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*in food*

Detection and control

Edited by N. Magan and M. Olsen



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# **Mycotoxins in food**

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Edited by  
**N. Magan and M. Olsen**



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# Preface

Recent research has increased awareness of chemical residues and natural contaminants in food. At the same time, consumer concerns about food safety have also grown. At a national and international level this has resulted in more stringent imposition of new, legislative limits for a range of mycotoxins which can contaminate food raw materials and enter the food chain. Mycotoxins are particularly important as they are very heat stable and difficult to destroy. Since prevention is better than cure, the former approach is now being utilised to reduce/eliminate the chances of mycotoxins entering the food and feed production chains. To facilitate the prevention approach, a wide range of complementary scientific information is required. This includes information on Risk Assessment of mycotoxins to enable effective and realistic limits to be set. To implement an HACCP approach, the pre-harvest, post-harvest, storage and processing components all need to be understood and the CCPs identified. Aspects such as sampling, monitoring systems and effective diagnostic technologies, including traditional analytical and newer molecular and antibody-based quantification systems; novel plant resistance breeding approaches; effective crop protection systems and an understanding of the ecological role of different mycotoxigenic fungi in the food chain are required. This book focuses on these aspects and includes detailed examination of some of the mycotoxins found in food. We believe that this is a timely volume which should contribute significantly to new information relevant to this key research area of significant importance to the food production and processing industries.

Naresh Magan and Monica Olsen

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# **Part I**

## **Measuring risks**

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# 1

## **Risk assessment and risk management of mycotoxins in food**

**T. Kuiper-Goodman, Bureau of Chemical Safety, Health Products and Food Branch, Health Canada**

### **1.1 Introduction to risk assessment of mycotoxins**

Many cereal and other crops are susceptible to fungal attack either in the field or during storage. These fungi may produce as secondary metabolites a diverse group of chemical substances known as mycotoxins. There can be wide year to year fluctuations in the levels of mycotoxins in foods, depending on many factors, such as adverse conditions favouring fungal invasion and growth. Many mycotoxins were initially identified after they had caused a variety of subacute health problems in livestock as well as humans, with many target organs and systems affected. With modern farming, storage and processing practices, the aim is to reduce obvious contamination, and much of our concern now focuses on chronic effects at low levels of exposure. In this regard, several mycotoxins are potent animal carcinogens and have been classified by the International Agency for Research in Cancer (IARC, 1993) as human carcinogens or potential (probable and possible) human carcinogens.

Although there are geographic and climatic differences in the production and occurrence of mycotoxins, exposure to these substances is worldwide, with much of the world food supply contaminated to some extent. Monitoring for the presence of mycotoxins is therefore needed. From time to time the presence of mycotoxins may render food commodities unsafe, requiring a variety of measures to reduce risk. As these toxicants can never be completely removed from the food supply,

## 4 Mycotoxins in food

many countries have defined levels in food (tolerances, guideline levels, maximum residue levels) that are unlikely to be of health concern. Initially, the lack of a unified and transparent approach resulted in a wide range of guidelines/regulations regarding mycotoxins among various countries (Stoloff *et al.*, 1991).

Once an issue involving mycotoxins has been identified as an area of potential health concern (issue identification), the current approach in most countries is to use a decision making framework for risk analysis, similar to that proposed by FAO/WHO (FAO/WHO, 1995).

For mycotoxins, risk analysis is an iterative process that considers risk assessment, risk management and risk communication and is updated as needed once critical new information becomes available. It is important that there is interaction between these major areas. This approach considers scientific principles related to human and animal health and includes a comparison to other risks as well as socioeconomic factors.

In terms of exposure and severity of chronic disease, especially cancer, mycotoxins appear at present to pose a higher risk than anthropogenic contaminants, pesticides (when used according to instructions), and food additives (Table 1.1). This was based on a comparison of tumor potency and exposure (Kuiper-Goodman, 1998). In addition, the implication of concurrent elevated exposure to several mycotoxins may need to be considered.

Considerable international efforts have been expended in assessing the health risks from just a few mycotoxins. Since the late 1980s, new mycotoxins have been discovered and characterized (fumonisins), and for other mycotoxins important new data related to toxicology, epidemiology and human exposure (due to better detection methods and the use of biomarkers) have become available. This has resulted in new and updated scientific evaluations by the IARC, IPCS, JECFA, EU-SCF, as well as national and other agencies such as ILSI (JECFA 1996a,b, 1998, 2000, 2001a,b,c; IARC 1991, 1993; IPCS 1990, 2000; ILSI, 1996; Scientific Committee for Food, European Commission ([http://www.europa.eu.int/comm/food/fs/sc/scf/index\\_en.html](http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html)); Kuiper-Goodman *et al.*, 1987; Kuiper-Goodman and Scott, 1989; Nordic Council of Ministers, 1991, 1998).

A number of mycotoxins have been found to be carcinogenic. Carcinogenesis is a complex multistage process that involves both genetic and epigenetic mechanisms. It is, therefore, useful to organize the data related to carcinogenic endpoints according to recently developed frameworks that help in synthesizing what is known about the mode and mechanism of action and the relevance of the observed effects to humans. Such information will aid future risk assessments and, undoubtedly, when gaps are identified it will stimulate further research.

Through risk analysis, the aim is to achieve practical solutions that balance the need to protect health with economic concerns. Scientific evaluations have now generally become the basis for recommendations regarding the international regulation on mycotoxins (aflatoxins, ochratoxins, patulin, zearalenone, and fumonisins) by the Codex Committee on Food Additives and Contaminants (CCFAC) as well as the European Union. Regulations may include guidelines regarding maximum residue levels or procedural guidelines aimed at prevention

**Table 1.1** Rating health risks from foods.\*

Acute		Chronic
	High	
Microbiological		Mycotoxins
Phycotoxins		Anthropogenic contaminants
Some phytotoxins		Some phytotoxins
Mycotoxins		Unbalanced diet
Anthropogenic contaminants		Phycotoxins
Pesticide residues		Food additives
Food additives		Pesticide residues
		Microbiological
	Low	

\* Kuiper-Goodman, 1998

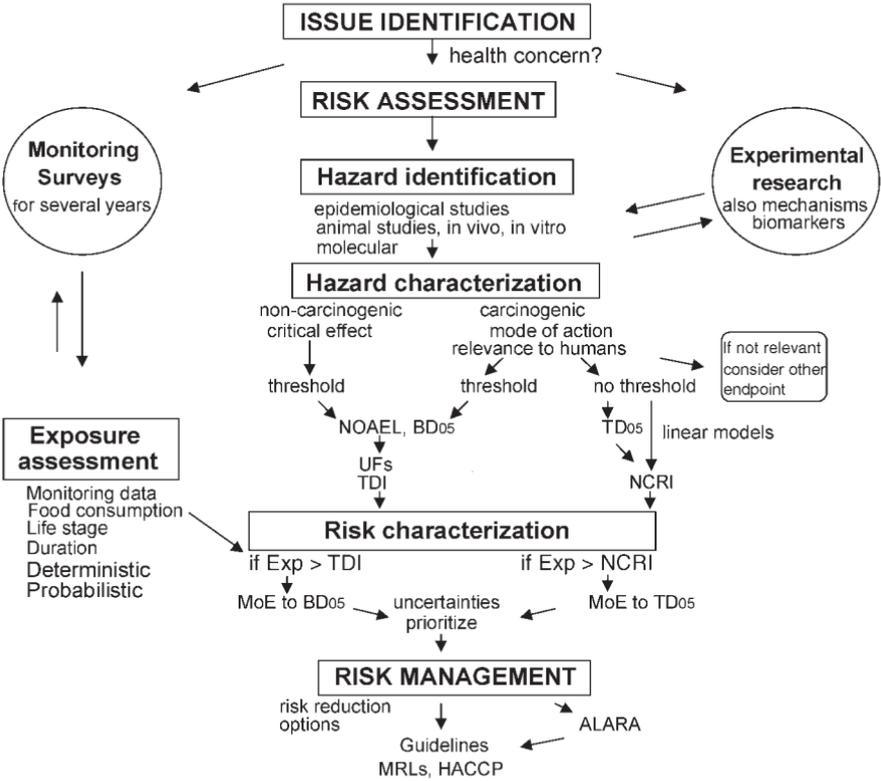
by using a Hazard Analysis of Critical Control Points (HACCP) (Codex, 2001), or a combination of these. The overall process needs to be transparent and should aid in the development of harmonized mycotoxin regulations and control procedures. Ideally, such guidelines are acceptable to countries producing as well as those importing food commodities.

Impediments to a globally harmonized approach to risk analysis relate in part to data interpretation and analysis and differences in food intake patterns between countries. It has been recognized that a unified terminology would help in communication and there have been efforts to bring this about (IPCS, 2001; OECD, 2002).

Currently, the term risk assessment, in the context of food safety, is used by many organizations to describe the process of assessing the health risks from a variety of agents that can be present in food. Thus, in order to derive potential human health risks, one assesses exposure to toxic substances in food and combines this with information on the hazards of these same substances, as identified in animal and human studies (WHO, 1999). For chemical agents such as mycotoxins, risk assessment consists of *hazard identification*, involving a complete toxicological and epidemiological assessment; *hazard characterization*, involving an assessment of dose response and extrapolation to humans; *exposure assessment*; and *risk characterization* (Fig. 1.1). For the purpose of our discussion, *hazard* is defined as the intrinsic property of a biological, chemical or physical agent to cause adverse health effects under specific conditions. This definition implies some certainty that, under similar conditions, the agent will cause similar adverse health effects. *Risk* is defined as an estimate of the likelihood/probability of the occurrence of an adverse health effect in humans, weighted for its severity, that may result from exposure to a biological, chemical or physical agent in food.

## 1.2 Hazard identification

Many mycotoxins and toxicogenic fungi of economic importance were discovered because they were causally associated with mycotoxicoses affecting humans



**Fig. 1.1** Showing the iterative process involved in Issue Identification, Risk Assessment (hazard identification, hazard characterization, exposure assessment, risk characterization) and Risk Management of mycotoxins in foods and the relationship to monitoring surveys and toxicological and epidemiological research.

(ergotism, liver cancer, yellow rice disease, alimentary toxic aleukia (ATA), Balkan endemic nephropathy (BEN), red mold toxicosis) or animals (turkey-X-disease from aflatoxin, porcine nephropathy from ochratoxin A, vulvovaginitis in pigs from zearalenone, equine leukoencephalomalacia and porcine pulmonary edema from fumonisins).

To gain a full understanding of the toxic properties of mycotoxins, short-term, sub-chronic, chronic and carcinogenic studies at various dosage levels need to be conducted in a variety of animal species, under controlled conditions. Also needed are studies to assess reproductive effects and a battery of genotoxicity studies (Barlow *et al.*, 2002). In addition, studies need to be conducted to determine the absorption, distribution, metabolism, and excretion of mycotoxins. Special studies, designed to gain a better understanding of the underlying mechanisms of the most significant health effects observed, may also be conducted. It is preferred that toxicity studies follow international testing guidelines, such as those established by the OECD (OECD, 2000).

Studies have shown that a number of mycotoxins have carcinogenic properties. Some of them are clearly DNA-reactive and for others DNA reactivity may not be the mode of action. When the endpoint is cancer, *in vitro* or *in vivo* studies may need to be designed to elucidate possible molecular events related to gene expression, modifications of relevant proto-oncogenes or tumor suppressor genes, and genomic instability, as this will help in gaining an understanding of the mode of action underlying the carcinogenic process and in the characterization of hazard (Fig. 1.3).

Mycotoxins may also cause developmental effects including birth defects, affect the reproductive system, affect the immune system, exhibit hormonal activity, affect specific target organs and may be neurotoxic. In addition to these diverse organ or site-specific actions, mycotoxins may affect the gastrointestinal system, cause skin irritation, have hematological effects and reduce growth.

In assessing the significance of the observed effects, toxicologists consider toxicity in several dimensions. The frequency or incidence of response depends on dose, duration and severity of the selected endpoint, as well as species or strain susceptibility, age and sex. Toxicologists also take account of the quality of the studies conducted, the relevance to humans of the endpoints affected, precursor lesions as well as the reversibility of effects, and the overall adequacy of the database. For each adequately conducted animal study one usually determines the 'no observed adverse effect level' (NOAEL), which is the highest dose for a specific endpoint at which no adverse effects are observed; also determined, for each specific endpoint, is the 'lowest observed adverse effect level' (LOAEL). It is useful to tabulate the results for several endpoints in different species and studies to provide information on species, strain, age, sex, group size, duration, dose (expressed as mg/kg bw per day), as well as the NOAEL and LOAEL. Such tabulations provide a clear overview, and help in determining the most relevant and sensitive (occurring at the lowest dose) endpoint, also known as the 'critical effect', the most appropriate species and the pivotal studies for further examination under hazard characterization (Fig. 1.1).

For animal carcinogenicity studies, such as those conducted under the auspices of the US National Toxicology Program (NTP), the resulting tumor incidence, including routine statistical analysis, for each organ system is compared to controls and historical controls. Also indicated are histopathological descriptions of the observed tumors, precursor lesions, indicated as 'key events' or 'critical events' under Environmental Protection Agency (EPA) carcinogenicity guidelines (EPA 1999, Anderson *et al.*, 2000), and associated lesions, as well as relevant clinical data.

A critical and sometimes controversial issue in conducting these studies is to ensure that one group of animals is tested at the maximum tolerated dose (MTD), defined as the dose that produces a maximum 10 % decrement in growth and body weight gain in the absence of other toxic manifestations (Dybing *et al.*, 2002). The MTD usually produces some toxic effects, without unduly affecting mortality from effects other than cancer or producing significant adverse health effects in the test animals. NTP normally conducts preliminary studies of shorter duration to find the

appropriate dose range that would include the MTD. There are a number of concerns with the MTD, one of which is that a number of detoxification pathways may be overwhelmed (Dybing *et al.*, 2002).

At the conclusion of the pathological assessment, the results of NTP carcinogenicity studies are reviewed by a panel of experts. A number of criteria are considered, such as common versus uncommon neoplasia, the ratio of carcinomas to adenomas, latency in tumor induction, multiplicity in site-specific neoplasia, occurrence of metastases, concurrent control and historical control incidence, and statistical significance of tumor response. These experts then rate the outcome of the studies as indicating 'clear', 'some', 'equivocal', 'inadequate' or 'no' evidence of carcinogenicity for each of usually two different species (F344/N rat and B6C3F<sub>1</sub> mouse) and both sexes (NTP, 1989). These categories refer to the strength of the experimental evidence and not to potency or mechanism. NTP studies have been conducted for zearalenone, ochratoxin A and fumonisins (NTP, 1982, 1989, 1999).

It generally is not possible to conduct studies in humans under controlled conditions but, as a first step, observational studies on disease in human populations associated with mycotoxins in their food are used in hypothesis generation; such studies then need to be followed by analytical studies such as case-control studies or cohort studies. For these latter studies, one ideally needs to measure exposure and effect at the level of the individual. A major difficulty with epidemiological studies on mycotoxins is obtaining data on historical exposure, since many of the effects observed are of a chronic nature. Even when using biomarkers, the estimate of exposure usually reflects only the recent past (van den Brandt *et al.*, 2002). Therefore, other measures need to be devised to obtain this information. Epidemiological studies on mycotoxins are generally conducted in countries where there is known to be high exposure to a particular mycotoxin. Different conditions such as those related to dietary patterns, prevalence of infections, or lifespan may influence the outcome of such studies.

Thus, mycotoxins may affect many diverse cellular processes and have a wide spectrum of toxicological effects. This diversity of biological effects demands a case by case evaluation and may require a variety of extrapolation techniques, as described under hazard characterization.

### 1.3 Hazard characterization

Hazard characterization can be described as the biological and mathematical extrapolation phase of risk assessment aimed at making a predictive characterization of the hazard to humans under a variety of exposure scenarios. Inherent in this process are many uncertainties. A range of different approaches may be needed, depending on the data available and the hazard or risk characterization issue (Edler *et al.*, 2002).

A general tenet in toxicology is that it is 'the dose that makes the poison', and it has been presumed that for many of the non-carcinogenic adverse effects observed in animals or humans there is a *threshold* dose, such as the NOAEL,

below which these effects are not observed. With regard to carcinogens, it is generally presumed that there is *no threshold* dose below which there is no induction of cancer initiation, and thus there would always be some risk, even at very low doses, unless it can be clearly established that the *mode of action* involves an *indirect* mechanism that may have a threshold. In the extrapolation to safe intake estimates, effects for which a *threshold* is presumed are treated differently from effects for which *no threshold* is presumed. Therefore, carcinogenic and non-carcinogenic mycotoxins will be considered separately under hazard characterization (Fig. 1.1).

### 1.3.1 Non-carcinogenic mycotoxins

The goal of hazard characterization for chemicals for which a threshold is presumed is the estimation of a 'safe dose', such as a provisional tolerable daily intake (PTDI), equivalent to the acceptable daily intake (ADI), used for food additives or pesticide residues, and the reference dose (RfD). The TDI is the dose that can be safely consumed daily over a lifetime without incurring appreciable adverse health effects, and which by implication therefore involves a biologically insignificant risk (WHO 1987, 1999, Edler *et al.*, 2002). In the term TDI, the word 'tolerable' indicates that mycotoxins generally do not serve a useful purpose to humans (Fig. 1.1).

#### *Biological aspects*

As an initial step, responses for selected endpoints in appropriate species, as identified under hazard identification, are examined using biological criteria for severity, reversibility and relevance to humans. This is followed by an examination of the relationship between dose and observed responses in pivotal studies, and an extrapolation from the animal species to humans. In pivotal studies, the NOAEL of the most sensitive but relevant adversely affected endpoint, called the *critical effect*, in the most sensitive species is selected, as it is argued that by protecting against its occurrence in humans, one also protects against more severe effects observed at higher dosage levels. For example, in the evaluation of deoxynivalenol, JECFA used the 10 % decrease in body weight observed in a chronic mouse study (Iverson *et al.*, 1996) as the critical effect on which to base its TDI. Immunomodulatory effects were observed at the same or higher dosage levels in several separate studies, and it was expected that these effects would not occur at exposures below the TDI (JECFA, 2001a).

The NOAEL represents only one point on the observational portion of the dose–response curve. Since the number of dose groups in a study, the spacing of dose groups and group size all influence the empirical derivation of the NOAEL, it has been argued that it may be more appropriate to derive mathematically the 'true' threshold (point of departure) in the dose–response relationship (Edler *et al.*, 2002).

One such estimate is the benchmark dose (BD), originally proposed by Crump (1984; 1995). The derivation of the BD, or its lower confidence interval (BDL),

uses all the experimental data in the observable range, and represents the dose, above background, where a response is observed in 5 or 10 % of animals ( $BD_{05}$  or  $BD_{10}$ , respectively). The BD can be used for both discrete and continuous data and, as it is in the observable range of the dose–response curve, one can choose various mathematical models to fit the data. The BD is not a true ‘point of departure’ on the dose–response curve, since it represents one point in a continuum of response incidences versus dose. Although the BD is, therefore, not a ‘true’ threshold, it is in the same region as the NOAEL, and can be treated in the same way.

Other mathematically derived estimates of the ‘true NOAEL’ such as the ‘no adverse effect level’ (NAEL) or ‘no effect level’ (NEL) have been suggested, which involve a statistical analysis and comparison of effects observed at various experimental doses over background (Kuiper-Goodman, 1990; Slob and Pieters, 1998; Edler *et al.*, 2002). Again these values are treated in an equivalent manner to the NOAEL.

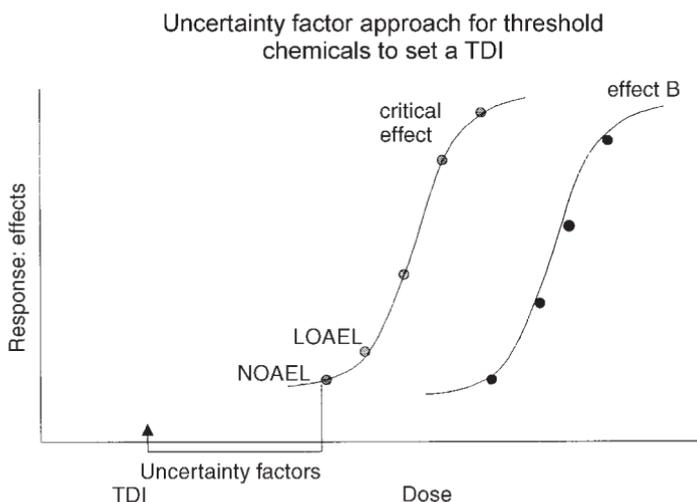
These mathematically derived estimates (BD, NAEL and NEL) derived from data in the observable range of the dose–response curve can be especially useful in situations where wide dose spacing has resulted in a NOAEL which is obviously too low, or in situations where only a LOAEL is available, but where this is not likely to be much greater than the NOAEL.

#### *Extrapolation when a threshold is presumed*

To derive a TDI, it has been common practice to divide the NOAEL, when derived from animal studies, by a default safety/uncertainty factor of 100 when extrapolating to humans. This factor was introduced 50 years ago at the FDA (Lehman and Fitzhugh, 1954). It takes into consideration a default factor of 10 for inter-species differences, and another 10-fold factor for inter-individual variation in susceptibility amongst humans (Fig. 1.2) (WHO, 1987; Edler *et al.*, 2002). The validity of these default uncertainty factors has been assessed and reviewed (Dourson *et al.*, 1996), and they have been widely accepted internationally.

A framework has been developed by which the 10-fold inter-species uncertainty factor is replaced by two factors, a 4-fold factor for toxicokinetic differences (related to the delivery of the chemical or its metabolites to the target site) and a 2.5-fold factor for toxicodynamic differences (related to how the target site reacts to the presence of the chemical or its metabolites); similarly the 10-fold inter-individual uncertainty factor is replaced by a 3.2-fold factor for toxicodynamic differences and a 3.2-fold factor for toxicokinetic differences (WHO, 1999; Renwick *et al.*, 2003). Comparative chemical specific data on the toxicokinetics and toxicodynamics, when available, may then be used to derive chemical specific adjustment factors which would replace some or all of these four default uncertainty factors for inter-species and inter-individual extrapolation (Edler *et al.*, 2002). However, such data, generally, have not yet become available for mycotoxins.

An additional uncertainty factor of 1–100 may need to be incorporated to



**Fig. 1.2** Showing the derivation of the TDI from the NOAEL for the critical effect. At higher doses more severe effects, each with their own dose response curve are observed.

account for uncertainties in the available experimental studies (i.e. data are sufficient to establish a LOAEL but not a NOAEL; subchronic studies rather than chronic studies are available; incomplete data such as the lack of studies on reproductive effects) (Dybing *et al.*, 2002). When significant irreversible and severe but thresholded effects (i.e. teratogenic effects) are observed, this may sometimes result in the use of an additional uncertainty factor of 1–10 for severity, if this is also the critical effect. The latter is designed to protect highly susceptible lifestages, such as pregnancy (EPA, 2003). Similarly, because of their possible greater vulnerability, it has been suggested that for infants and young children an additional 10-fold uncertainty factor should be used as a default (EPA, 1999), unless comprehensive data for the relevant age group are available (Renwick *et al.*, 2003). An ILSI sponsored workshop concluded that, in general, an ADI/TDI derived from adequate toxicological data should cover all age groups including infants from 12–16 weeks upwards (Larsen and Pascal, 1998; Østergaard and Knudsen, 1998). Younger infants are not covered by the multi-generation study, since their diet is a direct intake rather than an indirect intake through the dams milk in the surrogate rodent studies. With substances, such as natural toxicants, a full complement of reproductive and developmental studies may not be available and, in such cases, an additional uncertainty factor could be appropriate (Renwick *et al.*, 2003). Therefore, for the risk assessments of mycotoxins involving infants and very young children relevant toxicological data need to be reviewed on a case by case basis. The overall uncertainty factor should be less than 10 000, since larger factors indicate serious limitations in the database.

With different animal species and humans, a mycotoxin may cause several different adverse effects. Each situation may call for a different uncertainty factor, so that one needs to look at several combinations of effects and associated

uncertainty factors in an iterative fashion, before selecting an overall critical effect and uncertainty factor (Renwick *et al.*, 2003). Since there may be a number of uncertainties in the available data, experience and judgement are needed to decide upon the final uncertainty factor, and a rationale for this decision needs to be given. The benefit of the uncertainty factor approach is that the choice of the various factors is transparent and it may indicate where further research is useful. Sometimes the setting of a provisional/temporary (p/t)TDI is deferred until sufficient data are available. In such cases, and when dealing with unavoidable toxic substances, such as mycotoxins, one may determine the margin of exposure, relating actual human exposure to the NOAEL (see risk characterization – Section 1.5).

When a TDI is derived from a NOAEL in studies in humans who have suffered ill health as a result of (environmental) conditions of high exposure to mycotoxins, a default 10-fold uncertainty factor is used to take account of inter-individual variation in susceptibility amongst humans. This factor may be reduced if the human studies include all age groups and the data are considered robust.

As important information about the toxicity of mycotoxins is derived from observations at higher dose levels than those used for setting the TDI, it is important to consider not only the most sensitive endpoint (critical effect) but also the increased severity of effects seen at higher dose levels, as this will help in priority setting in situations where the TDI is exceeded (see risk management – Section 1.6). Categorical regression is a mathematical approach to hazard characterization whereby effects of various severities are rated and related in order to estimate potential health risks (Dourson *et al.*, 1997; Edler *et al.*, 2002).

Another measure, useful in risk management, is the acute reference dose ( $RfD_{acute}$ ). This value is estimated to deal with situations where there is high short-term or single exposure with the possibility of subsequent adverse effects. The  $RfD_{acute}$  is derived from appropriate animal or human data and is generally 2–10-fold greater than the TDI. In general, it should not be used for substances for which there is no threshold for the observed toxic effects.

### 1.3.2 Carcinogenic mycotoxins

#### *Biological aspects*

Many mycotoxins of economic importance have been found to be carcinogenic in animal studies. The processes underlying experimental carcinogenesis and carcinogenesis in humans are complex. To describe this process, the simplest model, used by many scientists over the last several decades, identifies three major stages – initiation, promotion and progression (Dragan *et al.*, 1993; Kinzler and Vogelstein, 1996) (Fig. 1.3):

- Initiation leading to an altered cell
- Promotion involving clonal expansion
- Progression to malignancy

Increasing genomic instability  
 Capacity to invade surrounding tissues  
 Metastasis to distant sites

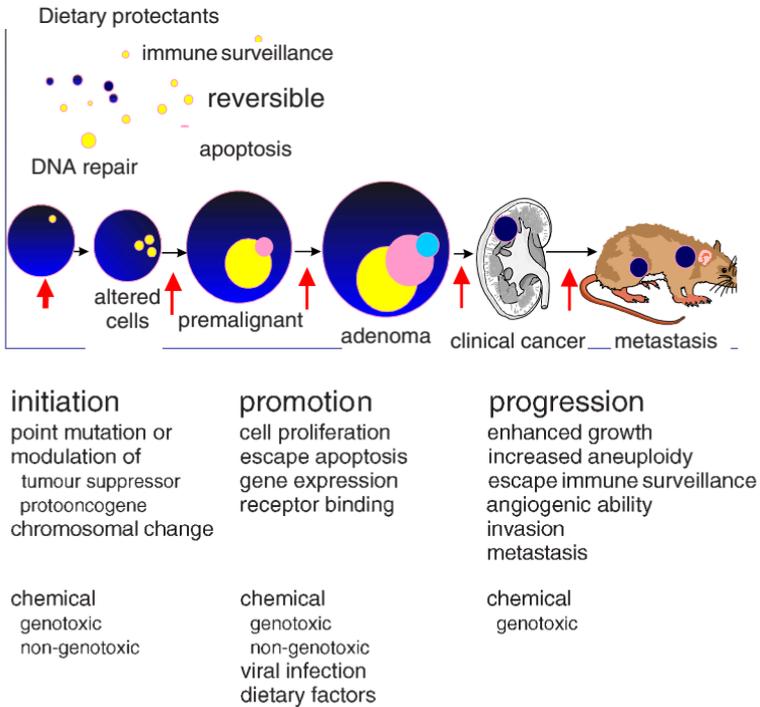
It has been presumed that initiation and progression involve DNA reactive events, whereas promotion is thought to involve several non-DNA reactive events, often described as epigenetic events (Fig. 1.3). It is becoming apparent that epigenetic events may play a larger role than first presumed, as gene expression may affect all three stages. The capacity to invade surrounding tissues and the ability to metastasize to distant sites are of great importance in human cancer, and each of these steps involves further genetic change. Genomic instability, whether induced by genetic or epigenetic mechanisms, is considered to play a key role in the process of carcinogenesis (Stoler *et al.*, 1999). Scientists still do not have a full understanding of all the processes underlying initiation, promotion, progression and factors affecting metastatic potential in carcinogenesis.

Many 'complete' DNA reactive carcinogens are capable of inducing both initiation and promotion, albeit at different rates, and several processes may occur concurrently (e.g. formation of DNA adducts, DNA damage and repair, apoptosis and escape from apoptosis, cell damage, cell proliferation, etc.) (Fig. 1.3). Individual assays that measure only one aspect or assessments that focus on one aspect do not cover the full process of carcinogenesis.

As it has been argued that initiation can take place only after a genetic change (which may lead to a mutation) has occurred, it has been common practice to determine the potential of a chemical to cause such changes in a battery of genotoxicity tests (Ashby *et al.*, 1996; Albertini *et al.*, 2000; OECD, 2000; Eisenbrand *et al.*, 2002). Results of such genotoxicity tests indicate a chemical's ability to induce point mutations (with or without metabolic activation), or cause direct or indirect DNA damage and chromosomal change, such as aberrations. However, the standard battery of short-term tests may fail to detect some rodent and human genotoxic carcinogens (Brambilla and Martelli, 2004). A large number of chemicals may also induce genome mutations, which involve numerical chromosome changes leading to aneuploidy (Eisenbrand *et al.*, 2002), a common property of all tumors.

It is generally assumed that for chemical carcinogens that are directly DNA reactive, there is no threshold dose below which this reaction does not take place. However, at very low doses, DNA repair, apoptosis and immunoprotective events could result in a practical threshold (see Fig. 1.3). Theoretically, the presence (or absence) of a threshold is deduced from an understanding of the underlying biological processes, such as discussed here, rather than from the shape of the dose-response curve.

Aflatoxin B<sub>1</sub> is an example of a DNA-reactive carcinogenic mycotoxin that appears to have both initiating and promoting properties and that may also contribute to tumor progression. Several other carcinogenic mycotoxins appear to be non-DNA-reactive or indirectly DNA-reactive, and have been considered by some organizations to operate predominantly through a mode of action involving



**Fig. 1.3** Showing a model of the multiple stages in the development of cancer. Arrows indicate where changes in genome may take place; the small circles within the large circles indicate new acquired traits, each of which gives greater autonomy to the growing tumor. DNA repair, apoptosis and immune surveillance are some of the protective mechanisms. The lower part of the text indicates agents that may influence each of the stages.

tumor promotion (Tennant, 1994). Epigenetic events underlying tumor promotion have been reviewed (Eisenbrand *et al.*, 2002; EPA, 2003) and may include:

- persistent cytotoxicity accompanied by cellular proliferation
- DNA methylation leading to altered gene expression
- chronic inflammation leading to free radicals and DNA damage
- direct and indirect effects of hormones
- receptor binding leading to cell division and blocked apoptosis

Some of these events can also interact with a variety of host factors, such as the

immune system, stress, virus infection such as hepatitis B and C (the presence of which interacts with aflatoxin), dietary factors, obesity, disease states, lifestyle, as well as species and strain susceptibility (Fig. 1.3). The carcinogenicity of the estrogenic mycotoxin zearalenone probably involves a direct effect on hormone balance (Kuiper-Goodman *et al.*, 1987). It has been reasoned that for many carcinogens operating *solely* through an epigenetic mode of action, there is a threshold dose below which such a sequence of events leading to cancer does not take place.

A controversial issue is whether sustained cytotoxicity in the target organ accompanied by cell proliferation is the *only* mode of action for some carcinogenic mycotoxins, such as ochratoxin A and the fumonisins. It is known that cell proliferation is a *necessary* 'key event' underlying all chemical carcinogenesis, as it leads to the clonal expansion of altered cells. Thus, extensive cytotoxicity and increased cell proliferation are also seen with DNA-reactive carcinogens, such as aflatoxin B<sub>1</sub>, and not all cytotoxic chemicals induce uncontrolled growth leading to carcinogenesis (Butterworth and Bogdanffy, 1999; Blagosklonny, 2002).

There is a need for better understanding of what is thought to happen as a result of exposure to a cytotoxic chemical. In normal tissue there is a balance between proliferation and controlled cell death (apoptosis). Effective DNA repair removes deleterious mutations, ensuring a low mutation rate (Fig. 1.3). It has been suggested that when normal cells are exposed to a cytotoxic chemical carcinogen, growth and survival are inhibited. In the ensuing regenerative response, a high mutation rate can accelerate adaptation and some of the cells become resistant to the toxin in a process akin to drug resistance (Blagosklonny, 2002). Resistance to the cytotoxic effects of a chemical may lead to 'non-oncogenic' and/or 'oncogenic resistance'. The former process involves a number of cytoprotective mechanisms that do not lead to cancer. Through genetic alteration, involving oncogenes or tumor suppressor genes, 'oncogenic resistance' can develop by which cells acquire a number of attributes that allow them to grow, despite the presence of a cytotoxic stimulus (Blagosklonny, 2002). Such 'altered' cells eventually overcome the ability to protect against transformation (Preston, 2000). In a milieu of genomic instability, and in a process akin to Darwinian selection, tumor cells eventually acquire at least six capabilities, considered to be 'hallmarks' of cancer (Hanahan and Weinberg, 2000), allowing them to become more and more aggressive and progress all the way to metastasis. These traits are:

- ability to proliferate independent of mitogenic growth signals
- insensitivity to antigrowth signals
- resistance to apoptosis
- ability to escape the intrinsic cell-autonomous program that limits their multiplication
- angiogenic ability (encourage blood vessel growth)
- capability for invasion and metastasis

The above model does not only apply to DNA-reactive carcinogens. Some non-

DNA-reactive chemical carcinogens, through a variety of mechanisms, appear to indirectly play a role in the epigenetic modulation of proto-oncogenes and tumor suppressor genes leading to initiation (Fig. 1.3). As an example, through altering a signalling pathway, one of these epigenetic mechanisms may involve changes in DNA methylation that lead to aberrant heritable over-expression of proto-oncogenes, such as the activation of the *ras* pathway, and contribute to transformation (Clark *et al.*, 1996). Similarly, hypermethylation may lead to silencing of tumor suppressor genes (Counts and Goodman, 1995; Feinberg and Tycko, 2004). Many alterations in tumor cells, as they escape normalcy, involve genetic and a variety of epigenetic events which occur side by side (Hanahan and Weinberg, 2000).

In assessing detailed mechanisms of action, such as discussed above, dose response is an important consideration. Are epigenetic amplifications of oncogenes and tumor suppressors only observed after high cytotoxic doses? In this regard, the presence of a threshold cannot be proven from experimental data, since the absence of a detectable effect at low doses could be either because the dose is below a threshold in the dose response, or because the response is below the level that can be detected in the test system (Renwick *et al.*, 2003).

