certain strains of *A. nidulans*, are the penultimate precursors of AFs (Yu *et al.* 2004; McDonald *et al*. 2005).

The overall biostynthesis pathway of STC is reported in Figure 3.

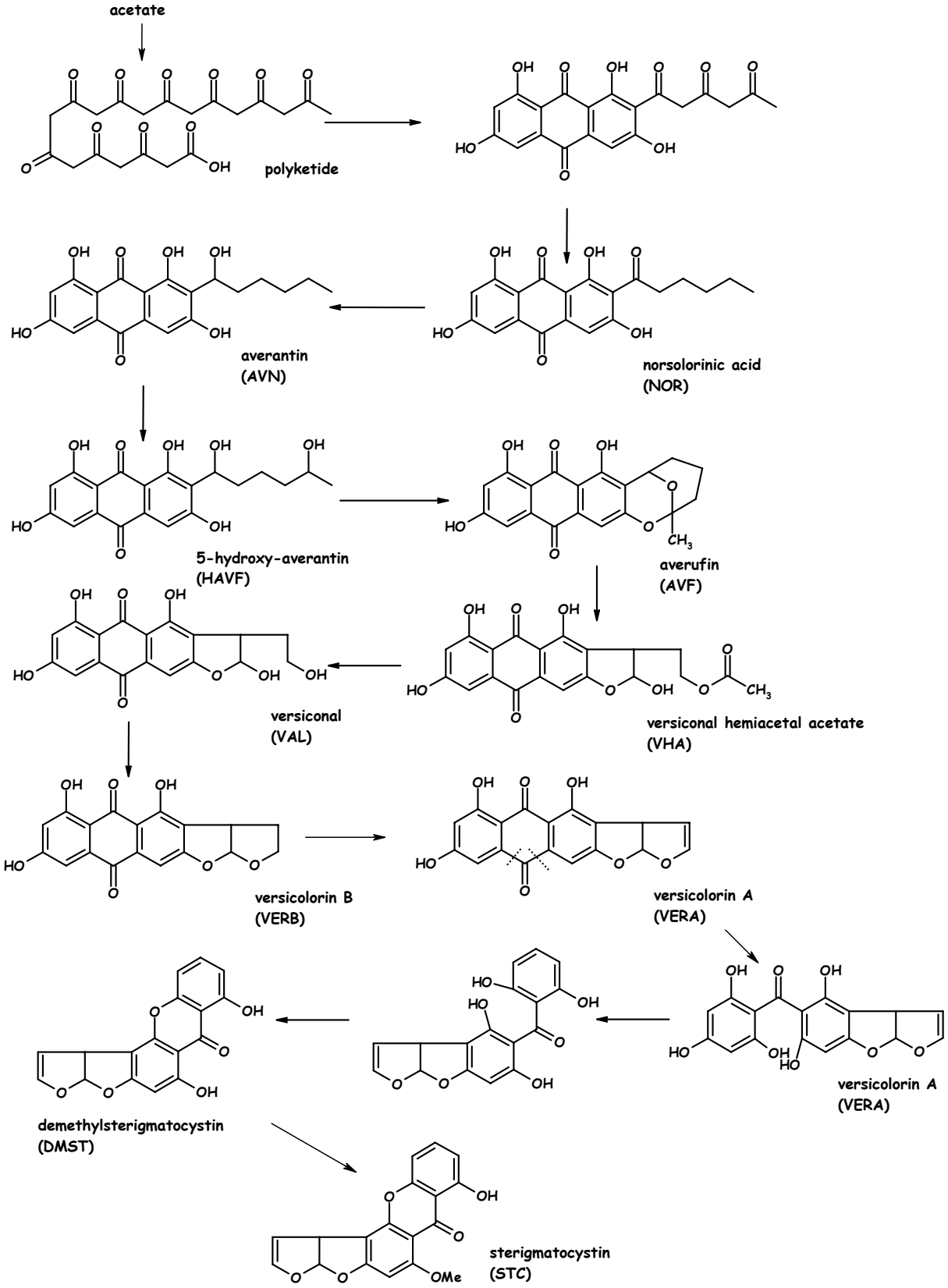

Figure 3. Complete biosynthesis pathway of STC.

Sterygmatocystin's remarkably complex biosynthetic origin begins with a poly-β-keto chain derived from a hexanoyl- CoA starter and seven malonyl-CoA extender units. This gives an anthraquinone
norsolorinic acid (NOR) by now-familiar condensation reactions, but the folding of the chain is rather different from that seen with simpler anthraquinones (Figure 4)

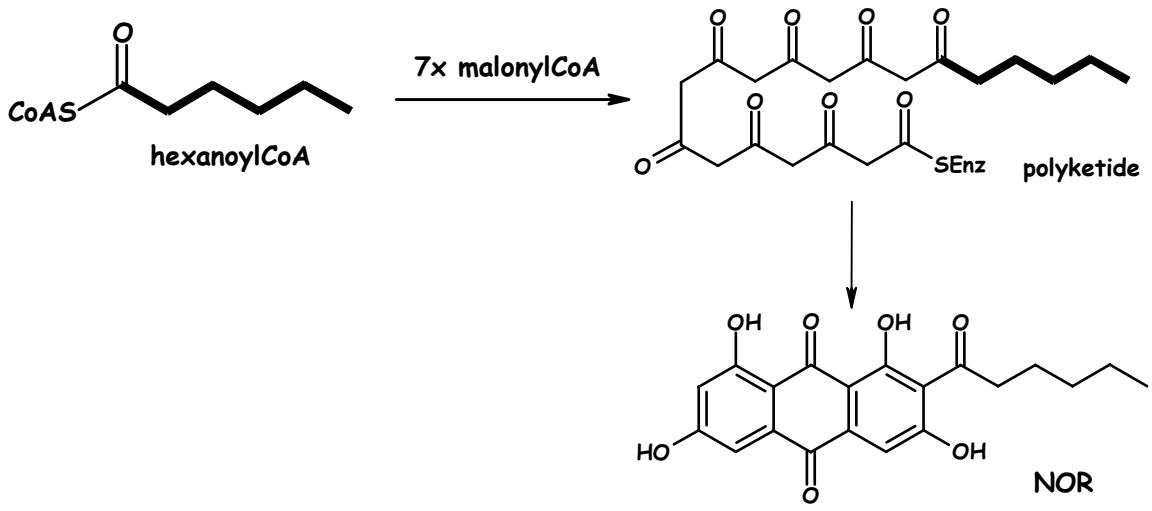

Figure 4. Formation of norsolorinic acid (NOR).

The six-carbon side-chain of norsolorinic acid is cyclized to give, in several steps, the ketal averufin. Versiconal acetate is another known intermediate, and its formation involves a Baeyer–Villiger oxidation, resulting principally in transfer of a two-carbon fragment (the terminal ethyl of hexanoate) to become an ester function (see Figure 5).

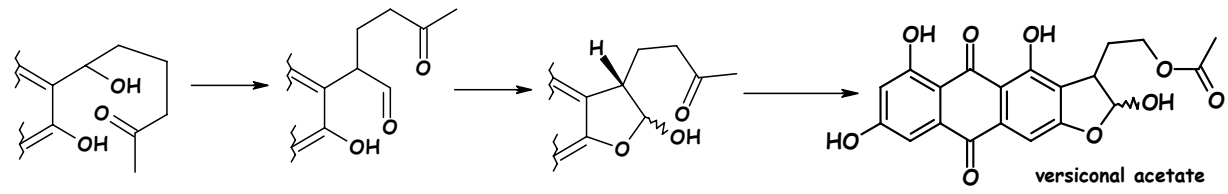

Figure 5. Cyclization of norsolorinc acid to versiconal acetate.

These two carbons can then be lost in theformation of versicolorin B, now containing the tetrahydrobisfuran moiety, oxidized in versicolorin A to a dihydrobisfuran system (Figure 6).

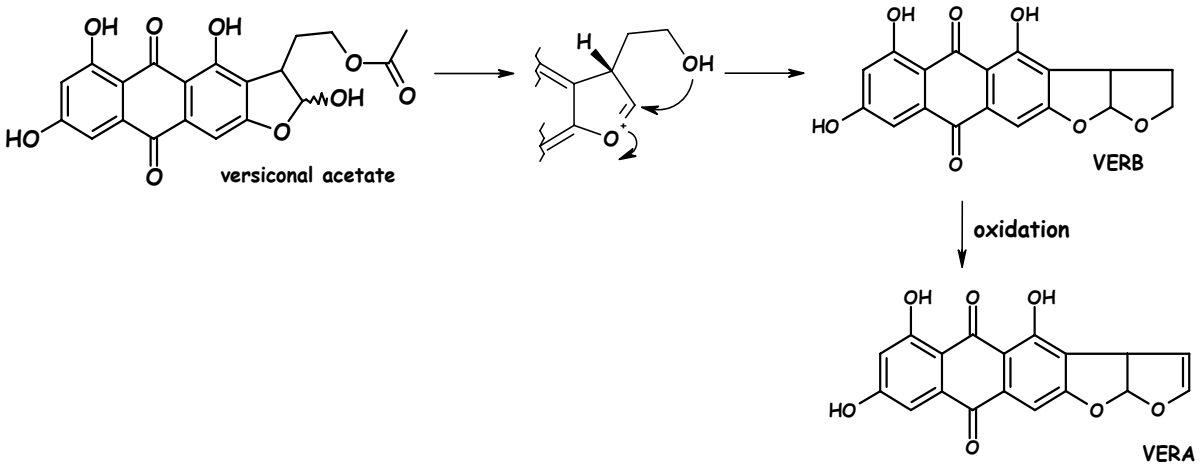

Figure 6. Formation of Versicolorin B (VERB) and Versicolorin A (VERA).

Sterigmatocystin is derived from versicolorin A by oxidative cleavage of the anthraquinone system involving a second Baeyer–Villiger oxidation, and recyclization through phenol groups to give a xanthone skeleton. Rotation of an intermediate leads to the angular product as opposed to a

linear product. One phenol group is methylated, and, quite unusually, another phenol group is lost, as reported in Figure 7.

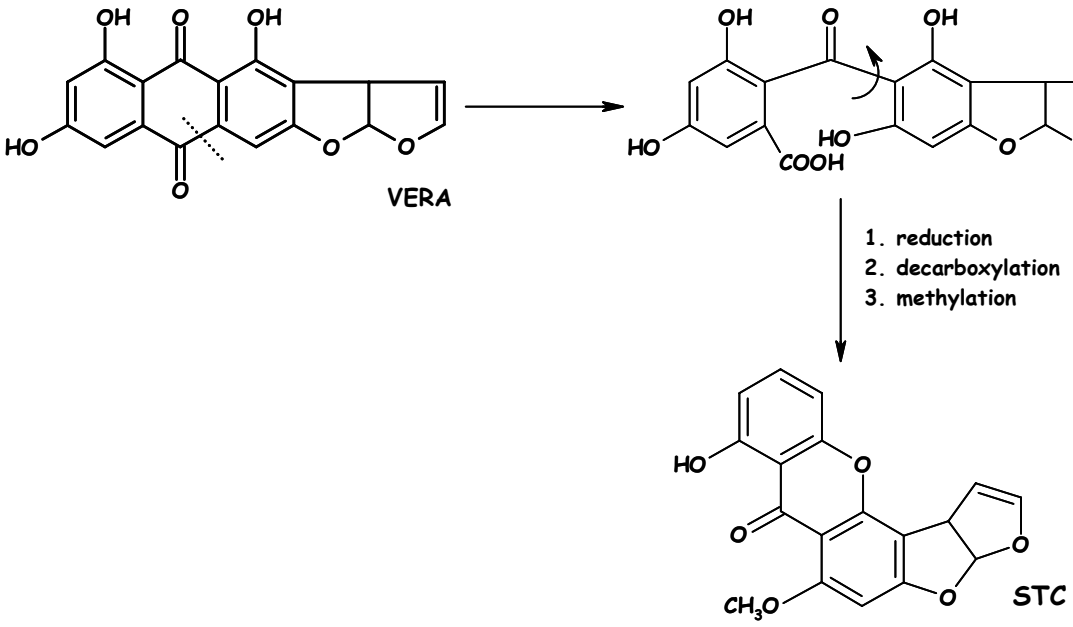

Figure 7. Final conversion of versicolorin A (VERA) to STC.

CHEMICAL ANALYSES

Source of standards

Septien *et al*., (1993) studied the solubility of STC in different organic solvents and the stability of these solutions during cold and frozen storage. STC was more soluble in chloroform and in pyridine. In cold storage STC was more stable in chloroform after both 7 and 30 days. In pyridine, STC was stable on day 7, but not on day 30. In frozen storage, 90% recovery was achieved only in chloroform. It is concluded that, of those studied, chloroform was the most suitable organic solvent to solubilise and store standard solutions of STC. In another study, Versilovskis and Mikelsone (2008) indicated that more suitable solvents for storage of STC standard solutions at -25 °C are acetonitrile and a mixture of acetonitrile and methanol (50:50, v/v), but not for longer than one week; for storage at 4 °C more suitable is a mix of acetonitrile and methanol, but not for longer than one week.

Sterigmatocystin standards are commercially available (Table 2).

Certified reference materials for method development and validation are not yet commercially available, even if Tanaka *et al*., (2008) reported the development of a method to produce a satisfactorily homogenous reference material for STC in brown rice.

Table 2. Sources of sterigmatocystin standards.

Compound	Concentration (μg/ml) or Purity (%)	Quantity	Supplier
Solid standard			
Sterigmatocystin	> 98%	1 mg/5 mg	Sigma-Aldrich
Sterigmatocystin	-	1mg/5 mg/10mg	Axxora Europe (Lausen, Switzerland)
Sterigmatocystin, *Aspergillus versicolor*	-	5 mg/10 mg	Fluorochem Ltd (Derbyshire, UK)
Sterigmatocystin	-	-	CHEMOS GmbH (Regenstauf, Germany)
Sterigmatocystin	98%	-	UK Green Scientific Co., Limited
Sterigmatocystin	-	1 mg/5 mg/25 mg	Apollo Scientific Ltd (Cheshire, UK)
Sterigmatocystin	98%	50 mg	Carbone Scientofic Co., Ltd (London, UK)
Sterigmatocystin	99.7 ± 0.3 %	5 mg	Romer Labs Diagnostic (Tulln, Austria)
Standard solution			
Sterigmatocystin	50 in acetonitrile	1 ml/5 ml	Romer Labs Diagnostic (Tulln, Austria)

Sampling

No specific research has been undertaken about sampling plans for STC in food and feed, a topic which should be faced in the near future.