Molecular data with regard to the activation of specific proto-oncogenes or tumor suppressor genes may not yet be available for a particular carcinogenic mycotoxin. Tumors induced through a non-DNA-reactive mode of action are typically less aggressive than those produced through a DNA-reactive mode of action (Dietrich and Swenberg, 1991; Tennant, 1994; Kuiper-Goodman, 1996) (Table 1.2). In this regard, ochratoxin A, considered by some to be non-DNA-reactive or indirectly DNA-reactive, is an enigma. A comparison of all NTP studies in which renal carcinogenesis was observed showed that ochratoxin A was not only the most potent renal carcinogen but, as indicated by the pathological description, the behaviour of the resulting tumor was more aggressive than that of any of the renal tumors induced by different genotoxic chemicals. Extensive karyomegaly (presence of large polyploid cells), possibly indicating genomic instability, and the very high incidence of metastases may suggest that additional modes of action other than promotion are likely (Kuiper-Goodman and Scott,

**Table 1.2** Non-DNA reactive carcinogens.\*

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Characteristics of induced tumors

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Often single species, site, sex  
 Few trans-species, common site  
 Low tumorigenic potency  
 Low incidence of tumors  
 Low proportion of carcinomas versus adenomas  
 Tumors are typically not very aggressive  
 Few metastases  
 Tumor does not reduce lifespan  
 Mutation frequency similar to spontaneous tumors

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\*Modified from Kuiper-Goodman, 1996

**Table 1.3** Evidence for carcinogenicity as rated by IARC in 1993.

Mycotoxin	Human evidence	Animal evidence	Overall classification
Aflatoxin B <sub>1</sub>	S	S	1
Aflatoxin M <sub>1</sub>	I	S	2B
Ochratoxin A	I	S	2B
<i>F. verticilloides</i> toxins	I	S	2B
Fumonisin B1		L <sup>1</sup>	
Deoxynivalenol		I <sup>2</sup>	
Zearalenone		L	3
Patulin	N	I	3

Note: S = sufficient; L = limited; I = inadequate; N = negative.

<sup>1</sup> Before the NTP study (NTP, 1999); rating will probably change to S.

<sup>2</sup> Before the Health Canada study (Iverson *et al.*, 1996); rating may remain as I, since only one species (mouse) was tested or it will change to N.

1989; NTP, 1989; Kuiper-Goodman, 1996; JECFA, 2001c). Karyomegaly in kidney tissue reported by NTP (NTP, 1989) was also observed in an earlier 90-day toxicity study concluded at Health Canada, and this did not completely disappear after a 90-day stop and recovery experiment (Munro *et al.*, 1974). In addition the large number of metastases, usually a rare event in rodent tumors, suggests that ochratoxin A probably operates through multiple modes of action and, at present, assuming an overall 'no threshold mode' is the more prudent course to follow.

A number of chemical carcinogens induce tumors in animals by a species-specific mechanism or mode of action that is likely to be irrelevant for humans (Meek *et al.*, 2003). Thus, taking all of the above under consideration, the finding of tumors in a two-year rodent bioassay requires careful interpretation, to determine whether such findings are relevant to humans. Nevertheless, the distinction between DNA-reactive and non-DNA-reactive carcinogens can be somewhat moot (Preston, 2000), and caution is advised when assigning a non-threshold or threshold mode of action to these two groups of carcinogens.

Most organizations, including the IARC, use a weight of evidence approach to consider all the available information on tumor induction potential, obtained under hazard identification, and, based on a balance of probabilities, characterize the biological significance of a carcinogenic hazard to humans (Table 1.3) (IARC, 1993; EPA, 1999). In the scheme currently used by the IARC, carcinogens are rated based on the overall animal and human evidence. Thus, as discussed in the preamble of each IARC issue (IARC 1993), animal data indicating a response in more than one species, in both sexes, and showing a clear dose response, are rated as providing 'sufficient evidence'. The IARC assigns the rating 'limited evidence' when the data suggest a carcinogenic effect, but are limited for making a definitive evaluation such as: only one experiment has been conducted; there are inadequacies in the design, conduct or interpretation of the study; the incidence of only benign neoplasms or predominantly spontaneous tumors in susceptible strains is increased. With only poor studies available, a rating of 'inadequate evidence' is given, and good studies in two species showing negative findings results in a rating

of 'no evidence'. Similarly, IARC rates evidence for human carcinogenicity based on epidemiological studies as 'sufficient' when causality has been clearly established, 'limited' when chance, bias and confounding factors cannot be ruled out, 'inadequate' for poor studies, and 'no' for good studies with negative results. The IARC then combines the ratings for animal and human data giving an overall rating of group 1 ('human carcinogen' – sufficient animal and human evidence); group 2A ('probable human carcinogen'); group 2B ('possible human carcinogen'); group 3 ('inadequate information'); and group 4 ('no evidence'). Expert judgment considering all evidence is required in assigning carcinogens to these groups and, as further data become available, assignments may change upon re-evaluation. Table 1.3 summarizes the IARC ratings for several mycotoxins of interest. While the IARC evidence ratings consider information on DNA reactivity, they generally do not include information on potency, and the IARC does not address the numerical aspects of risk assessment of carcinogens, nor does it give an opinion as to whether a carcinogen operates through a threshold or a non-threshold mode of action.

Recently, through efforts by the EPA and the International Programme on Chemical Safety (IPCS), approaches have been developed whereby, based on available data for specific chemicals, special attention is given to understanding the mode of action (MOA) underlying induced tumorigenesis, and whether this MOA is relevant to humans (EPA, 1999, 2003; Sonich-Mullen *et al.*, 2001) (Fig. 1.1). The MOA is used to describe 'key events' and processes underlying toxicity including carcinogenicity, whereas 'mechanism of action' implies a more detailed molecular description of events. Key events (necessary events that are on the causal pathway towards cancer) are distinguished from 'associated events' that may occur at the same time. Species and strain susceptibility play an important role in the MOA (EPA, 1999; Schlosser and Bogdanffy, 1999; Dybing *et al.*, 2002).

This approach was recently expanded into the Human Relevance Framework (HRF), which also uses a weight of evidence approach to hypothesize an MOA. It includes a qualitative and quantitative assessment of the relevance of the MOA to humans and further improves transparency (Fig. 1.1) (Meek *et al.*, 2003). Key events, data gaps and uncertainty are systematically considered. With this approach risk assessors can take a broad look before molecular information is available. The HRF is a four-part analysis which, for each tumor endpoint, features the following questions.

- Is the weight of evidence sufficient to establish the MOA in animals?
- Are key events in the animal MOA plausible in humans?
- Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans?
- Conclusion: statement of confidence; analysis; implications.

Under the HRF approach as well as under draft EPA guidelines, it is presumed that cancer induced in animals by both DNA-reactive and non-DNA-reactive chemicals is relevant to humans by default, unless the MOA indicates otherwise. It is recognized that any particular chemical may induce cancer through more than one

MOA, be responsible for tumors in more than one tissue and affect endpoints other than cancer (Meek *et al.*, 2003). Modified Bradford Hill criteria of causation are used to determine the strength of the evidence, by examining coherence, specificity and plausibility, and dose response as well as temporal relationships. It is anticipated that, as experience is gained in using such frameworks, further modifications will be made. Thus, rather than deciding on alternate MOAs for a particular tumor endpoint, it may be better to consider whether multiple MOAs are likely for that tumor endpoint. At present the focus of the HRF seems to be on the first stages of carcinogenesis. While approaches such as the HRF add transparency to decision making, it is important that all relevant information is used, including a critical use of pathological information on the resulting tumors and information on molecular markers, when available. Above all, when using the framework, one needs to be aware of additional models or hypotheses regarding the process of carcinogenesis, and one of the outcomes may include the need for further investigation.

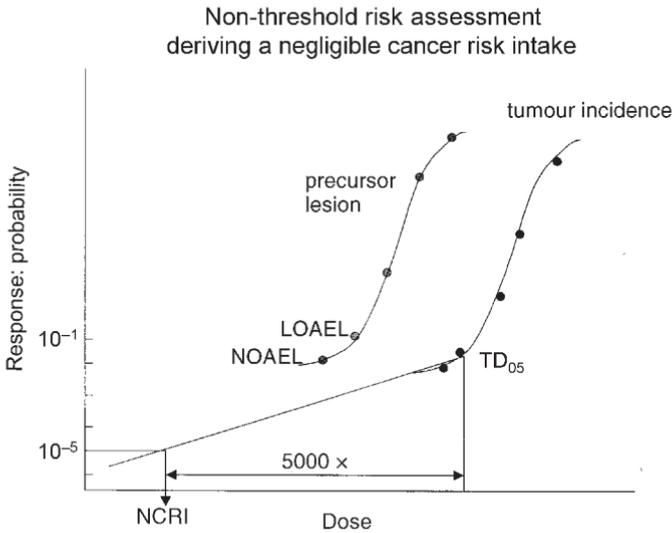
#### *Extrapolation approaches for carcinogens*

For DNA-reactive carcinogens the default position has been that there is no threshold dose below which effects, such as initiation of the carcinogenic process, will not occur, and a TDI, which is based on a threshold such as the NOAEL, is generally not determined. Non-threshold carcinogens are not allowed as food additives. When such chemicals cannot be completely avoided (i.e. some mycotoxins), a variety of approaches have been used to determine exposure levels that may be so low as to not be of concern.

Appropriate mathematical models, most of which presume a linear dose–response relationship at low doses, have been used to extrapolate from the observable part of the dose–response curve (usually consisting of only 2–3 data points) to low doses. Such models do not take into account relevant data on mechanisms of tumor induction or differences in toxicokinetics/dynamics between the animal species and humans, or differences in susceptibility between individuals. The dose corresponding to a response level of  $10^{-5}$  in animals (for mycotoxins) or  $10^{-6}$  in animals (i.e. for pesticides) has in some jurisdictions been considered to pose a risk to humans that is so small as to be negligible, compared with other risks faced by society, especially since, at such low doses, a variety of processes operate to counteract the risk (DNA repair, apoptosis, immune surveillance, etc.) (Fig. 1.3).

A starting point for linear extrapolation can be the tumorigenic dose (TD), or its lower confidence interval, which is derived from all the experimental data in the observable range, and represents the dose, above background at which 5, 10 or 25 % of the animals are responding, the  $TD_{05}$ ,  $TD_{10}$ , or  $TD_{25}$ , respectively. The  $TD_{05}$  is similar to the  $BD_{05}$ , and to derive it a polynomial regression method can be used on the data in the observable range (Fig. 1.1) (Crump, 1984, 1995).

Dividing the  $TD_{05}$ , which is not a threshold, by a factor of 5000, gives a value, which we have termed the negligible cancer risk intake (NCRI), and which is equivalent to a response level of  $10^{-5}$  in animals (Fig. 1.4). It provides a similar estimate of safe intake to that derived using low dose linearized models and is



**Fig. 1.4** Showing the linear derivation of a negligible cancer risk intake from the  $TD_{05}$ . The  $TD_{05}$  is derived from the animal tumor data in the observable range, and indicates a 5% response rate. Linear extrapolation to a response rate of  $1:10^{-5}$ , gives the negligible cancer risk intake (NCRI); dividing the  $TD_{05}$  by a factor of 5000 gives the same result. In some cases it may be decided to determine a TDI from the NOAEL of a precursor lesion seen at lower dose levels, and convergence between the two approaches may be seen (see text for details).

therefore appropriate for non-threshold carcinogens. The same approach can also be used for those threshold carcinogens for which there is some uncertainty regarding the mode of action (threshold versus non-threshold, e.g. ochratoxin A) (Kuiper-Goodman, 1991). The factor of 5000 can be decreased when additional biological information on the mode of action or quantitative aspects relating to human relevance indicate less concern. Alternatively, the  $TD_{05}$  can be compared with actual exposure data (Fig. 1.1) (Health Canada, 1994) (see risk characterization – Section 1.5).

Other jurisdictions, including JECFA, consider that for genotoxic carcinogens or genotoxic agents for which the carcinogenic potential has not been adequately studied, the appropriate way to regulate is to determine levels that are ‘as low as reasonably achievable’ (ALARA) (see Section 1.6 and Fig. 1.1).

When the MOA underlying the carcinogenic process does involve a threshold, the same approaches as outlined for non-carcinogenic mycotoxins can be used. However, rather than applying uncertainty factors to the NOAEL for actual tumor data, one can use an uncertainty factor approach on key events, such as a precursor lesion thought to be on the causal pathway (Fig. 1.4). Such an approach was used by JECFA in its evaluation of ochratoxin A and the fumonisins. Both of these mycotoxins were considered by JECFA to operate through a mode of action that

**Table 1.4** Provisional tolerable daily intakes (TDIs) for selected mycotoxins.

Mycotoxin	TDI ng/kg bw per day	Organization
Ochratoxin A	4	Health Canada, 1989 <sup>1</sup> ; 1996 <sup>2</sup>
	5	Nordic Council, 1991
	5	EU, 1998
	14	JECFA, 1996a, 2001c
Fumonisin	2000	EU, 2000a
	2000	JECFA, 2001b
	400	Health Canada <sup>3</sup> , 2001
Deoxynivalenol	3000	Health Canada <sup>4</sup> , 1985
	1000	Health Canada <sup>3</sup> , 2001
	1000	Nordic Council, 1998
	1000	EU, 1999
	1000	JECFA, 2001a
Zearalenone	100	Health Canada <sup>5</sup> , 1987
	100	Nordic Council, 1998
	500	JECFA, 2000
	200	EU, 2000b
Patulin	400	JECFA, 1996b
	400	EU, 2000c
	400	Health Canada <sup>3</sup> , 1996

<sup>1</sup>Kuiper-Goodman and Scott, 1989; <sup>2</sup>Kuiper-Goodman, 1996; <sup>3</sup>Kuiper-Goodman, personal comm; <sup>4</sup>Kuiper-Goodman, 1985; <sup>5</sup>Kuiper-Goodman *et al.*, 1987

involves cytotoxicity and sustained cellular proliferation. JECFA used a key event – cell damage – in the most sensitive species for this effect (pig rather than rat for ochratoxin A), and applied several uncertainty factors, including one for severity (JECFA, 2001c). In the case of ochratoxin A, this approach resulted in a TDI that was about 3.5-fold greater than an estimate of safe intake derived through linear extrapolation from the TD<sub>05</sub> for renal tumor induction (Kuiper-Goodman, 1996). The NOAEL and the BD<sub>05</sub> are in the same region of the dose–response curve. Since the BD<sub>05</sub> and the TD<sub>05</sub> can be derived in a similar manner, and since dividing by an uncertainty factor is actually a linear process, it can be seen that there can be a convergence between approaches that assume a threshold (such as for a precursor lesion) and a non-threshold mode of action (Figs 1.2 and 1.4).

Estimates of risk at low doses may also be derived from epidemiological studies when appropriate studies, such as for aflatoxins, are available (JECFA, 1998).

Provisional TDIs set by various organizations for selected mycotoxins are shown in Table 1.4. Although difficult to determine, the ‘true TDI’ or ‘estimate of safe intake’ can be viewed as an intrinsic property of mycotoxins, that takes into consideration both potency of the effect measured and biological factors regarding the severity, relevance and significance of the effects for humans.

## 1.4 Exposure assessment

Exposure to mycotoxins depends on the level of these substances in different foods and on the intake of those foods (Kroes *et al.*, 2002). For mycotoxins, actual monitoring data over several years in raw or finished food commodities usually provide the input for the data on occurrence levels.

### 1.4.1 Analytical data

Reliable and validated analytical methods to determine the occurrence of mycotoxins in food commodities are currently available for only a few mycotoxins. Recently, improved methods have been established with the development of specific antibodies for use in enzyme-linked immunosorbent assays (ELISAs) and use of immunoaffinity columns for sample clean-up. Problems exist with the non-homogenous distribution of mycotoxins in food commodities, thus requiring appropriate sampling (Gilbert, 1996; see also Chapter 4). There may also be matrix effects which can result in under-estimating the amount of mycotoxin, unless appropriate extraction methods for different foods are developed.

Mycotoxins in foods occur frequently at low concentrations, with a number of samples below the limit of detection (LOD) and limit of quantification (LOQ). How such 'non-detects' are treated is important in the exposure estimation, as whether such values are considered to be zero, 50 % of the LOQ or equal to the LOQ makes a big difference. For exposure assessments, it is useful also to provide data between the LOD and LOQ. Data on recovery may also be included.

### 1.4.2 Processing

Grain cleaning and further processing in mills can divert mycotoxins to various mill streams, and further processing such as baking may reduce mycotoxin levels. Thus, occurrence estimates may need to be refined by considering further modifications in the levels of these substances in the food, as actually consumed, by including information on manufacturing and processing both by industry and in the home and by applying appropriate correction factors.

### 1.4.3 Animal derived foods

It is also necessary to estimate the contribution to exposure from mycotoxins (including metabolites and conjugates) in animal derived food products. At high levels in feed these mycotoxins may cause loss or illness of farm animals, through development of animal mycotoxicoses as indicated under hazard identification (Section 1.2). At lower levels in feed these mycotoxins may have no apparent effect on livestock production, but their residues and related metabolites and conjugates may move up the food chain (Kuiper-Goodman, 1991). Carry-over

studies in livestock, to determine transmission from feed to tissue, have only been conducted for major mycotoxins and more information on bioavailability to humans of the parent compound and its metabolites including protein-bound and other conjugates is needed (Kuiper-Goodman, 1991).

#### 1.4.4 Food consumption patterns

There can be large national and regional differences in the intakes of foods, so that exposure assessments are country specific. This aspect has been addressed to some extent by the FAO through its Global Environmental Monitoring System (GEMS/Food) project, whereby diets for Far Eastern, African, South American and European (including North American) regions are assessed separately. The most reliable data come from national dietary surveys of individuals. Where such food consumption data are available, these can be combined with data on occurrence levels to estimate exposure. A full range of potentially exposed individuals including infants, the elderly, and ethnic minorities needs to be sampled. Intake by persons with a medical condition that may affect food consumption also needs to be addressed (Renwick *et al.*, 2003).

Traditionally, exposure estimates have been point estimates. These are based on the mean or the 90th percentile intake value of 'all persons' or on only the fraction of persons, 'eaters or consumers', that consume the food; these point estimates can also be stratified for different age groups or target groups, depending on the scenario being investigated. It has been found that exposure varies with age, with young children generally being exposed at a much higher rate, on a body weight basis. Thus for commodities such as milk and peanut butter, that may contain aflatoxins or their metabolites, exposure in young children compared to adults can be up to 7-fold and up to 4-fold, respectively.

The introduction of probabilistic exposure estimates in recent years allows one to examine the distribution of exposure from a convolution of all the underlying distributions (see also Chapter 2). A variety of commercial software packages have been developed that use the input of country-specific food intake data. In developing and using these approaches, a number of assumptions are made. Such assumptions need to be transparent as well as flexible, and they need to be carefully assessed against traditional approaches. Furthermore, for worldwide harmonization, international agreement is needed on how to derive the appropriate parameters, such as the appropriate percentiles of exposure.

#### 1.4.5 Biomarkers

Exposure assessments can also be based on measurements of serum or urinary biomarkers in humans, and exposure can then be estimated based on pharmacokinetic relationships. Biomarkers are available for aflatoxins and ochratoxin, and recently a biomarker has been developed for deoxynivalenol (Meky *et al.*, 2003), with the latter still needing to be validated.

## 1.5 Risk characterization and evaluation

Risk characterization is the last stage of risk assessment. It includes the qualitative and/or quantitative estimation, including the attendant uncertainties, of the severity and probable occurrence or absence of known or potential adverse health effects in an exposed population based on hazard identification, hazard characterization, and exposure assessment. Alternatively, for threshold chemicals, risk characterization involves a comparison of levels of daily exposure over a lifetime to the TDI, the intake for which the risk is considered to be insignificant. If long-term or short-term exposure exceeds the TDI, recommendations for risk reduction may be made, with priorities determined by the extent that the TDI is exceeded (Fig. 1.1).

For substances for which there are insufficient data for the establishment of a TDI, the margin of exposure, which is the ratio between human exposure and the NOAEL of the critical effect in animal species, may serve as an indication of the likelihood of ill effects occurring in humans and thus the need for risk reduction. To better characterize the risk, one can also make a series of comparisons between actual exposure and the NOAEL or even the LOAEL of several effects of increasing severity, as was done for fumonisins when the database was not sufficient to derive a TDI (Kuiper-Goodman *et al.*, 1996). Rather than estimating risk or assigning a risk level, a similar approach can be used for non-threshold mycotoxins as well.

Besides considering the average population, risk characterization also needs to consider lifestage and the most susceptible groups with regard to exposure or vulnerability, such as infants and young children (because of lower body weight and possibly increased susceptibility), as well as other groups for which there may be differences in bioavailability or metabolism, such as the elderly, or persons with specific genetic predispositions. In this regard, the adequacy of a 10-fold safety factor to address differences in human susceptibility as a reason for human variability may need to be examined on a case by case basis.

Since mycotoxins affect mainly staple foods, exposure in market-based economies, where sources of food commodities grown in different areas are mixed, tends to be lower, but usually is of long duration. In farming communities, where locally grown food may be consumed, high exposure of shorter duration combined with long-term low-level exposure may be encountered periodically. Neither the TDI nor the acute reference dose specifically address this issue (Renwick *et al.*, 2003). The TDI relates to lifetime exposure and provides a margin of safety that is large enough to allow for the occasional short-term exposure above the TDI, provided that average intake over longer periods of time does not exceed it (WHO, 1987; ILSI, 1999). For short-term exposure, one should consider whether toxicity is associated with the peak concentrations. For chronic exposure, the duration, the extent of the higher exposure, toxicokinetics, and mode of action determine whether such exposure would be considered to fall within the allowable short-term 'excursion' above the TDI (ILSI, 1999; Renwick *et al.*, 2003). For some substances, averaging exposure may give an indication of the risk. However, one has to specifically address high exposure during a vulnerable lifestage such as

embryonic development or infancy. Special consideration should also be given to the impact of high exposure of a non-threshold carcinogen. Probabilistic methods by which point estimates for the NOAEL and the uncertainty factors are replaced by distributions combined with a probabilistic exposure assessment may help in estimating the distribution of risk for various endpoints and for various segments of the population (Edler *et al.*, 2002; Renwick *et al.*, 2003), but one needs to carefully examine the underlying assumptions. This approach was used in the Netherlands when confronted with high deoxynivalenol levels (Pieters *et al.*, 2002).

The *indirect* intake of mycotoxins and related substances from the consumption of animal-derived food products may pose a health hazard to humans. Some mycotoxins (i.e. aflatoxins and ochratoxin) are known to be transmitted to edible tissues and therefore are of greater concern. For ochratoxins the risk to humans associated with indirect exposure from animal-derived food products is generally somewhat lower than that from direct exposure to cereal and other food crops (Gilbert *et al.*, 2001).

In summary, detailed risk assessments have been performed for only a few mycotoxins (aflatoxins, ochratoxin A, zearalenone, patulin, fumonisins, deoxynivalenol and some related trichothecenes) and these evaluations need to be reexamined from time to time with respect to new critical information on exposure, toxicology as well as a better understanding of the mechanism and mode of action.

## 1.6 Risk management of mycotoxins

For most of the major mycotoxins of economic importance (aflatoxins, ochratoxin, fumonisin, deoxynivalenol, zearalenone, ergot alkaloids, patulin) it is necessary to monitor their presence and, in at least some parts of the world, risk reduction may be necessary, to ensure that exposure does not pose a health risk.

Recommendations for risk reduction are made when exposure poses a possible health risk, as identified and described under risk characterization – Section 1.5 and Fig. 1.1. Risk management sets priorities for risk reduction in line with a variety of available options. Priorities depend on the extent and frequency that the TDI is exceeded, or on the size of the margin of safety or exposure (Health Canada, 1994). In these deliberations one considers the severity of the health effect, the impact of short-term elevated exposure on eventual health consequences, as well as the size and vulnerability of the population affected. Because of the many uncertainties involved in low dose extrapolation, estimating the size of the risk should be avoided.

With regard to mycotoxins there are a variety of risk management options that help to ensure a safe food supply. These range from prevention of mold growth through modifying agricultural practices, setting of regulatory limits for mycotoxins in grains destined for food and feed use, to diversion into alternate uses. There can be high economic costs associated with all of these, but prevention through adopting a HACCP approach is probably the most effective (Park *et al.*, 1999). In

recent years Codex has developed a draft code of practice for the prevention of mycotoxin contamination in cereals (Codex, 2001).

Over the years, most countries have established maximum residue limits for a variety of mycotoxins (see also Chapter 3). Initially, most regulations pertained to aflatoxins and, rather than a risk assessment, levels were based on technologically feasible levels or on limits of analytical detection (Stoloff *et al.*, 1991). Currently, risk assessment has become the basis for establishing regulations (Fig. 1.1). This ensures that exposure to mycotoxins, with regulations in place, should normally not exceed the TDI and should therefore pose an 'insignificant' risk. For non-threshold carcinogens, some jurisdictions prefer to use an approach that keeps residue levels 'as low as is reasonably achievable' (ALARA), but it is then difficult to derive an appropriate value for use in setting a maximum residue level (MRL). Other jurisdictions try to ensure that MRLs for unavoidable non-threshold carcinogens pose a 'negligible risk', which for mycotoxins is generally taken as the dose associated with a response of 1:100 000 animals. Compared to this, the EPA, using a similar derivation, uses a response level in animals of 1:1 000 000 for avoidable substances, such as pesticide residues, that pose a carcinogenic risk (EPA, 1999).

In recent years, Codex has taken the lead in developing an international consensus on deriving MRLs, based on scientific evaluations. Such MRLs should be acceptable to both producing and importing countries, and the setting of MRLs should not result in unnecessary trade barriers. Although MRLs are a cut-off for trade, levels of mycotoxins somewhat above this could be tolerated on a low incidence basis. However, in the final risk assessments, exposure assessments for mycotoxins ideally are based on actual residue levels and not on the presumption that the level is at the MRL, as a worst case scenario. Thus exposure tends to be lower than what would be predicted from the MRL. The concern of regulators has been that, if a higher MRL were to be allowed, this would become the acceptable level for industry and blending with contaminated grain upwards to this level would occur, thus increasing exposure. For this reason blending is not allowed in many jurisdictions.

## 1.7 Conclusion

There have been a wide variety of regulations pertaining to mycotoxins. Initially many of these regulations were not based on sound scientific evaluation. As of yet, there is a lack of agreement between countries on certain aspects of risk assessment and risk management but, in recent years, many organizations have been moving towards harmonization on various general aspects. Thus the CCFAC has proposed regulatory limits for several mycotoxins (aflatoxins, ochratoxins, patulin, zearalenone and fumonisins) and with comments from its member countries it is hoped that a consensus can be reached. The ultimate aim is to achieve optimal regulations for human safety, which at the same time do not become trade barriers. The role of risk assessment and risk analysis in achieving this goal is presented.

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## 2

# Modelling exposure to mycotoxins

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### 2.1 Introduction: quality and availability of data

The use of probabilistic modelling techniques within the field of exposure assessment is a subject of increasing interest. This chapter is aimed at illustrating the rationale for their use and the critical elements that need to be taken into consideration in order to develop and correctly interpret the results obtained with such models. Whenever available, examples have been taken from the literature to illustrate applications to the assessment of exposure to mycotoxins, mainly at European level. Clearly, the quality of assessment of exposure to hazardous substances depends largely on the data used rather than simply on the exposure model chosen. For this reason, the chapter begins with an analysis of the data on food consumption and chemical concentration in terms of their quality and availability. Deterministic conservative methods currently used to assess exposure are then illustrated and critically revised. Finally, the main characteristics of stochastic modelling are dealt with, i.e. simulation methods, correlation and dependencies, sensitivity analysis and validation. Both the limits and the potentialities of probabilistic modelling techniques are analysed.

#### 2.1.1 Problems related to the data used to assess exposure to hazardous substances

The levels of contaminants or pesticide residues in our food are an important aspect of food quality. These components may constitute a health hazard when overall intakes are too high. Ideally, dietary exposure to hazardous substances can be

assessed by combining data on concentration in all food products with data on their consumption. The food consumption data required for exposure assessments should, therefore, mirror the foods on which the concentration data are based. Unfortunately this ideal situation is difficult to realise. A brief analysis of the critical aspects of data currently available in the European Union (EU) in terms of food consumption and of food chemical concentration data is presented below.

### 2.1.2 Quality and availability of food consumption data

Uncertainty in food consumption data is mainly associated with the method of data collection and the design of the food consumption survey. A significant source of uncertainty in estimating intake of hazardous substances arises from difficulty in matching the food descriptions required for exposure assessments with those in existing food consumption databases (Gibney and Lambe, 1996; Langlais, 1996; European Commission, 1997). Most national food consumption surveys are conducted primarily to assess the nutrient intake of the population, using food coding schemes that differentiate on the basis of the nutrient composition of foods. Surveillance for pesticide residues and contaminants often provides chemical concentration data for food categories which are difficult to match with foods as coded in food consumption surveys. Analyses are mainly conducted on Raw Agricultural Commodities (RACs) which do not reflect food 'as eaten'. For example, wheat is not eaten as wheat but as bread, bakery wares, breakfast cereals, etc. Some foods are consumed in their raw state (e.g. apples, tomatoes), but almost always after some kind of processing (e.g. washing, peeling, as a part of different foods or recipes, such as apple pies, cider, tomato purée, soup, etc.). Some, but not all, food consumption databases have recipe databases attached which convert foods back to their equivalent value in RACs.

Moreover, the time period represented by the food consumption data in exposure assessment should reflect the timeframe considered by the safety limit for the substance in question. When a tolerable daily intake (TDI) is fixed, exposure assessments of hazardous substances require reliable estimates of long-term or habitual food consumption data (Lowik, 1996). In fact, this value represents the amount of hazardous substance that can be ingested daily over a lifetime without incurring any appreciable health risk. In these cases, it is important to appreciate and take into consideration the influence of survey duration on the various food intake parameters. For any food item, survey duration is strongly associated with the percentage of consumers and the average intake among 'consumers only'. The number of days of a food consumption survey does not affect mean total population intake (Lambe *et al.*, 2000). Consequently a short survey duration will be likely to lead to an under-estimation of the percentage of the population exposed and to an over-estimation of the higher percentiles of exposure.

At international level, the food consumption data most often used in chronic dietary exposure assessments are the model diets and the GEMS/Food regional diets based on Food Balance Sheet (FBS) data collected by FAO (WHO, 1997). The GEMS/Food regional diets are derived from the FBS from selected countries

to represent five regional dietary patterns, namely Middle Eastern, Far Eastern, African, Latin American and European. Note that European diet includes countries with European-type diets, such as Australia, Canada and the USA. Characteristics of these data are that they are not derived from consumption data but from disappearance data at national level and that they do not provide any information in relation to variability within the population. Although dietary patterns derived from such FBS are subject to many uncertainties and limitations, they constitute the best available source of data for international comparison.

### **2.1.3 Quality and availability of concentration data**

Levels of contaminants and pesticide residues can vary considerably within one commodity or over different commodities. Factors influencing the variability of chemical concentration levels in ready-to-eat food products are legislation, agricultural and storage practice and processing. As stated above, analyses of contaminants and pesticide residues in fruit and vegetables are mainly conducted in RACs including peel and non-edible parts. Processed or prepared foods are either not monitored or the number of samples is very small. This is due to the fact that in legislation, limits of residues are mainly set for RACs and the purpose of monitoring is usually to assess compliance rather than exposure. In the EU, concentration levels in RACs are primarily derived from monitoring programmes executed by member states. These data may be used to estimate dietary exposure, although they are not widely available. The raw data are present nationally in almost all EU countries, but are not easily accessible. Furthermore, there are differences in data collection and reporting, making it difficult to compare exposures between countries. This problem was recognized for pesticide residues by the EU which has set up a coordinated programme, Commission Recommendation 1999/333 EC (European Commission, 1999), aiming to work towards a system which makes it possible to estimate actual dietary pesticide exposure throughout Europe using a standardized method for collecting and reporting pesticide levels in products.

Another critical aspect of the available data is that sampling is not always random, but often focussed on those samples suspected to contain residue levels above the permitted limit (e.g. those products produced during wet seasons, under difficult conditions, or out of season). The use of these data to estimate dietary exposure may thus lead to an over-estimation of exposure (WHO, 1997). Moreover, it is difficult to obtain precise and accurate estimates of the true mycotoxin concentration of a bulk lot when using a mycotoxin-sampling plan that measures the concentration only in a small portion of the bulk lot. The sampling step is usually the largest source of error due to the extreme mycotoxin distribution among kernels in the lot (Whitaker, 2003). Moreover, a high percentage of the food samples analysed for contaminant levels have values below the Limit of Detection (LOD) (equivalent to Limit of Quantification, LOQ) (WHO, 2000). The influence of the value assigned to these samples on the output of the intake assessment depends on the percentage of non-detects and on the value of LOD. Results

reported as less than an LOD are usually treated in three different ways for the purpose of intake estimates: equal to the LOD (produces positive bias), equal to zero (produces negative bias) or equal to half the LOD (may produce positive or negative bias) (Hart *et al.*, 2003).

Mycotoxin regulations are generally implemented using a defined maximum limit and a sampling plan to detect mycotoxin-contaminated products and eliminate them from the food chain. Variations among maximum limits and sampling plans make it difficult for exporters and importers to market commodities in the world market. There are, therefore, a number of important driving forces for the development, evaluation and validation of analytical methods for mycotoxins. Standardization of mycotoxin sampling plans implies: developing a uniform sampling plan to be used by all buyers and sellers of agricultural commodities; and developing a uniform mycotoxin sampling plan that has a higher performance than most sampling plans currently being used in the marketplace. Performance of a sampling plan is generally related to several factors, such as the frequency with which false positives and false negatives occur and the degree of accuracy in the detection of contaminated lots.

In order to facilitate international trade and improve consumer protection, the world community, working through FAO/WHO, has readily acknowledged the need to harmonize or standardize mycotoxin-sampling plans and maximum limits. Standardizing mycotoxin-sampling plans will establish uniform levels for buyer's risk (false negatives) and seller's risk (false positives) associated with sampling plans used by all traders of agricultural commodities in the export market (Whitaker, 2003).

## 2.2 Deterministic methods for assessing mycotoxin exposure

Currently it is considered neither cost-effective nor necessary to collect detailed food consumption data for every hazardous substance (Lawrie and Rees, 1996). A stepwise procedure is commonly used to minimize costs and focus resources on the most important issues. The stepwise approach to dietary exposure assessment of food chemicals is such that, as the accuracy of dietary exposure assessments increases, the cost of collecting adequate data and resources needed to undertake the assessments also increases (WHO, 1997). The aim of the stepwise procedure is to direct dietary exposure estimates to chemicals which might be a health threat for average consumers or individuals belonging to certain at-risk groups. If the estimated exposure to a given pesticide residue, food additive, veterinary drug residue, contaminant exceeds its safety limit, e.g. ADI (acceptable daily intake) or PTWI/PTDI (provisional tolerable weekly/daily intake), a more accurate method of dietary exposure assessment should be applied.

Exposure is therefore first assessed by using methods following a deterministic approach based on conservative assumptions. Especially at this stage, the methodologies adopted to assess exposure from diet take into special consideration non-average individuals, and in particular those who consume relatively large

quantities of foods containing higher concentrations of substances that may potentially lead to a health risk. However, for such consumers in particular, food consumption data are not commonly available at individual level. Consequently, highly conservative methods are used. Estimates calculated under such conditions are often implausible. Therefore these methods are not suitable for predicting actual exposure since they are designed to cover the worst-case scenario. Nevertheless, they are useful and inexpensive screening tools for identifying those substances for which safe intake limits may be exceeded.

When consumption data are available at individual level but occurrence data are not, conservative approaches are usually adopted by assuming that the chemical is present in all the products in which these are legally permitted at maximum level. However, consumption data are often available only in aggregated food categories larger than those defined to establish the maximum levels. This is the case, for example, in most national household budget surveys. Commonly, with this kind of data, an estimate of the chemical intake is obtained by using the highest chemical concentration value allowed within the category, which is obviously a further conservative assumption.

Within the frame of exposure assessment, a major issue is that of what proportion of the population would have to exceed the at-risk dose before action is considered necessary to reduce intakes. This is both a scientific and a political/ethical question. From a scientific point of view, there are several possible approaches to evaluating the intake of a substance contained in food, but in many cases there are statistical difficulties in measuring adequate percentiles of intake. Thus, if methodological limitations do not allow the assessment of the 99<sup>th</sup> percentile of intake, the 95<sup>th</sup> percentile of intake under the safety limit can appear secure, but this result could not always be considered satisfactory. In fact, one per cent of the population at risk of an excessive intake of a certain substance is a serious political/ethical problem (European Commission, 1998).

Different deterministic methods are currently used to assess exposure to mycotoxins. A conservative approach was adopted by the working groups of experts set up by the European Commission to assess dietary intake of ochratoxin A (European Commission, 2002a) and patulin (European Commission, 2002b) by the population of EU member states. For both these mycotoxins, participants were asked to provide occurrence data in foods and beverages. Moreover the mean and the 95<sup>th</sup> percentile of consumption, for all the population and for specific groups of consumers, were requested for foods grouped according to the Codex Alimentarius System and/or for individual foods. Estimates of dietary intake from each food commodity were calculated by combining the mean and the 95<sup>th</sup> percentile of daily food consumption with the mean occurrence values. Two different approaches were used to calculate the mean occurrence values. In one case results lower than the LOD were set equal to LOD/2 and were considered to calculate the arithmetic mean whereas in the second case only positive values (above LOD) were used. For each member state, an estimate of total dietary intake was obtained by summing the average intake of food with the corresponding mean value, calculated using the first of the above approaches. The combination of the 95<sup>th</sup> percentile of food

consumption with the same mean occurrence value was used only to highlight the major contributor food commodity. Such figures were not summed since this would provide an over-estimation of total dietary intake.

A stepwise method for assessing exposure to contaminants and toxins was recommended in a FAO/WHO workshop held in Geneva in June 2000 (WHO, 2000). It was recommended that international estimates of dietary intakes should be first calculated by multiplying the mean or median concentration by the values for consumption of the commodity in the five GEMS/Food regional diets (WHO, 1998). Estimates should then be expressed in terms of the percentage of the PTWI. This step indicates whether the PTWI is likely to be exceeded and which regions have the highest potential exposure. In particular, dietary exposure estimates obtained with these GEMS/Food regional diets are used to identify food groups that contribute significantly to exposure. In 2001 this methodology was applied by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (IPCS, 2001) aimed at assessing the risk associated with the intake of five mycotoxins or groups of mycotoxins that may contaminate food commodities (aflatoxin M<sub>1</sub>, fumonisins B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, ochratoxin A, deoxynivalenol and T-2 and HT-2 toxins).

### **2.3 Probabilistic modelling techniques for assessing mycotoxin exposure**

In order to get a more realistic view of exposure to hazardous substances, risk managers are becoming more interested in probabilistic modelling (European Commission, 2000). A probabilistic approach could give the opportunity to take into account all sources of variability and uncertainty in estimates of exposure. It allows the utilization of all the available information on variability in the proportion of foods containing the substance, in the concentration of the substance present and in food consumption patterns. A better characterization of variability and uncertainty in risk assessment may lead not only to better risk management, but also to better risk communication (Thompson, 2002). Appreciating the variability in risk should lead to better understanding of its distribution and should increase the opportunities for risk managers to target those people at higher risk. A probabilistic treatment of exposure information could also help consumers to respond to risk communication with a greater degree of comprehension.

The primary goal of the probabilistic approach is to describe the exposure distribution for the whole population under consideration by quantifying the range of exposure and the degree of likelihood of each exposure level.

#### **2.3.1 Numerical simulation methods**

Simulation is a process of replicating the real world based upon a set of assumptions and models of reality (Cullen and Frey, 2002). Experimental simulations of exposure and risks can be costly or unethical; thus numerical simulations are often preferred. One set of methods for simulating the propagation

of variability and uncertainty of input variables through a model is based upon simulated random sampling. The most commonly used numerical simulation methods are the Monte Carlo and the Latin Hypercube.

It must be noted that simulations can be conducted in a wide variety of ways using different data, assumptions and algorithms. First of all, two different approaches can be used to perform simulations, the non-parametric and the distributional, the latest most often referred to as Monte Carlo simulations. Within the field of dietary exposure assessment to contaminants such as mycotoxins, the non-parametric method takes into account the consumption and contamination data in the simulations using random sampling of raw data; whereas the parametric method depends on the random samplings from probability distributions describing consumption and contamination data.

A probability distribution model is a description of the probabilities of all possible values in a sample space (Cullen and Frey, 2002). A probability model is typically represented mathematically as a probability distribution in the form of either a probability density function (*pdf*) or cumulative distribution function (*cdf*). The *moments* and the *maximum likelihood* methods are generally used to estimate probability distribution parameters starting from observed data sets. However, while it may be possible to obtain what appear to be reasonable values for these parameters (e.g. mean, variance, shape, scale, etc.), it may turn out that the selected probability model (e.g. normal, lognormal, gamma, beta, etc.) does not offer a good representation of the entire data set. Thus, other methods should be used to make inferences about the 'goodness-of-fit' of a probability model to a given data set. Graphical techniques can be used to identify the inadequacies of a particular probability model. For example, if the focus of interest is the upper tail of a probability distribution, a probability model which offers an excellent fit to the central ranges of the data but a poor fit to the upper tail might be deemed inadequate for the purposes of a particular assessment.

Two different approaches can be used for testing distributional assumptions based upon available data – the probability plotting and the goodness-of-fit tests. Probability plotting is a subjective technique for evaluating whether data contradict an assumed probability distribution. On the other hand, statistical goodness-of-fit tests tend to provide a more objective measure of how well a distribution function represents the data. However, with a small data set, statistical tests may often fail to reject a model that is in fact a poor fit to the data. Thus, even if statistical tests are used, one should also consider visualizing the data and the model outcomes to make a subjective evaluation of the goodness-of-fit. Furthermore, with very large data sets, goodness-of-fit tests may reject fits which may in fact be adequate for a given purpose. Probability plots are advantageous in situations in which one wishes to obtain a good fit for a particular portion of the distribution, such as the upper tail, and to retain a reasonable fit for the remainder of the distribution.

Figure 2.1 shows a hypothetical aggregate exposure from two potential sources of exposure (A and B). In this simple example, the intake of  $n$  individuals is simulated, generating, by the use of *pdfs*,  $n$  values of consumption ( $C_{A1}$ ,  $C_{A2}$ ,  $\dots$ ,

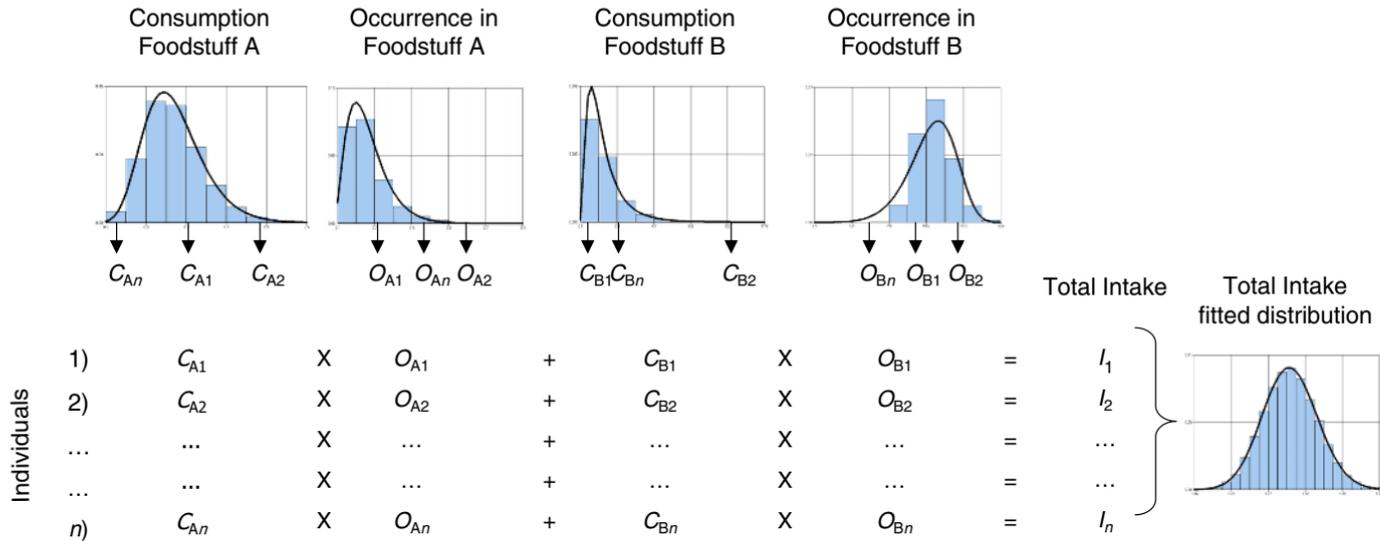


Fig. 2.1 Hypothetical aggregate exposure from two potential sources of exposure.

$C_{An}$ ) and  $n$  of contamination ( $O_{A1}, O_{A2}, \dots, O_{An}$ ) for foodstuff A and  $n$  values of consumption ( $C_{B1}, C_{B2}, \dots, C_{Bn}$ ) and  $n$  of contamination ( $O_{B1}, O_{B2}, \dots, O_{Bn}$ ) for foodstuff B. The intakes from each source are then combined to provide a profile for each individual. The  $n$  values of intake ( $I_1, I_2, \dots, I_n$ ) are finally used to estimate a *pdf* of exposure from both the sources.

A recent study (Gauchi and Leblanc, 2002) provided reliable calculations of exposure of the French population to the mycotoxin ochratoxin A with the conjugate means of a non-parametric-type method of simulation, a parametric-type method of simulation, and the use of bootstrap confidence intervals. The use of at least two methods, which validate each other, was needed for a more reliable estimation of high quantiles. In the non-parametric approach, no *pdf* hypotheses are made either on the consumption or on the contamination. Indeed, each consumption profile of the survey, normalized to the individual body weight, was taken into account and each type of consumed food was attributed a value of contamination randomly drawn from the available contamination data. Unlike the previous method, in the distributional approach a mixed *pdf* was fitted to each food consumption and a parametric *pdf* to each food contamination. In the same study, two pseudo-parametric bootstrap methods were also proposed to build confidence intervals of exposure estimates.

The principles of the non-parametric bootstrap are briefly presented here. Supposing a sample of  $n$  data, a bootstrap sample is obtained by randomly sampling  $n$  times, with replacement, from the  $n$  original data. A large number  $S$  of independent bootstrap samples is generated this way, each of them being of size  $n$ . For each bootstrap sample, a bootstrap replication of a given statistic  $s$  is obtained (for example, a standard error of the mean). With the  $S$  values of  $s$  we have at our disposal a bootstrap distribution of  $s$  that enables us to calculate empirical non-parametric bootstrap confidence intervals of  $s$ , for example, a confidence interval for the standard error of the mean. An example of non-parametric bootstrap is represented in Fig. 2.2, in which a bootstrap confidence interval of the average is estimated by generating  $S = 1000$  independent bootstrap samples from an original sample of  $n = 10$  data points. An in-depth description of the classical non-parametric bootstrap approach is reported by Efron and Tibshirani (1993).

The Monte Carlo simulation method was also used by the JECFA (IPCS, 2001) for the safety evaluation of certain mycotoxins in food. In this case, simulations of intake were performed by using different data, assumptions and algorithms according to the substance under consideration. Dietary intakes were also assessed based on the GEMS/Food regional diets and national food consumption and occurrence data provided by government and university institutions. Moreover, in a recent study the levels of fumonisin B<sub>1</sub> and B<sub>2</sub> in corn products were fitted with 10 alternative probability distributions (Humphreys *et al.*, 2001). The best model for each product, as judged by the same criteria used for weighing models (goodness-of-fit and number of parameters), was used to describe the distribution of this mycotoxin in each commodity. The consumption of corn products was evaluated using the raw data from a three-day food survey. An exposure period of 360 days was simulated by randomly re-sampling (bootstrapping) each of the

Original sample	
1)	A
2)	B
3)	C
4)	D
5)	E
6)	F
7)	G
8)	H
9)	I
10)	L

Bootstrap sample 1	
1)	L
2)	E
3)	A
4)	A
5)	C
6)	F
7)	G
8)	D
9)	C
10)	A

Bootstrap sample 2	
1)	C
2)	H
3)	D
4)	A
5)	F
6)	B
7)	B
8)	C
9)	I
10)	E

...  
...  
...  
...  
...  
...  
...  
...  
...  
...

Bootstrap sample 1000	
1)	I
2)	F
3)	H
4)	G
5)	I
6)	D
7)	A
8)	B
9)	F
10)	F

Average (original sample) = (A+B+C+D+E+F+G+H+I+L)/10 =  $A_0$

Average (bootstrap sample 1) = (L+E+A+A+C+F+G+D+C+A)/10 =  $A_{B1}$

Average (bootstrap sample 2) = (C+H+D+A+F+B+B+C+I+E)/10 =  $A_{B2}$

.....

Average (bootstrap sample 1000) = (I+F+H+G+I+D+A+B+F+F)/10 =  $A_{B1000}$

} Bootstrap confidence interval of the average

Fig. 2.2 Example of a non-parametric bootstrap.

three-day exposure records 360 times for each subject. The quantitative risk assessment of dietary exposure presented in this paper provides a quantitative estimate of the risk. This is especially useful when there is not a sufficiently large margin of exposure to conclude that the risk is insignificant or when the safety factor approach cannot conclude that there is no hazard, but the degree of that hazard, or risk, is unknown. In addition, it quantitatively describes the level of risk reduction achieved by alternative risk management points.

### 2.3.2 Variability and uncertainty

A challenge to the Monte Carlo methods is the breakdown of the input variables into components representing variability and uncertainty (Sanga *et al.*, 2001). Variability represents diversity or heterogeneity in a well characterized population. Being fundamentally a property of nature, variability is not reducible, but it can be better represented by further measurement or study. For example, different people have different body weights, no matter how carefully they can be measured. On the other hand, uncertainty represents partial ignorance or lack of perfect information about poorly-characterized phenomena or models. Being fundamentally a property of the risk analyst, uncertainty can be reduced through further measurements or study.

The structure of mathematical models employed to represent scenarios and phenomena of interest is a key source of uncertainty, due to the fact that models are often only a simplified representation of real-world systems. Significant approximations are often an inherent part of the assumptions upon which a model is built. But uncertainty arises also from a basic lack of knowledge regarding the input variables. The most commonly discussed and easily understood sources of uncertainty in exposure model inputs are the errors associated with measurements and analyses and the small sampling size of observed data (Cullen and Frey, 2002).

In principle, it is possible to represent a myriad of uncertain or variable factors probabilistically, but propagating uncertainty and variability together in a Monte Carlo simulation of exposures may complicate the interpretation of the results. If the objective is an assessment of exposure to identify at-risk subpopulations, it may be useful to separate uncertainty and variability. However, the separation of uncertainty requires a considerable amount of time and information, and involves relatively high computational costs (Sanga *et al.*, 2001). Frey and Burmaster (1999) described and evaluated two methods for quantifying both variability and uncertainty, the *bootstrap simulation* method and the *likelihood-based* method.

### 2.3.3 Correlation and dependencies

For simplicity and accuracy when using a conventional probabilistic simulation technique, the model should be structured in such a way that its input variables are as independent as possible. However, the presence or absence of moderate to strong correlations or dependencies between input variables is to be included in a

model and discussed. Intra-individual correlations include the dependency between events of food selection on a given day and on sequential days. There are well documented correlations between intakes on sequential days.

An illustration of the importance of such correlations can be found in studies by Arcella and colleagues (Arcella *et al.*, 2003; Leclercq *et al.*, 2003b). In these papers the impact of the inclusion of indicators on brand loyalty in a model of exposure to intense sweeteners from table-top sweeteners and soft drinks by Italian teenagers has been highlighted. Loyalty to a brand is responsible for a positive dependency between repeated events. Failure to recognize it could under-estimate the risk for high intakes: the hypothesis that a consumer may always consume the brand containing the highest concentration of a food chemical needs to be considered and the probability of this situation assessed. Brand loyalty is usually defined as the tendency to repurchase the same brand of a product. However, within an intake exposure model, the concept of brand loyalty could be seen in the broader context of product loyalty, i.e. tendency to repeat the consumption of a category of product that may contain a specific concentration of additives/pesticides/contaminants different from that of the other categories that can be consumed as an alternative to it. For example, loyalty to a specific type of coffee (containing higher levels of a mycotoxin) or loyalty to organic products (with no pesticides) among the group of vegetable products. Another kind of correlation is the inter-individual one. For example there are correlations between foods eaten by individuals in the same household. This case should be considered when using food consumption data from national household budget surveys.

The most obvious form of dependency in consumption data is between intakes of different food categories for the same individual. Most of these dependencies are likely to be negative because total consumption is limited, and so increased consumption of one type of food will often be associated with decreases in consumption of other categories. However, some dependencies may be positive, at least in short-term data sets, because some food types tend to be eaten together. Failure to recognize this correlation would under-estimate the risk for high intakes. One may avoid quantifying and incorporating all these interdependencies in exposure modelling if the amounts of different food categories consumed by the same individual are treated as a fixed vector and kept together, thus preserving any dependencies that may be present (Hart *et al.*, 2003).

In a study aimed at estimating, by Monte Carlo simulations, exposure to ochratoxin A in France, consumption dependencies were considered (Gauchi and Leblanc, 2002). Scatter plots were used to visualize the patterns of one food consumption versus another, normalizing the consumptions to body weight in kilograms. In order to quantify the dependencies, the Spearman correlation coefficient was preferred to the Pearson correlation coefficient since the former is more robust relative to outliers. Individuals who did not consume either of the two foods were excluded from the analysis. The Iman and Conover method (Iman and Conover, 1982) was then used to simulate correlations based on the Spearman coefficient. This method is not the only one that can be used to generate correlated random variables. In Haas (1999), alternative methods using copulas are

presented; in this paper it is also shown that the particular algorithms or assumptions used may have substantial effect on the output results.

### 2.3.4 Sensitivity analysis

The results of Monte Carlo simulations represent an estimate of exposure taking into account variability and uncertainty in input variables. Thus, sensitivity analysis is used to identify the parameters that contribute most to the estimates. Sensitivity analysis can also be used to assess the impact of various model assumptions and to determine whether there are any systematic model rules that are consistently biasing the results.

The results from probabilistic models of dietary exposure depend on many model inputs. Basic model inputs are the consumption and food chemical databases, but the way in which the model parameters are set and the choice of modelling options are also model inputs. Sensitivity analysis is concerned with quantifying the effects of changing some of these inputs on the model outcomes. It is clear that this can only be done for a limited set of inputs, and therefore the prior choice of inputs for which a sensitivity analysis will be done is an important matter.

The result of a sensitivity analysis will reveal how much the exposure estimates will vary if one or more inputs (data vectors, model parameters or modelling options) are changed. If more inputs have been investigated in an experimental design, then statistical methods can be used for quantifying the main effects and possible interaction effects resulting from simultaneously changing more than one input. No method of sensitivity analysis can be clearly considered the best for food safety risk models. Each method has its own key assumptions and limitations. Frey and Patil (2002) present ten sensitivity analysis methods, comprising four mathematical methods, five statistical methods, and one graphical method, for application to food safety risk assessment models. The selected methods are compared on the basis of:

- (i) their applicability to different types of models,
- (ii) computational issues, such as initial data requirement and complexity of their application,
- (iii) representation of the sensitivity, and
- (iv) the specific uses of these methods.

A general recommendation is to use two or more methods, preferably with dissimilar foundations, to increase confidence that the identification of key inputs is robust.

With respect to mycotoxins, an interesting and innovative application of a sensitivity analysis was performed to quantify the influences of the parameter variability of the consumption and contamination probability density functions on the high quantiles (95<sup>th</sup> and 99<sup>th</sup> quantiles) of exposure to ochratoxin A in French children (Albert and Gauchi, 2002). A full quadratic polynomial regression model was used.

### 2.3.5 Software

Modern computing technology allows us to perform simulations of potential exposures from multiple sources and even from multiple compounds. Statistical methods for conducting these types of analyses have been developed, tested, improved, and validated for a wide range of applications. Commercially available software programmes exist for stochastic modelling but they have largely been designed for use in other risk-laden areas, such as financial services, mining, environmental impact analysis, etc. In particular, Crystal Ball (Decisioneering, 2000) and @Risk (Palisade, 2003) are among the most widely used; being associated with spreadsheet software makes them user friendly. These software programmes are also available to produce correlated random variables with any set of marginal distributions and any specified value of the Spearman rank correlation coefficient. These correlated random variables are generated by using the above mentioned Iman and Conover procedure (Iman and Conover, 1982). However *ad hoc* simulation programs were also developed for food safety risk models by means of different computer languages. For example, simulations and calculations have been carried out by means of the Interactive Matrix Language (IML) and various procedures of the SAS software (Windows release 6.12, SAS Institute®, Cary, NC, USA) by Gauchi and Leblanc (Gauchi and Leblanc, 2002) whereas routines were written in the Microsoft™ macro language (Visual Basic for Applications) by Humphreys *et al.* (2001) to perform curve fitting and Monte Carlo simulation.

All computer-based models should be tested to ensure that the algorithms have been computed as intended. This can be accomplished by conducting simulations outside the software for comparison purposes (Petersen, 2000).

### 2.3.6 Model validation

There is a critical need to test and validate Monte Carlo models against actual exposure/dose data (Petersen, 2000). Within the EU funded ‘Montecarlo’ project, probabilistic models of dietary exposure were developed and validated for various food chemicals. Such models were considered valid if they provided more realistic (i.e. lower) estimates than the conservative deterministic methods currently in use without underestimating ‘true’ exposure, in particular at upper percentiles (Leclercq *et al.*, 2003a). Since ‘true’ intake value is unknown, procedures which can be used to calculate the best intake estimate of food additives, pesticide residues and nutrients were developed within the project in order to apply the validation criteria. Moreover, databases of ‘true’ intakes of food additives (based on brand level food consumption records and additive concentration data), pesticide residues (based on duplicate diet studies) and nutrients (based on biomarker studies) have thus been generated.

Estimates of exposure using methods based on food intake/food analysis could be subject to a large degree of error due to the large variability in contamination levels and to sampling difficulties. In particular, when these indicate exposure close to the TDI, alternative approaches to assessing mycotoxins exposure can be

used. Biological markers of exposure, such as blood and urinary metabolites, provide an opportunity for validation (Petersen, 2000). For example, a recent study demonstrated that the determination of ochratoxin A levels in plasma and urine samples is a viable approach to assessing dietary exposure (Gilbert *et al.*, 2001).

## 2.4 Conclusions

The food products market changes very rapidly in relation to both product formulation and consumer preferences. An important aspect within food safety research activity is, therefore, the development and refinement of ever more efficient statistical methods to periodically estimate the risk of an excessive intake. Often, in order to prevent consumers from being exposed to unexpectedly high intakes, estimates assessed by means of the above described deterministic conservative methods produce estimates of exposure which are improbable. In many cases, these estimates of potential exposure limit the ability of regulators, industry and consumers to determine which scenarios present a risk that is likely to occur and therefore needs to be addressed (Petersen, 2000).

Numerical simulation techniques provide powerful tools that will take advantage of all the available knowledge (empirical data, experts judgements, etc.) in order to provide realistic estimates of exposure. The results, however, are only as good as the input data, algorithms and assumptions. The critical aspects of food consumption and chemical concentration data presented in the first part of this chapter must therefore be taken into account also when using a probabilistic approach. Moreover, the impact of the assumptions should always be tested carefully and the results should be fully documented. A modelling tool must be structured so that all algorithms and assumptions inherent to the model can be identified and validated. Results from sensitivity analysis should be used to set priorities for risk reduction measures and to define the main sources of uncertainty in order to plan further studies and improve exposure estimates.

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# 3

## Current regulations governing mycotoxin limits in food

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### 3.1 Introduction: regulating mycotoxins in food

Food legislation serves to safeguard the health of food consumers and the economic interests of food producers and traders. In the early days of food legislation, protection of food was mostly local, and municipal ordinances were promulgated for that purpose. Later, when auxiliary sciences such as bacteriology, chemistry and microbiology developed, statutory regulations were planned in many countries, leading, at the beginning of the twentieth century, to the implementation of official food legislation and regulations.

At the time of writing, food regulations not only prohibit the introduction, delivery for introduction, or receipt in commerce of adulterated or misbranded food, but they often include specific regulations that impose limits or tolerances on the concentration of particular contaminants in foods. Currently, virtually all countries with fully developed market economies have established specific limits and regulations for mycotoxins in food. International inquiries on mycotoxin legislation in foodstuffs and feedstuffs have been carried out several times since the 1980s, yielding details about tolerances, legal bases, responsible authorities, and official protocols of analysis and sampling. Most of these surveys were

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Please note that at the time of publication the figures and data given were not yet final and may therefore be subject to further adjustments. The opinions expressed are those of the authors and do not necessarily represent the decisions or the stated policy of the National Institute for Public Health and the Environment.

conducted for the Food and Agricultural Organization (FAO) of the United Nations. The most recent survey was carried out in 2002/2003 and will be published in 2004 as an FAO Food and Nutrition Paper (FAO, 2004).

This chapter deals with developments in the mycotoxin regulatory area. First the various factors that play a role in the decision-making process for establishing mycotoxin regulations in food will be discussed. Some information will be provided then about the design of the international inquiry that was carried out in 2002. The results of the inquiry will be summarized and briefly discussed. Some observations for the various world regions will be presented, and information will be provided on the legal limits established worldwide for the most significant mycotoxins in food. Finally, some conclusions and recommendations will be made.

## 3.2 Factors affecting mycotoxin regulation in the food industry

Several factors, both of a scientific and socio-economic nature, may influence the establishment of mycotoxin limits and regulations. These include:

- availability of toxicological data
- availability of data on the occurrence of mycotoxins in various commodities
- knowledge of the distribution of mycotoxin concentrations within a lot
- availability of analytical methods
- legislation in other countries with which trade contacts exist
- need for sufficient food supply

The first two factors provide the necessary information for hazard assessment and exposure assessment respectively, the main ingredients for risk assessment. Risk assessment is the primary scientific basis for the establishment of regulations.

### 3.2.1 Risk assessment

Regulations are primarily made on the basis of known toxic effects. The Joint Expert Committee on Food Additives (JECFA), a scientific advisory body of the World Health Organization (WHO) and the FAO, has evaluated the hazards posed by the most significant mycotoxins (aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone and some trichothecenes including deoxynivalenol or DON). In February 2001, a special JECFA session was completely devoted to mycotoxins (FAO, 2001; WHO, 2002). The mycotoxins evaluated or re-evaluated at this 56<sup>th</sup> JECFA meeting included fumonisins B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub>, ochratoxin A, DON, T-2 and HT-2 toxins, and aflatoxin M<sub>1</sub>. The report addressed several concerns about each mycotoxin: explanation of the mycotoxin, absorption through excretion, toxicological studies, and final evaluation. Along with the mycotoxin evaluations, the committee put forth general considerations on analytical methods, sampling, associated intake issues, and control. The evaluation of aflatoxin M<sub>1</sub> is the most

interesting, as JECFA responded to a request by the Codex Committee on Food Additives and Contaminants at its 32<sup>nd</sup> session (Codex Alimentarius Commission, 2000) to 'examine exposure to aflatoxin M<sub>1</sub> and to conduct a quantitative risk assessment' to compare the application of two standards for contamination of milk (0.05 µg/kg and 0.5 µg/kg, limits currently applied in the EU and the USA respectively). The calculations showed that, with worst-case assumptions, the projected risks for liver cancer attributable to use of the proposed maximum levels of aflatoxin M<sub>1</sub> of 0.05 µg/kg and 0.5 µg/kg are very small, and that there is no significant health benefit from reducing a 0.5 µg/kg limit to 0.05 µg/kg.

### 3.2.2 Exposure assessment

In addition to information about toxicity, exposure assessment is another main ingredient in risk assessment. Reliable data on the occurrence of mycotoxins in various commodities and data on food intake are needed to prepare exposure assessment. The quantitative evaluation of the likely intake of mycotoxins is quite difficult. In its 56<sup>th</sup> meeting, JECFA stressed the importance of the use of validated analytical methods and the application of analytical quality assurance (see also the section on Methods of Analysis) to ensure that the results of surveys provide a reliable assessment of intake (WHO, 2002). In most of the JECFA reviews of mycotoxins, the analytical data on the levels of contamination from developed countries were often inadequate and those from developing countries non-existent. Because most mycotoxin contamination is heterogeneous, sampling is an important consideration in the development of information on the levels of contamination (Page, 2003) (see also Section 3.2.3).

In the EU, efforts to assess exposure are undertaken within SCOOP (Scientific Cooperation on Questions relating to Food) projects, funded by the European Commission. The SCOOP projects are targeted to make the best estimates of intake of several mycotoxins by EU inhabitants. In the 1990s these activities resulted in a report on the exposure assessment of aflatoxins (European Commission, 1997); later SCOOP reports for several other mycotoxins followed (for ochratoxin A (Miraglia and Brera, 2002), patulin (Majerus and Kapp, 2002) and several *Fusarium* toxins: trichothecenes, fumonisins and zearalenone (Gareis *et al.*, 2003). The SCOOP data have been used by the EFSA (European Food Safety Authority) for its evaluation and advisory work on the risks to public health arising from dietary exposure to certain mycotoxins.

### 3.2.3 Sampling procedures

The distribution of the concentration of mycotoxins in products is an important factor to be considered in establishing regulatory sampling criteria. It can be very heterogeneous, as is the case with aflatoxins in peanuts. The number of contaminated peanut kernels in a lot is usually very low, but the contamination level within a kernel can be very high. If insufficient care is taken to ensure representative sampling, the mycotoxin concentration in an inspected lot may therefore easily be

wrongly estimated. Also, consumption of peanuts could lead to an accidental high single dose of aflatoxins, rather than a chronic intake at a relatively low level. A similar situation could occur with pistachio nuts and figs. The risk to both consumer and producer must be considered when establishing sampling criteria for products in which mycotoxins are heterogeneously distributed.

The design of sampling procedures has been an international concern for a long time, for instance Codex Alimentarius (Codex Alimentarius Commission, 2000) and FAO (1993) have been active in this area. Discussions in working groups of these international organizations take place continuously to find a harmonized international approach. Examples of official sampling plans for mycotoxins are those for aflatoxins in peanuts and corn practised in the USA (Food and Drug Administration, 2004) and for peanuts in the EU (European Commission, 2002b). In the USA the US Department of Agriculture (USDA) requires three 22 kg laboratory samples, to average less than 15 µg total aflatoxins/kg for acceptance. In the EU one 30 kg laboratory sample is required to test less than 15 µg total aflatoxins/kg for raw peanuts destined for further processing, and three 10 kg laboratory samples to all test less than 4 µg total aflatoxins/kg (and 2 µg aflatoxin B<sub>1</sub>/kg) for finished peanuts sold for direct human consumption.

Although the approaches are different, the US peanut industry in cooperation with the USDA has recently developed an Origin Certification Program (OCP) with several key EU countries that import US peanuts into Europe. These key markets, in a memorandum of understanding, have agreed to recognize the sampling and testing of US peanuts for aflatoxin before being exported to these markets (Trucksess *et al.*, 2003). Documents showing positive lot identification and aflatoxin test results can be used to certify that the peanuts meet EU aflatoxin regulations. In the OCP the US exporter uses a first 22 kg sample test result for screening lots. A second USDA 22 kg sample is tested according to EU protocol for lot certification. The OCP will reduce lots rejected at the port of entry, reduce the disruption in supply for the importer, reduce economic losses for the exporter and the importer, and maintain EU standards for consumer safety. The OCP is an example of an agreement between two countries that is mutually beneficial to both while maintaining high standards for consumer safety (Adams and Whitaker, 2004).

### 3.2.4 Methods of analysis

Legislation calls for methods of control. Reliable analytical methods will have to be available to make enforcement of the regulations possible. Tolerance levels that do not have a reasonable expectation of being met are wasteful in the resources that they utilize, and they may well condemn products that are perfectly fit for consumption (Smith *et al.*, 1994). In addition to reliability, simplicity is desired, as it will influence the amount of data that will be generated and the practicality of the ultimate measures taken. The reliability of analysis data can be improved through use of methods that fulfil certain performance criteria (as can be demonstrated in inter-laboratory studies).

**Table 3.1** BCR reference materials developed for mycotoxins, situation August 2003.

Reference material	Available	Development
Aflatoxin M <sub>1</sub> in milk powder	x*	
Aflatoxin M <sub>1</sub> calibrant	x	
Total aflatoxins in peanut butter	x*	
Aflatoxin B <sub>1</sub> in peanut meal	x	
Aflatoxin B <sub>1</sub> in feedstuff	x	
Ochratoxin A in wheat	x	
DON in maize and wheat	x	
Zearalenone in maize	x	
Zearalenone calibrant	x	
Trichothecene calibrants		x

\* The initial batches of reference materials have come to exhaustion. Therefore newly prepared and certified batches have been produced for aflatoxin M<sub>1</sub> in milk powder, and are in the stage of production for aflatoxins in peanut butter.

AOAC International and CEN (the European Standardization Committee, the European equivalent of the ISO) have a number of standardized methods of analysis for mycotoxins available that have been validated in formal inter-laboratory method validation studies, and this number is gradually growing. The latest edition of Official Methods of Analysis of AOAC International (Horwitz, 2000) contains approximately 40 validated methods for mycotoxin determination. The CEN has produced a document that provides specific criteria for various mycotoxin methods (Comité Européen de Normalisation, 1999). This document gives information concerning method performance, which can be expected from experienced analytical laboratories. The CEN criteria are currently reflected as method performance requirements in official EU legislation on aflatoxins and ochratoxin A (European Commission, 1998; 2002a). They are expected to appear as well in future EU legislation for other mycotoxins in food.

In addition, the application of analytical quality assurance (AQA) procedures is recommended, including the use of (certified) reference materials, especially when a high degree of comparison and accuracy is required. Further developments in AQA and the use of reference materials are likely to emerge in the future for the control of mycotoxins in foods. Several (certified) reference materials for mycotoxins have been developed in projects funded by the European Commission's Standards, Measurements and Testing (SMT) Programme (previously named Bureau Communautaire de Référence [BCR]), or are currently being re-developed. In Table 3.1 an overview is given of the BCR (certified) mycotoxin reference materials that have been developed since the 1980s. Certified reference materials are relatively expensive and current supplies are limited. Therefore laboratories are advised to develop their own reference materials for routine use, the toxin content of which should be established on the basis of the certified materials.

Besides the application of (certified) reference materials, regular participation

in inter-laboratory comparisons, such as proficiency testing schemes, is becoming increasingly important as part of AQA measures that a laboratory must undertake to demonstrate acceptable performance. A number of proficiency testing schemes for mycotoxins exist at the international level, including those organized by FAPAS® (the Food Analysis Performance Assessment Scheme), operated from Europe by the Central Science Laboratory in the UK and those organized by the AOCS (American Oil Chemists' Society), operated from the USA.