Extraction and clean up

Sterigmatocystin is usually extracted from food and feed using a mixture of acetonitrile and water, with the organic solvent ranging from 80% to 90% (Versilovskis *et al*., 2007, Stroka *et al*.. 2004). When dairy products are considered, a defatting step with n-hexane is usually performed (Stroka *et al*., 2004). No immunoaffinity columns have been proposed for STC purification, thus the cleanup step is usually performed by solid phase extraction (SPE), using C18 or phenyl-bond SPE columns. Reference materials are not commercially available, although one attempt has been reported in literature to produce a rice reference sample for STC analysis (Tanaka *et al*., 2008).

Analytical methods

Methods have been described in literature, which allow the detection of STC at levels ranging from 2 to 100 μg/kg (Scudamore *et al*., 1996). However, it has been recognised that the surveillance of STC below μg/kg level was difficult in the past due to the weak fluorescence of this mycotoxin, and that a precise determination below this level requires mass spectrometric detection (Scudamore *et al*., 1996).

The most frequently applied analytical methods so far, are based on thin-layer chromatography (TLC) with fluorescence detection (AOAC Official Method 973.38, 1995; Athnasios and Kuhn, 1977; Gimeno, 1979; Valente Soarez and Rodriguez-Amaya, 1989). Because of its weak native fluorescence, a derivatisation procedure is generally used to visualize the STC on the developed TLC plates (Athnasios and Kuhn, 1977). The most common derivatization approach is spraying of the TLC plate with aluminium chloride ($AlCl_3$) solution, after development and heating of the plate. With such procedures, limits of detections (LODs) were reported to be 50 μg/kg (Athnasios and Kuhn, 1977) and 140 μg/kg (Gimeno, 1979), in cereal grains. A validated official method is also based on TLC with $AlCl_3$ spraying, and is reported to have a limit of quantification (LOQ) of 100 μg/kg (AOAC Official Method 973.38, 1995).

Other methods are based on high performance liquid chromatography (HPLC) with ultraviolet (UV) detection, and were applied for determination of STC in rice inoculated by *A. versicolor* at lower μg/kg levels (Neely and Emerson, 1990; Schmidt *et al*., 1981). Application of HPLC with post column derivatisation with $AlCl_3$ has also been reported (Neely and Emerson, 1990), with results comparable to the official TLC method (AOAC Official Method 973.38, 1995). The lowest detection limit of 1.7 μg/kg for maize and 1.9 μg/kg for bread was reported when liquid chromatography with mass spectrometric detection (LC–MS) was applied (Scudamore *et al*., 1996).

A method based on gas chromatography with mass spectrometric detection (GC–MS) reported an LOD of 5 μg/ kg for STC in wheat (Salhab *et al*., 1976).

In conclusion, currently the only methods with sufficient LOQs of STC at levels below 5 μg/kg are based on LC–MS and GC–MS.

For routine and monitoring purposes a reagent-free derivatisation procedure was also successfully applied for fluorescence induction of STC and method limit of detection was 2–11 μg/kg (Stroka *et al*., 2004). Now LC–MS/MS based methods are available for routine and monitoring purposes with LOD of 0.15 μg/kg in grains (Versilovskis *et al*., 2007).

A number of analytical methods have been published to examine STC in a cheese matrix. Thin layer chromatography (TLC) with LODs around 20 μg/kg. Ultraviolet light or post column fluorescence with $AlCl_3$ was described for LC detection. Aluminium chloride was also used as a spray reagent for two-dimensional TLC with densitometric detection (LOD=1 μg/kg in feed) (Domagala *et al*., 1997).

A sensitive method for STC detection in cheese was a liquid chromatography-atmospheric pressure chemical ionisation-mass spectrometry (LC-APCI-MS) method with a LOD of 2.4 μg/kg (Scudamore *et al*., 1996). Versilovskis *et al*. (2009) developed the first LC-MS/MS method with electrospray ionisation (ESI) that can be applied for routine and confirmation analysis. The LOD and LOQ were 0.03 and 0.1 μg/kg, respectively.

Validation and outlook

The Association of Official Analytical Chemists International (AOAC International) has validated a method for STC determination in barley and wheat (AOAC Official Method 973.38, 1995). Although it can perform reliably, it would not today be the favoured approach for many laboratories, being based on TLC.

Another method for STC determination in cheese by TLC (Francis *et al*., 1987) was evaluated by an interlaboratory study. However, because of the high variability at the 5 and 25 ppb levels, a recommendation for official first action status was deferred pending development of a more satisfactory determinative step.

In the near future, LC based validated analytical methods for the quantification of STC are needed as a prerequisite for a survey on its occurrence in food and also in feed.

OCCURRENCE DATA

Feed

Very little data are available concerning monitoring of foodstuffs for STC (Table 3).

One of the few surveys in Europe was carried out in 1983 in the United Kingdom. Out of 523 analysed cereal samples, about 3% were found positive. Although mould spoilage occurred in most hay samples, STC was detected in only one sample (Buckle, 1983). The drawback of this survey was that the method had a relatively high LOD of 20 μg/kg. This did not allow any conclusion concerning lower contamination levels, which are also of interest. Moreover this was not a random survey as many of the samples tendered for examination were suspected of being responsible for feed-related problems in animals or of inferior quality. In a survey of mouldy cereals in the UK, 7 out of 11 samples were positive for STC (Scudamore *et al.,* 1986).

Scudamore *et al*. (1997) analyzed a total of 330 samples of animal feed ingredients in United Kingdom. STC was present in only one wheat sample in a low concentration.

More recently STC has been detected in 2 silage samples in Egypt (El-Shanawany *et al*., 2005).

Food

The occurrence of STC in food has not often been reported (Table 3). The instances reported have usually been on mouldy or poor quality materials such as maize, hard cheese, pecan nuts (Schroeder and Hein, 1977) and green coffee beans (Purchase and Pretorius, 1973; Coman *et al*., 2006). While this lack of information may be due to deficiencies in the analytical methods, where surveys of good quality products have been carried out with reliable methodology, STC has rarely been found (EMAN, http://services.leatherheadfood.com/mycotoxins/item.asp?sectionid=1&mytype=basic&number=12&fsid=17).

A large survey was undertaken in Brazil (Valente Soarez and Rodriguez-Amaya, 1989) where STC was not detected in any of the food examined (whole grain, dried beans, rice, corn, corn meal, cassava flours, corn flour and corn grit). However, the LOD of the method applied was high (35 μg/kg). These rather high LOD values that were obtained with conventional (non-mass spectrometric) methods are rather unsatisfactory.

Sterigmatocystin has been reported in green coffee beans in Italy (1 sample out of 502, but at 1200 μg/kg) (DePalo *et al.,* 1977).

Sterigmatocystin has also been found in Egyptian spices, but in low concentrations ranging from 10 to 23 μg/kg (El Kady *et al*., 1995).

Surveillance by MAFF (1980) found STC in 2 out of 29 samples of maize and in one sample of flaked maize, although the levels were unknown. Further surveillance in 1987 (MAFF, 1987) failed to find STC in 20 retail samples of cheese and 20 samples of whole grain maize.

Using a chromatographic technique, 74 grain samples (24 wheat, 19 barley, 16 maize, 10 oats and 5 rye) from the 1980 and 1981 harvests were examined for STC (Bartos and Matyas, 1983). Two barley samples and 2 maize samples were positive; the maize samples contained approx. 50 μg/kg and the barley samples 200 and 400 μg/kg. One wheat sample had a trace amount of STC.

Versilovskis *et al*., (2008a) analysed 95 Latvian grain samples from the year 2006 and 120 samples from the year 2007. For the year 2006, 13.7% of the samples were positive for STC. The concentration levels were quite low from 0.7 to 83 μg/kg, but in some samples they were above maximum levels set for food products in the Czech Republic and Slovakia (20 μg/kg). Thirty five percent of the samples in 2007 were positive for STC. The concentration levels were variable: the highest levels were detected in wheat and barley, medium in buckwheat and quite low in oats and rye samples, but again almost half of thesamples showed STC levels above 20 μg/kg. There are no comparable results in literature, because of the high detection limits of the methods described and very scarce data about the presence of STC in grains. However, reported results indicate a possible health risk for consumers due to the ingestion of contaminated grain products. Because STC has been found in wheat grains, there is the possibility that it can be transferred to wheat derived products such as bread, crackers, cookies, etc. So, all of the above indicates yhe importance of the determination of STC in typical Latvian grains, as they are basic food products.

Another study by Versilovskis *et al*., (2008b) showed a low contamination of Latvian beer, where STC was found in only 2 of the 26 analysed samples (4 and 7.8 μg/l)

Cheese

In the last thirty years few studies on the occurrence and stability of STC in cheese has been carried out. Northolt *et al*. (1980) found that nine of 39 cheeses were positive for STC. These positive samples were obtained from different cheese categories with levels ranging from 5 to 600 μg/kg. Higher levels up to 9000 μg/kg were found in Gouda and Edam cheese scrapings (Northolt and Van Egmond, 1982). Retail samples of Edam Cake and Moravian Block cheeses contained low levels of STC in a Czechoslovakian survey (Bartos and Matyas, 1982). STC was usually detected in Dutch hard cheeses like Gouda or Edam (Van Egmond *et al*., 1982).

The occurrence of STC was also studied in Ras cheese in Egypt: 35% of the samples were found positive with a mean concentration of 22 μg/kg (Abd Alla *et al*., 1996).

Versilovkis *et al*., (2009) analysed different cheese samples (from soft to hard), produced in Latvia and Belgium, for their STC content. They found that most of the hard cheeses did not contain any typical moulds producing STC, so contamination could only be possible during cheese making or cheese storage if they are stored at temperatures above 7°C. Also, contamination of camembert-type cheese is only possible during cheesemaking and storage, because *Penicillium camemberti* does not produce either spores or STC. But in one Latvian camembert type cheese sample STC was detected (0.03 μg/kg), probably due to contamination during cheese-making or during storage. Higher concentrations were found in two Belgian soft cheeses (1.23 and 0.52 μg/kg). These results showed that a risk of cheese contamination with STC exists.

Distribution and stability of STC in cheese

Migration studies of STC in Wilstermarsch cheese after inoculation with *A. nidulans* and incubation at 15°C or room temperature for 40 days was limited to 0.5 cm from the cheese surface (Engel and Teuber, 1980). Similar results were obtained with 6 naturally contaminated Gouda cheeses on which *A. versicolor* was growing (Van Egmond *et al*., 1982).

Van Egmond and Paulsch (1986) checked the stability of STC in cheese and it was found to be stable for 3 months at freezer, refrigerator and warehouse temperatures. It was also shown that STC occurs in the outer layer of hard cheeses to a depth not further than ca 6 mm and that STC is distributed very unequally over the cheese surface. It appeared that the concentration of STC decreased rapidly from the outside to the inside.

Abd Alla *et al*., (1996) studied the distribution and stability of STC in Ras cheese in Egypt; when it was contaminated with spores of *A. versicolor*, toxin production started after 45 days of ripening, reaching a maximum after 90 days. Aged cheese (more than 6 months) inhibited toxin production.

Distribution studies need to be continued with more cheeses of various age categories, with the aim of providing sufficient data to design adequate sampling procedures for eventual regulatory purposes.

Table 3. Results of surveys for STC showing concentrations and distribution of contamination in food and feed commodities.

Country	Commodity	Year	N°. of samples	LOQ (μg/kg) or (μg/l)	*n* > LOQ	Mean (μg/kg)	Min/Max (μg/kg)	References	Sampling procedure
Feed									
Canada	Wheat	1968	29	na	1	na	300	Scott *et al*., 1972	na
UK	Cattle feed	na	na	na	na	na	100	Shreeve *et al*., 1975	na
Poland	Cattle feed	na	na	na	na	na	100	Juszkiewicz and Piskorska Pliszczynska, 1976	na
India	Maize	na	50	na	na	na	na	Devi and Polasa, 1982	na
Canada	Crumbled commercial feed	1979-1982	51	na	na	na	na	Abramson *et al*., 1983	450 g samples of suspect feed mainly associated with toxic disease symptoms, collected from provincial veterinary service laboratories
UK	Cereals	1976-1979	523	na	17	na	na	Buckle, 1983	na
	Hay		157	na	1	na	na		
Czechoslovakia	grain samples (wheat, barley, maize, oats and rye)	1980-1981	74	na	5	na	na/400	Bartos and Mathias, 1983	na
Hungary	Feed sample	1977-1982	7345	na	na	na	na	Sandor, 1984	na
USA	Dairy cattle feed	1983	1	na	na	na	na	Vesonder and Horn, 1985	feed associated with acute clinical symptoms of bloody diarrhea and death in dairy cattle
UK	Barley	1992	45	15[a]	0	-	-	Scudamore *et al*., 1997	Samples taken by sampling spears. Three incremental samples, mixed, quartered to obtain a final sample of 1 kg.
	Wheat		50	15[a]	1	-	18		
	Maize gluten, maize products		50	40[a]	0	-	-		
	Rice bran		40	20[a]	0	-	-		
	Cottonseed		21	40[a]	0	-	-		
	Sunflower		20	20[a]	0	-	-		
	Palm products		15	40[a]	0	-	-		
	Soya		20	20[a]	0	-	-		
	Dried peas and beans		15	20[a]	0	-	-		
Egypt	Silage	na	40	na	2	na	na	El-Shanawany *et al*., 2005	Samples of 500 g collected from farms, mills and retail markets

Country	Commodity	Year	N°. of samples	LOQ (μg/kg) or (μg/l)	*n* > LOQ	Mean (μg/kg)	Min/Max (μg/kg)	References	Sampling procedure
Food									
South Africa	Coffee bean	na	2	na	1	-	1143	Purchase and Pretorius, 1973	
Italy	Green coffe bean	na	502	na	1	-	1200	DePalo *et al*., 1977	
The Netherlands	Gouda, Edam cheese (surface layers)	na	39	na	9	na	na/600	Northolt *et al*., 1980	na
UK	Maize	na	29	na	2	na	na	Ministry of Agriculture, Fisheries and Food, UK, 1980	na
	Flake maize	na	2	na	1	na	na		
	Corn flour	na	13	na	0	na	na		
The Netherlands	Gouda cheese (surface layers)	na	na	na	6	na	na	Van Egmond *et al*., 1982	na
The Netherlands	Gouda, Edam cheese (scrapings)	na	67	na	11	na	na/9000	Northolt and Van Egmond,1982	na
Czechoslovakia	Hard cheeses	1981-1982	66	na	3	na	na/17.5	Bartos and Mathias, 1982	na
Egypt	Spices	na	120	na	10	na	na/23	El Kady *et al*., 1995	na
Egypt	Ras cheese	na	100	na	35	na	na/62.8	Abd Alla *et al*., 1996	Hard cheese rind (1 cm thick layer) collected from local markets
India	Rice	na	30	na	3	na	na/157	Weidenboerner, 2000	na
Japan	Rice	na	na	na	2	na	na/450		
Japan	Rice (moldy sample)	na	na	na		na	na/4300		
Japan	Rice	na	37	na	12	na	na/16300		
UK	Rice (chinese)	na	4		1	na	49		
Latvia	Beer	na	26	0.68	2	na	<0.68/7.8	Versilovskis *et al*. 2008a	na
Latvia	Grain samples (Wheat, barley, oat, buckwheat, rye)	2006	95	0.30 (wheat)	13	na	<0.30/83	Versilovskis *et al*. 2008b	na
	Grain samples (Wheat, barley, oat, buckwheat, rye)	2007	120	0.30 (wheat)	42	na	<0.30/47		
Latvia	Cheese	2008	8	0.1 (0.03[a])	0	na		Versilovskis *et al*. 2009	na
Belgium	Cheese	2008	13	0.1 (0.03[a])	2	na	0.52/1.23		

[a] : limit of detection
na : not available
n: number of samples

MITIGATION OF STERYGMATOCTSTIN

There are no examples in literature of mitigation practices targeted at controlling *A. nidulans* and *A. versicolor* and STC production in cereals, but there are a lot of strategies to limit the contamination of AFs in food and feed. Since STC is an AF precursor, it is possible to look at the mitigation practises used against AFs as useful tools also for STC reduction and control.