Good analytical methodology and AQA are prerequisites for adequate food law enforcement. Also important, especially in free trade areas, is how enforcement bodies handle an issue such as measurement uncertainty. Within the EU and the European Free Trade Area (EFTA) approaches are not yet harmonized, which may lead to different action levels, e.g. for aflatoxins. Therefore, the FLEP (Food Law Enforcement Practitioners) Working Party 'Mycotoxins' has recommended a uniform approach (Jeuring, 2004).

### 3.2.5 Trade contacts

It is preferable for regulations to be brought into harmony with those in force in other countries with which trade contacts exist. In fact, this approach has been applied both in the areas of the EU, MERCOSUR (a trading block consisting of Argentina, Brazil, Paraguay and Uruguay) and Australia/New Zealand, where harmonized regulations for some mycotoxins now exist. Strict regulative actions may lead importing countries to ban or limit the importing of commodities, such as certain food grains, which can cause difficulties for exporting countries in finding or maintaining markets for their products. For example, the stringent regulations for aflatoxin B<sub>1</sub> in animal feedstuffs in the EU (Commission of the European Communities, 1991) led European animal feed manufacturers to switch from groundnut meal to other protein sources to include in feeds; this had an impact on the export of groundnut meal of some developing countries (Bhat and Vasanthi, 1999). The distortion of the market caused by regulations in importing countries may lead to export of the less contaminated foods and feeds leaving those inferior foods and feeds for the local market. Some countries apply different limits for aflatoxins in certain products depending on the destination.

The World Bank issued a report in 2001 (Wilson and Otsuki, 2001), describing a study on the impact of adopting international food safety standards and harmonization of standards on global food trade patterns. Several scenarios led to estimates of the effects of aflatoxin regulatory standards in 15 importing (four developing) countries on exports from 31 (21 developing) countries. In one of the scenarios, the authors examined trade flow when all countries adopted an international standard for aflatoxin B<sub>1</sub> in food at 9 µg/kg (which would be equivalent to Codex guidelines of 15 µg/kg for total aflatoxins) in contrast to all importing countries remaining at the (generally lower) limits of 1998. This would lead to an increase of the cereal and nut trade among these countries by US\$6.1 billion (or 51 %).

### 3.2.6 Food supply

Regulatory philosophy should not jeopardize the availability of some basic commodities at reasonable prices. Especially in the developing countries, where food supplies are already limited, drastic legal measures may lead to lack of food and to excessive prices. People living in these countries cannot exercise the option of starving to death today in order to live a better life tomorrow. At the time of writing, for instance, the dramatic food security situation in parts of Africa leads to measures that prioritize food sufficiency above food safety. Mycotoxins are an important problem, as evidenced by occasional outbreaks of human mycotoxicoses and the role of aflatoxins in liver cancer in West Africa and fumonisins in oesophageal cancer in South Africa, as stated by Shephard (2004).

### 3.2.7 Synopsis

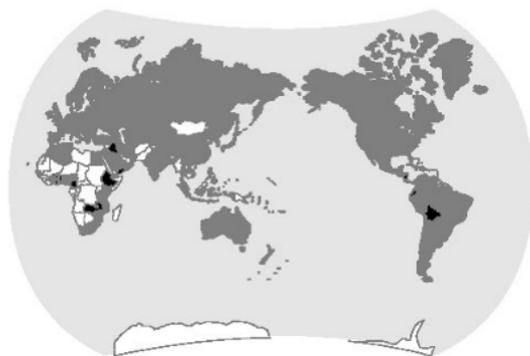
Weighing the various factors that play a role in the decision-making process of establishing mycotoxin tolerances is not trivial, and common sense is a major factor in reaching a decision. Public health officials are confronted with a complex problem: mycotoxins, and particularly the carcinogenic mycotoxins, should be excluded from food as much as possible. Since the substances are present in foods as natural contaminants, however, human exposure cannot be completely prevented, and exposure of the population to some level of mycotoxins has to be tolerated. Despite the dilemmas, mycotoxin regulations have been established in many countries, and newer regulations are still being drafted.

## 3.3 Case study: international inquiry into mycotoxin regulations in 2002/2003

Articles about limits and regulations for mycotoxins and their rationales have been published several times (Krogh, 1977; Schuller *et al.*, 1983; Gilbert, 1991; Stoloff *et al.*, 1991; Van Egmond, 1991; Van Egmond and Dekker, 1995; Boutrif and Canet, 1998; Rosner, 1998; Van Egmond, 1999). The most recent comprehensive review was based on an international inquiry carried out in 1994/1995. It was published as FAO Food and Nutrition Paper 64 (FAO, 1997). Since this publication appeared, many new limits and regulations for mycotoxins have come into force or are in development; therefore FAO decided to produce an update of this document, and contracted for this task the Laboratory for Food and Residue Analyses (ARO), of the National Institute for Public Health and the Environment (RIVM), in the Netherlands. Consequently the authors of this chapter conducted an inquiry, which took place in 2002 and early 2003.

### 3.3.1 Design of the International Inquiry 2002

In 2002, an international inquiry was held among the Agricultural Services of the Dutch Embassies around the world, with the request to gather up-to-date



**Fig. 3.1** Countries known to have regulations (grey), unknown whether or not they have regulations (white) and known to have no specific regulations (black) for mycotoxins in foodstuffs and feedstuffs.

information from the local authorities on the situation regarding mycotoxin regulations, in as many countries as possible. Where this procedure did not lead to the desired information, personal contacts were used.

The questions in the inquiry concerned:

- existence of mycotoxin regulations;
- types of mycotoxins and products for which regulations are in force or proposed, together with maximum permissible levels;
- authorities responsible for control of mycotoxins;
- use of official and published methods of sampling and analysis;
- disposal of consignments containing inadmissible amounts of mycotoxins.

By the end of 2002, data had been received from 86 countries. Together with information gathered in previous inquiries, detailed information was available about the existence or absence of specific mycotoxin limits and regulations in food and feed in 117 countries. All data were interpreted to the best of our knowledge and tabulated, with the project being finalized by the end of 2003 and the report at the preparation stage (FAO, 2004). Some provisional conclusions with respect to current limits and regulations for the most significant mycotoxins can be made from first impressions of the updated information. They are given below.

### 3.3.2 General observations

On a worldwide basis, at least 100 countries had mycotoxin regulations for food and/or feed in 2002 ( Fig. 3.1), an increase of approximately 30 % compared to 1995. The total population in these countries represents approximately 90 % of the world's inhabitants. In fact all countries with mycotoxin regulations at least have regulatory limits for aflatoxin B<sub>1</sub> or the sum of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> in foods and/or feeds, a situation that was also observed in 1995. For several other mycotoxins, specific regulations exist as well (i.e. aflatoxin M<sub>1</sub>; the trichothecenes

DON, diacetoxyscirpenol, T-2 toxin and HT-2 toxin; the fumonisins B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub>; agaric acid; the ergot alkaloids; ochratoxin A; patulin; phomopsins; sterigmatocystin and zearalenone). The number of countries regulating mycotoxins had significantly increased over the period. Comparing the situations in 1995 and 2002, it appears that in 2002 more mycotoxins were regulated in more commodities and products, whereas tolerance limits had generally tended to decrease. Regulations had become more diverse and detailed with newer requirements regarding official procedures for sampling and analytical methodology. At the same time, several regulations had been harmonized between countries belonging to economic communities (EU, MERCOSUR, Australia/New Zealand), or were in some stage of harmonization.

### 3.3.3 Specific observations per region

#### *Africa*

In Africa, 14 countries were known to have specific mycotoxin regulations in 2002. These countries cover approximately 54 % of the inhabitants of the continent. For the majority of the African countries, specific mycotoxin regulations (probably) do not exist. Several of these countries recognize that they have problems due to mycotoxins and that regulations should be developed. The mycotoxin issue in Africa needs to be viewed, however, in the overall context of local food safety, health and agricultural issues (Shephard, 2004). The establishment of mycotoxin regulations will have limited effects in terms of health protection in those countries where many farmers grow agricultural produce for their own consumption, which is the case in many African countries. Most of the existing mycotoxin regulations in Africa concern the aflatoxins, and Morocco seemed to have the most detailed mycotoxin regulations in 2002.

#### *Asia/Oceania*

Asia and Oceania cover a very large part of the globe, with most countries lying in the tropics and subtropics, so it is expected that most mycotoxin problems will be caused by fungi, which grow at higher temperatures (Pitt and Hocking, 2004). An exception is New Zealand, which has a temperate to cool climate and separate mycotoxin issues from Asia and northern Australia. In the whole region, 25 countries were known to have specific mycotoxin regulation in 2002 (89 % of the inhabitants of the region). Regulations for total aflatoxins dominate in food, whereas in feed it is those for aflatoxin B<sub>1</sub>. Australia and New Zealand have harmonized their mycotoxin regulations, which include limits for the 'exotic' mycotoxins agaric acid and phomopsins. By far the most extensive and detailed regulations can be found in China and Iran.

#### *Latin America*

The major Latin American agricultural crops (maize, wheat, coffee, cotton, soybeans, barley, sunflower, groundnuts and tree nuts, cocoa and dairy products)

are highly susceptible to fungal contamination and mycotoxin production (Pineiro, 2004) and 19 Latin American countries were known to have in force specific mycotoxin regulations for food and feed in 2002. This covers 92 % of the inhabitants of the region. Harmonized regulations for aflatoxins exist in MERCOSUR member states. Incidentally, other countries indicate that they follow MERCOSUR regulations. The aflatoxin regulations in food are often set for the sum of the aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>. Uruguay has the most detailed regulations, including limits for ergot alkaloids in feeds, which is rather unique in the mycotoxin regulatory world.

#### *North America*

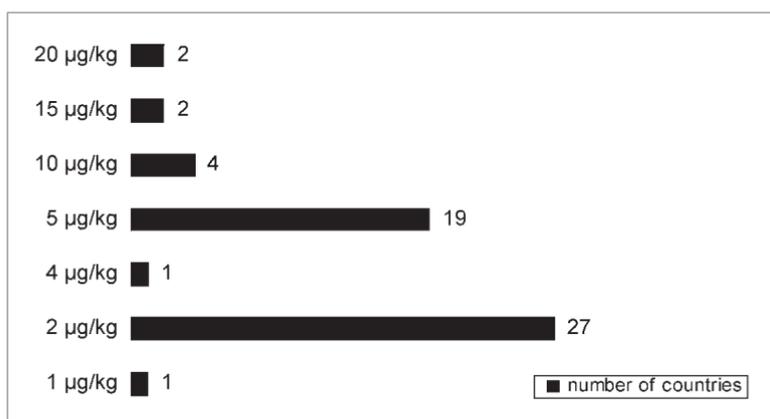
North America consists of only two countries, the USA and Canada. Both have had mycotoxin regulations for many years, and advanced techniques exist for sampling and analysis. In both countries, limits for aflatoxins are set for the sum of the aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>. In addition to limits for *Fusarium* toxins, Canada has also established tolerances for the percentage of *Fusarium* damaged kernels in wheat. In Canada limits also exist for the percentage of ergot in various crops for animal feed. In the USA detailed tolerance levels exist for the sum of the fumonisins B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub> in a wide variety of maize products.

#### *Europe*

In Europe, 38 countries were known to have specific mycotoxin regulations in 2002. These countries cover approximately 99 % of the inhabitants of the continent. Compared to the other regions, Europe has the most extensive and detailed regulations for mycotoxins in food. In the EU, now consisting of 15 countries but soon to be expanded with another 10 countries, harmonized regulations or guidelines exist for aflatoxins in various foodstuffs, aflatoxin M<sub>1</sub> in milk, ochratoxin A in cereals and dried vine fruits, DON in cereals and cereal products and for aflatoxin B<sub>1</sub> in various feedstuffs. It is of interest to note that many of the EU candidate countries have mycotoxin regulations that are often more detailed than those currently in force in the EU. In the future a significant further expansion of EU-harmonized mycotoxin regulations is expected for foods and feeds. For foods this concerns patulin in several fruit products; aflatoxin B<sub>1</sub>, aflatoxin M<sub>1</sub>, ochratoxin A and DON in infant formulae and follow-up formulae; ochratoxin A in coffee, wine, beer, spices, grape juice, cocoa and cocoa products; several *Fusarium*-produced mycotoxins, i.e. trichothecenes (nivalenol, T-2 and HT-2 toxins, in addition to DON), fumonisins and zearalenone in cereal-based foodstuffs. Also for feeds new limits are expected for several mycotoxins.

#### **3.3.4 Worldwide limits for aflatoxins in food**

Compared to the situation in 1995, the maximum tolerated levels for aflatoxin B<sub>1</sub> in food have not changed dramatically (Fig. 3.2). The range of limits has narrowed a little (1–20 µg/kg) and the median is at 2 µg/kg, a limit now in force in 27 countries (see Fig. 3.2). Most of these countries belong to the EU (where, since

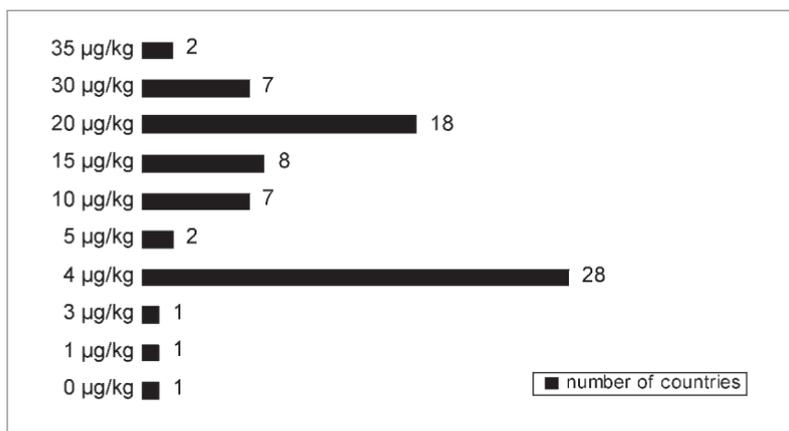


**Fig. 3.2** Frequency distribution of specific regulatory limits for aflatoxin B<sub>1</sub> in food in 56 countries, in 2002.

1998, harmonized limits for aflatoxin B<sub>1</sub> and the sum of the aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> have been in force), to EFTA and candidate EU countries. Many of the candidate EU countries have already brought their national regulations into harmony with the EU, in anticipation of their membership of the EU. Another major limit is visible at 5 µg/kg, followed by 19 countries, spread over Africa, Asia/Oceania, Latin America and Europe. The USA and Canada do not have a single limit for aflatoxin B<sub>1</sub>.

As in 1995, also in 2002 many countries regulated aflatoxins in food with limits for the sum of the aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>, sometimes in combination with a specific limit for aflatoxin B<sub>1</sub>. The limits range from 1–35 µg/kg, whereas the most frequently occurring limit (Fig. 3.3) is at 4 µg/kg (applied by 28 countries), again a limit found back in the harmonized regulations in the EU, EFTA and candidate EU countries, where dual limits both for aflatoxin B<sub>1</sub> and for total aflatoxins are enforced. Another major peak occurs at 20 µg/kg, applied by 18 countries, with half of them in Latin America (where it is also a MERCOSUR harmonized limit), and several in Africa. Also the USA, one of the first countries to establish an aflatoxin action limit, follows the 20 µg/kg limit. Over the years, the ‘popularity’ of a limit for total aflatoxins in foodstuffs has remained, resulting in 75 countries in 2002 applying this regulatory level (as compared to 56 countries with a specific limit for aflatoxin B<sub>1</sub>).

Whether a regulatory level for the sum of the aflatoxins, which requires more analytical work than for aflatoxin B<sub>1</sub> alone, contributes significantly to better protection of public health than a regulatory level for aflatoxin B<sub>1</sub> alone is debatable. Aflatoxin B<sub>1</sub> is the most important of the aflatoxins, considered from the viewpoints of both toxicology and occurrence. It is unlikely that commodities will contain aflatoxins B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> and not aflatoxin B<sub>1</sub>, and the concentration of the sum of the aflatoxins B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> is generally less than the concentration of aflatoxin B<sub>1</sub> alone. Typical occurrence ratios for aflatoxins B<sub>1</sub> and B<sub>2</sub> (mainly



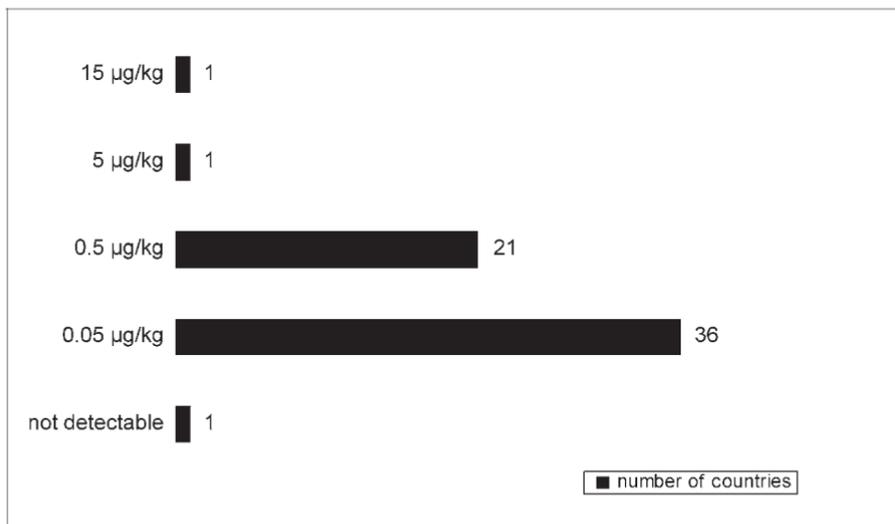
**Fig. 3.3** Frequency distribution of specific regulatory limits for the sum of aflatoxins  $B_1$ ,  $B_2$ ,  $G_1$  and  $G_2$  in food in 75 countries, in 2002.

produced by *Aspergillus flavus*) average approximately 4:1. Typical occurrence ratios for aflatoxin  $B_1$  and the sum of the aflatoxins  $B_2$ ,  $G_1$  and  $G_2$  (the G toxins are mainly produced by *Aspergillus parasiticus*) average approximately 1:0.8, although variations do occur for both ratios. Regulatory authorities in those countries that apply a regulatory level for the sum of the aflatoxins should critically inspect the analytical data of monitoring agencies to see how frequently the availability of data on the sum of the aflatoxins (above that on aflatoxin  $B_1$ ) has been essential to adequately protect the consumer. Analysis of one target component (aflatoxin  $B_1$ ) could be sufficient, more practical, and cheaper.

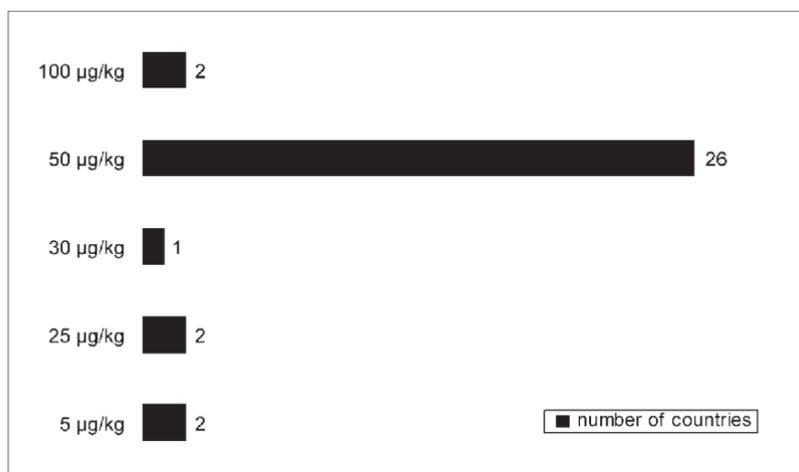
In 2002 regulations for aflatoxin  $M_1$  were seen in 60 countries, a more than three-fold increase as compared to 1995! It is again the EU, EFTA and the candidate EU countries that contribute in major part to the largest peak seen in Fig. 3.4 at 0.05 µg/kg, but some other countries in Africa, Asia and Latin America also apply this limit (Fig. 3.4). The other peaking limit is at 0.5 µg/kg. This higher regulatory level is applied in the USA and several Asian and European countries, and it occurs most frequently in Latin America, where it is established as a harmonized MERCOSUR limit. The ten-fold difference between the two most prevailing limits for aflatoxin  $M_1$ , which have existed already for many years, has given rise to debates within Codex Alimentarius, leading to their request to JECFA to re-evaluate the human health risk of aflatoxin  $M_1$  (see Section 3.2.1). Apart from these sub-ng/g regulatory levels, a few countries indicated in the 2002 inquiry that they regulate aflatoxin  $M_1$  in milk at levels of 5 and 15 µg/kg. These levels are not realistic and are probably mistakes.

### 3.3.5 Worldwide limits for other mycotoxins in food

Between 1995 and 2002 many more countries regulated patulin, mostly in fruit products such as apple juice, and these countries are remarkably united about the

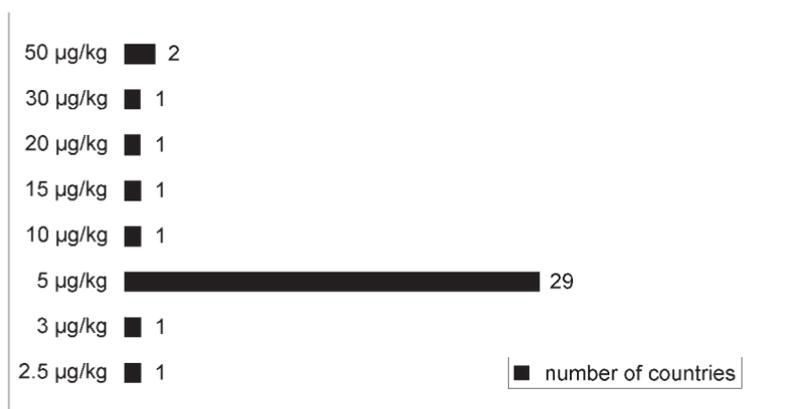


**Fig. 3.4** Frequency distribution of specific regulatory limits for aflatoxin  $M_1$  in milk in 60 countries, in 2002.



**Fig. 3.5** Frequency distribution of specific regulatory limits for patulin in fruit and fruit juices in 33 countries, in 2002.

desired limit (50 µg/kg, Fig. 3.5). The number of countries that regulate patulin in food increased significantly when harmonized EU limits for patulin came into force for various products in 2003, an action which may make patulin the most regulated mycotoxin in the world, second only to aflatoxins. Validated analytical methodology (AOAC, CEN) is readily available to determine patulin in fruit juice at a level of 50 µg/kg. However, the currently proposed EU limit of 10 µg/kg for baby food and infant formulae has caused additional research efforts by the EC's

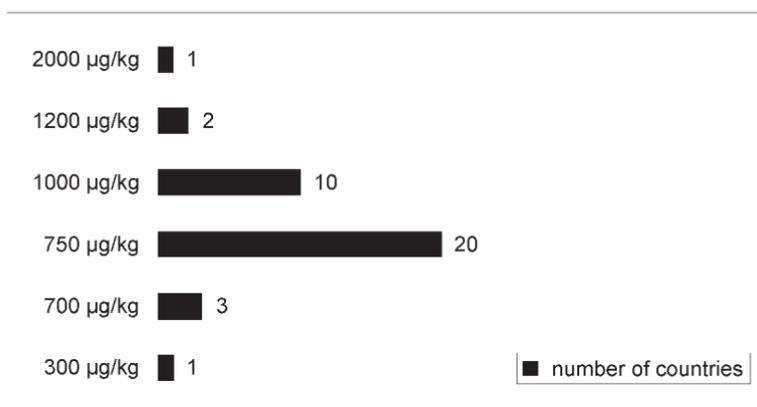


**Fig. 3.6** Frequency distribution of specific regulatory limits for ochratoxin A in cereals and cereal products in 37 countries, in 2002.

IRMM in Geel, Belgium, and a collaborative study has been carried out to prove that the newly developed methodology is fit for the purpose.

At a first glance, the developments in the area of regulations for ochratoxin A show strong similarities to those for patulin: a significant increase in the number of countries that apply limits, and a good agreement about the desired limit for cereals and cereal products (Fig. 3.6). Cereals are considered the major source of human exposure to ochratoxin A. There is a restriction with the presentation of the data in Fig. 3.6, however. Many countries have set a limit for ochratoxin A in cereals, many others for cereal products, and some have set separate (different) limits for each. For example, this latter situation occurs in the EU, where a limit of 5 µg/kg (the dominant peak in Fig. 3.6) is in force for raw cereals and a limit of 3 µg/kg (not presented in the figure) for processed cereals. To present all this in one figure was difficult, and therefore the approach followed was to preferentially include a country's limit for (raw) cereals in Fig. 3.6 and, where this did not exist, to include the limit (if any) for cereal products. The current and proposed limits for ochratoxin A may need to be reviewed in the near future, pending the outcome of an ongoing EC-supported project on 'Mechanisms of ochratoxin A induced carcinogenicity as a basis for an improved risk assessment'. This project is aimed at establishing whether or not the carcinogenicity of ochratoxin A is considered to arise through a threshold or non-threshold approach.

As is the case with patulin and ochratoxin A, a few dozen countries have set regulatory or guideline limits for DON (Fig. 3.7). Whereas in 1995 this trichothecene was only sporadically regulated in food, it has become a toxin of high concern in monitoring programmes and amongst regulatory authorities since the late 1990s when mg/kg concentrations were reported to occur in cereals and cereal products, particularly in Europe. Similarly as with ochratoxin A, it was difficult to summarize the most commonly occurring limits for DON in wheat and other cereals in one figure, and those interested in the full details of the many regulations



**Fig. 3.7** Frequency distribution of specific regulatory limits for deoxynivalenol in wheat (flour) in 37 countries, in 2002.

that now exist for DON should consult the full FAO document (FAO, 2004). The peak at 750 µg/kg is dominated by the countries of the EU that currently apply this (unofficial) guideline limit for DON in flour used as raw materials for several years.

Zearalenone, an oestrogenic mycotoxin, was regulated in 16 countries in 2002, as compared to six in 1995 (Fig. 3.8). Limits in maize and other cereals currently vary from 50 to 1000 µg/kg. Figure 3.8 betrays a tendency to set limits at higher rather than lower limits.

Fumonisin were discovered in the late 1980s. Whereas in 1995 fumonisins were the subject of regulations only in one country, this number had increased to 6 by 2002, with limits for maize ranging from 1000–3000 µg/kg (Fig. 3.9). Although proportionally a very significant increase, the number of countries regulating fumonisins is too small to draw meaningful conclusions about generally agreed limits. Regulatory authorities that are currently thinking about the constitution of legal limits for fumonisins should carefully consider whether they wish to do so for fumonisin B<sub>1</sub> only or for the sum of the naturally occurring fumonisins. A situation occurs here similar to that for aflatoxins, for which limits exist both for aflatoxin B<sub>1</sub> and for total aflatoxins (see discussion of Fig. 3.3 above).

### 3.4 Conclusion

Comparing the situations in 1995 and 2002, apparently in 2002 more countries were known to have regulations for more mycotoxins in more commodities and products. Regulations have become more diverse and detailed with newer requirements regarding official procedures for sampling and analytical methodology. This reflects the general concerns that governments have regarding the potential effects of mycotoxins on the health of humans and animals. At the same time,



**Fig. 3.8** Frequency distribution of specific regulatory limits for zearalenone in maize and other cereals in 16 countries, in 2002.



**Fig. 3.9** Frequency distribution of specific regulatory limits for fumonisins in maize in 6 countries, in 2002.

harmonization of tolerance levels is taking place in some free trade zones (EU, EFTA, MERCOSUR, Australia/New Zealand), and harmonization efforts are being undertaken for goods moving in international commerce (Codex Alimentarius). This harmonization is a slow process, because of the different views and interests of those involved.

Whereas harmonized tolerance levels would be beneficial from the point of view of trade, one might argue that this would not necessarily be the case from the point of view of (equal) human health protection around the world. Risk characterization is the product of hazard assessment and exposure assessment. The hazard of mycotoxins to individuals is probably more or less the same all over the world (although other factors sometimes play a role as well, e.g. hepatitis B virus infection in relation to the hazard of aflatoxins). Exposure is not the same, because of differences in levels of contamination and dietary habits in various parts of the world. Shephard (2004) exemplified this with the help of some calculations done for fumonisins. The JECFA established a group provisional maximum tolerable daily intake (PMTDI) for fumonisins B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub> of 2 µg/kg body weight per day. This PMTDI is readily exceeded by individuals on a maize-based diet in which maize consumption is of the order of 400 g/person per day. Shephard calculated

that, at a contamination level for fumonisins in maize of 2000 µg/kg (a level within the range of common limits: see Fig. 3. 9), dietary exposure for a 60 kg adult would be 13 µg/kg body weight per day or 650 % of the PMTDI! In the developed world, maize intakes are less than 10 g/person per day (Shephard *et al.*, 2002) and contamination levels as high as 12 000 µg/kg can be consumed before dietary exposure exceeds the PMTDI set by JECFA.

National governments or regional communities should encourage and fund activities that contribute to reliable exposure assessment of mycotoxins in their regions. Examples of such activities are the SCOOP tasks, undertaken in the EU in support of safety evaluations on some mycotoxins (see Section 3.2.2). The availability of inexpensive, validated and easily performed analytical methodology and the application of AQA are basic prerequisites for meaningful data on occurrence, and must therefore be stimulated.

Efforts to achieve improved hazard assessment should preferably be coordinated and funded at the international level. Chronic toxicity studies carried out under good laboratory practice conditions are very time-consuming, very expensive, and not necessarily bound to certain regions. These studies should be carried out in internationally recognized centres of excellence and their results evaluated by international groups of experts, e.g. the JECFA. An example of such an internationally concerted effort is the ongoing EC-sponsored project 'Mechanisms of ochratoxin A induced carcinogenicity as a basis for an improved risk assessment'. (See Section 3.3).

The regulations enacted for mycotoxins in food, and those under development, should be the result of sound cooperation between interested parties, drawn from science, consumers, industry and policy makers. Only then can realistic protection be achieved.

### 3.5 Sources of further information and advice

Some major and recent publications that readers may wish to consult about safety evaluation of mycotoxins, include:

- Food and Agriculture Organization (2001), *Safety Evaluation of Certain Mycotoxins in Food*. Prepared by the 56th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The FAO Food and Nutrition Paper 74, Rome, Food and Agriculture Organization of the United Nations.
- World Health Organization (2002), *Evaluation of Certain Mycotoxins in Food*. Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives, WHO Technical Report Series 906, Geneva, World Health Organization.

These two papers provide detailed insight into the process of safety evaluation of mycotoxins by the JECFA. The JECFA's evaluations are independent, authoritative and they provide the major scientific basis, key to establishing meaningful limits and regulations for mycotoxins.

Those who would like a detailed insight into the limits and regulations that existed for mycotoxins in food and feed in the mid 1990s are advised to consult FAO Food and Nutrition Paper: *Worldwide Regulations for Mycotoxins 1995. A Compendium*. This document provides tabulated information about specific mycotoxin regulations in 77 countries. A similar document, but now with full details about mycotoxin regulations in more than 100 countries in 2002/2003, is in preparation, and a relevant FAO Food and Nutrition Paper is expected in 2004.

### 3.6 Acknowledgement

The authors wish to thank the Food and Agriculture Organization of the United Nations for permission to make use of unpublished material collected during the international enquiry on worldwide limits and regulations for mycotoxins carried out by the National Institute for Public Health and the Environment in 2002 and 2003 under contract with FAO, as well as material published in 1997 in FAO Food and Nutrition Paper 64: *Worldwide Regulations for Mycotoxins 1995. A Compendium*. The detailed results of the 2002/2003 inquiry will be published by the FAO at a later stage.

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# 4

## Sampling for mycotoxins

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### **4.1 Introduction: mycotoxin sampling definition and uncertainty**

It is important to be able to detect and quantify the mycotoxin concentration in foods and feeds destined for human and animal consumption. In research, quality assurance, and regulatory activities, correct decisions concerning the fate of commercial-bulk lots can only be made if the mycotoxin concentration in the lot can be measured with a high degree of accuracy and precision. The mycotoxin concentration of a bulk lot is usually estimated by measuring the mycotoxin in a small portion of the lot or a sample taken from the lot. The mycotoxin concentration in the bulk lot is assumed to be the same that in the sample. Then, based on the measured sample concentration, some decision is made about the edible quality of the bulk lot or the effect of a treatment or a process on reducing aflatoxin in it. For example, in a regulatory environment, decisions will be made to classify the lot as acceptable or unacceptable based upon a comparison of the measured sample concentration with a legal limit. If the sample concentration does not accurately reflect the lot concentration, then the lot may be misclassified and there may be undesirable economic and/or health consequences. Fortunately, sampling plans can be designed to minimize the misclassification of lots and reduce the undesirable consequences associated with regulatory decisions about the fate of bulk lots. In this chapter, sampling plans will be defined, sources of uncertainty associated with a mycotoxin sampling plan will be identified, risks associated with misclassifying lots will be discussed, and methods that reduce misclassification of lots will be described.

### 4.1.1 Definition

A mycotoxin-sampling plan is defined by a mycotoxin test procedure and a defined accept/reject limit. A mycotoxin test procedure is a multi-stage process and generally consists of three steps: sampling, sample preparation, and analysis (quantification). The sampling step specifies how the sample will be selected or taken from the bulk lot and the size of the sample. For granular products, the sample preparation step is also a two-part process where the sample is ground in a mill to reduce particle size and a subsample is removed from the comminuted sample. Finally, in the analytical step, the mycotoxin is solvent extracted from the comminuted subsample and quantified using approved procedures.

The measured mycotoxin concentration in the sample is used to estimate the true mycotoxin concentration in the bulk lot or compared to a defined accept/reject limit that is usually equal to a legal limit. Comparing the measured concentration to an accept/reject limit is often called acceptance sampling, because the measured concentration value is not as important as whether the measured concentration (and thus the lot concentration) is above or below a legal limit. In quality assurance and research activities, a precise estimate of the true mycotoxin concentration of a lot becomes important.

### 4.1.2 Uncertainty

Because of the uncertainty associated with a mycotoxin-sampling plan, the true mycotoxin concentration of a bulk lot cannot be determined with 100 % certainty; nor can all lots be correctly classified into good and bad categories with 100 % accuracy. There are generally two types of uncertainties associated with sampling procedures, accuracy and precision.<sup>1</sup> Accuracy is defined as the closeness of measured values to the true value. Precision is defined as closeness of measured values to each other. Accuracy is associated with a bias. Biases have the potential to occur in the sample selection process and in the quantification process. Biases are the easiest to control and eliminate, but are the most difficult to measure because of the difficulty in knowing the true mycotoxin concentration of the lot. Sample selection equipment and analytical methods are continuously performance tested to minimize any biases. Precision is associated with variability. Variability can occur with each step of the mycotoxin test procedure and is usually associated with the mycotoxin distribution among contaminated particles in the lot. As will be shown later, increasing the quantity of material inspected usually reduces variability or improves precision. The various components of a mycotoxin-sampling plan are discussed in greater detail below.

## 4.2 Methods of sample selection

Procedures used to take a sample from a bulk lot are extremely important. Every individual item in the lot should have an equal chance of being chosen (called random sampling). Biases are introduced by sample selection methods if

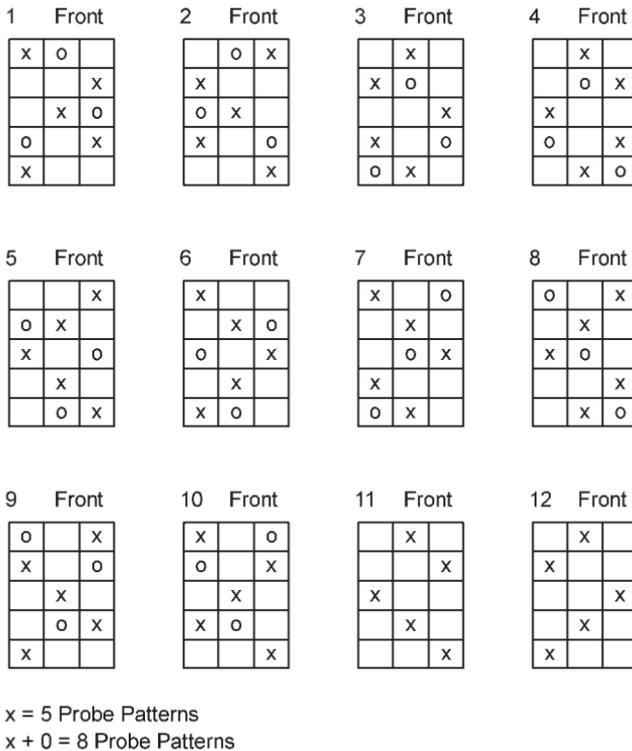
equipment and procedures used to select the sample prohibit or reduce the chances of any item in the lot from being chosen.<sup>2</sup> If the lot has been blended thoroughly from various material handling operations, then the contaminated particles are probably distributed uniformly throughout the lot.<sup>3</sup> In this situation, it is probably not too important from what location in the lot the sample is drawn. However, if the lot is contaminated because of moisture leaks that cause high moisture clumps or for other localized reasons, then the mycotoxin-contaminated particles may be located in isolated pockets in the lot.<sup>4</sup> If the sample is drawn from a single location, the contaminated particles may be missed or too many contaminated particles may be collected. Because contaminated particles may not be distributed uniformly throughout the lot, the sample should be an accumulation of many small portions taken from many different locations throughout the lot.<sup>5,6</sup> The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) recommends that each incremental portion be about 200 g and one incremental portion be taken for every 200 kg of product.<sup>7</sup> The accumulation of many small incremental portions is called a bulk sample. If the bulk sample is larger than desired, it should be blended and subdivided until the desired sample size is achieved. The smallest sample size that is subdivided from the bulk sample and comminuted in a grinder in the sample preparation step is called the test sample. It is generally more difficult to obtain a representative (lack of bias) test sample from a lot at rest (static lot) than from a moving stream of the product (dynamic lot) as the lot is moved from one location to another. Sample selection methods differ depending on whether the lot is static or dynamic.

#### 4.2.1 Static lots

Examples of static lots are commodities contained in storage bins, railcars, or many small containers such as sacks. When drawing a sample from a bulk container, a probing pattern should be developed so that product can be collected from different locations in the lot. An example of several probing patterns used by the US Department of Agriculture (USDA) to collect samples from peanut lots is shown in Fig. 4.1.<sup>8,9,10</sup> The sampling probe should be long enough to reach the bottom of the container when possible. Attempts should be made to use a sampling rate similar to the 200 g per 200 kg mentioned above. However, it may not be possible to achieve the suggested sampling rate because of the design of the sampling equipment, size of the individual containers, and the size of the lot.

When sampling a static lot in separate containers such as sacks or retail containers, the sample should be taken from many containers dispersed throughout the lot. When storing sacks in a storage facility, access lanes should be constructed in order to allow access to sacks at interior locations. The recommended number of containers sampled can vary from one in four in small lots (less than 20 metric tonnes) to the square root of the total number of containers for large (greater than 20 metric tonnes) lots.<sup>7</sup>

If the lot is in a container where access is limited, the sample should be drawn when the product is either being removed from or being placed into the container.



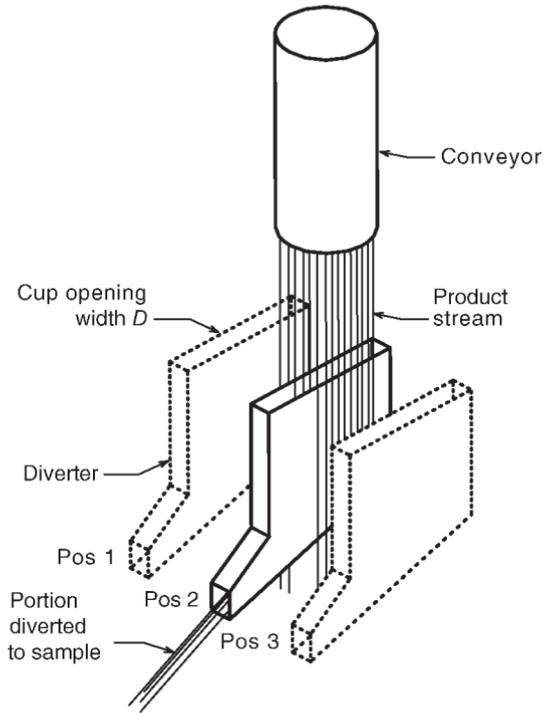
**Fig. 4.1** Example of several five- and eight-probe patterns used by the USDA to sample farmers stock peanuts for grade and support price.

If the accumulated bulk sample is larger than required, the bulk sample should be thoroughly blended and reduced to the required test sample size using a suitable divider that randomly removes a test sample from the bulk sample.

**4.2.2 Dynamic lots**

True random sampling can be more nearly achieved when selecting a bulk sample from a moving stream as the product is transferred (i.e. conveyor belt) from one location to another. When sampling from a moving stream, small increments of product should be taken along the entire length of the moving stream and across the entire cross-section; then all the increments of product composited to obtain a bulk sample; if the bulk sample is larger than required, then the bulk sample is blended and subdivided to obtain the desired size test sample.

Automatic sampling equipment such as cross-cut samplers (Fig. 4.2) is commercially available with timers that automatically pass a diverter cup through the moving stream at predetermined and uniform intervals. When automatic equipment is not available, a person can be assigned to manually pass a cup through



**Fig. 4.2** Automatic cross-cut sampler.

the stream at periodic intervals to collect the bulk sample. Whether using automatic or manual methods, small increments of product should be collected and composited at frequent and uniform intervals throughout the entire time the product flows past the sampling point.

Cross-cut samplers should be installed in the following manner:

- (a) the plane of the opening of the sampling cup should be perpendicular to the direction of flow;
- (b) the sampling cup should pass through the entire cross-sectional area of the stream; and
- (c) the opening of the sampling cup should be wide enough to accept all items of interest in the lot. As a general rule, the width of the sampling cup opening should be two to three times the largest dimensions of the items in the lot.

The size of the bulk sample,  $S$  in kg, taken from a lot by a cross-cut sampler is

$$S = (D)(L)/(T)(V) \quad [4.1]$$

where  $D$  is the width of the sampling cup opening in cm,  $L$  is the lot size in kg,  $T$  is the interval or time between cup movement through the stream in seconds, and  $V$  is cup velocity in cm/sec.

Equation 4.1 can also be used to compute other terms of interest, such as the time between cuts,  $T$ . For example, the required time,  $T$ , between cuts of the sampling cup to obtain a 10 kg sample from a 30 000 kg lot where the sampling cup width is 5.08 cm (2 inches), and the cup velocity through the stream 30 cm/sec is, from Eq. 4.1,

$$T = (5.08 \text{ cm} \times 30\,000 \text{ kg}) / (10 \text{ kg} \times 30 \text{ cm/s}) = 508 \text{ s}$$

If the lot is moving at 1000 kg per minute, the entire lot will pass through the sampler in 30 minutes and only three or four cuts will be made by the cup through the lot. This may be considered too infrequent, because too much product passes the sampling point between the times the cup cuts through the stream. The interaction among the variables in Eq. 4.1 needs to be fully understood in terms of the amount of sample accumulated and the frequency of taking product.

### 4.2.3 Bulk versus test sample

Because contaminated seed may not be uniformly dispersed throughout the lot, many incremental portions are taken from many different locations throughout the lot and accumulated to form a bulk sample. As a result, the bulk sample is usually larger than the desired test sample size used to estimate the lot mycotoxin concentration. For granular material, the test sample is the smallest sample of granular product ground in a mill in the sample preparation step. For finely ground materials (corn flour) or liquids (milk), the test sample is the smallest sample used in the analytical step to quantify the mycotoxin. When the bulk sample is larger than the test sample, dividers are used to remove the desired test sample from the bulk sample. If a test sample is to be removed from the bulk sample, then it is best to use a mechanical device that gives random divisions, such as a Boerner or riffle divider.<sup>9</sup> When using a divider that gives random divisions, the bulk sample doesn't have to be blended before the test sample is removed. However, if the test sample is to be removed from the bulk sample using quartering or a manual device such as a cup or scoop, then the bulk sample should be blended before the test sample is removed.

If the test sample is a granular product such as shelled corn or nuts, then the test sample should not be further reduced in size before particle size reduction associated with the sample preparation step. As the test sample becomes smaller, the uncertainty associated with estimating the true lot mycotoxin concentration becomes greater. As will be shown later, the size of the test sample put through the grinder should be as large as possible.

## 4.3 Reducing random variation in the mycotoxin test procedure

Even when using accepted sampling, sample preparation, and analytical procedures,<sup>11-16</sup> there are errors (the term error will be used to denote variability)

**Table 4.1** Distribution of aflatoxin test results for ten 5.4 kg samples from each of six lots of shelled peanuts.\*

Lot number	Sample test result										Mean (ppb)	SD <sup>1</sup> (ppb)	CV <sup>2</sup> (%)
	(ng/g)												
1	0	0	0	0	2	4	8	14	28	43	10	15	150
2	0	0	0	0	3	13	19	41	43	69	19	24	126
3	0	6	6	8	10	50	60	62	66	130	40	42	105
4	5	12	56	66	70	92	98	132	141	164	84	53	63
5	18	50	53	72	82	108	112	127	182	191	100	56	56
6	29	37	41	71	95	117	168	174	183	197	111	66	59

\* Aflatoxin test results are ordered by aflatoxin concentration (ng/g).

<sup>1</sup> SD = standard deviation.

<sup>2</sup> CV = coefficient of variation =  $SD \times 100/\text{mean}$ .

associated with each of the above steps of the mycotoxin test procedure.<sup>17-24</sup> Because of these errors, the true mycotoxin concentration in the lot cannot be determined with 100 % certainty by measuring the mycotoxin concentration in a test sample taken from the lot. For example, 10 replicated aflatoxin test results from each of six contaminated shelled peanut lots are shown in Table 4.1.<sup>25</sup> For each test result in the table, the mycotoxin test procedure consisted of (a) comminuting a 5.45 kg test sample of peanut kernels in a USDA subsampling mill developed by Dickens and Satterwhite,<sup>14,26</sup> (b) removing a 280 g subsample from the comminuted test sample, (c) solvent extracting aflatoxins from a 280 g subsample as described by AOAC Method II,<sup>11</sup> and (d) quantifying the aflatoxins densitometrically using thin-layer chromatography (TLC).<sup>11</sup> The 10 aflatoxin test results from each lot are ranked from low to high to demonstrate several important characteristics about replicated aflatoxin test results taken from the same contaminated lot.

First, the wide range among replicated sample results from the same lot reflects the large variability associated with estimating the true mycotoxin content of a bulk lot. In Table 4.1, the variability is described by the standard deviation (SD) and the coefficients of variation (CV).

The maximum sample result can be four to five times the lot concentration (the average of the 10 sample results is the best estimate of the lot concentration). Secondly, the amount of variation among the 10 sample results appears to be a function of the lot concentration. As the lot concentration increases, the standard deviation among sample results increases, but the standard deviation relative to the lot mean, as measured by the CV, decreases. Thirdly, the distribution of the 10 sample results for each lot in Table 4.1 is not always symmetrical about the lot concentration. The distributions are positively skewed, meaning that more than half of the sample results are below the true lot concentration. However, the distribution of sample test results becomes more symmetrical as the lot concentration increases. This skewness can be observed by counting the number of aflatoxin test results above and below the lot concentration in Table 4.1 (average of the 10

sample test results). If a single sample is tested from a contaminated lot, there is more than a 50% chance that the sample test result will be lower than the true lot concentration. While it can't be shown in Table 4.1, the skewness is greater for small sample sizes and the distribution becomes more symmetrical as sample size increases.<sup>27</sup> The above characteristics described by Table 4.1 for aflatoxin in peanuts are also generally found for other mycotoxins and other commodities.<sup>23,24,28,29</sup> The sampling, sample preparation, and analytical steps associated with the aflatoxin test procedure each contribute to the total variability observed among aflatoxin test results shown in Table 4.1.

### 4.3.1 Sampling variability

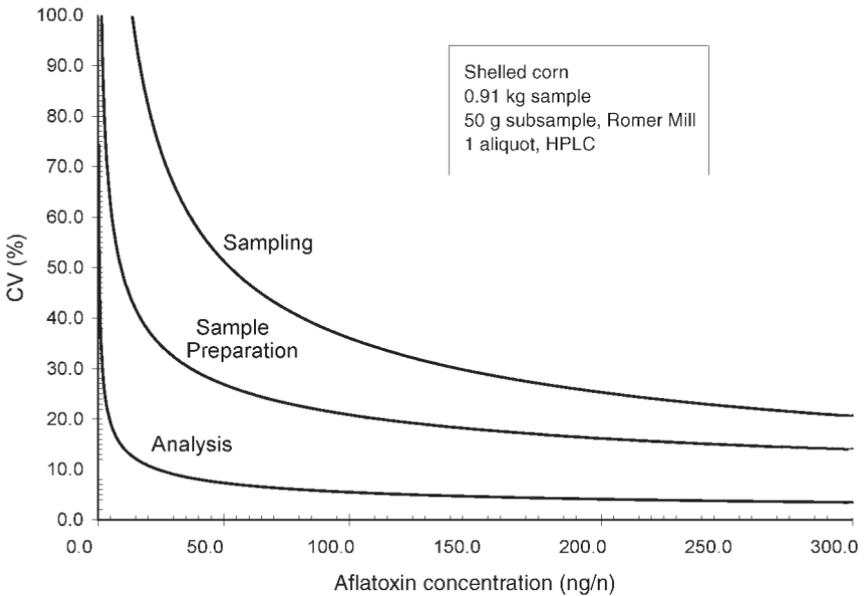
Studies by researchers on a wide variety of agricultural products (peanuts, cottonseed, shelled corn and pistachio nuts) indicate that, especially for small sample sizes, the sampling step is usually the largest source of variability associated with the mycotoxin test procedure.<sup>18–24</sup> Even when using accepted sample selection equipment and random sample selection procedures, sampling error is large because of the extreme distribution among contaminated particles within a lot. Studies by researchers on a wide variety of agricultural products, such as peanuts and shelled corn, indicate that a very small percentage (0.1 %) of the kernels in the lot are contaminated and the concentration on a single kernel may be extremely high.<sup>30,31</sup> Cucullu *et al.* reported aflatoxin concentrations in excess of 1 000 000 ng/g (parts per billion, ppb) for individual peanut kernels and 5 000 000 ng/g for cottonseed.<sup>32,33</sup> Shotwell *et al.* reported finding over 400 000 ng/g of aflatoxin in a corn kernel.<sup>34</sup>

Because of this extreme range in aflatoxin concentrations among a few contaminated kernels in a lot, variation among replicated sample test results tends to be large. As an example, the sampling variance,  $VS$ , associated with testing shelled corn was estimated empirically and is shown in Eq. 4.2 for any sample size  $ns$ .<sup>28</sup>

$$VS = (12.95/ns)M^{0.98} \quad [4.2]$$

where  $M$  is the aflatoxin concentration in the lot in nanograms of total aflatoxin per g of corn (ng/g) or parts per billion (ppb),  $ns$  is the mass of shelled corn in the sample in kg (kernel count per gram was 3.0). From Eq. 4.2 one can see that the sampling variance is a function of the lot concentration  $M$  and sample size  $ns$ . The sampling variance among 0.91 kg (2 lb) samples taken from a lot of shelled corn at 20 ppb is 268.1. The coefficient of variation is 81.8 %. The sampling CV over a range of aflatoxin concentrations is shown in Fig. 4.3.

Researchers have developed equations to describe the sampling variance for several commodities and mycotoxins.<sup>17–24</sup> The equations are specific for the mycotoxin type and type by product studied, but generally show that sampling variance is a function of concentration and increases with it.



**Fig. 4.3** Coefficients of variation (CV) for sampling, sample preparation and analysis when testing shelled corn for aflatoxin.

### 4.3.2 Sample preparation variability

Once the test sample has been taken from the lot, the sample must be prepared for aflatoxin quantification. Since it is not practical to extract the mycotoxin from a large test sample, it is usually extracted from a much smaller portion of product (subsample) taken from the test sample. If the commodity is a granular product such as shelled corn, it is essential that the entire test sample be comminuted in a suitable mill before a subsample is removed from it.<sup>11</sup> Removing a subsample of whole seed from the test sample before the comminution process is simply a sample size reduction process and eliminates the benefits associated with the larger size sample of granular product. After the sample has been comminuted in a mill to reduce particle size, a subsample is removed for mycotoxin extraction. It is assumed that the distribution of contaminated particles in the comminuted sample is similar to the distribution among contaminated kernels found in the lot. As a result, there is also variability among replicated subsamples taken from the same test sample. However, the sample preparation variance is not as large as the sampling variance due to the large number of comminuted particles in the subsample. An example of sample preparation variance for aflatoxin and shelled corn,  $VSS$ , is shown below in Eq. 4.3 for any subsample size  $nss$ .<sup>28</sup>

$$VSS = (62.70/nss)M^{1.27}$$

[4.3]

where  $M$  is the aflatoxin concentration in the test sample in ppb,  $nss$  is the mass of shelled corn in the subsample in grams. The variance in Eq. 4.3 also reflects the use of a Romer mill that produces a particle size where most of the particles will pass through a number 20 screen (0.85 mm opening). From Eq. 4.3, it can be seen that the sample preparation variance is also a function of the aflatoxin concentration in the sample and the subsample size. The sample preparation variance associated with a 50 g subsample taken from a sample at 20 ppb is 56.3 and the CV is 37.5 %. The sample preparation CV over a range of aflatoxin concentrations is shown in Fig. 4.3.

Researchers have developed equations to describe the sample preparation variance for several commodities, types of mills, and mycotoxins.<sup>17-24</sup> The equations are specific for the type of mycotoxin, type of mill (particle size), and the type of product used in the study. The type of mill affects the particle size distribution. If the average particle size decreases (number of particles per unit mass increases), then the subsampling variances for a given size of subsample decrease. Particle size reduction can be achieved either with dry grinding or blending the test sample in a fluid.

### 4.3.3 Analytical variability

Once the subsample is removed from the comminuted test sample, the mycotoxin is solvent extracted. Analytical methods usually involve several steps, such as solvent extraction, centrifugation, drying, dilution, and quantification.<sup>35</sup> As a result, there can be considerable variation among replicated analyses on the same subsample extract. The analytical variance,  $VAh$ , associated with high-performance liquid chromatography (HPLC) techniques used to measure aflatoxin in corn is given by Eq. 4.4 for any number of aliquots  $na$ .

$$VAh = (0.143/na)M^{1.16} \quad [4.4]$$

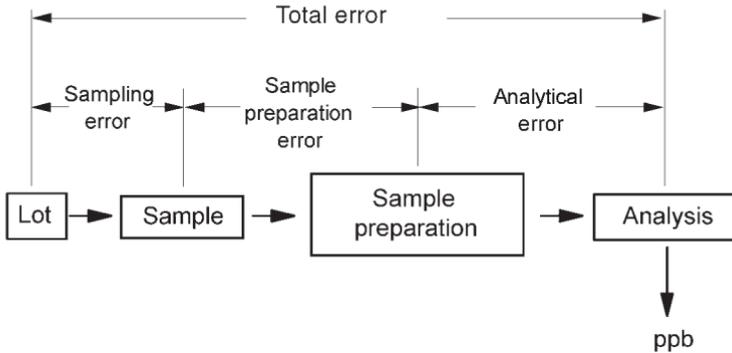
where  $M$  is the aflatoxin concentration in the subsample in ppb,  $na$  is the number of aliquots quantified by HPLC methods. The analytical variance and CV associated with using HPLC to measure aflatoxin in a comminuted subsample of corn at 20 ng/g, is 4.6 and 10.7 %, respectively. The analytical CV over a range of aflatoxin concentrations is shown in Fig. 4.3.

High-performance liquid chromatography tends to have less variability than other analytical technologies such as TLC and immunoassay (ELISA) methods.<sup>36</sup> Using precision estimates from collaborative studies, the analytical variances associated with TLC ( $VA_t$ ) and ELISA ( $VA_e$ ) methods to measure aflatoxin in corn are shown in Eqs 4.5 and 4.6, respectively.

$$VA_t = (0.316/na)M^{1.744} \quad [4.5]$$

$$VA_e = (0.631/na)M^{1.293} \quad [4.6]$$

The coefficients of variation associated with measuring aflatoxin in a corn subsample at 20 ppb with the TLC and ELISA methods are 38.3 and 27.5 %, respectively.



**Fig. 4.4** Sources of variability associated with a mycotoxin-test procedure.

respectively. The variability associated with HPLC (10.7 %, Eq. 4.3) is lower than either TLC or ELISA.

All of the analytical variance information described above reflects results from single laboratories and does not reflect inter-laboratory variances. As a result, some laboratories may have higher or lower variances than those reported in Eqs 4.4, 4.5, and 4.6. Inter-laboratory variance is about double the within-laboratory variance.<sup>36</sup>

#### 4.3.4 Total variability

As shown in Fig. 4.4, the total variability,  $VT$  (using variance as the statistical measure of variability) associated with a mycotoxin test procedure is equal to the sum of the sampling ( $VS$ ), sample preparation ( $VSS$ ), and analytical ( $VA$ ) variances associated with each step of the mycotoxin test procedure.

$$VT = VS + VSS + VA \quad [4.7]$$

Examples of the magnitude of this variability associated with each step of a mycotoxin test procedure (Eq. 4.7) are shown below. The expected total variance associated with testing a shelled corn lot at 20 ppb when using a 0.91 kg sample, grinding the test sample in a Romer mill, taking a 50 g subsample from a comminuted sample, and quantifying aflatoxin in one aliquot by immunoassay methods can be estimated by summing the variances in Eqs 4.2, 4.3, and 4.6 associated with each step of the aflatoxin test procedure.

$$VT = 268.1 + 56.3 + 30.4 = 354.8 \quad [4.8]$$

The variance, standard deviation, and CV associated with the total aflatoxin test procedure described above when measuring a lot at 20 ppb are 354.8, 18.8, and 94.2 %, respectively. The sampling, subsampling, and analytical variances account for 75.6, 15.9, and 8.5 % of the total mycotoxin testing variance, respectively.

As the above example demonstrates, the sampling step accounts for most of the variability (uncertainty) associated with the total variability of a mycotoxin test procedure because of the extreme distribution among contaminated seed in a lot. For shelled corn, it is estimated that only six kernels in 10 000 are contaminated in a lot at 20 ppb.<sup>31</sup> Because of this extreme mycotoxin distribution among seed in a contaminated lot, it is easy to miss the contaminated seed with a small sample and underestimate the true lot concentration. On the other hand, if the test sample contains one or more highly contaminated seeds, then the test sample will overestimate the true mycotoxin contamination in the lot. Even using proper sample selection techniques, the variation among test sample concentrations is large due to the mycotoxin distribution described above.

#### 4.3.5 Reducing variability of a mycotoxin test procedure

The only way to achieve a more precise estimate of the true lot concentration is to reduce the total variability of the test procedure. Minimizing the variability associated with each step of the mycotoxin test procedure can reduce the total variability of the test procedure. Increasing the size of the sample can reduce sampling variability. Increasing the size of the subsample and/or increasing the degree of comminution (increasing the number of particles per unit mass in the subsample) reduces the sample preparation variability. Increasing the number of aliquots quantified by the analytical method and/or using a more precise quantification method (i.e. using HPLC instead of TLC) will reduce the analytical variability. If the variability associated with one or more of these steps can be minimized, then the total variability among mycotoxin test results from the same lot can be reduced.

The range of mycotoxin test results associated with any size sample and subsample and number of analyses about the lot concentration  $M$  can be estimated from the standard deviation SD (square root of the total variance) associated with the mycotoxin test procedure. Approximately 95 % of all test results will fall between a low of  $(M - 1.96 \cdot SD)$  and a high of  $(M + 1.96 \cdot SD)$ . The two expressions are only approximate because they are only valid for a normal distribution where test results are symmetrical about the mean. The distribution among aflatoxin test results is usually skewed, but will approach a symmetrical distribution as sample size becomes large.

The effect of increasing sample size on the range of test results when testing a contaminated lot of shelled corn that has 20 ppb aflatoxin is shown in Table 4.2. It can be seen that the range among the smallest and largest sample test results doesn't decrease at a constant rate as sample size increases. For example, doubling sample size has a greater effect on decreasing the range at small sample sizes than at large sample sizes. This characteristic suggests that increasing sample size beyond a certain point may not be the best use of resources and that increasing subsample size or number of analyses may be a better use of resources in reducing the range of test results once sample size has become significantly large. Increasing the sample size by a factor of five from 0.91 to 4.54 kg will cut the sampling

**Table 4.2** Range of aflatoxin estimates for 95 % confidence limits when testing a contaminated lot of shelled corn with 20 ng/g and using different sample sizes.

Sample size (kg)	Standard deviation <sup>1</sup> (ppb)	Low <sup>2</sup> (ppb)	High <sup>3</sup> (ppb)
1	17.5	0.0	54.2
2	13.5	0.0	46.5
4	11.0	0.0	41.6
8	9.6	1.3	38.7
16	8.7	2.9	37.1
32	8.3	3.8	36.2

<sup>1</sup> Standard deviation reflects sample size shown in table plus a 50 g subsample ground in a Romer mill, and high-performance liquid chromatography analytical method. Sample preparation plus analytical standard deviation = 7.8 and is constant for all sample sizes.

<sup>2</sup> Low = 20 - 1.96(standard deviation).

<sup>3</sup> High = 20 + 1.96(standard deviation).

variance in Eq. 4.2 by a factor of five to 53.6. The total variance with the 4.45 kg sample now becomes

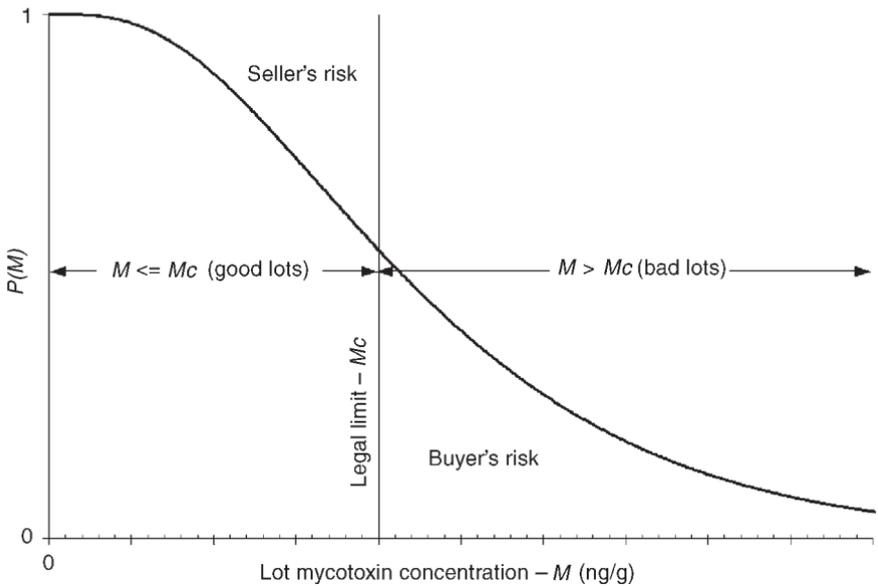
$$VT = 53.6 + 56.3 + 30.4 = 140.3 \quad [4.9]$$

The variance, standard deviation, and CV associated with the total testing procedure have been reduced to 140.3, 11.8, and 59.2 %, respectively. Sampling now accounts for 38 % of the total error.

#### 4.4 Designing mycotoxin sampling plans

Because of the large variability among test results (Table 4.1), two types of mistakes are associated with any mycotoxin-sampling program. First, some good lots (lots with a concentration less than or equal to the legal limit) will test bad and be rejected by the sampling program. This type of mistake is often called the seller's risk or false positives since these lots will be rejected at an unnecessary cost to the seller of the product. Secondly, some bad lots (lots with a concentration greater than the legal limit) will test good and be accepted by the sampling program. This type of mistake is called the buyer's risk or false negatives since contaminated lots will be processed into feed or food, causing possible health problems and/or economic loss to the buyer of the product. In order to maintain an effective regulatory and/or quality control program, the above risks associated with a sample design must be evaluated and reduced in magnitude if considered too large. Based upon these evaluations, the costs and benefits (benefits refers to removal of mycotoxin contaminated lots) associated with a sampling program can be evaluated.

A lot is termed bad when the sample test result is above an accept/reject limit and the lot is termed good when the sample test result is less than or equal to an accept/reject limit. While an accept/reject limit is usually equal to the legal limit,

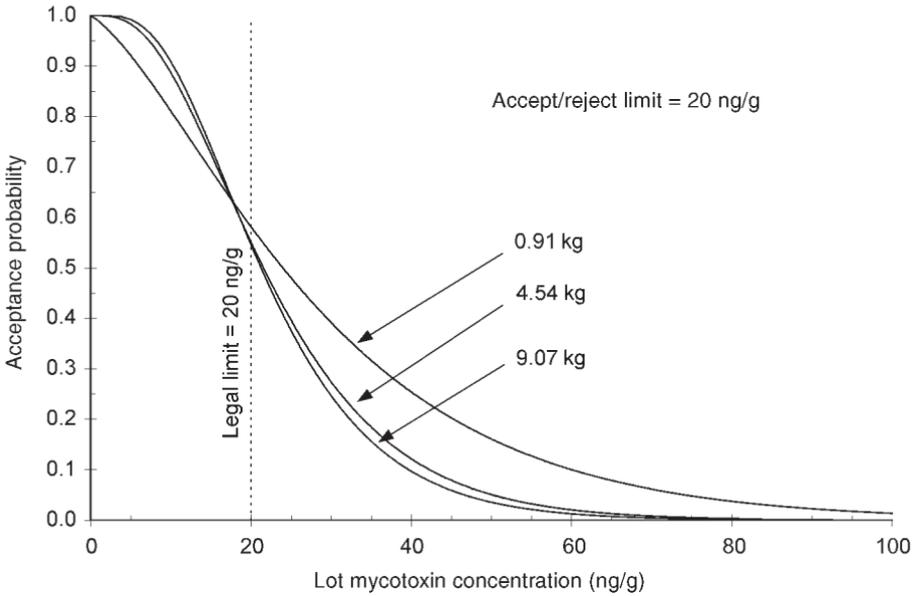


**Fig. 4.5** Typical shape of an operating characteristic curve used to predict portion of lots accepted,  $P(M)$ , and to evaluate the buyer's and seller's risks associated with a mycotoxin-sampling plan.

the accept/reject limit can be greater than or less than the legal limit. For a given sample design, lots with a mycotoxin concentration  $M$  will be accepted with a certain probability  $P(M)$  by the sampling plan. A plot of the acceptance probability  $P(M)$  versus lot concentration  $M$  is called an operating characteristic (OC) curve. Figure 4.5 depicts the general shape of an OC curve. As  $M$  approaches 0,  $P(M)$  approaches 1, and as  $M$  increases,  $P(M)$  approaches zero. The shape of the OC curve is uniquely defined for a particular sampling plan design with designated values of sample size, degree of comminution, subsample size, type of analytical method, and number of analyses and the accept/reject limit.

For a given sampling plan, the OC curve indicates the magnitude of the buyer's and seller's risk. When  $M_c$  is defined as the legal limit or the maximum lot concentration acceptable, lots with  $M > M_c$  are bad and lots with  $M \leq M_c$  are good. In Fig. 4.5, the area under the OC curve for  $M > M_c$  represents the buyer's risk (bad lots accepted) while the area above the OC curve for  $M \leq M_c$  represents the seller's risk (good lots rejected) for a particular sampling plan. The lots rejected  $R(M)$  is  $1 - P(M)$ .

Because the shape of the OC curve is uniquely defined by the sample size, degree of comminution, subsample size, the number of analyses and the accept/reject limit, these parameters can be used to reduce the buyer's and seller's risks associated with a sampling plan. Methods have been developed to predict the seller's and buyer's risks, the total number of lots accepted and rejected, the

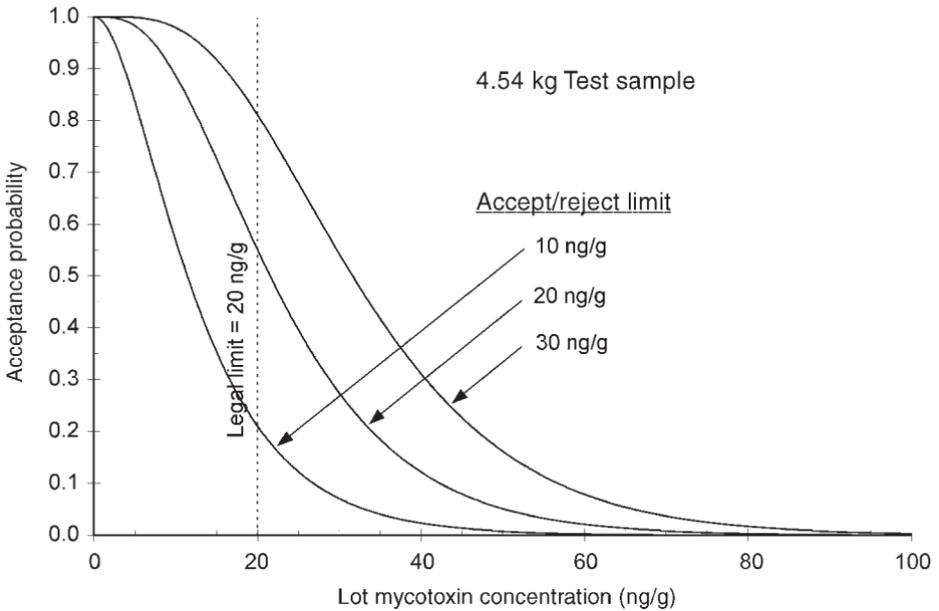


**Fig. 4.6** Effect of sample size on the buyer's and seller's risks associated with sampling shelled corn for aflatoxin.

amount of mycotoxin in the accepted and rejected lots, and the costs associated with mycotoxin inspection programs for several commodities.<sup>15,37,38,39,40</sup> The basic information needed to evaluate the performance of a mycotoxin-sampling plan is the variability associated with the mycotoxin test procedure and the distribution among sample test results. The effects of sample size and accept/reject limit on the performance of several aflatoxin-sampling plans for shelled corn are shown below using OC curves.

The effect of increasing sample size on the shape of the OC curve when testing shelled corn lots for aflatoxin is shown in Fig. 4.6 where the accept/reject limit is equal to the legal limit of 20 ppb. As sample size increases from 0.91 to 9.1 kg, the slope of the OC curve about legal limit increases, forcing the two areas associated with each risk to decrease. As a result, increasing the sample size decreases both the buyer's and seller's risks. The same effect can be obtained by increasing either the degree of sample comminution, subsample size, or number of analyses.

The effect of changing the accept/reject limit, relative to the legal limit, on the two risks when testing shelled corn lots for aflatoxin is shown in Fig. 4.7. If the legal limit is assumed to be 20 parts per billion (ppb), then changing the accept/reject limit to a value less than 20 ppb shifts the OC curve to the left. Compared to the sampling plan where the accept/reject limit is 20 ppb, the buyer's risk decreases, but the seller's risk increases. If the accept/reject limit becomes greater than 20 ppb, the OC curve shifts to the right. As a result, the seller's risk decreases



**Fig. 4.7** Effect of accept/reject limit on buyer's and seller's risks associated with sampling shelled corn for aflatoxin.

but the buyer's risk increases. Changing the accept/reject limit relative to the legal limit reduces one risk while increasing the other.