Regarding cheese contamination, an improvement in hygienic conditions can prevent the formation of moulds and possibly STC production; nevertheless, it has to be taken into account that the toxin will be concentrated in the surface layer of the product, which mostly will be cut away before consumption (Schuddeboom, 1983)

The use of resistant cultivars

No examples are available in literature. However, antifungal enzymes, such as chitinase and β-1,3-glucanase (Roberts and Selitrennikoff, 1986; Nelson *et al*., 1969; Gnanamanickam, 2002), have been found in a number of plant seeds, and they may act as a defense against pathogenic fungi, since chitin and glucan are major polymeric components of many fungal cell walls. Such polysaccharides in the fungal cell wall may be enzymically hydrolysed into smaller products resulting in damage or the killing of fungal mycelia or spores. It is foreseen that seeds rich in such antifungal enzymes likely resist the infestation of fungi.

Chemical and biological control

There are no examples of chemical or biological control against *A. nidulans* and *A. versicolor* in literature, but some results from studies on AF mitigation practices can be taken into account.

Interesting results have recently beenreported on the application of certain fungicides and non conventional chemicals against *Aspergillus* spp and AF contamination in rice (Reddy *et al*., 2008), but most efforts have regarded the competitive exclusion of toxigenic strains of *A. flavus* by atoxigenic strain (Cotty *et al*., 2008). The greatest successes to date have been achieved with this approach to reduce AF contamination in cotton (Cotty, 1994), peanuts (Dorner *et al*., 1998; Dorner, 2004, 2008; Pitt and Hocking, 2006) and more recently in maize (Abbas *et al*., 2006). Two products of nontoxigenic strains have received U.S. Environmental Protection Agency (EPA) registration as biopesticides to control AF contamination in cotton and peanuts in several states of the USA (Dorner, 2004).The selected strains could be tested against *A. nidulans* and *A. versicolor*, but specific trials are needed. Nevertheless, literature suggests that biocontrol is more interesting than chemical control when aspergilli are the target fungi.

Kotan and colleagues (2009) found that the cell suspensions and/or culture filtrates of 4 strains of *Pantoea agglomerans* (RK-80, RK-86, RK-153 and RK-160), 2 of *Achromobacter piechaudii* (RK-137 and RK-157), one of *Bacillus subtilis* (RK-6), 1 of *Burkholderia cepacia* (RK-277), 1 of *Erwinia rhapontici* (RK-135) and one of *Erwinia chrysanthemi* (RK-67) may be useful as potential biocontrol agents against *A. flavus*.

Several bacterial species, such as *Bacillus subtilis*, *Lactobacilli* spp., *Pseudomonas* spp., *Ralstonia* spp. and *Burkholderia* spp., have shown the ability to inhibit fungal growth and production of AFs by *Aspergillus* spp. in laboratory experiments. Palumbo *et al.* (2006) reported that a number of *Bacillus*, *Pseudomonas*, *Ralstonia* and *Burkholderia* strains isolated from California almond samples could completely inhibit *A. flavus* growth. Several strains of *B. subtilis* and *Pseudomonas solanacearum* isolated from the non-rhizophere of maize soil were also able to inhibit AF accumulation (Nesci *et al*., 2005). In most cases, although these strains were highly effective against AF production and fungal growth under laboratory conditions, they do not have good efficacy in field trials because it is difficult to bring the bacterial cells to the *Aspergillus* infection sites on commodities under field conditions (Dorner, 2004).

Some saprophytic yeast species (such as *Candida krusei* and *Pichia anomala*) have shown promise as biocontrol agents against *A. flavus*. Similar to bacterial agents, these yeast strains were able to greatly inhibit *Aspergillus* growth in laboratory conditions (Hua *et al*., 1999; Masoud and Kaltoft, 2006). Although they were considered to be potential biocontrol agents for the management of AFs, further field experiments are necessary to test their efficacy in reducing AF contamination under field conditions.

Decontamination

There are no specific studies related to the decontamination and detoxification of STC. A number of physical methods including extraction, adsorption, irradiation, heat treatment, ultraviolet light, ozone gas, solar irradiation and gamma rays can be used to reduce mycotoxin contamination (Ruston, 1997). Physically, fungi-contaminated seeds can be removed by hand picking or photoelectric detecting machines. These methods are time-consuming and expensive.

Many common chemicals have been used to test the effectiveness in detoxification of AF (Table 4). The chemical reactions of detoxification of AF are primary addition of the double bond of the furan ring and oxidation involving phenol formation and opening of the lactone ring. In the presence of acid, AFB and AFG will be converted into their 2-hydroxy derivatives.

STC, similarly to AF, has a lactone group in the molecule that can be similarly destroyed by alkaline condition using ammonia, sodium hydroxide and sodium bicarbonate.

Organic solvents (chloroform, acetone, hexane and methanol) have been used to extract AFs for agricultural products, but mainly in vegetable oil refining processes (Vorster, 1985).

Certain conditions such as moisture content, heat, ultraviolet or gamma irradiation, sunlight and pressure at different treatment-periods have been simultaneously combined with chemicals for the enhancement of detoxification.

Inactivation methods can be achieved by mixing, packing, fumigation and immersion with the chemical used.

Table 4. Chemicals tested on aflatoxin detoxification.

Chemical	**References**
acetic acid	(Pons *et al*., 1981)
ammonia gas or NH_4OH	(Brekke *et al*., 1977; Moerch *et al*., 1980)
ammonium salts, 3-5%	(Brekke *et al*., 1977)
calcium hydroxide	(Codifier *et al*., 1976)
formaldehyde	(Codifier *et al*., 1976; Mann *et al*., 1970)
hydrogen peroxide	(Spreenivasamurthy *et al*., 1967)
methylamine	(Park *et al*., 1983)
ozone gas	(Dwaratanath *et al*., 1968)
phosphoric acid	(Mann *et al*., 1970)
phosphine gas	-
sodium bicarbonate	(Mashaly *et al*., 1983)
sodium bisulfite	(Moerch *et al*., 1980)
sodium bisulfite	(Moerch *et al*., 1980; Mashaly *et al*., 1983)
sodium hypochlorite	(Yang, 1972)

Fate of STC during food processing

There are no specific studies related to the fate of STC during food processing, but there are several studies about the fate of AFs.

Heating and cooking under pressure can destroy nearly 70% of AF in rice while under atmospheric pressure only 50% are destroyed (Coomes *et al*., 1966). Dry and oil roastings can reduce about 50-70% of AFB_1 (Feuell, 1966). Only about 10% of the total 1242 μg/kg of AFB_1 decreased in naturally contaminated peanut by heating at up to 100°C (Wangjaisuk,

1989). Since AF resist higher temperatures up to 260°C, long-time cooking and overheating would destroy essential vitamins and amino acids in treated foods.

Ionizing radiation such as γ-rays can stop the growth of food spoilage organisms, including bacteria, moulds and yeasts. It also inactivates pathogenic organisms including parasitic worms and insect pests. It has been reported that gamma-irradiation (5-10 M-rad) caused reduction of AF (Sommer and Fortlage, 1969). The irradiation, however, could not completely destroy the toxin, in r laboratory, only about 30% of the total 600 ppb at AFB_1, either pure toxin or in contaminated peanut, was destroyed by 1 and 5 Mrad or gamma irradiation (Chipley and Uraih, 1980). The treatment combination of γ irradiation and ammoniation should be therefore attempted for more AF decontamination.

PHARMACOKINETICS

Absorption and Distribution

Exposure to STC may occur via enteric, inhalation, or direct contact to skin and mucosa (Anyanwu, 2008).

Owing to the insolubility of the toxin, experimental doses given to animals are absorbed to a small extent only. Sterigmatocystin can be administered by the oral, intraperitoneal or subcutaneous routes (Sreemannarayana *et al*., 1988; IARC, 1972; Ueda *et al*., 1984). In a study of Sreemannarayana *et al*., (1988) STG was administered by intra-abdominal injections (0.5 and 0.7 mg per injection). Ueda *et al*., (1984) administrated this mycotoxin by intraperitoneal injection of 10^{-1} mM (31.2 mg)/kg body weight of STG .

A study indicated that the volume of distribution of STC shows age and sex differences (Walkow *et al*., 1985). Oral administration to immature (50-150 g) and mature (200-300 g) rats of both sexes of ^{14}C-STC (8 mg/kg/13 days in feed and on the 14th day was orally intubated into the rats) resulted in plasma levels in male rats which were consistently higher than those found in the females.

Another study described the distribution of labelled STC in rats (Wang *et al*., 1991). The highest concentration of radioactivity in serum appeared 3 h after administration of 0.5 microCurie/g bw. The half-life of distribution was 0.51 h. Radioactivity was concentrated mainly in the liver, stomach, kidney, duodenum and lung, and to a lesser extent in fat, muscle, testis, rectum and bone.

Metabolism

Sterigmatocystin is a compound in which a substituted anthraquinone is fused to the bisdihydrofuran ring. An unsaturated 2, 3 bond in the bis-dihydrofuran ring is closely related

to the biological activities of STC (Terao, 1983). Sterigmatocystin is one of the late intermediates in AF biosynthesis (Hsieh *et al*., 1973; Beasley, 1999; Singh and Hsieh, 1976; Barnes *et al*., 1994). In contrast to the considerable literature on AFB_1, little information has been published on the metabolism and biochemical effects of STC.

Like AFB_1, STC has been found to be metabolized by the cytochrome P4503A4 (Yamazaki *et al*., 1995) system to an active epoxide, which can then interact with nucleic acids to form a DNA-adduct at the N^7-guanyl position in the target cells (Essigmann *et al*., 1979, 1980, 1982; Terao, 1983).

AFB_1 has been shown to be 10 times more potent than STC in inducing tumors in Fischer rats (Olson *et al*., 1993 a). Data on the amount of DNA adducts formed at certain doses are consistent with the carcinogenic potential of both mycotoxins. For example, Fischer rats administered 0.6 mg of AFB_1/kg had the same adduct levels as rats given 6 mg of STC/kg. Thus, rats need a STC dose 10-fold higher that of AFB_1 to form the same amount of DNA adduct.

Sterigmatocystin has a phenolic group for direct conjugation; in contrast, AFB_1 must be first hydroxylated before conjugation. Thus, STC may be more efficiently conjugated and eliminated than AFB_1 and prevent much of the STC from forming an active epoxide (Olson *et al*., 1993 a).

By chemical synthesis of DNA adducts via an epoxide generated by peroxidative activation, STC produced about 10-fold more adducts than AFB_1, suggesting that STC readily forms the epoxide and is just as reactive if not more than the AFB_1 epoxide. Since the reactivity of the STC epoxide is not less than that of AFB_1 epoxide, other factors must account for the greater toxicity of AFB_1 (Olson *et al*., 1993 a).

A paper by Essigmann *et al*. (1979) indicates that the stereochemistry of the STC epoxide is identical to that of AFB_1 and that a major target for both epoxides is the N7 atom of guanine, which is located in the major groove of DNA in a position apparently readily accessible to attack by bulky chemicals. Moreover, it is conceivable that the toxicological similarities and differences between STC and AFB_1 may be attributed to their mechanisms of epoxidation and interaction with DNA. A number of reasonable mechanisms can be proposed. In rat liver, for example, the toxic and tumor-producing potency of STC is much lower than that of AFB_1. It is possible that the ultimately carcinogenic form of STC, conceivably the epoxide, is formed in rat liver in lower amounts than that of AFB_1 or that it reacts more readily with other cellular nucleophiles (e.g., glutathione) to produce detoxification products. Alternatively, differences in potency might be attributable to differences in the relative intragenomic distribution of binding by STC and AFB_1, determined perhaps by structural characteristics that might limit

the access of one or the other to specific areas of the genome. It is also possible that structurally similar DNA adducts of STC and AFB_1 have different efficiencies in initiating carcinogenic events, with AFB_1 generally being more effective than STC in this regard. These carcinogens may also differ in the kinetic features of adduct removal and repair of their respective modified DNAs, as has been shown to be the case with DNAs modified in vivo with alkylating agents. Finally, it cannot be overlooked that STC and AFB_1 possibly differ in toxicological properties by virtue of quantitatively minor interactions with DNA, because of interactions with other cellular components by as yet unexplained mechanisms or simply because of differences in the relative distribution of the toxins in various organs after administration (Essigmann *et al*., 1979).

Excretion

Very little is known about the metabolic pathways that lead to the elimination of STC.

After oral administration of ^{14}C-labeled STC(100 mg, 0.145 microCurie) to a male vervet monkeys (5.43 Kg), a major urinary metabolite with a glucuronide conjugated at the phenolic group of STC was isolated; 50% of the recovered radioactivity in the urine (collected 40 h after dosage) was due to this metabolite (Thiel and Steyn, 1973).

In a study, a linear dose-response relationship for the elimination of STC in rats injected with 1-16 mg of STC/kg of body weight (bw) was observed (Olson *et al*., 1993 b). Approximately 3% of the total STC administered was eliminated in the first 24 h. This value is considerably lower than the data obtained by Walkow *et al*. (1985), when ^{14}C-labeled STC was used (8 mg/kg/13 days, administered in the feed); they found as much as 7.5 % of the total radioactivity in the first 24-h urine sample.

Wang *et al*., (1991) described the excretion of radioactively labelled STC in rats after administration of 0.5 microCurie/g bw. This study showed that the half-life of excretion was 43.9 h and by 48 h, 56.4% had been excreted in faeces and 20.1% in urine. Biliary excretion may be the major route of excretion of STC.

It has been shown (Beasley, 1999; Essigmann *et al*., 1979) that a large portion of the dose administered orally to monkeys is converted to a STC-glucoronide and that metabolic activation is required for the toxicity and mutagenicity of STC in bacteria and some cultured cells.

Since STC-glucuronide was found to be the only urine metabolite, the UDP-glucuronide transferase may play a major role in metabolization and detoxification of STC.

Carry over

The presence of STC in many foods leads to the assumption that it might be found in milk and milk products like, for instance, AFM_1. Model experiments did not yield a final conclusion, althought, the transfer into cheese curd seems possible. When 100 ml milk containing 100 ng STC was coagulated with 10-50 mg of rennet at 20-45 °C, or with citric or lactic acid at pH 4.2-4.8, virtually all (89.3-99.1%) of the STC was found in the curd and none in the whey, due to its higher water-insolubility and its increased protein binding. Further experiments with more precise methods seem to be needed (Kiermeier and Kraus, 1980).

TOXICITY

The toxic effects of STC-fed laboratory animals have included kidney and liver damage and diarrhoea. Skin and hepatic tumours are induced in rats by dermal application. Cattle exhibit bloody diarrhoea, loss of milk production and in some cases death (Vesonder and Horn, 1985). Sterigmatocystin is considered to be a potent carcinogen, mutagen and teratogen. In fact this biogenic precursor to AF is hepatotoxic and hepatocarcinogenic, but is less common than AF.

It has been proved that STC forms guanyl DNA adduct in the liver of animals (Essigmann *et al*., 1979, 1980, 1982; Terao, 1983). During the process of biotransformation to form the guanyl adduct, an enhanced production of reactive oxygen species (ROS) is reported (Heinonen *et al*., 1996). Free radical damage initially induced by mycotoxins can be propagated and magnified by lipid peroxidation chain reactions (Atroshi *et al*., 2000). Lipid peroxidation is one of the factors responsible for the damage and necrosis of the liver induced by mycotoxins (Souza *et al*., 1999). Lipid peroxidation can be considered as a secondary mechanism of STC toxicity (Sivakumar *et al*., 2001).

Mycotoxicosis should be suspected when the history, signs, and lesions are suggestive of feed intoxication. Chronic or intermittent exposure can occur in regions where grain and feed ingredients are of poor quality, and feed storage is substandard or prolonged. Impaired production can be an important clue to a mycotoxin problem, as can improvement due to correction of feed management deficiencies.

Acute toxicity

Sterigmatocystin has a low acute toxicity due to its poor absorption rate from the digestive tracts (Terao, 1983).

Abdelhamid *et al.,* (1988) described the toxicity symptoms of dietary contamination with STC in catfish and common carp, since STC naturally contaminates grains and feeds. Polluted

diets caused a gradual decrease in growth rate as well as in muscular protein content and a gradual increase in mortality, serum transaminases activity and muscular dry matter and ether extract contents in addition to some pathological findings in carp in proportion to the dietary levels of STC. The LD_{50} was estimated to be as 211 μg/kg STC in carp diet. Three months feeding of catfish on STC (250 μg/kg) led to loss of body weight, increased mortality rate and muscular contents of ether extract, decrease of muscular content of protein as well as to some pathological findings in addition to the presence of residual STC in the fish muscles.

Beasley (1999) reported that the LD_{50} of STC administered intraperitoneal to albino rats were 60 to 65 mg/kg. In another study (Purchase and Van Der Watt, 1969) the oral LD_{50} in rats was 120-166 mg/kg, but part of this toxicity may be attributable to dimethylformamide used as solvent. When STC was dissolved in dimethylsulfoxide, the LD_{50} (intraperitoneal) in vervet monkeys was 32 mg/kg (Harwig and Munro, 1975; Hsieh and Atkinson, 1991).

Chronic toxicity

Animals

Symptoms after chronic STC exposure include the induction of hepatomas in rats, pulmonary tumours in mice, renal lesions and alterations in the liver and kidneys of African Green monkeys.

In a chronic study 224 newborn mice were separated into 4 groups. Animals in groups 1, 2, and 3 received an injection of 0.03 ml suspension containing 5, 1, or 0.5 μg/g bw of STC. Group 4, the control, received 0.03 ml of 1% gelatin solution. A single injection gave rise to high incidences of lung and liver adenomas when the animals were killed at 1 year old. The incidence of both tumors in mice at the dose of 5 μg/g bw was statistically significant, and the incidences of lung tumor in female mice and of liver tumor in male mice at the dose of 1 μg/g bw were also statistically significant, compared with tumors in control mice. Other tumors (two malignant lymphomas and one adenoma of the submaxillary gland) were also induced to STC in mice (Fujii *et al*., 1976).

Oral dosing of monkeys with 20 mg/kg bw once each fortnight for 4 to 6 months resulted in chronic hepatitis; after a 12-month exposure, hyperplastic liver nodules were observed (Engelhart *et al*., 2002)

A study (Vesonder and Horn, 1985) described STC (7.75 μg/g of feed) detection in dairy cattle feed. The researcher reported that cattle eating feed with STC exhibited bloody diarrhea, with subsequent loss of milk production and death in some cases (no data are

avaible about time frames). When the dairy herd was fed a different ration, these symptoms disappeared.

Humans

No data are available regarding humans.

Genotoxicity/carcinogenicity/cytotoxicity

In spite of its low acute toxicity, STC is a potent mutagen as well as a carcinogen.

Sterigmatocystin is classified by IARC in group 2B (possibly carcinogenic to humans). Sterigmatocystin has the potential to cause human liver cancer and its carcinogenicity has been demonstrated with organ specificity varying with species, and the route and frequency of administration. Sterigmatocystin induced malignant tumors not only in the target organs, but also at the site where the mycotoxin was applied (Terao, 1983). As a hepatocarcinogen, stenigmatocystin is about 150 times less potent than AFB_1. However, it is at least 10 times more potent than the other well-known hepatocarcinogens (Terao, 1983).

Carcinogenicity requires the presence of the double bond at the 1,2 position and the activated species is presumed to be the 1,2 epoxide of STC. Various studies (Essigmann *et al*., 1979; 1980; 1982; Terao, 1983) postulated an intermediate cationic adduct at the N7 of guanine to be the primary adduct with DNA. The epoxide has not been isolated. However, a laboratory of Columbia University has successfully chemically synthesised the epoxide of AFB_1 and showed that it can bind DNA without metabolic activation to form the N-guanine adduct previously identified from biological systems (Baertschi *et al*., 1988).

As for its carcinogenicity, the target organ of STC in rats is the liver, and those in mice are the lung and blood capillaries. Sterigmatocystin induced malignant tumors not only in the target organs, but also at the site where the mycotoxin was applied (Terao, 1983)

Sterigmatocystin is carcinogenic in mice and rats following oral administration. Oral administration to mice and rats at doses of 0.15 to 2.25 mg/animal/day produced pulmonary adenomas in mice and hepatocellular carcinomas in rats. Skin papillomas and carcinomas have been reported in rats given skin applications of 1 mg STC in dimethylsulphoxide or acetone. Twice weekly subcutaneous administration of 0.5 mg in arachis oil for 24 weeks resulted in the development of local sarcomas and hepatomas in rats (Fujii *et al*., 1976). Levels of STC as low as 15 µg/day fed continuously, or a single 10 mg dose, caused liver cancer in 30% or more of male Wistar rats (Terao, 1983). Incorporation in the diet of rats to provide a dose of 0.15-2.25 mg/day results in hepatocellular carcinoma (Harwing and Munro, 1975). Sterigmatocystin also produced lung tumours in mice (Fujii *et al*, 1976).

Sterigmatocystin was tested for its carcinogenic effects on rat skin by Purchase and Van der Watt (1973). The shaved skin of rats was treated with 1 mg of STC in dimethyl sulfoxide or in acetone twice weekly for 70 weeks. At 40 weeks papillomas developed, and by 70 weeks all treated rats had either papillomas or squamous cell carcinomas. In 17 out of the 20 rats treated with STC, liver lesions, consisting of regenerative changes or hepatocellular carcinomas, were present. The observation that STC is carcinogenic to rat skin emphasises the danger of skin contamination to laboratory workers.

A study (Ma *et al*., 2003) examined the effects of *Helicobacter pylori* and STC in male Mongolian gerbils (n=196). Mongolian gerbils were treated with *H. pylori* supernatants (10 ml/1000 mg) mixed with diet or inoculated intragastrically with *H. pylori* alone or with STC (100 or 1000 μg/kg), and then sacrificed 27 months later. *Helicobacter pylori* caused gastritis, ulcer and intestinal metaplasia. Sterigmatocystin enhanced the development of intestinal metaplasia and increased gastrin levels in *H. pylori*-infected Mongolian gerbils.

Sterigmatocystin was reported toxic to *Salmonella-typhimurium* TA-1530 in the presence of bioactivation; frameshift mutations were also induced in this test. In the umu assay (a method which has been used to detect DNA damage), STC showed higher response than AFB_1 (Baertschi *et al*., 1989)

Aflatoxins and related dihydrofurans such as STC are not active, but require oxidation to epoxides in order to react to form covalent bonds with DNA and induce genetic damage (Guengerich and Kim, 1990).

Mammalian cells in culture exposed to STC (2 mg/l of toxin in 0.1% of DMSO for 24 hours) display nucleolar aberrations, inhibited mitosis, inhibited uptake of thymidine and uridine, and stimulated DNA repair synthesis (Engelbrecht and Altenkirk, 1972; Stich and Laishes, 1975). Sterigmatocystin has also been demonstrated to inhibit RNA synthesis in rat liver. Intraperitoneal injection of STC is reported to inhibit the incorporation of orotic acid into liver RNA.

The cytotoxic and mutagenic effects of STC on cultured Chinese hamster cells were investigated by Noda *et al*., (1981) in the presence or absence of a metabolic activation system. Sterigmatocystin directly applied to the cells induced cytotoxicity and drug-resistant mutations in a time- and concentration-dependent manner. Analyses of the effects of STC showed that treatment for shorter periods was more effective than for longer periods. In the presence of an activation system, the cytotoxic and mutagenic effects of STC were strongly enhanced. Analyses by equitoxic comparison showed that there was little difference in mutagenic activity between direct treatment and treatment using an activation system.

Xie *et al*., (2000) indicated that the carcinogenic effects of STC (exposed to cells for 12 or 24 h) seem to be mediated by failure of p53-mediated G1 checkpoint.

The results of a study (Abdel-Wahhab *et al*., 2005) showed that STC was toxic and clastogenic to fish as indicated by the significant decrease of body weight and the increase in frequencies of micronucleated red blood cells and chromosomal aberrations in the kidney. In the study, fish were treated orally with 1.6 µg Stg/kg body weight twice a week for four successive weeks resulting in an overall cumulative dose of 12.8 µg/kg bw

Sterigmatocystin can induce apoptosis of human peripheral blood lymphocytes *in vitro* and may have some negative effects on the human immune system (Sun *et al*., 2002). Sterigmatocystin inhibited the secretion of IL-2 of human peripheral blood mononuclear cells *in vitro* (Huang *et al*., 2002). The inhibiting effects at low concentrations (0.03125-0.125 mg/l) and high concentrations (1-2 mg/l) were stronger than at other concentrations. The time-effect analysis (STC 1 mg/l) showed that the inhibiting effects of STC on IL-2 secretion could be seen to a variable degree from 1 to 64 h after STC treatment.

Because of the lack of a sensitive and specific method for monitoring STC in foods and body fluids and the lack of a systematic epidemiological study, the role of STC in human carcinogenesis is still not known (Olson *et al*. ,1993 b; Essigmann *et al*., 1979).

Despite its potent toxic and carcinogenic properties in animals, the importance of STC as a human health hazard is unknown because surveillance programs have detected its presence in foods only infrequently and at low concentrations even though sterigmatocistyn-producing fungi are widely distributed (Engelhart *et al*., 2002).

LEGISLATION ON STERIGMATOCYSTIN

The Food and Drug Administration continuously follows the development of newer data regarding STC, thereby constantly evaluating the need to set regulatory standards (CAST, 2003).There are no current specific regulations, or mandatory or recommended maximum limits for STC in food and in feed. However, some Eastern European countries did set limits in legislation prior to becoming members of the UE. The Czech Republic set maximum limits of 5 or 20 µg/kg, depending on the nature of the product (Lawley *et al*., 2008). The Czech Republic and Slovakia, have set relatively low maximum levels for STC (e.g., level of 5 µg/kg for rice, vegetables, potatoes, flour, poultry, meat, milk, and of 20 µg/kg for other foods) (Stroka *et al*., 2004). The California Department of Health Services used TD_{50} values from the Cancer Potency Database to produce 'no significant risk' intake levels for humans. The level set was 8 µg/kg bw/day for a 70 kg adult (Lawley *et al*., 2008). The Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment declared that in the

absence of any detectable contamination of foodstuffs there is no evidence of any risk to human health from STC. However, it acknowledged that levels of contamination by any mycotoxin in particular crops may be variable, depending on factors such as climatic conditions during growth and harvesting or environmental conditions during storage. The Food and Drug Administration did not detect STC in an analysis of more than 500 samples of U.S. cereal grains conducted in 1974 to 1975 (Vesonder and Horn, 1985).

CONCLUSION

Sterigmatocystin, produced by the fungi *A. nidulans* and *A. versicolor,* is a toxic metabolite belonging to group 2B of IARC-classification. It is structurally closely related to AFs and consists of a xanthone nucleus attached to a bifuran structure. It has been reported in mouldy grain, wheat, green coffee beans and cheese. Acute and chronic disorders, loss of milk production, teratogenic effects, weight reduction, cancer and even death may develop after exposure to this mycotoxin. There are no legal limits for STC in food and in feed. However, some Eastern European countries did set limits in legislation prior to becoming UE members (5-20 μg/kg).