The Agricultural Marketing Service of the US Department of Agriculture (USDA/AMS) and the peanut industry has made use of the evaluation method to design an aflatoxin-sampling plan for shelled peanuts.<sup>37</sup> The FAO has also used the evaluation method to design an aflatoxin-sampling plan for corn and peanuts traded in the international market.<sup>15</sup>

## 4.5 Conclusions

Because of the variability associated with a mycotoxin test procedure, it is difficult to determine with 100 % certainty the true concentration of a bulk lot. Even when using acceptable sample selection procedures, there will be variability associated with the mycotoxin test procedure. The variance associated with a mycotoxin test procedure is the sum of sampling, sample preparation, and analytical variances. For small sample sizes, sampling is usually the largest source of variability due to the extreme distribution among contaminated seed in the lot. Increasing sample size, the degree of sample comminution, subsample size, and the number of aliquots quantified can reduce the variability associated with a mycotoxin test procedure. Reducing variability of the mycotoxin test procedure will reduce the

number of lots misclassified by the sampling plan. Methods have been developed for several mycotoxins and several commodities to evaluate and design sampling plans that minimize the buyer's and seller's risks associated with mycotoxin-sampling plans.

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## Mycotoxin analysis: current and emerging technologies

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### 5.1 Introduction: controlling and analysing mycotoxins

According to Pitt (1996), mycotoxins are defined as ‘fungal metabolites which when ingested, inhaled or absorbed through the skin cause lowered performance, sickness or death in man or animals, including birds’. The main group of moulds and mycotoxins of world-wide concern are the *Aspergillus* spp. (produce aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, ochratoxin A and patulin), *Fusarium* spp. (produce T-2 toxin, deoxynivalenol (DON), zearalenone and fumonisin B<sub>1</sub>) and *Penicillium* spp. (ochratoxin A). Mycotoxins are frequently formed in field crops as a result of contamination and growth of toxigenic plant pathogenic fungi. Commodities frequently contaminated include cereals (e.g. corn, wheat, barley, maize, oats and rye), nuts (peanuts, pistachios), dried fruit (figs), spices and pulses. In order to help control mycotoxin contamination in the foodchain, HACCP (Hazard Analysis of Critical Control Point) systems are now widely available and used by the industry. For further information on practical control strategies, the reader is referred to the following source (Anon, 2001a; [www.mycotoxins.org](http://www.mycotoxins.org)).

Analysis of mycotoxins forms an important tool in the control strategy and is required for reasons that include the following.

- To help comply with mycotoxin legislation. For details on EC Regulations and Directives which include tables on maximum limits for mycotoxins in specified commodities, the reader is referred to Fact Sheet 6 on ‘Mycotoxin legislation within the European Community’ ([www.lfra.co.uk/eman/fsheet6\\_1.htm](http://www.lfra.co.uk/eman/fsheet6_1.htm)).

Analytical methodology must allow determination of mycotoxin at least down to the specified regulatory levels. For example, maximum aflatoxins B<sub>1</sub> and B<sub>1</sub> + B<sub>2</sub> + G<sub>1</sub> + G<sub>2</sub> at 2 and 4 µg/kg, respectively, in groundnuts, nuts and dried fruits intended for direct human consumption or as an ingredient in foodstuffs; and maximum ochratoxin A at 3 µg/kg in all products derived from cereals (including processed cereal products and cereal grains intended for direct human consumption).

- It is crucial in establishing HACCP for control of mycotoxin contamination in food production, including sourcing of raw materials, identification of critical control points and establishing critical limits, and for use in verification once a HACCP system is in place.
- Fit-for-purpose tests are also valuable elements in a company's overall due diligence programme, including trouble shooting. In conjunction with HACCP, these systems can significantly help in preparing food products that meet the required standards. As a consequence, a company can save significant costs, for example in the case of an incident or a product recall.

Classical analytical methods for mycotoxins include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC); gas chromatography (GC) and mass spectrometry (MS). In recent years, most of these techniques have been coupled with immunoaffinity techniques to simplify extraction and improve mycotoxin recovery and measurement from foodstuffs. The reader is referred to the following references on mycotoxin methodology for further information (Anon, 2001b; Josephs *et al.*, 2001; Krska and Josephs, 2001; Bony, 2000; Chu, 2000; Miraglia and Brera, 2000; Stroka *et al.*, 2000a,b). For detailed information on all aspects of mycotoxin research, including structure–function, test kits and methods, toxicology, fungal metabolism, epidemiology and surveillance, HACCP and prevention, the reader is referred to the following websites and links within ([www.eman@leatherheadfood.com](mailto:www.eman@leatherheadfood.com); [www.mycotoxin-prevention.com](http://www.mycotoxin-prevention.com));).

The overall aim of this report is to provide a review of the current status in mycotoxin analysis, focusing on commercially available immunological tests, recently reported alternative methods for mycotoxins and examples of other innovative tests reported in the literature that have the potential for developing new format, real-time tests in the field of mycotoxins.

## 5.2 Commercial and alternative techniques for analysing mycotoxins

In addition to the use of proper sampling procedures, reliable and robust results can only be obtained from a fit-for-purpose method that has undergone rigorous optimization, evaluation and validation studies. Only then can the results be of value in making informed decisions, e.g. risk assessment in relation to food safety, identification of critical control points as part of HACCP and product verification, monitoring and control of imported raw materials and products, etc. For further

information on method validation aspects, including international validation schemes and validated methods, proficiency testing, use of certified reference materials and laboratory accreditation, the reader is referred to the following references (Boenke, 1998; Boenke *et al.*, 2002; Gilbert and Anklam, 2002).

### 5.2.1 Commercial techniques

Commercial immunological techniques for mycotoxins are based on specific monoclonal and polyclonal antibodies produced against the toxin, and divided broadly into immunoaffinity (IAC) column-based analysis and enzyme-linked immunosorbent assay (ELISA) (Table 5.1).

A list of commercial immunological kits, their specifications and reported evaluation/validation data is summarized in Table 5.2. The IAC are used effectively to clean-up complex matrices and allow isolation and concentration of the specific toxin. The protocol involves addition of a sample extract to the column containing the immunoaffinity matrix, comprising a solid phase (e.g. agarose bead) to which anti-mycotoxin antibodies are covalently-coupled (Fig. 5.1). The toxin in the sample binds to the corresponding immobilized antibody. Subsequent steps involve removal of the unbound matrix components, including any co-extractants, elution of the toxin by changing the solvent composition and, finally, detection of the toxin using analytical techniques. Alternatively, the mycotoxin bound to the column can be eluted and measured directly by fluorometry, based on the intrinsic fluorescence of mycotoxins or, alternatively, quantified by using a range of analytical techniques, including HPLC (Stroka *et al.*, 2000b), TLC (Stroka *et al.*, 2000a) and MS (Rosenberg *et al.*, 1998).

In the case of ELISAs, the clean-up procedures are generally not as intensive as the other analytical techniques. A sample homogenate containing toxin is either directly quantified using a standard microtitre plate or tube assay format ELISA or else a membrane-based format ELISA is used for the purpose of screening for the presence of the specific toxin. ELISAs are generally used to screen for the presence above a certain level (or absence) of a mycotoxin in the test sample, and a range of qualitative, semi-quantitative and quantitative tests are available. Based on the results, the suspect samples are then confirmed with classical analytical techniques. Many different ELISA formats are commercially available for mycotoxin analysis (e.g. single disposable membrane-based test, microtitre plate and tube assays). Typically, the ELISAs are based on a competitive assay format that uses either a conjugate of an enzyme-coupled mycotoxin or a primary antibody specific for the toxin analyte (Fig. 5.2).

A typical sequence of reactions using the former reagent in a microtitre plate format is as follows:

- (1) a pre-optimized, toxin-coupled enzyme is added to the sample extract;
- (2) the mixture is added to the corresponding surface-immobilized antibody (e.g. microtitre plate sensitized with antibodies);
- (3) the amount of toxin-coupled enzyme that binds to the immobilized antibody is

**Table 5.1** Commercially available immunological techniques for mycotoxins.

Mycotoxin	Immunoaffinity column	ELISA		
		Membrane	Microtitre plate	Tube
Total aflatoxin	+	+	+	
Total aflatoxin/ Ochratoxin A	+	+		
Aflatoxin B <sub>1</sub>	+		+	+
Aflatoxin B <sub>1</sub> , B <sub>2</sub>	+			
Aflatoxin G <sub>1</sub> , G <sub>2</sub>		+		
Aflatoxin M <sub>1</sub>	+	+	+	
Aflatoxin M <sub>1</sub> , M <sub>2</sub>		+		
Ochratoxin A	+		+	
Deoxynivalenol (DON)	+		+	
Fumonisin B <sub>1</sub> , B <sub>2</sub> , B <sub>3</sub>	+		+	
T-2 toxin	+		+	
Zearalenone	+		+	

+ = products commercially available.

dependent on the amount of toxin in the sample; the higher the amount of toxin in a sample, the lower will be the amount of the enzyme conjugate bound to the surface and *vice versa*; and finally

- (4) the enzymic activity of the conjugate bound to the surface is determined by adding a corresponding substrate which results in a chromogenic product, the concentration of which is inversely proportional to the concentration of the toxin in the sample.

Table 5.2 summarizes the commercial immunological kits available at the time of writing for the analysis of a wide range of mycotoxins, including details of the company address, commercial kit trademark, assay format, applicable matrix, the reported lower limit of detection (sensitivity), official approval of kits, references showing independent data on kit evaluations and the nature of analysis (quantitative, semi-quantitative or qualitative).

## 5.2.2 Alternative techniques for mycotoxin analysis

In this section, emerging technologies that are not yet widely available commercially are considered. They are categorized as follows.

### *Biosensors*

A range of biosensors has been reported for mycotoxin analysis, including those based on optical (e.g. surface plasmon resonance, SPR, and evanescent wave fibre optic) and surface acoustic wave (e.g. quartz crystal microbalance) principles. Biospecific interaction analysis (BIA) is based on the 'real-time' measurement of the interaction of a receptor with the corresponding analyte (e.g. antibody-antigen,

**Table 5.2** Commercial immunological kits (and specifications) for the analysis of mycotoxins.

Manufacturer	Mycotoxin type/ Trade name	Immunoassay format	Matrix	Sensitivity*	Approval status	Reference**	Analysis
Vicam, LP, 313 Pleasant Street, Watertown, MA 02472, USA www.vicam.com	Aflatoxins B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub> , G <sub>2</sub> and M <sub>1</sub> (AflaTest)	Immunoaffinity column (IAC)	Feeds, foods, grains, nuts, dairy products, dried fruit, cocoa, tea	>10 ppb by LC; corn, peanut butter 10–100 ppt by HPLC; milk 2 ppb by LC; peanut, fig, maize, gluten >2.4 ppb by LC, peanut butter, pistachio/fig paste	AOAC – –	Trucksess <i>et al.</i> , 1991 Ioannou-Kakouri <i>et al.</i> , 1995; Barmark & Larsson, 1994	Quan Quan
	Aflatoxin B <sub>1</sub> (Afla B)	IAC	As above	>1 ppb by LC, peanut butter, pistachio/fig paste	AOAC	Stroka <i>et al.</i> , 2000a	Quan
	Aflatoxin M <sub>1</sub> (Afla M <sub>1</sub> )	IAC	Liquid milk	> 20 ppt by reversed phase LC, milk	AOAC AOAC	Stroka <i>et al.</i> , 2000a Dragacci <i>et al.</i> , 2001	Quan Quan
	Aflatoxins B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub> , G <sub>2</sub> & Ochratoxin A (AflaOchra HPLC)	IAC	Feeds, foods, grains, nuts, coffee, beer	0.25 ppb	–	D/A	
	Ochratoxin A (Ochra Test)	IAC	As above	0.2 ppb by HPLC; >1.2 ppb by HPLC, coffee < 45 ppt by HPLC; wines 10 ppt by reversed phase HPLC; beers/wine	AOAC – –	Pittet <i>et al.</i> , 1996; Entwisle <i>et al.</i> , 2001 Castellari <i>et al.</i> , 2000 Visconti <i>et al.</i> , 2000b & 2001a	Quan Quan Quan
				0.8 ppb by HPLC; wheat, oats < 3 ppb by HPLC; wheat	– –	Solfrizzo <i>et al.</i> , 1998 Scudamore & MacDonald, 1998	Quan Quan
	Deoxynivalenol (DON; DONtest TAG)	IAC	As above	0.5 ppm by fluorometry	–	D/A	
	(DONtest HPLC)	IAC	As above	100 ppb by HPLC; wheat 1–4 ppb by HPLC; maize products and cornflakes; 0.005 ppm, cornflakes	–	Cahill <i>et al.</i> , 1999 Meister, 1999; Visconti <i>et al.</i> , 2001b & c	Quan Quan
	Fumonisin B <sub>1</sub> , B <sub>2</sub> (FumoniTest)	IAC	Corn, feeds	>0.5 ppm by HPLC; cornflakes, muffins, extruded, corn, infant formula	– –	Solfrizzo <i>et al.</i> , 2001 Visconti <i>et al.</i> , 2000a	Quan Quan

				10 ppb by LC; infant cereals	–	Scott <i>et al.</i> , 1999	Quan
				16 ppb by HPLC; 250 ppb by fluorometry; corn	–	Duncan <i>et al.</i> , 1998	Quan
	T-2 (tricothecene) (T-2 TAG)	IAC	Grains, feeds	0.15 ppm by fluorometry	–	D/A	
	Zearalenone (Zearala Test)	IAC	Feeds, cereals	100 ppb by fluorometry, or 2.5–10 ppb by LC; corn	–	Kruger <i>et al.</i> , 1999	Quan
				3 ppb by HPLC; corn samples	–	Visconti & Pascale, 1998	Quan
Rhone Diagnostics Technologies Ltd West of Scotland Science Unit 306 Kelvin Campus Maryhill Road, Glasgow G20 0SP, UK www.rhone-diagnostics.co.uk	Aflatoxins B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub> , G <sub>2</sub>		As previous	>2.4 ppb total (or >1 ppb B1) by LC; >0.1 ppb	AOAC	Stroka <i>et al.</i> , 2000a	Quan
	(Aflaprep & Easi-extract aflatoxin)	IAC		by TLC, peanut butter, pistachio and fig paste, etc.	AOAC	Stroka <i>et al.</i> , 2000b	
				<1 ppb by HPLC; feeds and maize	–	Roos <i>et al.</i> , 1997	Quan
				2.8 ppt by HPLC; olive oil	–	Daradimos <i>et al.</i> , 2000	Quan
	Ochratoxin A (Ochraprep)	IAC	As previous	20–45 ppt by HPLC; wines	–	Castellari <i>et al.</i> , 2000	Quan
				0.2 ppb by HPLC; dried vine fruit	–	MacDonald <i>et al.</i> , 1999	Quan
				0.2 ppb by LC; barley	AOAC	Entwisle <i>et al.</i> , 2000	Quan
				< 3 ppb by HPLC; wheat	–	Scudamore & MacDonald, 1998	Quan
				0.2 ppb by HPLC; >1.2 ppb by HPLC, coffee	AOAC	Leoni <i>et al.</i> , 2000; Entwisle <i>et al.</i> , 2001	Quan
		Total aflatoxins (Aflascan)	Glass blender and UV light	As previous	1 ppb	–	D/A
	Aflatoxin M <sub>1</sub> (Aflaprep M)	IAC	As previous	50 ppt by membrane-based ELISA, milk	IDF, AOAC	Sibanda <i>et al.</i> , 1999	Q
				> 20 ppt by reversed phase LC, milk	AOAC	Dragacci <i>et al.</i> , 2001	Quan
	Aflatoxin B <sub>1</sub> (Aflaplate)	Microtitre plate ELISA	As previous	1.5 ppb by HPLC, peanuts, maize	–	Marley <i>et al.</i> , 2001	Quan
	Aflatoxin B <sub>1</sub> (Aflacard 2 ppb)	membrane-based card test	As previous	2 ppb, peanuts, maize	–	Marley <i>et al.</i> , 2001	Q
	Total aflatoxin (Aflacard total)	membrane-based card test	As previous	4 ppb	–	N/A	Q

Table 5.2 cont'd

Manufacturer	Mycotoxin type/ Trade name	Immunoassay format	Matrix	Sensitivity*	Approval status	Reference**	Analysis
	Fumonisin B <sub>1</sub> , B <sub>2</sub> , B <sub>3</sub> (Fumoniprep)	IAC	As previous	400 ppb by HPLC, infant food, cornflakes	–	Boonzaaijer <i>et al.</i> , 2001	Quan
	Zearalenone (Easi- extract)	As above	As previous	0.12 ppb by HPLC-MS; maize, corn	–	Rosenberg <i>et al.</i> , 1998; CRM***, D/A	Quan
	Fumonisin B <sub>1</sub> , B <sub>2</sub> , B <sub>3</sub> (Fumoniplate)	Microtitre plate ELISA	As previous	0.3 ppm	–	D/A	Quan
	Zearalenone (Zearaplate)	As above	As previous	25 ppb	–	N/A	Quan
	Ochrascan	Glass blender and UV light	Coffee	0.1 ppb, green, roasted and instant coffee	–	Hoyland <i>et al.</i> , 2001	
International diagnostic systems corp., 2620 S Cleve- land Ave., Suite 100 P.O. Box 799, St. Joseph, MI 49085, USA www.ids- kits.com	Total aflatoxins (Afla-cup)	Immunoblot (membrane ELISA)	Agricultural products	> 20 ppb, cottonseed & peanut butter; > 30 ppb corn & peanut	AOAC	Trucksess <i>et al.</i> , 1989	Q
	Aflatoxin B/G			> 20 ppb, corn <5 ppb, maize	AOAC	Trucksess & Stack, 1994 Casanova <i>et al.</i> , 1995	Q Q
	Aflatoxin M <sub>1</sub>	1-Step micro- titre plate ELISA	Agricultural products	0.5 ng/ml	–	N/A	
	Aflatoxin M <sub>1</sub>	Microtitre plate ELISA	As previous	N/A	–	N/A	Quan
	Fumonisin B <sub>1</sub> DON	As above	As previous	250 ng/ml	–	N/A	Quan
		As above	As previous	250 ng/ml	–	N/A	Quan
Diffchamb Ltd, Unit 12, Block 3, Old Mill Trading Estate, Old Mill Lane, Mansfield Woodhouse Nottinghamshire NG19 9BG, UK www.diffchamb.com	Aflatoxin B1 (Transia Tube assay)	Tube-based ELISA	As previous	Various (1, 5, 20, 50 ppb)	–		Q/SQ
	Aflatoxin B <sub>1</sub> (Transia plate assay)	Microtitre plate ELISA	As previous	< 0.5 ppb	–		

Neogen Corporation, 620 Leshher Place, Lansing MI 48912, USA www.neogen.com	Aflatoxins (qualitative) (Agri-Screen field and lab kit)	Microtitre plate ELISA	As previous	> 20 ppb, roasted peanut	AOAC	Park <i>et al.</i> , 1989	Q/SQ
	Aflatoxins (Veratox, Veratox-AST & Veratox-HS)	Microtitre plate ELISA	As previous	< 5 ppb, peanuts	AOAC (Veratox-AST) Veratox-AST	Dorner <i>et al.</i> , 1993	Quan
	Aflatoxin column 5 DON (Agri-Screen)	IAC	As previous	< 4 ppb	–	D/A	Quan
		Microtitre plate ELISA	As previous	< 500 ppb	USDA	D/A	Q
	DON (Veratox)	Microtitre plate ELISA	As previous	0.2–40 ppm, wheat, barley, malt	AOAC	Tacke & Casper, 1996;	Quan
	Fumonisin (Agri-Screen)	As above	As previous	0.3 ppm, grain and grain products	AOAC	Anon, 1995	Quan
				5 ppm	–	D/A	
	Fumonisin (Veratox)	As above	As previous	0.2 ppm in corn samples and feed	–	Abouzied <i>et al.</i> , 1996	Quan
	Zearalenone (Agri-Screen)	As above	As previous	> 800 ppb, corn, wheat and pig feed	AOAC	Bennett <i>et al.</i> , 1994	Q/SQ
	Ochratoxin (Veratox)	As above	As previous	2 ppb	–	D/A	Quan
T-2 toxin (Veratox)	As above	As previous	25 ppb	–	D/A	Quan	
Idexx Laboratories Ltd, Milton, Churchfield Rd., Chalfont, St Peter, Bucks. SL9 9EW www.idexx.com	Aflatoxin M <sub>1</sub> (SNAP test)	Immuno-chromatographic membrane test	Milk	0.5 ppb	–	D/A	Q

Table 5.2 cont'd

Manufacturer	Mycotoxin type/ Trade name	Immunoassay format	Matrix	Sensitivity*	Approval status	Reference**	Analysis
Tepnel Bio- systems Ltd, 1 Newtech Square, Deeside Industrial Park, Deeside, Flintshire CH5 2NT, UK www.tepnel.com	Ochratoxin A (Biokits)	Microtitre plate ELISA	As previous	< 1 ppb	–	D/A	Quan
	Aflatoxins (total, Biokits)	As above	As Previous	9 ppb, peanut butter	AOAC	Patey <i>et al.</i> , 1992	Quan
Charm Sciences Inc., 659 Andover Street, Lawrence, MA 01843, USA www. charm.com	Aflatoxin B <sub>1</sub> (Rosa format)	Lateral flow strip test	Feed/grain	20 ppb, feed/grain	–	D/A	SQ
	Aflatoxin B <sub>1</sub> (Rosa format)	Lateral flow strip test	Feed/grain	2 ppb, feed/grain	–	D/A	Q
	Aflatoxin B <sub>1</sub> (Charm II)	Radio- immunoassay	Grains	1–2 ppb, grains	–	D/A	Q
	Aflatoxin G <sub>1</sub> /G <sub>2</sub> (Charm II)	Radio- immunoassay	Milk products	< 0.25 ppb, milk products	–	Saitanu, 1997	Q
	Aflatoxin M <sub>1</sub> (Rosa format)	Lateral flow strip test	Milk	< 0.25 ppb (SQ); 0.05 ppb (Q), milk	–	D/A	SQ & Q
R-Biopharm Ltd., 65 High Street, Wheatley, Oxford OX33 1XT, UK www.r-bio pharm.co.uk	Aflatoxin B <sub>1</sub> (Ridascreen)	Microtitre plate ELISA	As previous	0.2 ppb, nuts/products, seeds	–	Leszczynska <i>et al.</i> , 2000	Quan
	Aflatoxin M <sub>1</sub> (Ridascreen)	As above	As previous	< 10 ppt, milk	–	D/A	Quan
	Total aflatoxin (Ridascreen)	As above	As previous	< 100 ppt, cheese 0.9 ppb, nuts/products,seeds	–	D/A Leszczynska <i>et al.</i> , 2000	Quan Quan
	DON (Ridascreen)	As above	As previous	1.25 ppb, cereals/feeds 6 ppb, beer	– –	D/A Ruprich & Ostry, 1995	Quan Quan

	Fumonisin B <sub>1</sub> , B <sub>2</sub> , B <sub>3</sub> (Ridascreen)	As above	As previous	10 ppb in corn products	–	Dreher & Usleber, 1996	SQ
	T-2 Toxin (Ridascreen)	As above	As previous	3.5 ppb, cereals	–	D/A	Quan
	Zearalenone (Ridascreen)	As above	As previous	1250 ppt, cereals/feed	–	D/A	Quan
				250 ppt, beer	–	D/A	Quan
	Ochratoxin A (Ridascreen)	As above	As previous	400 ppt, cereals/feed	–	D/A	Quan
	Aflatoxin B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub> , G <sub>2</sub> (Ridascreen EXPRESS)	As above	As previous	Standards: 15/20 ppb	–	D/A	SQ
	Citrinin (Ridascreen FAST)	As above	Cereals, feed	15 ppb	–	D/A	Quan
	DON (Ridascreen EXPRESS)	As above	Cereals, malt, feed	Standards: 0.5, 1, 2, 5 ppm	–	D/A	SQ
	Fumonisin B <sub>1</sub> , B <sub>2</sub> , B <sub>3</sub>	As above	Cereals, feed	9 ppb, corn-based commodities	–	Ostry & Ruprich, 1998	Quan
	Rida Aflatoxin column (Aflatoxin B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub> , G <sub>2</sub> , M <sub>1</sub> )	IAC	Food/feed	1 ppb by HPLC; nuts, nut products, seeds	–	D/A Leszczynska <i>et al.</i> , 2000	Quan Quan
	Rida Ochratoxin A column	IAC	As above	10 ppt by HPLC; meat and meat products	–	Gareis & Scheuer, 2000	Quan
ELISA Technologies, Inc., 4581-L NW 6th Street, Gainesville, Florida 32609, USA www.elisa-tek.com	Total aflatoxin (ELISA-Tek)	Microtitre plate ELISA	As previous	N/A	N/A	N/A	N/A
	Ochratoxin A (ELISA-Tek)	As above	As previous	N/A	N/A	N/A	N/A

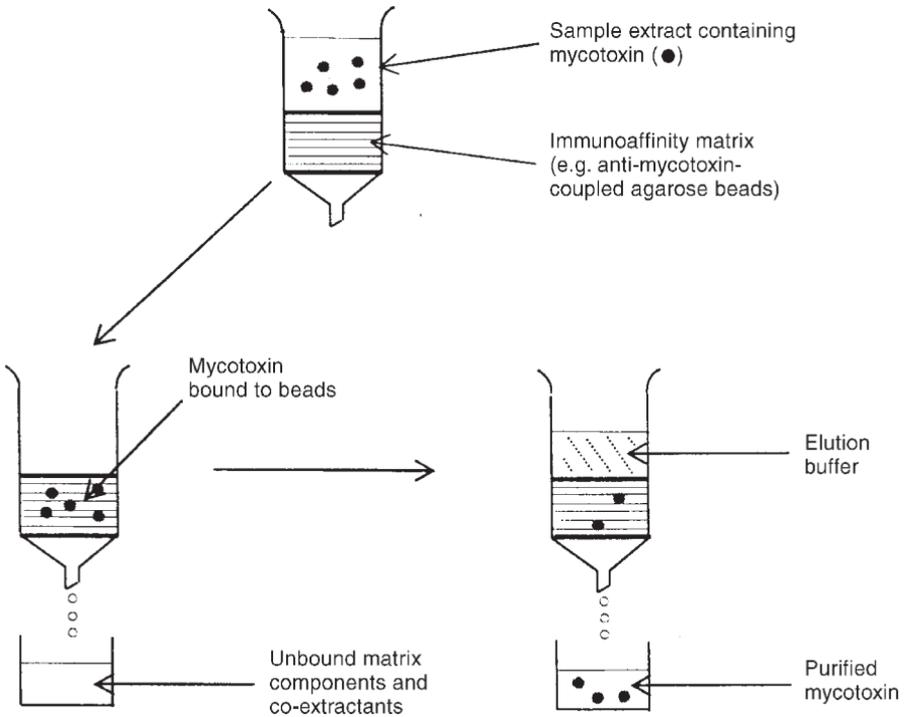
*Note:* Quan = quantitative; SQ = semi-quantitative; Q = qualitative.

\* = According to kit manufacturer or published reference as given in the table.

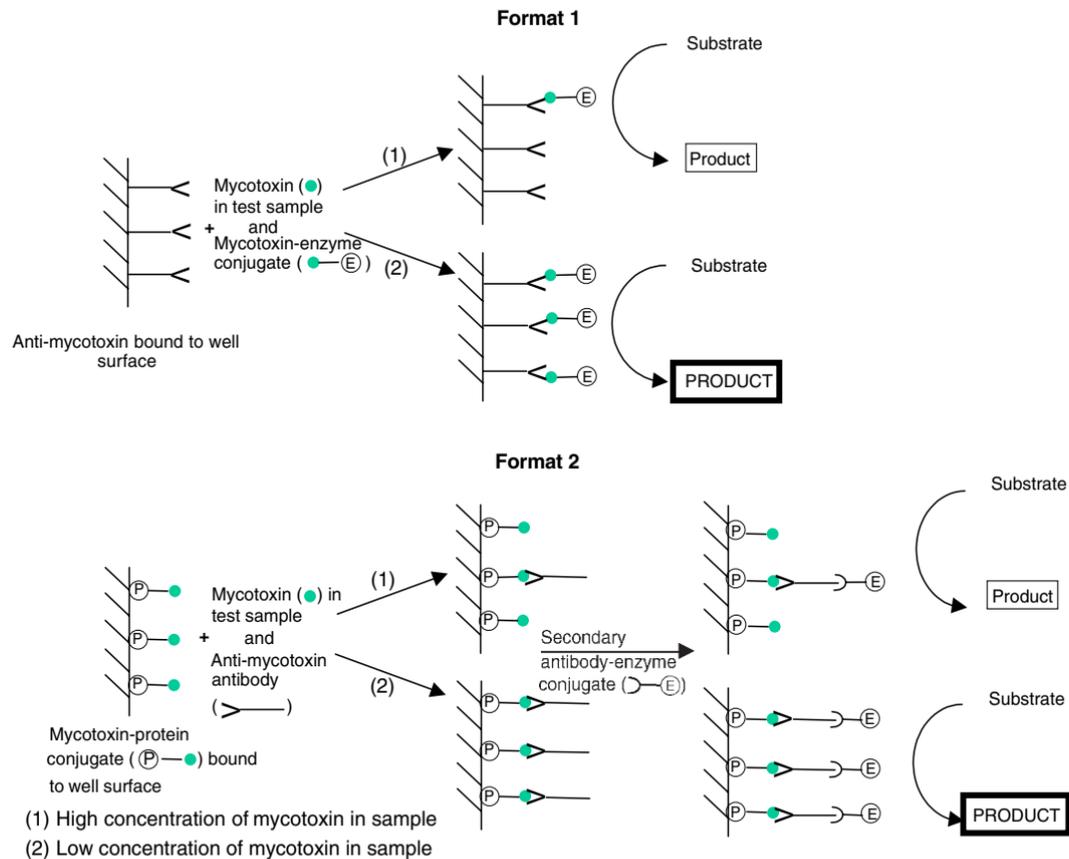
\*\* = Useful articles on immunological techniques for mycotoxins are listed separately.

\*\*\* = Certified reference material for DON in maize.

N/A = not available at time of preparation of this factsheet; D/A = external publication not available yet but unpublished data available on request from the manufacturer.



**Fig. 5.1** Principle of immunoaffinity column purification of mycotoxins.



**Fig. 5.2** Principle of competitive ELISAs for mycotoxins.

lectin–glycoprotein and nucleic acid to complementary sequence). BIA is widely exploited in commercially available optical biosensor instrumentation for the measurement of analytes of importance or concern to a number of industrial sectors (e.g. medicare, agrofood and water; see review by Patel, 2002). The SPR biosensor technique developed for aflatoxin B<sub>1</sub> utilizes the following principle (Daly *et al.*, 2000). It continuously detects changes in refractive index close to the sensor surface during the interaction of an aflatoxin-conjugate (covalently immobilized to the sensor surface) with the excess anti-aflatoxin antibody that results following competition of free aflatoxin (in the test sample) and the immobilized aflatoxin-conjugate with the corresponding antibody. The assay for aflatoxin was reported to exhibit a linear range of 3–98 ng/ml with good reproducibility. A similar biosensor assay developed for deoxynivalenol in wheat showed a working range between 0.13 and 10 µg/ml with a detection limit of 2.5 ng/ml (Schnerr *et al.*, 2002).

The evanescent wave-based fibre optic immunosensor was studied for the detection of fumonisins and aflatoxins in maize (Maragos and Thompson, 1999; Maragos, 2002). In the competitive format, fumonisin B<sub>1</sub> (FB<sub>1</sub>) was measured in both spiked and naturally contaminated maize as follows. Fumonisin monoclonal antibodies were covalently coupled to an optical fibre and the competition between FB<sub>1</sub> and FB<sub>1</sub>-labelled with fluorescein (FB<sub>1</sub>-FITC) for the limited number of binding sites on the fibre was determined. The fluorescence signal generated in the assay was inversely proportional to the FB<sub>1</sub> concentration in the sample. In a comparative study, the immunosensor was found to correlate well with an HPLC method for naturally contaminated maize samples except when large amounts of other fumonisins that cross-react with the immunosensor were present. The authors concluded that this type of sensor has the potential to provide a rapid screening assay for maize samples (sensitivity 3.2 µg/g) but requires coupling with a clean-up technique (sensitivity 0.4 µg/g) to be truly effective.

An alternative biosensor utilizes the principle of measurement of mass changes at the surface or bulk of quartz resulting from interaction of an analyte with the corresponding receptor immobilized at the surface of quartz crystal. This type of approach has been reported for the determination of ochratoxin A in liquid food products (Hauck *et al.*, 1998). The assay was based on competition of free ochratoxin A (test sample) and a sensor surface immobilized conjugate of ochratoxin with the corresponding antibody, the resulting binding of excess antibody to the surface being detected at a resonance frequency of 20 MHz. The method has a linear range of 2–100 ng/ml.

#### *Capillary electrophoresis (CE) with fluorescence detection*

The principle of this technique comprises

- (i) resolution of a mycotoxin, based on its overall charge, from potential cross-reacting (e.g. other mycotoxins) and interfering (e.g. proteins) species present in a test sample, and
- (ii) subsequent detection at trace levels of the resolved mycotoxin by laser-

induced fluorescence, either exploiting the fluorescence characteristics of the molecule or derivatization of the mycotoxin with a fluorophore [e.g. fluorescein isothiocyanate (FITC)]. Examples include the separation and quantification of fumonisins in corn samples (Maragos *et al.*, 1996). Overall, the negatively charged fumonisins (contains two carboxylic acid residues) were separated and detected from corn samples by capillary zone electrophoresis coupled with laser-induced fluorescence (CZE-LIF). The steps involved were:

(i) corn samples were contaminated with fumonisins and then extracted with methanol/water;

(ii) fumonisins were then isolated using an IAC column;

(iii) following derivatization of the extracts with FITC, the fumonisins were analyzed further by CZE-LIF. Recoveries from corn fortified with 0.25–5 ppm FB<sub>1</sub> averaged 89 % (range 71–102 %) with a sensitivity of 0.05 ppm FB<sub>1</sub> in corn. The CZE-LIF method was comparable in sensitivity to that of an HPLC method.

### *Automation*

For high throughput analysis, it is cost-effective to automate the analysis of mycotoxins, in particular the labour-intensive components of the analytical process. In one study, the IAC-based sample preparation procedures for the determination of aflatoxins B<sub>1</sub>, B<sub>2</sub> and G<sub>1</sub>, G<sub>2</sub> in several food matrices and aflatoxin M<sub>1</sub> in milk were automated by using modular automation or robotics (Zymate I liquid handling robot) (Carman *et al.*, 1996). After IAC clean-up aflatoxins were separated by reversed-phase LC and determined subsequently by fluorescence without derivatization. The results of automated techniques were found to be comparable with an AOAC official method.

## **5.3 Applying new technologies to the analysis of mycotoxins**

There are a number of recent developments that have potential for application in the field of mycotoxin analysis. Some of these are briefly considered below.