Several analytical methods have been described in literature, but LC based validated analytical methods for the quantification of STC are needed as a prerequisite for a survey on its occurrence in food.

STC has been detected in cereals, but a risk of cheese contamination exists. An improvement in hygienic conditions can prevent the formation of moulds, even if it has to be taken into account that the toxin will be concentrated in the surface layer of the product, which mostly will be cut away before consumption. Nevertheless, distribution studies need to be managed with cheeses of various age categories, with the aim of providing sufficient data to design adequate sampling procedures for eventual regulatory purposes.

The presence of STC in many foods leads to the assumption that it might be found in milk and milk products, but model experiments did not yield a final conclusion. Further experiments with more precise methods seem to be needed.

FUTURES

- Methods of analysis and current specific regulations, mandatory or recommended maximum limits for STC in food and in feed should be developed.
- The absorption and distribution of STC should be elucidated.
- Epidemiological studies should be performed on the possible effects of no detected levels of STC.

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Chapter 8 - Phomopsins in food and feed

Keywords: Phomopsin, *Phomopsis leptostromiformis*, *Diaporthe woodii*, Lupin, Lupinosis, Methods of analysis, Validation, Toxicity, Mitigation, Resistant cultivars.

INTRODUCTION

Phomopsis leptostromiformis (Kühn) Bubák is considered an important limiting factor to more extensive sowing of lupins. This organism has been reported to cause stem blight in young lupins (*Lupin luteus* L.) (Ostazeki and Wells, 1960) and to produce phomopsins (Culvenor *et al*., 1977). These mycotoxins, a number of compounds capable of binding to tubulin (Tonsing *et al.,* 1984), cause the animal liver disease known as lupinosis (Van Warmelo *et al*., 1970; Gardiner, 1975; Allen and Wood, 1979). The ingestion of a high dosage of toxin causes liver damage which can result in disorientation, blindness, lethargy and eventually death in severe cases. It also results in significant production losses without any other obvious symptoms. Any animals grazing lupin stubble may be affected, but sheep are affected most severely because they are most sensitive to the toxins (Seymour, 2009).

The discovery of ascomata of a *Phomopsis* found growing on lupins stubble in Western Australia led to the teleomorph being described in culture and named as *Diaporthe woodii* Punith. (Punithalingam, 1974; Punithalingam ans Gibson, 1975).

PHOMOPSIS LEPTOSTROMIFORMIS AND ITS HOSTS

Phomopsis leptostromiformis (Kühn) Bubàk ((Ainsworth *et al*., 1973; Punithalingam and Gibson, 1975)) is the anamorph of *Diaporthe woodii* Punith (Punithalingam and Gibson, 1975). The anamorph produces both alpha and beta conidia (van Jaarsveld and Knox-Davies, 1974a). The teleomorph is homotallic (Punithalingam and Gibson, 1975) and occurs naturally on infected lupin stubble in Western Australia (Wood and Brown, 1975), but has not yet been reported from other countries where only the *Phomopsis* state is known. It is not known whether this is due to differences in strains of the fungus, or to an environmental effect. *Phomopsis* has been recorded on lupins in Denmark (Lind, 1913), Poland (Kochman, 1957; Pape, 1927), Russia (Strukinskas, 1971) South Africa (Van Warmelo *et al*., 1970), USA (Ostazeski and Wells, 1960) and Australia (Ali *et al*., 1982; Clarke and Kellock, 1979; Gardiner and Petterson, 1972; Stovold *et al*., 1983-84; Wood *et al*., 1973; Wood and Brown, 1975).

The first report of the fungus *D. woodii* on a *Lupinus* species was on *L. luteus* L in Germany by Khün in 1880 (Fischer, 1893).

The disease was first found in Poland on yellow lupine plants early in the twentieth century by Kochman (1957). He described only the stromatic conidiomatal stage of the fungus, called pycnidium on infected stems (Marcinkowska, 2003). The perfect stage was described by Punithalingam and Gibson (1975) who named the teleomorph *D. woodii.*

Diaporthe woodii has been described as a stem pathogen, infecting *L. luteus* (Kochman, 1957; Ostazeski and Wells, 1960), *L. cosentini* (Wood *et al*., 1975) and *L. angustifolius* (Wood and Brown, 1975). It has also been reported to infect pods and seeds of *L. albus* (Van Warmelo *et al*., 1970; Wood and Petterson., 1985) and of *L. angustifolius* (Ali *et al*., 1982; Clarke and Kellock, 1979; Petterson and Wood, 1986; Wood and Brown, 1975; Wood and Petterson, 1985, 1986). Leaf infection by *D. woodii* has also been reported in Western Australia (Brown, 1984; Wood and Hamblin, 1981) even if lesions have only rarely been observed on lupin leaves during the growing season.

Diaporthe woodii has also been isolated from lupin roots (Punithalingam and Gibson, 1975). In Western Australia, however, during the course of investigations of fungi associated with lupin root rots, it has rarely been detected on *L. angustifolius* (Wood and Sivasithamparam, 1989).

In Australia, seed infection of *L. angustifolius* by *D. woodii* is widespred. Levels of infection of between 2% and 12% have been recorded in Victoria (Clarke and Kellock, 1979) and up to 20% in South Australia (Ali *et al*., 1982). The pathogen was also found to be seed-borne in white and yellow lupines (Ostazeski and Wells, 1960; Strukinskas, 1971) as well as in seeds of *L. angustifolius* in Poland (Nowicki, 1995).

More recently, in the 2007 growing season, elongated whitish-greyish patches covered with black stromatic conidiomata were visible on lower stem parts above the soil on some yellow lupine plants in Sulejów (Marcinkovska, 2007). Mycological study of the conidiomata revealed that *P. leptostromiformis* was responsible for the disease. Identification was performed according to the description of Punithalingam and Gibson (1975) and Marcinkowska (2003). Infected plants occurred in a post-registration experiment. The experiment covered 10 lupine genotypes which were tested for their susceptibility to *Fusarium* wilt pathogen. Sometimes on blighted stems, besides patches with conidiomata characteristic of *P. leptostromiformis*, also sporodochia of *F. oxysporum* f.sp. *lupini* were found. Reappearance of the fungus in yellow lupine fields, especially on new Polish cultivars, might be a threat of *P. leptostromiformis* epidemic occurrence. On the other hand, the presence of the fungus on same plants, as *F. oxysporum* f.sp. *lupini,* seems to be very interesting. Further studies on the relationship between both fungi, the causing agents of lupine blight, are recommended.

Infection cycle

Diaporthe woodii remains viable in woody stem fragments for at least two years (Wood and McLean, 1982). Stromatal development commences soon after the appearance of stem lesions (Wood and Brown, 1975). These then differentiate into pycnidia and ascostromata.

In a field study managed in 1985 spore traps were used to study spore dispersal. Alpha conidia, capable of infecting lupin seedlings, were first detected in June (early winter) and continued to be liberated from the infected stubble of the previous season until November (late spring); ascospores were similarly released over the period July to November (Wood, 1986). Stylospores or beta conidia of *P. leptostromiformis* have previously only been reported from South Africa (Van Jaarsveld and Knox-Davies, 1974a) from lupin stems incubated in the laboratory. However in Western Australia they were detected in field on lupin stems from the previous season as early as February (late summer) and continued to be released until August (Wood, 1986). It was shown that inocula of alpha and beta conidia and ascospores were all capable of infecting lupin seedlings in glasshouse experiments, even if Wood (1986) concluded that ascospore inoculum is important only in long distance spread of the fungus.

In Western Australia, the appearance of beta conidial inoculum in the field on infected lupin trash from the previous season is relevant for the survival of the fungus. As self-sown seedlings are commonly found in summer following rainfall from tropical cyclonic disturbances, beta conidia from lupin trash, either in the same paddock, or nearby, are then available to infect these seedlings.

Leaves of *L. angustifolius* seedlings have occasionally been observed in the field to have symptoms of infection by the anamorph *P. leptostromiformis* (Wood and Hamblin, 1981). In Western Australia, symptoms of *D. woodii* infection in lupin stems do not usually appear until after harvest. Brown (1984) found defoliated leaves of seedlings with advanced pycnidial development. As already noted, however, defoliation of a healthy crop does not commence until just prior to senescence. The role of leaf infection as a mechanism of secondary spread of the fungus within a crop is therefore not considered to be important.

Ecology

Phomopsis leptostromiformis can be readily isolated and cultured on either 1.5% malt extract agar with 100 μg/l of sodium novobiocin (Ali *et al*., 1982; Van Warmelo *et al*., 1970) or potato marmite dextrose agar (Wood *et al*., 1975; Wood and Petterson, 1985). On both media, a dense white surface mycelium is formed with irregular stromatic masses developing in the myceliar layer after three weeks on a laboratory bench at 25°C. The fungus can also

be readily cultured on autoclaved lupin seeds, rape, sorghum, vetch, linseed and runner beans (Wood *et al*., 1978) although in nature it has only been recorded on lupins. It can also be grown on liquid media (Lanigan *et al*., 1979).

Diaporthe woodii can be easily isolated from seeds and stems of *L. angustifolius*. Its saprobic nature is evident in the facility with which it can be isolated as a pure culture from infested stubble. In Western Australia the anamorph *P. leptostromiformis* can be separated into two distinct morphological types designated biotype A and B (Wood, 1986). Biotype A is isolated in over 95% of cases, and invariably produces toxin in artificial flask culture on sterilized wheat seed. Cultures grown on Oxoid® potato marmite dextrose agar (PMDA) at 25°C form floccose mycelium with little pigmentation. Stromata form profusely on the agar surface within 28 days. In contrast, biotype B isolates form a pigmented adpressed mycelial mat on PMDA with only sparse stromatal formation (Wood, 1986). None of the biotype B isolates studied has produced toxin in artificial flask cultures.

Gardiner (1966) postulated the presence of an essential substrate in the lupin plant which was necessary for toxin production by the fungus. Wood *et al*. (1978) however, produced the toxin by culturing the fungus on a wide range of substrates. It also grows and produces toxin on a range of liquid media (Lanigan *et al*., 1979). However toxin production on artificial media is temperature sensitive, with little toxin being produced above 28°C or below 22°C, even though there is little effect of temperature on growth within the range 20°C to 30°C (Wood and Sivasithamparam, 1989).

Generally, lupin stubble heavily colonized by *D. woodii* are not toxic until they become moistened from either summer rainfall or a series of heavy dews (Allen *et al*., 1980). If rain falls on the senescing crop just prior to harvest, however, toxicity can be present at harvest (Allen *et al*., 1979). The precise conditions of moisture levels of the stubble, incubation period and field temperature necessary for toxin production are not known and warrant investigation. However after at least 10 mm of heavy soaking rain immediately followed by cloud cover with high relative humidity, toxin production in field stubbles has been known to produce symptoms of lupinosis in sheep in less than 48 hours (Wood and Sivasithamparam, 1989). Generally, once lupin stubble becomes toxic it remains so, regardless of subsequent weather conditions (Allen *et al*., 1979).

Plant-pathogen interaction

Phomopsis stem blight of lupins has many features of a latent disease (Wood and Sivasithamparam, 1989).

Some studies (Kochman, 1957; Van Jaarsveld and Knox-Davies, 1974b) have been conducted into the early stages of infection on various lupin species, without reporting the mode of penetration or the means by which the fungus survives in symptomless plants as a latent infection. Williamson *et al*., (1991) reported for the first time the existence of subcuticular coralloid hyphae in lupin stems infected with *P. leptostromiformis*. Conidia of *P. leptostromiformis* do not form long germ tubes on narrow-leafed lupin stems, but germination is accompanied by the formation of a stain-absorbing swelling at the point of attachment to the cuticle. Penetration of the cuticle occurs directly below conidia through this thickened point of attachment. This unusual behaviour explains in part why previous workers (Kochman, 1957; Van Jaarsveld and Knox-Davies, 1974b) have failed to detect the latent infection structure.

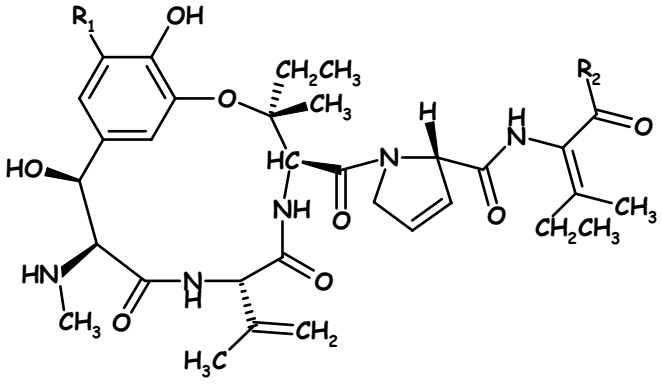
Field observation by Williamson *et al*., (1991) indicated that coralloid hyphae are rare in field-infected plants, but this is not surprising as not all plants show symptoms of Phomopsis stem blight at the end of the growing season, and some environments result in very little disease at all (Cowling *et al*., 1988, 1987; Cowling and Wood, 1989). However, the observations on stubble provided the first evidence that normal colonizing hyphae grow directly from coralloid hyphae and invade the dead stem tissue upon senescence.

PHYSICO-CHEMICAL CHARACTERISTICS

Phomopsins are a family of macrocyclic hexapeptide mycotoxins produced by the fungus *Phomopsis leptostromiformis* (Culvenor *et al.,* 1977; Allen and Hancock, 1989). The chemical structure of the phomopsins A, B and D, identified so far is shown in Figure 1. The compounds, phomopsinamine A and octahydrophomopsin A (phomopsin D), are chemical derivatives of phomopsin A. Both have similar biochemical activity as phomopsin A. A third phomopsin, phomopsin C, has been partially identified but a full structure is not yet available (Edgar, 1991).

Mass spectral and X-ray crystallographic studies have shown that the phomopsins are linear peptides but with a 13-membered ring formed by an ether bridge in place of 2 hydroxyl groups (Edgar *et al.,* 1986, Mackay *et al.,* 1986). Phomopsins contain the unusual amino acid residues 3,4-didehydroproline, 2,3-didehydroisoleucine, 2,3-didehydroaspartic acid, 3,4-didehydrovaline, 3-hydroxyisoleucine and N-methyl-3-(3'-chloro-4',5'-dihydroxyphenyl) serine (Edgar, 1985; Edgar *et al.,* 1985; Edgar *et al.,* 1986)

Phomopsin A is soluble in water above pH 7.5 and below pH 1.0, reasonably soluble in aqueous alcohols, but only sparingly soluble in lipid solvents.