### **5.3.1 Lab-on-a-chip (LOC) and microarrays**

The 'science of miniaturization' encompasses numerous terminologies, including LOC,  $\mu$ TAS (micrototal analytical systems), MEMS (microelectromechanical systems), MOEMS (microoptoelectromechanical systems), MST (microsystems technology), spectrometer-on-a-chip, chemistry-on-a-chip and PCR-on-a-chip. The technology in the broadest sense has arisen from the manufacturing tools based on batch thin and thick film fabrication techniques commonly used in the electronics industry. Most of the reported applications in this area comprise miniaturization of separation processes based on electrophoresis (e.g. CE, free flow electrophoresis (FFE) and gel electrophoresis). An alternative format is a microarray, in which microdots (spotted onto silicon substrate) comprising receptor

molecules (e.g. short DNA or RNA sequences or primarily antibodies) are used in a simple and high-throughput approach to identify complementary sequences of DNA (or RNA) and target analytes in test samples. These techniques are applied in a range of fields, including gene-expression profiling and gene identification (DNA microarrays, Al-Khalidi *et al.*, 2002), screening of potential pharmacologically active compounds using protein arrays (Cooper *et al.*, 2001) and detection of analytes using protein microarray-based immunoassay (Delehanty and Ligler, 2002).

Some examples of miniaturized CE/FFE are presented. Effenhauser *et al.* (1993) developed a micromachined CE system on glass chips with integrated sample injection and demonstrated the fast (in seconds) and efficient separation of FITC-labelled amino acids and phosphorothioate oligonucleotides followed by an external laser-induced fluorescence (LIF) detection. The authors specify the many advantages of miniaturization (e.g. quasi-continuous monitoring of chemical and biochemical species and highly reproducible and efficient separations), and predict future developments leading to improved generation of bench-top CE instrumentation and integration of CE into on-line at-site monitors.

Jacobson *et al.* (1994) showed baseline resolution of the fluorescent dyes fluorescein and rhodamine B (at 20  $\mu\text{M}$  concentrations) within seconds, indicating their significant potential for biosensor applications. The use of confocal microscopy in combination with a microchip device allowed, for the first time, single molecule detection of rhodamine 6G (1.7 pM) and rhodamine B (8.5 pM) within 35 s (Fister *et al.*, 1998). More recently, Walker *et al.* (1998) constructed a glass microchip etched serpentine channel with on-chip monitoring of the herbicides paraquat and diquat by normal Raman spectroscopy. Since the microchip was directly coupled to a Raman microprobe, no interfacing was required. As low as  $2.3 \times 10^{-7}$  M (60 ppb paraquat and 80 ppb diquat) in deionized water was detected using Raman isotachopheresis. The authors comment that further enhancement of the sensitivity (down to micromolar concentrations) could be achieved using resonance or surface enhancement, based on direct incorporation of surface-enhanced Raman spectroscopy (SERS)-active surface onto the microfabricated device.

Application of CE to immunoassays (also generally referred to as 'on-chip immunoassay') is a newly developing field. Two assay formats are commonly used. In the direct assay, a fluorescent labelled analyte is mixed with the corresponding antibody prior to resolution of the labelled immune complex from the reactants by CE. The fluorescent labelled complex is then directly detected by LIF. In the competitive assay format, a fluorescent labelled analyte is added to a sample containing an unknown amount of the same analyte. Following incubation, the antianalyte antibody is added. The final labelled immune complex, which is inversely related to the initial analyte concentration in the sample, is resolved by CE prior to measurement of the fluorescence. Chiem and Harrison (1997) developed a rapid (within a minute or less) CE-LIF based competitive assay for theophylline with a calibration range from 2.5 to 40  $\mu\text{g}/\text{ml}$  (a useful therapeutic range). However, the total analysis time, including incubation of the sample with the reagents, was approximately 15 min.

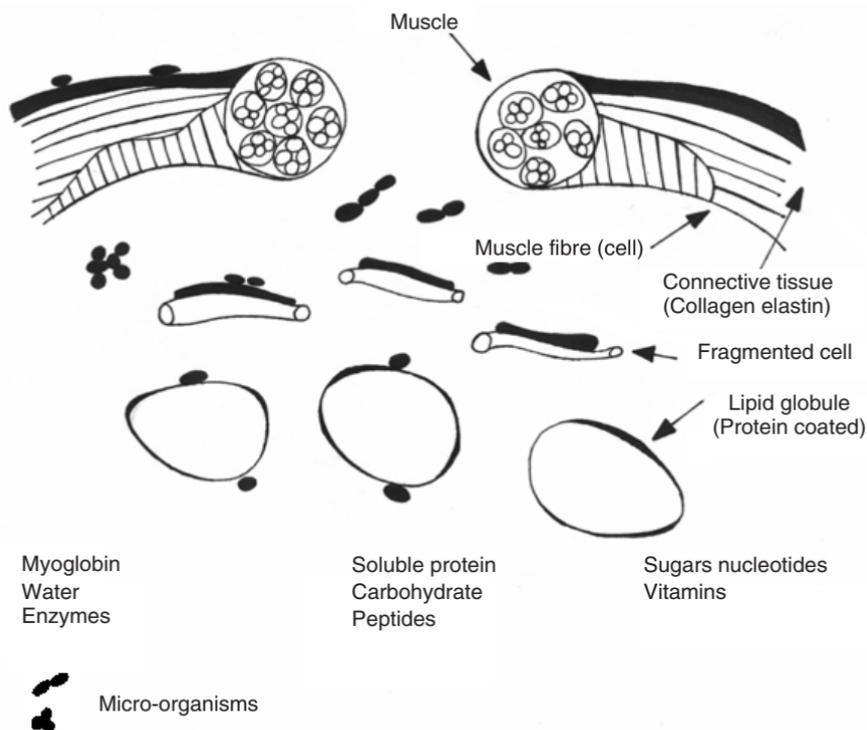
### 5.3.2 Food matrix effects: scope of innovative technologies

Sample extraction and preparation remain the most time-consuming and error-prone steps in the analytical process, but these are crucial procedures because food scientists need to isolate and concentrate a wide variety of analytes from complex and varied matrices. Advances in sample extraction and preparation in chemical analysis have only in the past several years been given critical consideration as an important component in obtaining reliable and robust analytical results.

For illustration purposes, a typical meat homogenate is shown in Fig. 5.3. It contains a wide range of insoluble (e.g. fragmented muscle fibres, micro-organisms and lipid droplets), coloured and soluble components (e.g. haemoglobin and other soluble muscle proteins, salts and nutrients) amongst which a trace level of the target contaminant may be present. To ensure reliability of the analytical results, it is crucial to quantitatively isolate (and concentrate) the analyte from the potential background interference prior to measurement using compatible detection systems. Evidence exists on the interaction of aflatoxins with peanut proteins (Monteiro *et al.*, 1996). In a comparison study of different extraction and clean-up procedures for determination of fumonisins in maize and maize products, De Girolamo *et al.* (2001) concluded that the extraction solvent and the clean-up procedures were two critical factors that affected the analysis of fumonisins.

Conventionally, this has been carried out using labour-intensive and complex procedures (e.g. fat and protein extraction, solvent extraction, rotary evaporation and HPLC) that result in inevitable loss of the target analyte during every step, prior to detection (e.g. UV of fluorescence depending on the type and concentration of the analyte). More recently, the process of isolation and concentration has been made more efficient using immunoaffinity columns as described in the previous sections. An alternative to the immunoaffinity systems is the use of molecular imprinted polymer (MIP). Unlike biological antibodies in the immunoaffinity systems, MIPs are based on chemical synthetic polymers which are highly stable (i.e. do not degrade by enzymic action, processing conditions such as heat and low pH, and organic solvents), can be regenerated extensively, require low cost reagents to prepare MIPs and can be reproducibly prepared in larger quantities. Molecular imprinting involves preparation of a 'mould' comprising polymerizable functional monomers around a print molecule (e.g. mycotoxin). Initially, the monomer (methacrylic acid) is allowed to establish bond formation with the print molecule and the resulting complexes or adducts are then co-polymerized with cross-linkers (e.g. ethylene glycol dimethacrylate) into a rigid polymer. The print molecule is extracted to leave specific recognition sites in the polymer that are structurally and functionally complementary to the print molecule. The resulting so-called 'plastic antibodies' can be functionally as effective as the biological antibodies. For further details on MIP technology, the reader is referred to the review paper by Patel (2001) while the development and use of MIP for deoxynivalenol and zearalenone has been reported recently (Weiss *et al.*, 2003).

There are other novel technologies reported that have potential for simple and rapid purification of an analyte from complex matrices, including the application of immunomagnetic systems, particularly those based on colloidal ferrofluids



**Fig. 5.3** Schematic diagram representing a typical meat homogenate (from Patel, 2000).

which can be used in either batch or flow-through configurations. A range of innovative isolation and concentration techniques have been described previously (Patel, 2000).

### 5.3.3 Multianalyte screening

This is an area that is receiving significant interest from analysts and some of the developments in this area are considered here. Van der Gaag *et al.* (2003) reported development of an immunochemical biosensor assay for the detection of multiple mycotoxins (aflatoxin B<sub>1</sub>, zearalenone, ochratoxin, DON and fumonisins). The assay involved covalent binding of a different mycotoxin to each of the four flow cells on the sensor chip surface. A mixture of the anti-mycotoxin antibodies is added to a sample containing the test mycotoxins. Following reaction, the excess antibodies are detected as a function of the refractive change on the sensor surface as the antibody binds to the corresponding immobilized mycotoxin. The detection limits of the multiple assay are 0.2 ng/g (aflatoxin B<sub>1</sub>), 0.01 ng/g (zearalenone), 0.1 ng/g (ochratoxin A), 50 ng/g (fumonisin B<sub>1</sub>) and 0.5 ng/g (DON).

A microelectrophoresis-based immunoassay has been reported for the simultaneous detection of fluorescein-labelled staphylococcal enterotoxin B and cholera toxin B on an electronic chip (Ewalt *et al.*, 2001). An array of microlocations was transformed into an immunoassay array in which the biotinylated capture antibodies were attracted (under an influence of an electric field) to the microlocations containing immobilized streptavidin. A mixture of the fluorescein-labelled toxins, applied in the electric field, resulted in each of the toxins binding to the corresponding microlocation containing the capture antibody within 1 min. The toxins were then detected using an epifluorescent microscope. The technique allowed detection of staphylococcal enterotoxin in the range 1.8–180 nM, whilst the cholera toxin was detectable down to 18 nM.

A microarray biosensor for the simultaneous detection of cholera toxin, staphylococcal enterotoxin B, ricin and *Bacillus globigii* at detection limits of 8, 4 and 10 ng/ml, and 62 000 cfu/ml, respectively, was reported by Delehanty and Ligler (2002). This comprised multiple arrays of immobilized biotinylated capture antibodies on the surface of an avidin-coated glass slide. A six-channel flow module fed analyte containing solutions over the array of capture antibody microspots and bound analyte was detected using fluorescent tracer antibodies. The pattern of fluorescence could be visualized using a scanning confocal microscope.

## 5.4 Conclusions

The overall aim of this article was to give a perspective on rapid alternative techniques for mycotoxin analysis, focusing on commercially available immunological techniques, emerging non-commercial rapid and innovative technologies reported in the literature that have potential for application to mycotoxin measurement. The findings show that, as yet, no commercial method for mycotoxin analysis is available that can simply and rapidly measure, at trace levels, several mycotoxins simultaneously from a given sample.

Based on this investigation, it is also apparent that a number of innovative technologies have been reported for analytes other than mycotoxins that can be exploited to develop such types of methods required for analysis of mycotoxins. For instance, linking miniaturized separation techniques for the isolation and concentration of mycotoxin from a sample matrix followed by application of a suitable transduction system (e.g. optical, electrochemical, amperometric and impedance) with multianalyte detection capability would be a valuable new platform for mycotoxin analysts in the future.

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# 6

## Rapid detection of mycotoxigenic fungi in plants

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### 6.1 Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi that have deleterious effects on human and animal consumers. Mycotoxins are structurally diverse, deriving from a number of biosynthetic pathways and their effect upon consumers is equally diverse ranging from acutely toxic to immunosuppressive or carcinogenic. The production of a particular mycotoxin is restricted to a limited number of fungal species and, in some instances, may be limited to particular strains within a species. Although over 300 mycotoxins have been described, relatively few are of major concern with respect to human and animal health. Fungi from five genera (*Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps*) are responsible for the production of the great majority of the mycotoxins that are of agricultural relevance (Geisen, 1998; D’Mello *et al.*, 1998). The majority of these fungi infect plants prior to harvest and can be regarded as phytopathogens while others become active following harvest where storage conditions are permissive of fungal growth. Not all infections can be regarded as pathogenic, however, as infection may not result in symptoms of disease, e.g. *Aspergillus flavus* infection of maize. Table 6.1 lists the fungal genera of greatest agricultural importance along with their relevant mycotoxins.

**Table 6.1** Fungal genera and species of major significance and their associated mycotoxins.

Fungi	Mycotoxins
<i>Aspergillus</i> species (e.g. <i>A. flavus</i> , <i>A. parasiticus</i> and <i>A. nomius</i> )	Aflatoxins, predominantly B1 and B2 ( <i>A. flavus</i> ) and, in addition, G1 and G2 ( <i>A. parasiticus</i> ). B1 is metabolized to M1 which is excreted in milk of humans and animals
<i>Aspergillus</i> species (e.g. <i>A. flavus</i> , <i>A. parasiticus</i> , <i>A. nidulans</i> )	Sterigmatocystin
<i>Fusarium</i> species (e.g. <i>F. graminearum</i> ( <i>Gibberella zeae</i> ), <i>F. culmorum</i> , <i>F. sporotrichioides</i> , <i>F. poae</i> and <i>F. cerealis</i> )	Trichothecenes: Type A (e.g. T-2, HT-2, diacetoxyscirpenol (DAS), neosolaniol) produced by <i>F. sporotrichioides</i> , <i>F. poae</i> and/or <i>F. equiseti</i> Type B (e.g. nivalenol (NIV), deoxynivalenol (DON) and acetylated derivatives) produced by <i>F. graminearum</i> , <i>F. culmorum</i> and/or <i>F. cerealis</i>
<i>Fusarium proliferatum</i> and <i>F. verticillioides</i>	Fumonisin
<i>Fusarium</i> species (e.g. <i>F. proliferatum</i> and <i>F. avenaceum</i> )	Enniatins and beauvericin
<i>Penicillium</i> and <i>Aspergillus</i> species (e.g. <i>P. verrucosum</i> , <i>P. ochraceus</i> and <i>A. carbonarius</i> )	Ochratoxin A
<i>Penicillium</i> and <i>Aspergillus</i> species (e.g. <i>P. expansum</i> )	Patulin
<i>Penicillium</i> species	Citrinin and roquefortine
<i>Claviceps purpurea</i> , <i>Aspergillus</i> , <i>Penicillium</i> and <i>Acremonium</i> species	Ergot alkaloids
<i>Alternaria</i> species	Alternariol, altertoxin, tenuazonic acid

## 6.2 Agriculturally significant mycotoxins and their associated fungal species

Currently, aflatoxins are probably the most significant mycotoxins worldwide, with an estimated 20 000 deaths linked to aflatoxin-induced liver cancer per annum in Indonesia (Lubulwa and Davis, 1994). Aflatoxins are polyketide compounds produced by several members of the *Aspergillus* section *Flavi* (Varga *et al.*, 2003a). Some strains of *A. flavus* and most strains of *A. parasiticus* and *A. nomius* produce aflatoxins (Moreno and Kang, 1999) with *A. parasiticus* and

*A. flavus* being the two most agriculturally important species. Infection of maize, peanuts, pistachio nuts, figs, cottonseed meal and other commodities by these species may result in contamination with aflatoxins (Shapira *et al.*, 1996). Infection may occur in the field, particularly where the plant experiences water, temperature or nutrient stress and accumulation of mycotoxin may increase if harvested material is inappropriately stored (Moreno and Kang, 1999). *Aspergillus flavus* produces aflatoxins B1 (AFB1) and B2 (AFB2) while *A. parasiticus* also produces aflatoxins G1 (AFG1) and G2 (AFG2) (Xu *et al.*, 2000). Aflatoxin B1 is considered to be the most potent naturally occurring carcinogen known (Squire, 1989). Aflatoxin M1 is a derivative of AFB1 that is formed and excreted in the milk of humans and animals following consumption of foodstuffs contaminated with AFB1. AFB1 is derived from sterigmatocystin (ST), which is itself carcinogenic (O'Brian *et al.*, 2003). Although very few species produce aflatoxins, ST is produced by many *Aspergillus* species, including *A. nidulans*, as well as fungi from other genera (Cole and Cox, 1981; Barnes *et al.*, 1994).

Trichothecenes are sesquiterpenoid compounds produced by fungi from several genera (e.g. *Fusarium*, *Myrothecium*, *Stachybotris*, *Trichoderma*, *Trichothecium*, *Cephalosporium*, *Cylindrocarpon* and *Vertimonosporium*) (Trapp *et al.*, 1998). Trichothecenes are structurally diverse and over 180 naturally occurring trichothecenes have been described to date (Brown *et al.*, 2001). The *Fusarium* species are of significance with respect to food safety because many are important pathogens and colonizers of plants, particularly cereals. Four classes of trichothecenes are recognized with types A and B being produced by *Fusarium* species. Type A trichothecenes include T-2 toxin, HT-2, diacetoxyscirpenol (DAS) and neosolaniol (NEO) while type B trichothecenes include deoxynivalenol (DON) (also known as vomitoxin), 3-acetyl and 15-acetyl derivatives of DON (3-ADON and 15-ADON), nivalenol (NIV) and 4-acetyl NIV (also known as fusarenon-X). The type of trichothecene produced is characteristic of a particular species. The chief producers of type A trichothecenes are *F. sporotrichioides*, *F. poae* and *F. equiseti* while *F. graminearum*, *F. culmorum* and *F. cerealis* are producers of the type B trichothecenes that are most often associated with infected plant products. Trichothecenes are potent inhibitors of eukaryotic protein synthesis and have been associated with diseases such as alimentary toxic aleukia (ATA) that led to the deaths of thousands of Russians in the 1940s as a result of consuming grain that had become contaminated with *Fusarium* species while over-wintered in the field (Joffe, 1986). *Fusarium sporotrichioides* and *F. poae* strains isolated from such grain were found to produce high levels of T-2 toxin (Joffe, 1986). Currently, the contamination of cereals with DON and NIV is the focus of attention in many parts of the world because of the increased incidence of fusarium head blight (FHB) of wheat and barley (Gilbert and Tekauz, 2000). While a large number of trichothecene producing *Fusarium* species have been associated with FHB relatively few are of overall significance (Parry *et al.*, 1995; Bottalico and Perrone, 2002). *Fusarium graminearum* is the major pathogen worldwide while *Fusarium culmorum* and *F. poae* are more often associated with FHB in cooler regions, such as Northern Europe. Although these species are of the greatest

overall significance, others may be important in particular situations (Sugiura *et al.*, 1993; Miller, 1994; Tekauz *et al.*, 2000). FHB constitutes a disease complex in which trichothecene producing and non-producing species may co-exist. For example, *F. avenaceum* is frequently associated with FHB in cooler regions along with *Microdochium nivale*. *Fusarium avenaceum* produces non-trichothecene mycotoxins including moniliformin, enniatins and beauvericin, while *M. nivale* is not known to produce any mycotoxins. These species cause similar symptoms to those that produce trichothecenes making it impossible to determine whether there is a risk of trichothecenes, or other fusarium mycotoxins, accumulating in a particular crop.

Fumonisin are another group of mycotoxins produced by *Fusarium* species, chiefly those associated with pink ear rot of maize. Consumption of fumonisin-contaminated maize has been linked to high rates of oesophageal cancer (Nelson *et al.*, 1993). Fumonisin consist of a linear carbon backbone substituted at various positions with hydroxyl, methyl and tricarboxylic acid groups. The forms generally present in naturally contaminated maize are members of the B series, FB<sub>1</sub>, FB<sub>2</sub>, FB<sub>3</sub> and FB<sub>4</sub> (Nelson *et al.*, 1993). Three species are most commonly associated with pink ear rot, *F. proliferatum*, *F. verticillioides* and *F. subglutinans* (Logrieco *et al.*, 2002). Only *F. verticillioides* and *F. proliferatum* are able to produce fumonisins while *F. subglutinans* and *F. proliferatum* can produce moniliformin and beauvericin.

Ochratoxins are cyclic polyketides, the most potent of which is ochratoxin A (OTA) (Varga *et al.*, 2001). OTA has been associated with human endemic necropathy occurring in the Balkan region (Krogh, 1987). This mycotoxin was first detected in *Penicillium ochraceus* (van der Merwe *et al.*, 1965), but a large number of other *Penicillium* and *Aspergillus* species have since been reported to be OTA producers (Varga *et al.*, 2001). While *Penicillium* species are the predominant OTA producers in temperate regions, *Aspergillus* species are more significant in warmer regions. Although many species of both genera can produce OTA relatively few may be agriculturally significant for a particular crop or region. For example, *P. verrucosum* is the main OTA producing species in European cereals (Olsen *et al.*, 2003) while *A. carbonarius* is the chief producer in grapes (Cabañes *et al.*, 2002) and *A. ochraceus* is the organism most often found contaminating coffee (Schmidt *et al.*, 2003).

Patulin is a polyketide lactone that is produced by a number of *Aspergillus* and *Penicillium* species as well as fungi from other genera (Varga *et al.*, 2003b). *Penicillium expansum* is probably the species of greatest agricultural significance. Patulin can occur in mouldy fruits; major sources of human contamination are apples and apple juice (Jones and Toal, 2003). The ability of phylogenetically dissimilar species to produce patulin or OTA indicates that the genes for the biosynthesis of these mycotoxins have been gained (or lost) several times during evolution (Varga *et al.*, 2003a).

Ergot alkaloids are also produced by a range of fungi, including species within the genera *Acremonium*, *Balansia*, *Aspergillus* and *Penicillium* (Boichenko *et al.*, 2001). *Calviceps purpurea* is perhaps the best-known species having been

associated with epidemics of St Anthony's fire, a severe disease of humans, in the Middle Ages. Ergot alkaloids have a tetracyclic ergovaline ring that has homology to neurotransmitters. Peptide alkaloids have strong affinity for adrenaline receptors while clavines and D-lysergic acid amides have strong affinity for serotonin receptors (Tudzynski *et al.*, 2001).

As well as the mycotoxins described above, a range of fungal species produces several other agriculturally important mycotoxins. These include fusaproliferin produced by *F. proliferatum* (Bottalico, 1998), beauvericin and enniatins produced by *Fusarium* species including *F. avenaceum*, *F. proliferatum* (Bottalico, 1998), and roquefortine and citrinin produced by *Penicillium* species (Scott, 1994). In addition, *Alternaria* species also produce a number of mycotoxins, some of which have been detected in plant products. Among these are toxins such as alternariol (AOH), alternariol methyl ether (AME), altertoxin (ATX), tenuazonic acid (TeA) and the sphingosine-like derivative, AAL (Zur *et al.*, 2002). Zearalenone is a non-steroidal oestrogen that is responsible for reproductive disorders, particularly in pigs. Zearalenone is produced by many *Fusarium* species, including those that produce trichothecenes, and has been referred to as a mycotoxin. Zearalenone, however, is not intrinsically toxic and should be regarded as a mycooestrogen rather than a true mycotoxin (Hagler *et al.*, 2001).

### 6.3 Conventional methods for identifying mycotoxigenic fungi in plants

Visual assessment of infected plants or plant products is often insufficient to diagnose the causal agent of the disease, particularly where different organisms can induce similar symptoms. Conventional methods for the identification of species in plant tissues generally involve isolation of the fungus, or fungi, into axenic culture. In some instances, selective media have been developed to enable the target species to be isolated from material where it is only a relatively minor component of the microflora present (Booth, 1971). Once isolated, further culturing may be required before the organism can be identified. This is generally done on the basis of morphological characteristics of the colony, conidia and conidiogenous cells. Reliable identification requires considerable expertise and is greatly complicated by the plasticity of species such as *Fusarium* and *Penicillium*. Such difficulties are illustrated by the example of the 'Quorn' mycoprotein fungus *F. venenatum* (ATCC20334), that has been identified as four different species by different taxonomists (Mishra *et al.*, 2003). Such cultural and diagnostic methods are time-consuming and require considerable expertise in fungal taxonomy. In addition, these procedures have implications for the conclusions that can be drawn from such studies. Whatever the medium used, it will influence the isolation of particular fungal species and compromise attempts to assess the relative abundance of each species. Such techniques can only reveal what may be isolated from a tissue and not, necessarily, what is present within the tissue.

In some instances the detection and identification of the causal agent(s) may be

secondary to other considerations. For example, it may be more important to quantify the amount of pathogen present rather than just determine its identity. Ergosterol and, to a lesser extent, glucosamine (derived from chitin) have been used to estimate fungal quantities in plant tissues (Ride and Drysdale, 1972; Gretenkort and Ingram, 1993). Colonization of wheat by *F. culmorum* estimated by ergosterol content was found to be correlated with FHB symptoms and mycotoxin (DON) content of grain (Snijders and Kretching, 1992; Meidaner and Perkowski, 1996). Such non-specific assays are useful only for experimental systems in which the presence of other fungi is negligible, such as the grain of artificially inoculated cereals (Miller *et al.*, 1983). However, the use of glucosamine and ergosterol to estimate fungal biomass has been questioned even for these situations. Interspecific variation in ergosterol content has been observed and the ergosterol:biomass ratio has been found to vary with culture age in some fungal species (Birmingham *et al.*, 1995).

Several approaches have been taken to develop diagnostic assays that overcome the above difficulties. The main approaches divide, broadly, into immunological and DNA-based systems, the latter generally being polymerase chain reaction (PCR). The former has been reviewed by Barker (1996) and Li *et al.* (2000). DNA-based detection has been reviewed by Mills (1996), Geisen (1996) and, more recently, by Edwards *et al.* (2002).

#### **6.4 Using immunological and nucleic acid hybridization assays to detect mycotoxigenic fungi**

Although polyclonal antibodies (PABs) derived from immunizing animals have largely been superseded by the use of monoclonal antibodies (MAbs), this procedure can still provide the basis for useful assays. While the cost and time required to develop MAb-based immunoassays is considerable, this is offset by the potential to produce, indefinitely, unlimited quantities of a specific MAb. A broad range of immunoassay formats have been developed, although the enzyme-linked immunosorbent assay (ELISA) is the most widely used for the detection of plant pathogens (Li *et al.*, 2000). Immunoassays have also been adapted for on-site field work in dip-stick or dot-blot formats providing a user-friendly system for rapid pathogen detection and disease diagnosis (Dewey *et al.*, 1990). More recently, lateral flow devices have been produced that simplify detection of the target molecule in a one-step procedure (Danks *et al.*, 2003). In addition to detection, such immunoassays can be used for quantification of the target species.

Many antigens appear to be common to fungi of different species or genera such that polyclonal antibodies that are raised against a particular fungus may cross-react with another that is taxonomically distinct (Banks *et al.*, 1996). The specificity of the antibodies may reflect that of the antigens against which they were raised. For example, the immunodominant extracellular polysaccharide (EPS) antigens are more highly conserved than soluble macromolecules (exoantigens) obtained

from surface washings of mycelium (Li *et al.*, 2000). Such conservation may be exploited where fungi from more than one genus produce a particular mycotoxin. For example, the EPS of *Aspergillus* and *Penicillium* species both contain galactofuranoside residues so that antibodies raised against these targets may react to both. Indeed PABs raised against culture filtrate of *A. parasiticus* cross-reacted with *Aspergillus* and *Penicillium* species but not with *Fusarium* species (Shapira *et al.*, 1997) and a monoclonal antibody raised against *A. flavus* EPS also cross-reacted with high specificity to *Aspergillus* and *Penicillium* species (Kwak *et al.*, 2001). A novel approach to detecting aflatoxin producing species was reported by Shapira *et al.* (1997). Polyclonal antibodies were raised to products of two genes, *ver-1* and *apa-2* involved in aflatoxin biosynthesis following their expression in *Escherichia coli*. Polyclonal antibodies to these chimeric proteins were highly specific towards *A. flavus* and *A. parasiticus* and did not cross-react with the other species tested. The authors, however, suggested that such assays may not react with mycelium in which the mycotoxin is not being synthesized and may also cross-react with related enzymes from other fungi that are not involved in mycotoxin biosynthesis (Shapiro *et al.*, 1997).

Polyclonal antibodies raised to soluble protein fractions of *F. culmorum*, detected *F. culmorum*, *F. graminearum* and *F. poae* when used in an ELISA but did not cross-react with *Microdochium nivale* or *Tapesia* species (Beyer *et al.*, 1993). However, the assay could not differentiate between the three *Fusarium* species. In a separate report, PABs raised against *F. graminearum* and *F. sporotrichioides* were found to cross-react with other *Fusarium* species while those to *F. poae* were species specific (Gan *et al.*, 1997). Samples of grain of hard and soft wheat suffering from FHB were assayed with the *F. sporotrichioides* antiserum and the results found to correlate well with the levels of ergosterol and DON (Gan *et al.*, 1997). Monoclonal antibodies have been raised against a number of *Fusarium* species including *F. culmorum* and *F. avenaceum* (Banks *et al.*, 1996) and, more recently, *F. graminearum* (P. Jennings, CSL, UK, personal communication). However, they have been used only in preliminary studies for the detection of these fungi within plant tissues. While the MAb-based assays for detection of these *Fusarium* species are still under development, it is anticipated that ELISA-based formats utilizing the monoclonal antibodies to the *Fusarium* species described above will be available in the near future for the detection of these pathogens in plant tissues.

In addition to disease diagnosis, antibodies have considerable potential in epidemiological studies. For example, fluorescently labelled antibodies to *Fusarium vasinfectum* have been used to detect the pathogen in host tissues and soil (Kumar *et al.* 1986).

A third method, phage display libraries, has been developed for the production of antibodies (McCafferty *et al.*, 1990; Hoogenboom and Winter, 1992). The gene segments encoding the light and heavy variable antibody domains are rearranged *in vitro* which, when expressed in *E. coli*, results in the production of a single polypeptide chain that forms a functional antibody fragment (scFv). The whole sequence can be expressed, fused to the minor coat protein pIII, on the surface of

a filamentous phage. Populations (libraries) of phage have been produced containing in excess of  $10^8$  different scFv clones (Nissim *et al.*, 1994). There are very few reports of the use of this approach to produce assays to detect mycotoxigenic fungi but an scFv has been used to detect melanin within *Alternaria alternata* (Carzaniga *et al.*, 2002).

#### 6.4.1 Nucleic acid hybridization

While relatively few assays have been developed for the immunological detection of mycotoxigenic fungi, a large number of assays have been produced to detect these fungi on the basis of nucleic acid sequences that are specific to the target organism. Nucleic acid hybridization assays involve the selection, cloning and chemical labelling (e.g. biotin, digoxigenin,  $^{32}\text{P}$ ) of sequences specific to the target organism. These are then used as probes to detect RNA or DNA of the pathogen in extracts or tissue squashes of plant material. The assay may involve immobilization and detection of nucleic acid on a membrane (e.g. Gilbertson *et al.*, 1989) or, in some instances, utilize a microplate format similar to that used in immunoassays (e.g. Palkovics *et al.*, 1994). The development and use of nucleic acid hybridization assays to detect and identify plant pathogenic fungi has been limited, although species-specific DNA probes have been developed towards several *Fusarium* species, including *F. culmorum* (Koopmann *et al.*, 1994; Nicholson *et al.*, unpublished), *F. graminearum* (Nicholson *et al.*, unpublished) and *F. avenaceum* (Turner *et al.*, 1998). Hybridization is relatively insensitive and there are few instances where this method has been used to detect fungi directly in extracts from plant tissues. Hybridization assays are sufficiently sensitive for the identification of fungi cultured from plant tissue (bio-amplification) but this, of course, incurs the problems associated with selection during isolation (e.g. Pettitt *et al.*, 1993) and greatly increases the time required to complete the analysis.

### 6.5 Polymerase chain reaction (PCR)-based assays for detecting mycotoxigenic fungi

In contrast to hybridization, the PCR has found widespread use throughout plant pathology, and a significant number of PCR-based assays have been developed for use with species associated with the production of mycotoxins. PCR involves the enzymatic amplification of a target DNA sequence by a thermostable DNA polymerase such as *Taq* from *Thermus aquaticus* (Saiki *et al.*, 1988). PCR generally involves the denaturing of DNA strands and annealing of two oligonucleotide primers to their homologous sequences that flank the region to be amplified. The DNA polymerase extends the primers across the region and so generates a copy. The process of melting, annealing and extension is repeated up to 50 times, so producing many millions of copies (amplicons) of the target region (see Mills, 1996). The sensitivity of the PCR process lies in the

amplification aspect while the specificity is determined by the choice of primers used in the reaction. Where sufficient specificity is designed into the primers, individual isolates of a particular species can be detected within complex DNA mixtures.

Although PCR is an extremely sensitive technique, it is inherently unsuitable for quantification in most situations. Minor differences in amplification efficiencies, such as might occur where DNA samples contain different amounts of inhibitory substances, result in large differences in amplicon yield. Two approaches have been taken to overcome such problems. The first is competitive-PCR (Diviacco *et al.*, 1992) in which a known quantity of 'competitor' template DNA is added to the sample before PCR. The 'competitor' and fungal target DNA have identical primer sites but differ in the length of sequence between them. During the PCR the fungal 'target' and 'competitor' template sequences compete for reagents such that the ratio of fungal 'target' DNA to 'competitor' DNA amplified increases with increasing amounts of fungal DNA. Following PCR, the two products can be size separated and the relative amount of each determined with standard image analysis software. In uninfected plant tissue only the 'competitor' DNA is amplified confirming that the PCR is functioning normally. There are relatively few competitive PCR assays for mycotoxigenic phytopathogens (Nicholson *et al.*, 1998, 2004; Edwards *et al.*, 2001), perhaps because of the difficulty of producing and maintaining competitor templates for relatively large numbers of fungal species.

'Real-time' PCR, in which the amount of amplicon is assayed at each cycle, provides a second means of quantifying the amount of DNA of a particular species or fungal target. In its simplest form the amplicon is quantified on the basis of fluorescence produced by an intercalating dye, such as SYBR®Green I, binding to double-stranded DNA. Such a system is not, however, able to determine whether the fluorescence is due to amplification of the correct amplicon. In order to address this, a number of fluorogenic probe-based assays have been developed to reduce interference from amplification of non-target fragments. These include TaqMan (Holland *et al.*, 1991), Molecular Beacons (Tyagi and Kramer, 1996) and Scorpion primers (Thelwell *et al.*, 2000). In all cases, the use of such assays should be accompanied by an internal control to account for differences in amplification efficiency between samples. Quantitative assays, whether 'competitive' or 'real-time', permit the sensitive detection, identification and quantification of individual species/targets within host tissues and other substrates.

PCR can also be used in a one- or two-step reverse-transcriptase (RT) PCR protocol to detect genes that are being expressed rather than to detect the presence of the DNA that encodes a particular gene. Reverse transcriptase is used to generate a DNA copy (cDNA) from RNA (mRNA or total RNA) present in a sample. The cDNA is then subjected to PCR using primers designed for the target sequence. When designed to genes characteristic of a particular species or to genes involved in mycotoxin biosynthesis and assayed in a real-time or competitive PCR format the mRNA level of target genes can be estimated in a manner similar to that for DNA (Riedy *et al.*, 1995).