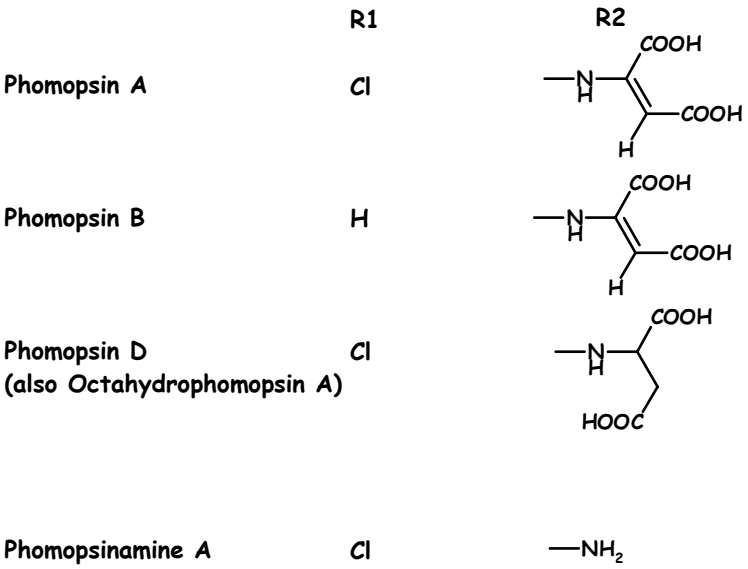


Figure 1. The chemical structure of phomopsins.

BIOSYNTHESIS

Phomopsin A, the main mycotoxin isolated from cultures of *P. leptostromiformis* and the cause of lupinosis disease, is a linear hexapeptide containing 3-hydroxy-L-isoleucine, 3,4-didehydrovaline, N-methyl-3-(3-chloro-4,5-dihydroxyphenyl)serine, E-2,3-didehydroaspartic acid, E-2,3-didehydroisoleucine, and 3,4-didehydro-L-proline.

The sequence of the amino acids was established by heteronuclear 13C{1H} selective population inversion experiments and by fast atom bombardment mass spectrometry of phomopsin A and its derivatives; the structure was also confirmed by X-ray chrystallography (Culvenor *et al*. 1989).

Several studies have been performed in order to investigate the chemical structure of phomopsin A and its derivatives (Culvenor *et al*., 1978, Culvenor *et al*., 1983, Frahn *et al*., 1983, Edgar *et al*., 1985; Edgar *et al*., 1986, Cockrum *et al*., 1994), but no specific investigation has been reported to date about biosynthesis.

To our knowledge, only one study has reported some information about phomopsin analogues biosynthesis (Kobayashi *et al*., 2003). In particular, the authors supposed that these compounds seem to be biogenetically synthesized via a biological Diels–Alder reaction similar to the biosynthesis of solanapyrones and other decalin derivatives.

CHEMICAL ANALYSES

Source of standards

Phomopsin A standards are commercially available (Table 1), while no certified reference materials for method development and validation are available.

Table 1. Sources of phomopsin standards.

Compound	Concentration (μg/ml) or Purity (%)	Quantity	Supplier
Solid standard			
Phomopsin A from *Phomopsis leptostromiformis*	-	5 mg	Sigma-Aldrich
Phomopsin A	98.0%	-	3B scientific Corporation (Illinois, USA)
Phomopsin A	> 98.0%	1 mg/5mg	Bioaustralis (Smithfield, Australia)

Sampling

No specific research has been undertaken about sampling plans for phomopsins in food and feed, a topic which should be faced in the near future.

Extraction and clean-up

Phomopsin extraction from lupin seeds is usually performed by soaking the ground sample in a methanol-water (4:1, v/v) solution overnight at room temperature. After a homogenization step, the paste is stirred for 2 h and the supernatant containing the analytes is separated by centrifugation prior to analysis. No specific clean up systems have been proposed (Reinhard *et al*., 2006).

Analytical methods

Analytical methods used for phomopsin analysis are mainly enzyme-linked immunosorbent assay (ELISA) assays or chromatographic separations. In particular, liquid chromatography with UV detection (HPLC-UV) has been used successfully for these analytes. Usually a gradient elution is obtained using methanol and *o*-phosphoric acid solution as eluents (Reinhard *et al*., 2006). UV detection was performed at 210 nm and 290 nm, since phomopsins exhibit typical UV absorption maxima in this range when methanol is used as a solvent medium (Culvenor *et al*., 1977, Reinhard *et al*. 2006). Electrochemical detection was also proposed as an alternative to UV detection, by using an amperometric analytical cell operating in DC mode (cell potential 600 mV, gain range 100 nA) (Reinhard *et al*., 2006).

Limits of quantification (LOQ) of 200 and 500 μg/kg have been reported for the HPLC detection of phomopsins in lupin grain and stubble respectively (Hancock *et al*., 1987). Confirmatory structural information can be obtained using liquid chromatography – mass spectrometry (LC/MS) methods.

Typical mass spectrometric conditions involve a gradient elution based on methanol and 0.1% formic acid in water as mobile phases. An electrospray ionization (ESI) source operating in positive ion mode is usually used (typical ionization voltage: 5kV). Quantification is based on Multiple Reaction Monitoring (MRM) transitions. Typical phomopsin A transitions are: 789→323*m*/*z* and 789→226*m*/*z*. Collision energies to generate the product ion 323 and 226*m*/*z* were 39 and 57 eV, respectively (Reinhard *et al*., 2006).

A lower limit of about 50 ng of phomopsin A injected onto a reverse phase HPLC column can be detected using ESI/LC/MS. This would equate to about 25 μg/kg in feed if 0.5 μg of phomopsin A were recovered from 20 g of feed sample and a 10 μl analytical volume was injected on to the column from a 100 μl sample volume. The potentially detrimental effects of co-extractives from various types of field samples, on this lower level of detection of phomopsins, remains to be determined. Australian food safety authorities have set an upper limit of 5 μg/kg of phomopsins in food destined for human consumption. This presents an analytical challenge in that, even as the sensitivity of the physicochemical methods is being improved (HPLC/ESIMS/MS for example), the sample turnaround is slow and the technological requirements are high.

Immunochemical methods such as ELISA are the main technique for phomopsin detection. For ELISA kit preparation, polyclonal phomopsin antibodies were induced by injecting sheep with a phomopsin-derived hapten-keyhole limpet haemocycanin conjugate (Than *et al*., 1992). The conjugate was prepared according to protocols described by Carter and Meyerhoff (1985). Thus, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1.9

mg, 9.91 μmol)) in a solution of dry *N,N*-dimethylformamide (DMF) and dry dimethylsulfoxide (DMSO) (1:2, 0.2 ml), and *N*-hydroxysuccinimide (NHS, 1.13 mg, 9.82_mol) in dry DMF:dry DMSO (1:2, 0.2 ml), and phomopsins (6.5 mg, 8.25 μmol, phomopsin A 85–90%) were added together and stirred for 8 h at room temperature and then overnight at 4°C. The resultant phomopsin-NHS ester solution (0.4 ml) was added slowly in 50 μl aliquots to keyhole limpet haemocyanin (6.5 mg in 500 μl of 0.1M sodium bicarbonate pH 9.2). The protein solution was kept at 0°C on ice during the addition of the ester mixture, then at 4°C overnight with stirring in the dark. After the reaction period, unconjugated phomopsins were removed by ultrafiltration using a YM30 membrane filter of 10,000 molecular weight cut-off (MWCO). The retentate was redissolved in water (13 ml) and stored as 1 ml aliquots at −18 °C. One-year-old sheep were subcutaneously immunised at the back of the neck with the phomopsins-haemocyanin protein conjugate (1 ml) emulsified in an equal volume of Freund's complete adjuvant. Three subsequent booster injections, at 21 days intervals, of the same amount of conjugate emulsified in Freund's incomplete adjuvant were followed by boosters at 6 month intervals when necessary to maintain antibody titres. Blood from the jugular vein was taken prior to each immunisation and 2–3 weeks after the injections. The production of antibodies against phomopsins was assessed using an indirect ELISA in which the wells of a microtitre plate were coated with phomopsins and the captured antibodies detected using donkey, anti-sheep IgG horseradish peroxidase (HRP) (Than *et al*., 1990). For analytical application, the purified (Protein G affinity) ovine polyclonal IgG antibodies, which were equally cross-reactive with both phomopsin A and its deschloro analogue phomopsin B, were used to coat wells in an ELISA microtitre plate. The coated wells were then incubated with a solution of the sample extract (or known calibration standards of phomopsin A) and a phomopsin–horseradish peroxidase conjugate (prepared in a similar way to the antigenic conjugate but using horseradish peroxidase instead of haemocyanin) in a direct, competitive ELISA format. Following post-incubation washing of the wells, tetramethylbenzidine (TMB), the substrate for the horseradish peroxidase, was added to develop colour in inverse proportion to the amount of phomopsins in the sample being analysed. The resultant 1 μg/kg lower level of detection, the simplicity of the assay, the rapid sample turnaround time and high sample throughput makes this assay suitable for monitoring animal feed and animal-derived food products for phomopsin contamination. This ELISA technology has been transferred to analytical laboratories in and outside Australia for assessment and/or application. A combination of HPLC analysis and ELISA of collected fractions from the HPLC analysis has proved to be very useful in the identification of new phomopsins and/or phomopsin-related compounds in extracts.

Validation and outlook

None of the mentioned methods, including LC-methods, has been validated by interlaboratory studies and there are no certified reference materials or proficiency studies available for the determination of phomopsins.

OCCURRENCE DATA

The survey data available for phomopsins is limited to Australian data and restricted to lupin seed only (Table 2).

Surveys have found that up to 20% of harvested seed can be infected by *P. leptostromiformis* (Clarke and Kellock 1979; Ali *et al.,* 1982; Wood and Petterson, 1985).

Phomopsins were detected in 17 of 43 samples of lupin seed from the 1981 harvest in three Australian states (Western Australia, Victoria and New South Wales) (Petterson *et al.,* 1985). The phomopsin content, assessed by a nursling rat bioassay, ranged from <6 µg/kg to 360 µg/kg. However, the extraction efficiency in these assays was found to vary from about 15% at low concentrations (ca. 10 µg/kg), to 50-60% at high concentrations (40-60 µg/kg), so the values reported were probably underestimated.

A detailed study was made on the *P. leptostromiformis* infection and phomopsin A content of seed in a lupin crop having a high level of visible pod infection (Wood *et al*., 1987). Over 96% of the visibly infected seeds contained viable *P. leptostromiformis*. Visually clean, or whole white seeds, from the primary pods had a higher level of fungal infection (56%) than comparable seeds from the other pods (11%). The discoloured seed content of the primary pods was always higher than for the other pods. The phomopsin A content of the seeds was higher than previously reported. Although the cracked and discoloured seed fractions contained from 200 to 2300 µg of phomopsin A/kg, the highest amount of this toxin detected in whole white seeds was 6 µg/kg.

In a survey of unsorted lupin seeds from the 1991-1992 harvests in Western Australia (Than *et al*., 1994), the mean level of contamination by phomopsins was found to be 6.1 µg/kg (enzyme immunoassay used). If the seed was sorted on the basis of discolouration, the mean level of phomopsins in the clean seed was 1.3 µg/kg (much lower than the acceptable limit of 5 µg/kg set by the Australian National health and medical Research Council Food Standards Code), whereas, in the discoloured portions, the mean level was 355.1 µg/kg, with concentrations up to 4522 µg/kg. The results of this study demonstrate that seed sorting is an effective means of reducing phomopsin contamination of seed.

There is no data available on the levels of phomopsin carried over to lupin flour. Therefore, it is not clear to what extent the milling process may remove phomopsin contamination. As it is

known that phomopsins are concentrated in the seed coat initially, special attention needs to be directed towards gathering survey data for lupin hulls, which may be included as a fibre supplement in breads.

No data is available for other potential sources of exposure such as other lupin products, offal, milk etc.

Table 2. Results of surveys for phomopsins showing concentrations and distribution of contamination in food and feed commodities.

Country	Commodity	Year	N°. of samples	LOQ (μg/kg)	*n* > LOQ	Mean (μg/kg)	Min/Max (μg/kg)	References	Sampling procedure
Australia	Lupin seed	1981	43	na	*17*	na	<6/360	Petterson *et al*., 1985	na
Australia	Cracked and discoloured lupin seed	na	na	na	na	na	na/2300	Wood *et al*., 1987	na
	Whole white lupin seed	na	na	na	na	na	na/6		
Australia	Lupin seed non-sorted	1991/1992	88	na	na	6.1	nd/86	Than *et al*., 1994	Samples (5 kg)of mixed Phomopsis-resistant and susceptible seed from 88 receival points, then hand sorted

na : not available
nd . not detectable

MITIGATION OF PHOMOPSINS

Not many strategies have been developed to limit contamination of lupin seed.

In established lupin growing areas of western Australia infection of *L. angustifolius* by *D. woodii* is endemic (Wood and Brown, 1975). Infested stubbles from previous crops invariably occur beneath subsequent ones, and due to the woody nature of the stems, retain infectivity even after two years of rotation (Wood and Sivasithamparam, 1989). This retention of trash occurs mainly as a consequence of the practice in Western Australia of minimum cultivation to preserve soil structure (Jarvis *et al*., 1986). Sheep grazing stubble during the summer also tend to avoid the thicker and less palatable stem bases (Allen *et al*., 1980). In any case, at the first onset of lupinosis during the summer, the recommendation is for animals to be removed from and not re-introduced to the y affected paddock. Consequently, large amounts of stem material infected with *D. woodii* can remain. Stromata of the fungus differentiate into both pycnidia and ascostromata during the following season (Wood, 1986), thus providing an inoculum source for the infection of lupin crops in and outside the paddock. During the second year, further differentiation of stromata into pycnidia occurs, but only rarely does

further formation of ascostromata occur (Wood and Sivasithamparam, 1989). The fungus does not appear to survive beyond two years. Infected stem material therefore provides a source of inoculum for the infection of lupin crops over a two year period, and under standard agricultural practices in western Australia large amounts build up, especially with lupin cropping on a short rotation. Sanitation practices such as cultivating trash to bury it, or burning of trash, are not acceptable due to the danger of erosion and degradation of soil structure.

The fungus can be introduced to new areas of lupin production through the use of infected seeds. However, the presence of asymptomatically infected seeds is almost always indicated by the concurrent presence of discoloured infected seeds (Wood and Sivasithamparam, 1989).

The practice of slashing and fodder-rolling the green crop can dramatically reduce levels of the fungus in the stems of *L. angustifolius* (Allen *et al*., 1978a; Allen and Wood, 1979). It appeared that the rapid drying of slashed stem material produced substrate changes with which *D. woodii* was unable to cope. It resulted in a reduction in the level of infestation by the fungus and in toxicity compared with that of untreated lupin material.

Studies of *P. leptostromiformis* have shown that it is mainly restricted to the coarser central and lateral stems of lupin plants. However, in field trials in 1976, removal of coarse lupin material from *L. angustifolius* cv. Marri stubble paddocks did not reduce the incidence or severity of liver damage caused by phomopsin. Sheep grazing paddocks from which coarse lupin material had been removed did not show changes in the body weight (bw) (Allen *et al*., 1978b).

A further agronomic practice is the cropping of a susceptible lupin crop in combination with a non-susceptible host. This may be either a lupin cultivar resistant to the pathogen (Hamblin *et al*., 1981; Wood and Hamblin, 1981) or a cereal such as oats (Wood and Sivasithamparam, 1989). As well as reducing the level of fungal infection of the susceptible lupin feed component, the practice of polyculture also provides the other non-infected feed component as a toxin-diluting factor.

The effect of calcium (Ca) supply on infection of narrow-leafed lupins (*Lupinus angustifolius*) by *D. toxica*, was investigated. The susceptible cv. Yandee and the resistant breeding lines CE2:435 and 75A:258 were grown in solution culture at varying levels of Ca supply. Epidermal Ca concentration increased with increased Ca supply in both resistant and susceptible narrow-leafed lupins. There was an inverse relationship between the concentration of epidermal Ca and the frequency of infection by the fungus (Williamson *et al*., 1994).

The use of resistant cultivars

Considerable effort to control lupinosis in Australia has focused on the development of lupins cultivars resistant to *Phomopsis* infection and consequently with reduced levels of phomopsin in the stubble.

Some reports indicated that lupin species varied in resistance to *Phomopsis* stem blight, but few demonstrated variations in resistance within species. In field and glasshouse studies in Poland, yellow lupin (*L. luteus* L.) was the only lupin species attacked by *P. leptostromiformis* (Kochman, 1957), but it was later reported that the fungus was highly aggressive to *L. luteus*, *L. angustifolius* L. and *L. albus* L. (Kochman and Kubicka, 1974). Yellow lupin was also highly susceptible in a report from the USA (Ostazeski and Wells, 1960), but symptoms could not be reproduced on *L. luteus* or *L. albus* without wounding, and never on *L. angustifolius*. In glasshouse and field experiments in South Africa, it appeared that *L. albus* was more susceptible than *L. angustifolius*, *L. luteus* and *L. mutabilis* Sweet (Van Jaarsveld and Knox-Davies, 1974b), but symptom development did not vary among five *L. albus* cultivars, and similarly there was no variation among five *L. angustifolius* cultivars (Van Jaarsveld and Knox-Davies, 1974b).

In contrast, field studies in Australia showed that *L. albus* cv. Ultra had very low levels of infection compared to *L. angustifolius* and *L. cosentinii* Guss, cultivars that were all very susceptible (Wood and Allen, 1980). Recently, in south-eastern Australia, the incidence and severity of *Phomopsi*s in *L. albus* crops has increased and lead to further research on this disease (Cowley *et al*., 2008).

A wild *L. angustifolius* line collected from Spain (P22750) was identified as resistant and used as a parent to produce the first resistant cvs. Gungurru and Merrit (Cowling *et al*., 1987). A breeding line 75A:258 was shown to have an extremely resistant phenotype which can be traced to a wild parent from Morocco (P22872). Screening of F1, F2 and F3 families from crosses between 75A:258, Merrit and susceptible cv. Unicrop showed that 75A:258 had a single dominant allele Phr1 which conferred resistance, whereas Merrit carried a different incompletely dominant resistance allele Phr2 (Shankar *et al*., 2002). A co-dominant locus specific molecular marker has been produced based on an MFLP polymorphism linked to the Phr1 gene (Yang *et al*., 2002). A marker for Phr2 is under development. The European *L. albus* cvs. Ultra and Kiev Mutant and the *L. luteus* cvs. Teo and Motiv 369 have a good resistance to *Phomopsis* stem blight in Australia. Resistance reduced the lupinosis toxicity in stubbles grazed by sheep, even if variations in toxicity were not fully explained by variations in resistance or frequency of isolation of the fungus (Cowling *et al*., 1988).

Resistance to infection of *L. angustifolius* by *D. toxica* was shown to be the result of an incompatible reaction between host and pathogen during the latent phase of the disease that appeared to occur after cuticle penetration. Conidia were attached firmly to the cuticle by an extracellular substance presumably exuded from the conidia and penetrated the cuticle directly via an infection peg, forming subcuticular coralloid hyphae. Resistant plants had a high frequency of small coralloid hyphae (10-80 µm length), while susceptible plants had a high frequency of large coralloid hyphae (80-400 µm length). Colonization of tissues below the cuticle began immediately after excision of stems from susceptible plants, but was delayed in resistant plants (Shankar *et al*., 1998a). Conidial germination and penetration of the cuticle are not affected by host resistance, and resistance appears to be the result of preexisting or induced resistance responses in epidermal cells below the infection site (Shankar *et al*., 1996).

Breeders have been able to exploit natural epidemics and have assessed resistance by rating the frequency and intensity of stem lesions on stubble immediately prior to the crop being harvested (Cowling *et al*., 1987).

Williamson *et al*. (1991) developed an artificial inoculation, tissue staining and microscopic examination procedure to visualise subcuticular coralloid mycelia which are a latent infection structure. Shankar *et al*. (1996) standardised the inoculation and incubation procedures and was able to show that breeding lines known to be resistant or susceptible under field conditions had small or large coralloid structures, respectively. These last two studies were hampered by the lack of a non-destructive glasshouse test for resistance. Shankar *et al*., (2002) developed a non-destructive glasshouse test, based on inoculating one of the branches of the plant with a conidial suspension, while the other branch was protected from infection and maintained for seed production. An ELISA test, based on polyclonal antibodies to *D. toxica*, was also developed to distinguish large (susceptible) and small (resistant) pathogen biomass as an alternative to microscopic examination (Shankar *et al*., 1998b).

Cowley *et al*., (2008) developed and refined a screening assay using detached leaves and pods to screen for resistance to *D. toxica* in *L. albus*. The pod assay allows a better control of experimental conditions and ensures that all pods being tested are at a similar physiological stage. In contrast with pods, *Phomopsis* infection in leaves is not considered to be an agronomically important character in albus lupins, but may play a role in epidemics (Brown, 1984). This method of assay has been undertaken to ascertain if foliar symptoms correlate with either pod or stem resistance. Assessing the leaves rather than pods has appeal in that it enables rapid screening of genotypes, without the need to grow plants till maturity. The authors found significant differences between genotypes for both assays ($P < 0.001$). The

correlation between the two assays was only moderate (r = 0.36), but significant (P < 0.05), however, they only accounted for 13% of the variability. Genotypes that were resistant in one assay were not always resistant in the other. Therefore they postulate that there may be different phases of the disease in albus lupins, with resistance to each phase under independent genetic control.

Chemical and biological control

Only a few studies have been done on the chemical control of *Phomopsis* on lupins.

In a trial managed in 4 successive years in Western Australia, blue fodder lupin (*L. cosentini*) was sprayed with water or solutions of 4 fungicides (Benomyl, Thiabendazole, Folpet, Captafol) or fumigated with methyl bromide. The rate of infestation with *P. rossiana* was noted, sheep grazed the test plots, and the toxicity of the lupin was estimated with mice. Results of fungicide sprays varied from failure in 1969-70 to partial control of lupinosis in 1970-71. Fumigation with methyl bromide was effective. None of the fungicides could be considered practical for controlling lupinosis (Wood *et al*., 1975). Moreover, in view of the extended infection period of lupins, from the seedling stage until senescence, the number of fungicide applications necessary to achieve control would not be economic. Captafol and methyl bromide are no longer permitted in Europe and other compounds are not specifically authorised in lupins.

Treatment of cut lupins with formalin, before rolling or bailing, did not result in fungal control (Allen *et al*., 1978a), even if, in one site of their trial, increasing the rate of application of formalin appeared to proportionally reduce the degree of *Phomopsis* infection on the surface of the rolls.

No examples of microbial biocontrol are available in literature. A preliminary field experiment showed that lupin plots inoculated with lupin stubble infected with biotype B of *L. leptostromiformis* reduced the level of natural infection by biotype A (Wood, 1986). Further work on biotypes could include attempts at biological control of toxigenic biotype A strains, possibly using more aggressive strains of biotype B as cross-protecting agents. Basic knowledge on the effect of environmental conditions on the activity of biotype B is needed for its successful establishment on lupins (Wood and Sivasithamparam, 1989).

Decontamination

There are not many examples in literature of decontamination or detoxification of phomopsins; moreover, they have been shown to be resistant to destruction by extensive processing, including cooking (Cockrum *et al.,* 1994).

Two studies to evaluate the effectiveness of treating *L. angustifolius* stubbles with sodium hydroxide in the field to reduce their mycotoxin toxicity and to kill the fungus *P. leptostromiformis* were carried out in 1986 and 1987 (Croker and Allen., 1990). Treatment of the stubble with a sodium hydroxide spray at rates between 6 and 18 kg/100 kg of stubble material did not significantly reduce the presence of *D. woodii* in either study. In 1984, when phomopsin levels were high, the phomopsin A concentration was lower for the samples obtained after the 2 highest rates of application (12 kg NaOH/100 kg with 1200 or 4800 l of water/ha), but these levels were not considered safe for grazing sheep. Phomopsin C was also present. Treatments did not significantly alter the digestibilities of materials from the plot nor did any of them raise the pH of the stems above 7. In 1986 and 1987, when phomopsin levels were lower, using between 6 and 18 kg NaOH/100 kg of stubbles, sprays had no significant effect on the concentrations of phomopsin A in the materials. The authors dismiss the use of higher concentrations of NaOH as impractical and dangerous. It remains to be seen whether NaOH applied as a spray on lupin stubbles after harvest can achieve similar results.

PHARMACOKINETICS

Absorption, Distribution, Metabolism and Excretion

No information was found on the absorption, metabolism, distribution or excretion of ingested phomopsins in animals.

In vitro studies using artificial rumen preparations have shown that phomopsin does not undergo any significant metabolism by rumen microorganisms (Peterson, 1986).

Peterson (1986) reports that at high levels of ingestion, the toxicity of phomopsin is limited by its rate of absorption. Cytotoxic effects in the liver of rats have been observed one hour after intraperitoneal injection of phomopsin (Peterson, 1978). This suggests that once absorbed, phomopsin may undergo very rapid transport to the liver, probably through the portal vein. There is some evidence from kidney effects observed in toxicity studies that phomopsin, or an active metabolite of phomopsin, may be excreted via the kidneys (Peterson, 1986; Peterson, 1990; Peterson and Lanigan, 1976).

Carryover

Lupin flour can be used in bread, biscuits, and pasta. Lupin hulls are also used as a fibre component in high fibre white bread and muffins. Whole seeds may also be used in a mashed form in a number of processed foods, or to produce milk and lactic beverages. Therefore it is possible to find phomopsins in all these food categories

There is no data available on the levels of phomopsins in the tissues of livestock, in milk or in other animal products (ANZFA, 2001).

TOXICITY

Phomopsins are compounds capable of binding to tubulin (Tonsing *et al*, 1984), and this binding is considered to be responsible for the acute toxicity of phomopsin by preventing the polymerisation of tubulin at concentrations of less than 1 µM (Lacey *et al*, 1987; Ngo *et al*, 2009; Peterson, 1990). This action leads to an inhibition of crucial functions such as spindle formation during mitosis and the intracellular transport of lipids.

In vivo mitotic arrest in hepatocytes induced by phomopsin is followed by cell death in most cases (Peterson and Lanigan, 1976). This suggests that the effects resulting from the binding of phomopsin to tubulin are not reversible. However, results from studies using cultured cells have provided conflicting results (Brown and Bick, 1986; Tonsing *et al*, 1984).

Cell membranes may also be affected by phomopsin, where changes in the activity of some membrane-associated enzymes and increased fluidity have been observed in fractions of hepatocyte plasma membrane after phomopsin treatment (Peterson, 1986).

A redistribution of Golgi apparatus membranes has also been observed after phomopsin exposure (Tonsing *et al*, 1984).

Acute toxicity

Mice

Experiments in mice exposed intraperitoneally to crude preparations of phomopsin have been carried out (Papadimitriou *et al*, 1974; Peterson and Lanigan, 1976), however, the phomopsin dose used in these experiments does not appear to have been quantified. Pathological examinations revealed the liver as the main target organ with major changes in the central zones of the hepatic lobes. A variety of observed changes in the nucleus of liver cells were directly proportional to the dose administered. Changes in the activities of various liver enzymes were also observed.

Alkaline phosphatase, 5'-nucleotidase, β-glucuronidase and acid phosphatase activities were elevated, whereas, succinic dehydrogenase, glutamic oxaloacetic transaminase, lactate dehydrogenase and glucose-6-phosphatase activities were decreased. These changes persisted for several days but gradually returned to normal after 4 weeks. An increase in the number of parenchymal cells undergoing mitosis in the liver was seen after 26 hours; this reached a maximum between the second and third day and then declined. Similar mitotic effects were also seen in the kidneys and occurred much later than in the liver. Evidence of

mitotic arrest was not observed in the duodenum, lung or spleen.

Rats

Lethal doses (LD_{50}) of phomopsin in rats, from various routes of exposure have been reported by Peterson (1986). The reported values are 24 - 52.5 mg/kg for oral exposure, 4.4 - 8.0 mg/kg for subcutaneous injection and 1.2 - 2.0 mg/kg for intraperitoneal injection. Groups of 2 week old male rats (5 rats/dose) were given a single intraperitoneal dose of a crude preparation of phomopsin (Peterson, 1978). This preparation was subsequently found to contain about 4% toxin on a dry weight basis, therefore the appropriate adjustments have been made to the doses reported for the study. Nursing rats were selected over adult rats because they were expected to exhibit a high level of mitotic activity, and hence be a better model for studying the effects of phomopsin on the cell cycle. The doses used varied, depending on the experiment, but were within the range of 0-2.64 mg/kg. Animals were sacrificed at various intervals (up to 28 days) after injection. The response in male and female rats (5 rats/sex/dose) was also compared at the dose levels of 0.026 mg/kg and 0.53 mg/kg at 18 hours and 7 days after injection. No sex-specific differences were observed. The intraperitoneal LD_{50} for this study was estimated to be about 1mg/kg, causing death in 4 to 8 days. This value is consistent with other reported LD_{50} values for this exposure route (see above). The principal effect associated with low doses of phomopsin (<0.04 mg/kg) was metaphase arrest in liver parenchymal cells. This could be seen within one hour of injection and reached its peak within 2 to 4 days after which its occurrence declined rapidly. At higher doses (>0.17 mg/kg), fatty changes and fibrosis developed in the liver and the rats became jaundiced. The fatty changes to the liver could be observed 18 hours after injection and reached their maximum at 3 to 4 days after injection. Depletion of cortical cells in the thymus, depletion of haematopoietic tissue in the spleen, reduced gastric activity, and retarded growth rates were also observed. The severity of these responses increased with increasing dosage. Mitotic arrest was also observed in the kidney and pancreatic acinar, but only at high doses (0.7 mg/kg for kidneys and 1mg/kg for the pancreas), and not in any other tissues.

Sheep

In sheep, the ingestion of phomopsin-contaminated lupin stubble is associated with the occurrence of a disease known as lupinosis. The classical clinical signs of lupinosis are inappetence, loss of condition, lethargy and jaundice (Gardiner, 1975; Gardiner and Parr, 1967; Van Warmelo *et al*, 1970). The gross pathology of lupinosis depends on whether the disease is acute, subacute or chronic.

Groups of male sheep (1-3 sheep/dose) were given a single subcutaneous dose of phomopsin over the dose range of 1.25 – 98 µg/kg, and animals were observed over 28 days (Jago *et al*, 1982). All sheep given ≥ 75 µg/kg died within 3-5 days. Those given between 10 and 37.5 µg/kg died in 10-26 days, except for two animals who were showing clinical signs of recovery at the end of the experiment. All sheep receiving 5 µg/kg or less survived the 28 days of the experiment. The first clinical sign observed was inappetence, which rapidly progressed to anorexia in all sheep that subsequently died during the experiment. Reduction in food intake was observed within one day of administration of doses at 10 µg/kg or higher, and within 3 days after 2.5 µg/kg. Weight loss could not be correlated with dose. Clinical signs of toxicity were not observed in animals receiving the lowest dose of 1.25 µg/kg. In sheep given lethal doses of phomopsin, both total serum protein and albumin levels fell by 10-17% within 4 days, with albumin levels continuing to fall, consistent with liver failure. There were too few animals to establish an LD_{50} dose. Survival time was shown to have an approximate inverse relationship to dose, ranging from 3 days after 94 µg/kg to 26 days after 10 µg/kg.

Sub-Chronic toxicity

Rats

Groups of 10 week-old male and female Long-Evans rats were administered phomopsin by subcutaneous (SC) injection at the dose level of 30 µg/kg bw for 5 days per week for 2, 6 or 17 weeks (Peterson, 1990). Matched groups of treated and control rats were killed at scheduled intervals during the treatment period and up to 32 weeks after the start of dosing. In addition, some male and female rats were allowed to survive until about 2 years of age. Control rats received equal volumes of physiological saline.

During the treatment period, no effect was observed on the behaviour of the rats. Phomopsin was shown to decrease survival times in a manner which was related to the duration of the treatment and was statistically significant for the groups treated for 6 and 17 weeks. All rats administered phomopsin for 17 weeks developed permanent, irreversible liver damage, characterised by nodular cirrhosis and extensive biliary hyperplasia, which continued to progress after the treatment ceased. On the contrary in some rats in the 6 week dose group the cessation of treatment was followed by almost complete regression of the liver lesions, with only a small amount of fibrous tissue evident 2 years after the last injection. This indicates that both the biliary and parenchymal damage may be reversible after a relatively short period of exposure to phomopsin. This was also confirmed by the livers of animals administered phomopsin for 2 weeks which exhibited full recovery within a few weeks after

treatment was ceased.

This study also looked at the incidence of tumours at 2 years of age, and these findings are summarised in Table 3.

Table 3. Incidence of tumours in rats at 2 years following subcutaneous injection with phomopsin at 30 µg/kg bw/day.

Treatment Duration	No. of animals	Cholangioma	Cholangiocarcinoma	Hepatocellular carcinoma
Untreated	59 (34M, 25F)	0	0	0
2 weeks	20 (20M)	0	0	0
6 weeks	34 (20M, 14F)	10	1	0
17 weeks	37 (27M, 10F)	22	2	3

M=male, F=female

The tumours most commonly observed in the phomopsin-treated rats were localized in the liver. The occurrence of liver tumours following such a short exposure period suggests that some of the phomopsin-induced liver lesions may continue to progress in the absence of further exposure to the toxin.

Sheep

Groups of male sheep (3 animals/dose) were administered single or multiple doses of phomopsin by either the subcutaneous (SC) or intraruminal (IR) routes (Peterson *et al*, 1987). IR injection was used to simulate the retention of plant material in the rumen and is considered equivalent to gavage.

Mild suppression of appetite was observed in 1 out of 3 sheep administered a single SC dose of 2.0 µg/kg, and was completely suppressed in all 3 sheep given a single SC dose of 10 µg/kg, with two of the animals dying 4 days after exposure. The surviving sheep exhibited slow recovery of appetite. The overall clinical, biochemical and histological responses closest to the effect seen from a single SC dose of 10 µg/kg resulted from a single IR dose of 1000 µg/kg. The same total dose administered at daily IR rates of 50 or 200 µg/kg was more toxic, resulting in the death of all sheep. A single IR dose of 500 µg/kg was associated with significant liver damage, but no deaths. Single IR doses of 125 and 250 µg/kg and repeated daily IR doses of 12.5 µg/kg over 16 weeks, were not associated with any detectable tissue damage, but were associated with a loss of appetite. Of the four groups that received a total IR dose of 1000 µg/kg (either singly or through multiple doses), treatment by a single dose was less toxic than separate doses of 200 or 50 µg/kg.

The main changes were related to the liver. In particular, in the sheep that died early, the livers were very fatty. With lower doses of phomopsin or longer survival, liver fat was less evident. Histopathological examination revealed that in sheep surviving for longer periods (> 4 days), the fatty changes in the liver were progressively replaced by fibrosis and a variable degree of proliferation of biliary tissue. Pigmented macrophages containing ceroid and haemosiderin were abundant in the fibrosed tissue. A SC dose of 10 µg/kg may approximate the LD_{50} for this exposure route in sheep.

Cows

A group of 14 Holstein-Friesian cows in mid-lactation were dosed orally with phomopsin A in water at 2.88 mg/cow/day (calculated to be approximately 5.2 µg/kg bw/day based on an initial average body weight of 556 kg) for 8 weeks (Hough and Allen, 1994). Controls were dosed orally with equal volumes of water only. Cows were returned to pasture for 4 weeks after the exposure period before cessation of the study. Milk yield, milk composition, body weight and body condition were measured weekly for the 8 week treatment period and for 4 weeks after treatment. Liver damage was monitored by the measurement of plasma γ-glutamyltransferase (GGT) and glutamate dehydrogenase (GLDH) activities, and plasma bilirubin concentrations were determined weekly during the 8 week treatment period. Administration of 5.2 µg phomopsin A/kg bw/day was not associated with any measurable effect on milk yield, fat, protein or total solids content. No significant difference was found between the treatment and control groups with respect to body weight, plasma GGT and GLDH activities or bilirubin concentrations.

Chronic toxicity

No chronic studies were found for phomopsin. The only data available are qualitative observations in cases of chronic lupinosis in sheep. In these animals the liver is small, hard, coppery or tan in colour and often misshapen. The rumen contents are watery, and the abomasum and small intestine contain very little solid matter. The caecum may contain hard, dry, impacted faecal material. Ascites may be present and there may be evidence of general muscle wastage (Gardiner, 1965; Gardiner, 1967).

Cytotoxicity

No studies were found on citotoxicity of phomopsin

Reproductive toxicity

No studies were found on the reproductive toxicity of phomopsin.

Developmental toxicity

In a study by Peterson (1983), the effect of phomopsin on pregnant hooded rats and their embryos was examined. In a first experiment, 40 pregnant rats in 4 treated and 2 control groups (number per group not specified) were injected intraperitoneally (IP) with 30 or 90 µg/kg bw/day on days 6-10 or 11-15 of pregnancy. In a second experiment, 25, 100, or 400 µg/kg of phomopsin was administered as a single IP dose to 200 pregnant rats in 15 treated and 5 control groups (number per group not specified, presumably 10) on days 6, 8, 10, 12 or 14 of pregnancy. On day 20 of pregnancy the dams were sacrified and the foetuses were examined.

A dose of 90 µg/kg bw/day was associated with the death of 40% of the dams. Liver damage was also observed in all the dams that had received phomopsin treatment, regardless of the dose or duration of exposure. A single dose of 400 µg/kg or a dose of 90 µg/kg bw/day for 5 days was associated with high embryo lethality. Doses of 30 µg/kg bw/day for 5 days were found to be associated with embryo lethality only when administered over days 6-10 of the pregnancy. Foetuses which survived the higher dose rate of 90 µg/kg bw/day were severely retarded in their growth and their skeletal ossification was irregular. Notably, the livers of the foetuses were apparently unaffected, and there was an absence of metaphase arrests in any of the embryonic tissue examined. This suggests that the embryonic deaths may not be associated with direct phomopsin action on their tissues but may instead be the indirect result of maternal toxicity.

Genotoxicity

Very little information can be found on the genotoxicity of phomopsin. Negative results have been obtained for phomopsin in the Ames test and in the Chinese hamster ovary chromosome aberration and HGPRT locus mutation tests (BIBRA, 1986). However, Brown and Bick (1986) have shown that phomopsin can induce chromosomal aberrations consisting of chromatid and isochromatid deletions and chromatid exchanges in the Chinese hamster DON cell line.

Carcinogenicity

No long-term carcinogenicity studies have been found upon using oral exposure to phomopsin. However, a sub-chronic study in rats, where a single dose level was

administered to rats by subcutaneous injection for up to 17 weeks, demonstrated an unequivocal association between phomopsin treatment and the occurrence of liver tumours, which first appeared at 36 weeks (see Table 3).

Human studies

None found.

LEGISLATION ON PHOMOPSINS

Australia and New Zealand are the only countries to include phomopsins in their mycotoxin regulations, with a limit of 5 μg/kg in lupin seeds and products of lupin seeds (Van Egmond and Jonker, 2004). This appears to be due to the emergence of lupin flour as a human food ingredient and the availability of data on the phomopsin content of lupins in Australia (ANZFA, 2001; Petterson *et al.*, 1985; Than *et al.*, 1994; Wood and Petterson, 1986). In fact in 2002 New Zealand and Australia initiated a joint food regulatory approach, codified in the Australia New Zealand Food Standards Code. Prior to drafting of the joint Code, a series of risk assessments were carried out by Food Standards Australia New Zealand (FSANZ; later called Australia New Zealand Food Authority). This included a review of the maximum permitted concentrations of non-metals in food, including some mycotoxins.

CONCLUSIONS

Phomopsis leptostromiformis has been reported to cause stem blight in young lupins and to produce phomopsins. These mycotoxins, a family of compounds, are capable of binding to tubulin and cause the animal liver disease known as lupinosis.

The phomopsins are a family of macrocyclic hexapeptide mycotoxins. Phomopsin A, B and D are identified so far. The compounds, phomopsinamine A and octahydrophomopsin A (phomopsin D), are chemical derivatives of phomopsin A and have similar biochemical activity. A third phomopsin, phomopsin C, has been partially identified but a full structure is not yet available.

Phomopsin A standards are commercially available while no certified reference materials for method development and validation are available.

ELISA and HPLC based analytical methods are reported, but none of the mentioned methods has been validated by interlaboratory studies and there are no certified reference materials or proficiency studies available for the determination of phomopsins.

The survey data available for phomopsins is limited to Australian data and restricted to lupin seed only. No data are available on derived food and feed products even if the risk for their

contamination exist. No data are available either regarding the presence of phomopsins in animal derived products.

FUTURES

It is proposed that the current level of 5 µg/kg be maintained but that further analyses of phomopsin levels in lupins used for direct consumption should be investigated as well phomopsin levels in flours prepared from lupins. Further work on the mechanism of phomopsin toxicity would also be beneficial.

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General summary and comments

As a general conclusion, it can be underlined that for all compounds considered there is a common lack of knowledge that can be summarised as follows:

:

- Detailed knowledge on fungi involved and their interaction with host crops
- Validated analytical methods and reference contaminated materials
- Detailed surveys in different countries and different years
- Specific sampling methods
- Detailed and updated knowledge on pharmacokinetics and toxicity

Analitycal methods developed are summarised as follows:

	LC/MS	GC/MS	GC/FID and GC/ECD	HPLC/UV	HPLC/FL	CE	TLC	ELISA	NIR
Morphine	+	+	–	+	–	+	+	–	–
Ergot alkaloids	+	+	–	+	+	+	+	+	+
Alternaria toxins	+	–	–	+	+	–	+	–	–
Moniliformin	+	+	–	+	+	–	+	–	–
Nivalenol	+	+	+ and +	+	+	–	–	+	–
Diacetoxyscirpenol	+	+	+ and +	+	+	–	+	+	–
Sterigmatocystin	+	+	–	+	–	–	+*	–	–
Phomopsins	+	–	–	+	–	–	–	+	–

* = Validated method
CE: Capillary Electrophoresis

Target organs for compounds toxicity are summarised as follows**:**

	Kidney	Liver	Heart	IS	NS	Lung	Mouth	Esophagus	GI tract	Skin
Morphine					+	+*			+	+
Ergot alkaloids			+		+					
Alternaria toxins					+	+		+	+	+
Moniliformin	+	+	+							
Nivalenol				+						
Diacetoxyscirpenol				+	+		+		+	
Sterigmatocystin	+	+				+				+
Phomopsins		+								

* = Only acute toxicity
IS = Immunitary system
NS = Nervous system
GI tract = Gastro-intestinal tract