### 6.5.1 Detection of mycotoxigenic species by PCR

A number of approaches have been taken to develop assays with the desired level of specificity, whether to detect a single species or to detect strains from different species with the potential to produce a common mycotoxin. Two broad approaches have been adopted to developing assays to detect individual fungal species. The first has been by using 'universal' primers to isolate specific DNA regions followed by comparison of the sequence of the target species with that of other species within databases. The coding portions of many fungal 18S, 5.8S and 28S rDNA genes are highly conserved and primers to these regions have been generated (White *et al.*, 1990). These allow the isolation of the internal transcribed spacer sequences (ITS-1 and ITS-2), which lie between the coding regions, from a wide range of fungi. The ITS region is amplified from the target fungus and sequenced to identify regions of DNA unique to the fungus of interest. Polymorphism within the ITS region is generally (but not always) at the level of species, rather than between isolates of the same species, making it an ideal target for the development of species-specific PCR assays. For example the ITS-1 and -2 of *F. avenaceum* was found to differ markedly from that of *F. graminearum* and *F. culmorum* and primers to this species were obtained which did not cross-react with DNA from a range of other fungal or plant species (Schilling *et al.*, 1996). The ITS region was also used to design primers to differentiate between *Alternaria* species infecting carrot (Konstantinova *et al.*, 2002). While the assays could differentiate between *A. alternata*, *A. radicina* and *A. dauci*, two of the assays (*A. alternata* and *A. dauci*) cross-reacted with other *Alternaria* species using the reported PCR conditions. Where such species are not associated with a particular plant or plant product, such reactions may not pose a practical problem, but they may limit the use of the assay on other crops or food products.

More highly conserved regions within the ITS are also suitable for designing primers to differentiate one genus from another such as was done for *Fusarium* (Bluhm *et al.*, 2002). However, where it is desired to detect and differentiate closely related species there may be insufficient polymorphism in the ITS to permit the design of PCR primers to this region. This was found to be the case for *F. culmorum* and *F. graminearum* where no differences were detected between the ITS-1 of the two species and the differences within ITS-2 were insufficient to produce specific primers (Schilling *et al.*, 1996).

In instances where the ITS region is of limited use, other characterized regions of the genome may be used. For example, galactose oxidase is produced by only a few fungal species and Niessen and Vogel (1997a) used this to produce PCR primers specific to *F. graminearum* based upon the sequence of the galactose oxidase (*gaoA*) gene. As the amount of sequence information from fungi deposited in databases increases, the number of potential targets for the design of PCR assays based upon characterized genes will increase.

An alternative approach is to develop assays based upon polymorphisms detected within anonymous DNA regions. Two methods are commonly employed to detect such polymorphisms between fungal species. Random amplified polymorphic DNA (RAPD) analysis (Williams *et al.* 1990) has been used by a

large number of workers to study variability within and between species, including many *Fusarium* species (e.g. Bentley *et al.*, 1995; Amoah *et al.* 1996). RAPD fragments characteristic of particular species are selected and used in Southern blots to ensure differential hybridization to the target and non-target species. Suitable fragments are cloned and sequenced. Primers are then designed for use in conventional PCR. Such an approach has been used to obtain primers specific to a number of *Fusarium* species. Schilling *et al.* (1996) derived primers specific to *F. graminearum* and *F. culmorum* using this method. Other workers have also used RAPD products specific to particular species to develop primers specific to *F. graminearum*, *F. cerealis* (*F. crookwellense*), *F. venenatum*, *F. torulosum*, *F. sambucinum* s.s. (Yoder and Christianson, 1997) and *F. proliferatum* (Möller *et al.*, 1997). Where plant material may contain a number of target species the primers can be designed with similar melting temperatures to enable all targets to be detected using a single PCR protocol. Furthermore, if the size of the PCR product for each target is different, two or more species can be detected in a single (multiplex) PCR assay. Such assays have been developed for several *Fusarium* species, including *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. poae* (Parry and Nicholson, 1996; Nicholson *et al.*, 1998; Turner *et al.*, 1998). Such refinement of the PCR assays can be useful where large-scale screening or experimental procedures are required or envisaged.

The second method to generate anonymous markers is the amplified fragment length polymorphism (AFLP) assay, first described by Vos *et al.* (1995). This method has been used to develop a species-specific assay for *A. ochraceus* that did not cross-react with other *Aspergillus* or *Penicillium* species tested (Schmidt *et al.*, 2003). The development of truly species-specific PCR assays for mycotoxigenic fungi is entirely dependent upon a robust taxonomic foundation. Primer sets should be tested against closely related species to ensure specificity. Unfortunately, it is not always obvious from traditional, morphology-based taxonomies to determine which species are closely related. For example PCR assays to *F. avenaceum* designed from ITS (Schilling *et al.*, 1996) and RAPD (Lees, 1995) sequences were found subsequently to cross-react with *F. tricinctum* (Turner *et al.*, 1998). *F. tricinctum* is placed in the *Sporotrichiella* section while *F. avenaceum* is in the section *Roseum* and these two species might not be considered to be closely related. However, subsequent evidence from multiple molecular analyses indicates that the *Sporotrichiella* section contains unrelated species and should be revised or abolished (Bateman *et al.*, 1996; Altomare *et al.*, 1998; Turner *et al.*, 1998). Molecular analyses are also revealing, sometimes, unsuspected distinctions within species. For example, sub-groups/lineages have been recognized within *F. graminearum* (Carter *et al.*, 2000, 2002; O'Donnell *et al.*, 2000). Sequence analysis has revealed that the product of the Fg16F/R primer pair (Nicholson *et al.*, 1998) is diagnostic of the lineage/groups tested to date and hence this primer pair may be used to detect *F. graminearum* and simultaneously determine lineage/group (Nicholson *et al.*, unpublished). Furthermore, because particular lineages/groups appear to be associated with geographic regions and mycotoxin chemotypes, the assay could be used to aid the detection of migrants and monitor pathogen movement.

Although a number of PCR assays have been developed against mycotoxigenic species there are relatively few reports of their use to detect pathogens in plant material (e.g. Schilling *et al.*, 1996; Parry and Nicholson, 1996; Niessen and Vogel, 1997a; Doohan *et al.*, 1998). However, PCR assays are sufficiently sensitive that *Fusarium* and other cereal pathogens can be detected, even in symptomless plants (Parry and Nicholson, 1996; Doohan *et al.*, 1998; Nicholson *et al.*, unpublished). Thus species-specific PCR-based assays to mycotoxigenic species are potentially both specific and highly sensitive.

## 6.6 Using mycotoxin biosynthetic gene clustering for identifying mycotoxins

In some instances, detection to species level is not always of primary concern. It may be more relevant to determine whether the producers of particular mycotoxins are present rather than to identify exactly what the species are. This is particularly important where a mycotoxin can be produced by a number of species. Many mycotoxin biosynthetic genes are present within gene clusters, and some of these appear to have undergone horizontal transfer from one species to another and are now present in several species (Sidhu, 2002). If the sequences of genes associated with the biosynthesis of particular mycotoxins are available then a more generic approach is possible. Regions of homology within mycotoxin biosynthetic genes from the different species can be used to develop primers to detect the genes from all of the relevant mycotoxigenic species. Where mycotoxin biosynthetic genes are present in clusters, identification of one gene frequently facilitates the identification of other genes involved in the pathway (Alexander *et al.*, 1997; Proctor *et al.*, 2003). Such an approach has been broadly successful for a number of mycotoxins.

### 6.6.1 Trichothecenes

Although our understanding of the biosynthesis of trichothecene mycotoxins is incomplete, many steps in the pathway are known, and a large number of genes that are involved in trichothecene biosynthesis have been isolated (Brown *et al.*, 2001; Kimura *et al.*, 2001; Meek *et al.*, 2003). While a significant number of genes appear to be present within a 23 kb gene cluster, several genes involved in trichothecene biosynthesis lie outside this region, and it is not known whether they form a second cluster or are present within a distal portion of the same cluster (Kimura *et al.*, 1998; Meek *et al.*, 2003).

The first gene in the pathway (*Tri5*), which encodes trichodiene synthase, is highly conserved among trichothecene-producing *Fusarium* species (Fekete *et al.*, 1997). This has facilitated the development of PCR assays to detect species that produce trichothecenes (Niessen and Vogel, 1997b; Doohan, 1998). The primer pair developed by Niessen and Vogel was shown to be specific to trichothecene producing *Fusarium* species and did not cross-react with other trichothecene

producing species such as *Trichothecium roseum*, *Trichoderma virens*, *Myrothecium roridum* or *Stachybotris chartarum*.

Both 'real-time' and 'competitive' PCR assays have been developed to *Tri5* (Schnerr *et al.*, 2001; Edwards *et al.*, 2001). Generic assays to detect trichothecene producing *Fusarium* species have also been developed to other genes within the cluster. *Tri6* encodes a transcriptional regulator of trichothecene biosynthetic genes and a PCR assay to this gene has been used to detect *F. graminearum* in cornmeal and to differentiate it from a fumonisin producing species (*F. verticillioides*) (Bluhm *et al.*, 2002). In addition, RT-PCR assays have been developed against *Tri5* to study regulation of trichothecene biosynthesis in *F. culmorum* and *F. graminearum* (Doohan *et al.*, 1999; Draeger, unpublished). These assays have been used to study biosynthesis of trichothecenes during colonization of cereal hosts and in response to fungicides (Doohan, 1998; Draeger, pers.comm.)

Generic assays to detect trichothecene producing *Fusarium* species have limited use with respect to risk assessment and the focusing of additional analysis. The most common trichothecene in blighted grain is DON which often occurs along with acetylated derivatives (3-ADON or 15-ADON) that are less toxic (Kimura *et al.*, 1998). In other instances the predominant toxin produced by the isolate or species present may be nivalenol (NIV). This compound is believed to be more toxic than DON or its acetylated derivatives and hence is of importance with respect to food safety (Ryu *et al.*, 1988). Nivalenol and its acetylated derivatives are produced by some isolates of *F. graminearum* and *F. culmorum* and also by *F. cerealis*, *F. poae* and *F. equiseti* (Sugiura *et al.*, 1993; Liu *et al.*, 1998; Langseth *et al.*, 1999). Type-A trichothecenes (DAS, T-2, HT-2) produced by *F. sporotrichioides* and *F. armeniacum* are significantly more toxic to human and animal consumers than any of the type-B trichothecenes mentioned above (Krska *et al.*, 2001). Analysis of 28S ribosomal DNA sequences revealed that type-A and type-B producing species clustered together in two monophyletic groups (Mulé *et al.*, 1997). Sequence divergence in *Tri4* between producers of type-A and type-B trichothecenes has been exploited to develop an assay to specifically detect producers of type-A trichothecenes (Nicholson *et al.*, 2004). The primer set to type-A trichothecene producing species did not amplify from any of the type-B trichothecene producing species tested. A second assay to type-B trichothecene producers amplified a product from all the type-B trichothecene producing species but also amplified a product of the expected size from an isolate of *F. sambucinum* (type-A producing species). Thus this assay requires further refinement for use where this species is of significance.

Where individual isolates of a single species (e.g. *F. graminearum* and *F. culmorum*) can produce trichothecenes of different toxicity (NIV or DON) further refinement is required. Recent work by Lee *et al.* (2001, 2002) and Brown *et al.* (2001, 2002) has identified the two genes responsible for the conversion of DON to NIV (*Tri13*) and the acetylation of NIV to 4-acetyl nivalenol (4-ANIV) (*Tri7*). Both genes are non-functional in DON producing isolates (Lee *et al.*, 2001, 2002). A PCR assay to *Tri7* based on product size polymorphism between DON

and NIV chemotypes of *F. graminearum* has been described (Lee *et al.*, 2001). However, the assay does not identify DON producing isolates that carry alternative mutations or mutations that lead to failure to amplify any product (Chandler *et al.*, 2003). A number of positive–negative PCR assays to both *Tri7* and *Tri13* have since been developed to characterize isolates of *F. graminearum*, *F. culmorum* and *F. cerealis* in terms of their NIV and DON chemotype (Chandler *et al.*, 2003). The PCR results obtained from *F. graminearum* and *F. culmorum* isolates concurred with known toxin production in all cases. Understanding the molecular genetic basis of trichothecene biosynthesis has been used to develop assays with differing specificities to detect species capable of trichothecene production or to producers of a particular class of toxin or even a specific toxin.

### 6.6.2 Fumonisin

The first fumonisin biosynthetic gene to be isolated, *Fum5* from *F. verticillioides*, encodes a polyketide synthase (Proctor *et al.*, 1999). As with many other mycotoxins, the genes involved in fumonisin biosynthesis appear to be clustered (Seo *et al.*, 2001; Proctor *et al.*, 2003). At least 15 genes within the fumonisin cluster are coregulated and have patterns of expression that correlate with production of fumonisin (Proctor *et al.*, 2003). Some of these genes provide potential targets for the development of additional assays to detect fumonisin-producing species. A PCR assay developed to sequence from this gene has been used in a multiplex format along with assays to *Tri5* and ITS to provide genus-level detection combined with determination of mycotoxin production potential (trichothecene and fumonisin) (Bluhm *et al.*, 2002).

### 6.6.3 Aflatoxins and sterigmatocystin

Twenty five transcripts were found to be coregulated within the sterigmatocystin gene cluster of *Aspergillus nidulans* (Brown *et al.*, 1996). Although the arrangement of the genes within the aflatoxin biosynthetic cluster is different, many of the gene products are conserved at the amino acid level (Woloshuk and Prieto, 1998). Recently, it was shown that 24 genes were differentially expressed during aflatoxin biosynthesis by *A. flavus* and *A. parasiticus* (O'Brian *et al.*, 2003). The sequence of many of the genes in both pathways is known, and this information has been used to design a number of PCR assays to detect aflatoxin producing fungi and to monitor biosynthesis of aflatoxins. Multiplex assays were designed by Geisen (1996) and Shapira *et al.* (1996). The former assay targeted *nor-1*, *ver-1* and *omt-A* while the latter selected *ver-1* and *omt-1* and a regulatory gene, *apa-2* (now called *aflR*). The *omt-1* (*omt-A*) gene encodes the *O*-methyltransferase that converts sterigmatocystin to *O*-methylsterigmatocystin, and its inclusion in multiplex assays can be used to differentiate between fungi that produce aflatoxins and those that can only synthesize sterigmatocystin.

Both assays were broadly successful in differentiating aflatoxin-producing isolates from other fungi, but difficulties were encountered in obtaining the

required level of specificity. The *nor-1*, *ver-1* primer sets cross-reacted with an isolate of *Penicillium roquefortii* (Geisen, 1996) while the *aflR* assay only amplified a product from *A. parasiticus* (Shapira *et al.*, 1996). Similar assays have been developed more recently in which four biosynthetic genes have been multiplexed (Criseo *et al.*, 2001; Chen *et al.*, 2002). While all aflatoxigenic isolates produced four products in both instances, the majority of non-aflatoxin producing isolates produced three, or fewer, amplicons. The lack of amplification of one or more amplicons indicates that the biosynthetic genes within the non-aflatoxin producing isolates have undergone mutation and may no longer be functional. However, some non-aflatoxigenic isolates and most isolates of *A. sojae* and *A. oryzae* (non-aflatoxigenic 'domesticated' species used in fermentation) produced amplicons of the expected size with all four primer sets (Criseo *et al.*, 2001; Chen *et al.*, 2002). It may be that conditions for aflatoxin production by the isolates differ from those tested or that mutations exist within the aflatoxin biosynthetic or regulatory genes of these isolates.

It is thought that biosynthesis of members of the aflatoxin G-family (produced only by *A. parasiticus*) occurs via a separate pathway to that for the B-family branching from sterigmatocystin (Woloshuk and Prieto, 1998). Currently, there are no assays designed, specifically, to differentiate between producers of the two types of aflatoxin.

A real-time PCR assay has been developed towards *nor-1* to detect aflatoxigenic fungi (Mayer *et al.*, 2003), but in all probability this assay will also be unable to differentiate between non-aflatoxin and aflatoxin producing isolates. Furthermore, this assay was not tested against sterigmatocystin producing *Aspergillus* species. A real-time RT PCR assay has been reported for *pks-A* and *aflR* (Kondo *et al.*, 2001) and has been used to study the effect of nutritional factors and inhibitors of aflatoxin production on these two genes. A second gene (*aflJ*) is involved in the regulation of aflatoxin biosynthesis. This gene has not been identified in the sterigmatocystin gene cluster, indicating that production of these toxins may be differentially regulated. This gene may provide a suitable target for differentiating between fungi producing the two types of mycotoxin.

#### 6.6.4 Patulin

The biosynthesis of patulin involves at least ten biosynthetic steps and has been relatively well characterized at the biochemical level (Gaucher *et al.*, 1981). In contrast the organization of the genes involved in patulin biosynthesis is not known (Varga *et al.*, 2003a). A PCR assay was, however, designed to the iso-epoxydon dehydrogenase (*IDH*) gene from the patulin biosynthetic pathway of *Penicillium expansum* (Paterson *et al.*, 2000). The assay also reacted to all isolates of *P. brevicompactum*, although no patulin was detected in cultures from this species. It was suggested that isolates of this species might produce patulin under different conditions and evidence was presented to support this in later work (Paterson *et al.*, 2003). The primers to the *IDH* gene have also been shown to amplify from *Aspergillus* species able to produce patulin (Varga *et al.*, 2003b) and thus may

provide a generic tool to detect patulin producing species. A second gene involved in biosynthesis of patulin has also been targeted for use in PCR assays. A portion of the 6-methylsalicylic acid synthetase gene (6-MAS), the first step of the patulin biosynthetic pathway, has been cloned from *P. expansum* (Edwards *et al.*, 2002), but a PCR assay for the specific detection of this gene from patulin producing species has yet to be reported.

### 6.6.5 Ergot alkaloids

The first step in the biosynthesis of ergot alkaloids is the formation of 4-dimethylallyltryptophan (DMAT) from L-tryptophan and dimethylallyldiphosphate. This step is catalyzed by DMAT synthase (DMATS) encoded by the gene *cpd1* (Tudzynski *et al.*, 1999). PCR primers designed towards this gene have been shown to amplify a product from *Penicillium* and *Claviceps* species able to produce clavine alkaloids (Boichenko *et al.*, 2001).

### 6.6.6 Other mycotoxins

Genes involved in the biosynthesis of several other mycotoxins and fungal secondary metabolites have been isolated (e.g. Haese *et al.*, 1993; Johnson *et al.*, 2000). Where such information is obtained, and suitable regions of sequence are present within the gene, it should be possible to develop PCR assays of sufficient specificity and robustness to detect the presence of fungi capable of producing particular mycotoxins within plant tissues and plant products. Polyketide synthases are involved in the biosynthesis of several mycotoxins, and the genes encoding several of those involved in mycotoxin biosynthesis have been identified, including fumonisin (*Fum5*) and aflatoxin (*pks-A*). Several research groups have been attempting to utilize this information to isolate gene(s) encoding polyketide synthase(s) involved in ochratoxin biosynthesis. To date, however, those genes isolated appear to function in pathways not associated with ochratoxin biosynthesis (Edwards *et al.*, 2002). In cases where genes involved in mycotoxin biosynthesis have yet to be identified, such as is currently the case for ochratoxin, the most effective route may be through the identification of either generic (e.g. ITS) or anonymous (RAPD or AFLP) sequences and the development of assays to individual species.

## 6.7 Combination assays and alternatives to PCR

PCR is reliant upon the isolation of DNA of the target species from contaminating substances that may compromise the reaction. A combination of antibody and PCR assays can be used to both purify and concentrate the target in order to enhance sensitivity. Immunocapture PCR uses antibodies to purify the target organism from complex substrates (Hartung *et al.*, 1996). The DNA is isolated from the bound organism and used in subsequent PCR assays. The antibody step aids

purification, thus enhancing sensitivity while the specificity resides in the PCR component of the assay. While the majority of antibodies to mycotoxigenic fungi react to several species or genera, when combined with species/mycotoxin-specific PCR assays they may be used for the sensitive, specific detection of mycotoxigenic species within complex substrates. Immunocapture-PCR has been used to detect phytopathogenic bacteria (Hartung *et al.*, 1996) and may be useful for detecting resting structures, such as chlamydospores, in soil.

The ligase chain reaction (LCR) is an alternative to PCR (Barany, 1991) that also employs a temperature cycling reaction. In this process oligonucleotide primers anneal to adjacent points in the DNA of the target organism. The primers are then ligated using a thermostable DNA ligase. If the target DNA is present, the primers anneal and are covalently joined by the ligase. The new molecule then acts as a template for a second set of complementary primers which are included in the reaction. The ligated product is exponentially produced in a process analogous to that of PCR. LCR can exploit single base pair differences between the target organism and other species if the primers are designed such that the 3' end of one of them is complementary to the critical nucleotide. Although LCR has not been used, to date, for the detection of mycotoxigenic fungal species it has been used for the detection of bacterial plant pathogens including *Erwinia stewartii* (Wilson *et al.*, 1994).

A number of other nucleic acid-based technologies have been developed and these may also be of use in studies involving *Fusarium* species. Nucleic acid sequence-based amplification (NASBA) or self-sustained sequence replication (3SR) employs the concerted action of three enzymes (Rnase H, AMV RT and T7 RNA polymerase) to amplify RNA targets in an isothermal reaction (Gingeras *et al.*, 1990; van Gemen *et al.*, 1993). This assay has been used to detect RNA of the *Fum5* gene involved in fumonisin biosynthesis and *Tri5* from trichothecene producing *Fusarium* species (Waalwijk *et al.*, unpublished). NASBA, however, like RT-PCR is only able to detect fungi when they are actively producing mycotoxins and is thus of limited use where the fungi are quiescent or growing under conditions not suitable for mycotoxin production. These, and other, molecular techniques are, however, rapidly being adopted to form part of the tool kit employed by many *Fusarium* researchers.

## 6.8 Future trends

Although PCR is a very sensitive assay, various limitations have hindered the widespread uptake of this technology for the control of plant disease for the detection of mycotoxigenic fungi. The plant material has to be processed to extract and purify the DNA. The DNA is then subjected to PCR and, unless using 'real-time', then PCR products are size separated prior to analysis. Sample sonication can greatly speed up the DNA extraction process. Sonication has been successfully used to prepare DNA of *Fusarium* species from wheat grain for PCR in only five minutes (Knoll *et al.*, 2002). Immunocapture of the pathogen or DNA offers an

alternative, rapid method to purify DNA for PCR analysis. Recently, portable PCR machines have been developed and are now being marketed (e.g. Smart Cycler® II TD (transportable device) system). This device comprises 16 independently programmable modules and incorporates four-colour real-time PCR detection (Lévesque, 2001; Schaad and Frederick, 2002). This allows simultaneous detection of several target organisms, even where the PCR protocols differ.

The advent of DNA microarrays is poised to revolutionize many aspects of plant pathology, disease diagnosis and disease management, including the detection of mycotoxigenic fungi. Microarrays consist of DNA or oligonucleotides bound (or synthesized), at discrete locations, on a glass slide. Originally, DNA microarrays were designed to permit analysis of gene expression of a large part or the whole genome (transcriptome) in a single assay. For example the *Arabidopsis thaliana* array produced by Affymetrix Inc. comprises 24 000 oligonucleotide probes on a single chip. This technology is being adapted for use in the examination of environmental samples where many target organisms may be present. Microarrays based upon fragments of the 16S rDNA of bacteria have been used to characterize microbial communities (Stine *et al.*, 2003). DNA arrays on membranes require more reagents but are a step towards true microarrays. Such membranes based on 16S and 16S–23S intergenic spacer sequence have been used to detect and differentiate between bacteria pathogenic on potato (Fessehaie *et al.*, 2003), and arrays to ITS 1 have been used to detect and differentiate between oomycetes (Lévesque *et al.*, 1998). Multiplex PCR can be used to amplify numerous sequences simultaneously, and these can be hybridized to DNA microarrays as has been achieved for bacterial pathogens (Chizhikov *et al.*, 2001). Arrays based upon sequence within the *TEF1 $\alpha$*  gene have been designed to differentiate between *Fusarium* species (Holst-Jensen *et al.*, unpublished). The resolving power of the hybridization process is such that probes that differ by two or more nucleotides can be differentiated (Fessehaie *et al.*, 2003). In a further advance, the detection of hybridization can be achieved electronically (Umek *et al.*, 2001). Electronic detection of hybridization may permit further miniaturization of equipment and increase portability of assays.

An alternative form of microarray consists of biotinylated antibodies immobilized onto streptavidin-coated slides. Where antibodies are available to both the mycotoxin producing species and to the mycotoxin itself, it is possible to detect the organism(s) and the metabolites in a single assay. Flow-through devices currently permit the use of 88 capture antibodies and provide analysis within minutes rather than hours (Delehanty and Ligler, 2002).

While improved, rapid DNA extraction procedures, 'real-time' PCR and the development of portable PCR machines enhance our ability to detect and quantify mycotoxigenic fungi the future lies with the microarray technology. The physical capacity of current DNA microarrays would permit all mycotoxigenic fungi to be detected in a massive parallel analysis of any plant tissue or product. The robustness of such assays could be increased by inclusion of anonymous species-specific DNA fragments and phylogenetically informative sequence alongside sequence for genes involved in mycotoxin biosynthesis. Whatever the assay, it relies upon

robust phylogenetic information and a detailed understanding of the molecular genetic basis of mycotoxin biosynthesis.

## 6.9 Sources of further information and advice

Recent reviews have been produced on the use of PCR and antibody-based detection of mycotoxin producing fungi (Li *et al.*, 2000; Edwards *et al.*, 2002). More general reviews of plant pathogen diagnostics have also been published relatively recently (O'Donnell, 1999; Lévesque, 2001; Schaad and Frederick, 2002). Further information related to mycotoxigenic fungi and their detection is available at websites of EU funded projects. For example the project 'Early detection of toxigenic *Fusarium* species and ochratoxigenic fungi in plant products' can be accessed via <http://www.detox.ba.cnr.it>. The European Mycotoxin Awareness network (<http://www.lfra.co.uk>) contains information related to a broad range of mycotoxin issues and includes a large number of links to sites and groups involved in mycotoxin-related research. In addition information on DNA microarrays can be accessed at <http://www.Gene-Chips.com> and also at <http://www.affymetrix.com> while information relating to portable PCR equipment can be located at <http://www.cepheid.com>.

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## **Part II**

### **Controlling risks**

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# The use of HACCP in the control of mycotoxins: the case of cereals

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## 7.1 Introduction: hazards and HACCP systems

### 7.1.1 Nature of the problem

Cereals are probably the most important source of intake of mycotoxins. Intake of *Fusarium* toxins, such as trichothecenes and fumonisins, is almost solely due to consumption of cereals. Aflatoxins and ochratoxin A are associated with a wider range of food commodities, but significant amounts of aflatoxins may occur in maize. Ochratoxin A occurs mainly in small grains such as wheat, rye and barley, and intake estimates show that more than 50 % comes from these cereals. The hazard these toxins constitute, and the estimates showing that the tolerable daily intake may occasionally be exceeded, have triggered the establishment of regulations. It is therefore important, both from a consumer health aspect and from an economic point of view, to prevent mould growth and subsequent mycotoxin production in cereal crops.

### 7.1.2 What is HACCP

Quite simply, HACCP (Hazard Analysis Critical Control Point) is a food management system designed to prevent safety problems, including food poisoning. It was originally developed some 30 years ago in a collaboration between NASA, the Pillsbury company and the US army laboratories to produce 'absolutely safe food'

for US astronauts. However, the system proved so successful that it was quickly taken up by the food processing industry at large and today represents a global standard for safe practice. This is mainly because it provides for a proactive approach by means of process and materials control, rather than a reactive reliance on end-product quality control (QC) testing, which it has largely replaced. HACCP is a vastly more efficient tool for preventing food-borne hazards that typically occur at low incidence because it eliminates the need for exhaustive end-point testing. The HACCP approach involves conducting a detailed analysis of every step in a food processing operation by following seven clearly defined stages or 'principles'. These collectively describe the development and implementation of a complete HACCP plan.

## **7.2 Preparing a HACCP plan**

### **7.2.1 Initial stages**

Prior to the development of the HACCP plan proper, a number of elements need to be in place. The first of these will be an appropriate team of people who are suitably qualified to produce an effective plan. In conventional food manufacture this team will typically comprise management, technical, scientific and production staff, so that all aspects of the production system are represented. The team will probably also include a HACCP specialist, and a team leader whose job will be to keep this diverse assemblage of experts on-task!

The second element required is a full definition and description of the product, including customer specifications and the intended use and the user group(s). This will also typically include information on such parameters as physical/chemical properties, storage conditions, shelf-life and packaging details. This information is used by the HACCP team when they assess the possible dangers associated with the product.

The third element required is a full description of the operation under review. This normally involves preparation of a 'process flow diagram' (PFD), which describes all stages in the production process. This must be sufficiently detailed to form the basis on which an effective HACCP plan can be devised and implemented. It is important to note here that the PFD is likely to be unique to each production site even when comparing very similar products, so that the use of 'generic' PFDs for particular product types is not considered good practice, except for guidance purposes. Clearly the production of a suitably detailed PFD will require input from a number of members of the HACCP team, and is, in fact, one of the most important tasks that the team will normally have to perform. Production of the PFD should be followed by site or plant visits for verification purposes. This may be an exhaustive process in itself, involving checking all parameters associated with materials and processes.

## 7.2.2 The seven Principles of HACCP

### *Principle 1: Identification of hazards; assessment of risk; description of control measures*

Before we can proceed with a discussion of Principle 1, we need to define the terms ‘hazard’ and ‘risk’.

*A hazard is something which can have a negative effect on health.*

In terms of HACCP this means something that can cause a food item to be unsafe. In general we are able to identify three types of hazards:

- biological: e.g. pathogenic micro-organisms or their toxins, including mycotoxins;
- chemical: e.g. pesticide residues;
- physical: e.g. materials such as glass or metal fragments.

*Risk is the probability of an adverse effect resulting from a hazard.*

Principle 1 therefore consists initially of a thorough hazard analysis of the steps in the PFD. It essentially involves thinking of all of the things that could go wrong. This is likely to be based on prior experience, the experience of others in similar industries, and an imaginative assessment of what else may conceivably happen under various sets of circumstances. This is followed by the risk assessment, i.e. the likelihood of the hazards previously identified being realized. The purpose of this is to identify the most significant risks in the system, and perhaps to eliminate others. Principle 1 also involves describing the possible control methods needed to prevent the hazards identified from occurring.

Principle 1 could reasonably be described as the most crucial part of any HACCP plan. The ultimate success of the exercise will clearly hinge on the thoroughness and accuracy of these early analyses, which themselves rely on the mix of skills in the HACCP team. The perception of risk in particular is an area where knowledge and experience are essential, and in some instances expert opinion will need to be sought outside of that available within the core HACCP team.

### *Principle 2: Identification of the critical control points (CCP)*

Before we can discuss Principle 2, we need to define the term ‘critical control point’ (CCP).

*A critical control point is a step in a process where control is possible, and loss of control may lead to an unacceptable health hazard. A CCP may be a raw material, a location, a practice, a procedure or a process stage, but it must be specific.*

The process of designating critical control points involves consideration of *each* step in the PFD for each identified hazard. If an identified hazard *must* be controlled at a given step to give a safe final product (based on the risk assessment),

this then represents a CCP for that particular hazard. If a hazard exists at a step, and no control is in place, then controls must be designed, at this stage or perhaps some other stage, to give a safe final product. One very important point regarding CCPs is that they must contain an element that is quantifiable in real-time. This includes simple parameters such as temperature, time and moisture content that can be monitored and adjusted on-line.

The task of identifying CCPs can be carried out with the help of a 'decision tree'. There are a number of examples available. One version is shown in Fig. 7.1.

In practice, the questions in the decision tree are applied to each step, for each hazard. The tree then helps to identify the steps where the hazard is relevant, and whether control *must* be imposed at that step. The decision tree effectively identifies two situations where CCPs exist:

- Where a step is *specifically* in place to control a hazard (Q. 2).
- Where a step is vulnerable to the hazard, and control is not available elsewhere in the system (Q. 4).

A classic example of a CCP is the pasteurization step used first in the dairy industry. This step is in place specifically to destroy dangerous micro-organisms that may be present in the system (biological hazard), and is crucial to the safety of the final product.

*Principle 3: Establishment of critical limits*

*Critical limits are the tolerances applied to CCPs which maintain a safe condition.*

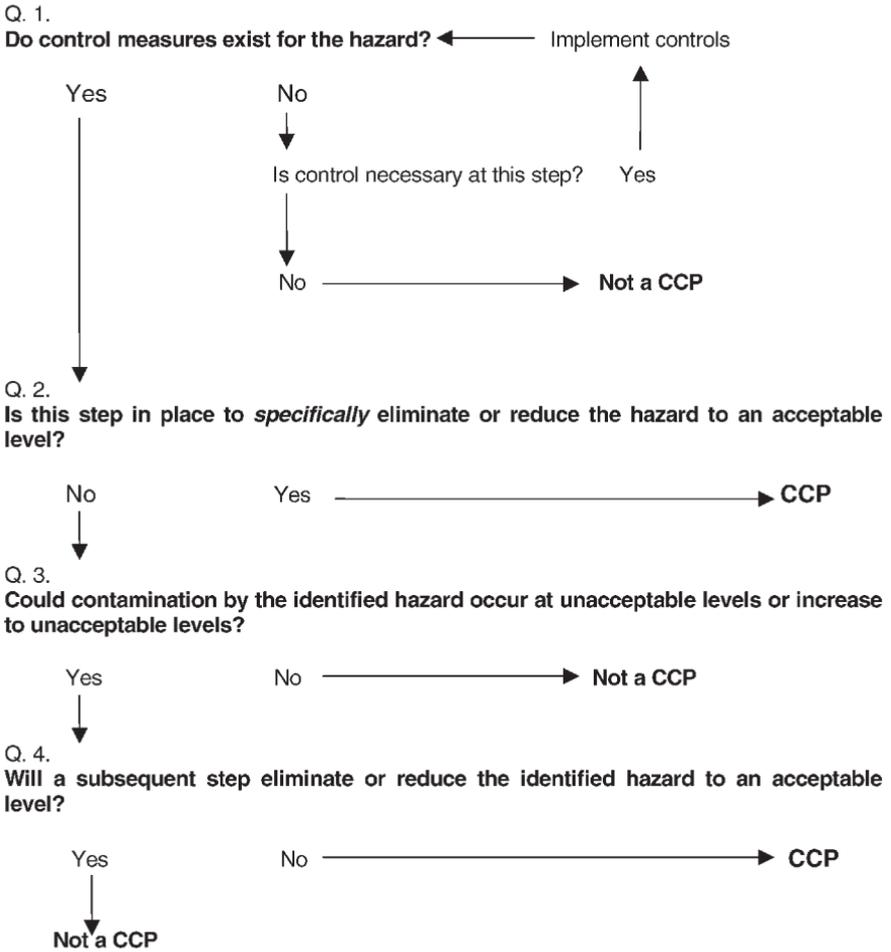
The critical limits are usually simple measurable parameters, such as temperature and time, which will ensure that control is maintained at the CCP. However, regulatory mycotoxin limits may in some occasions be used as critical limits, such as at mill intake.

*Principle 4: Establishment of procedures for monitoring of CCPs and*

*Principle 5: Establishment of corrective actions*

The design of monitoring procedures is an extremely important aspect of the HACCP approach, since effective monitoring is key to maintaining control of each CCP. The establishment of appropriate corrective actions (where monitoring reveals that critical limits are not being achieved) then follows directly from this. Corrective actions will also specify procedures for handling material that has been subject to a loss of control.

Monitoring and correction of control parameters is carried out on-line, so the parameters need to be relatively simple and rapidly adjustable. The establishment of monitoring procedures and corrective actions involves producing detailed written protocols, including consideration of operating procedures, instrumentation and staff training.



**Fig. 7.1** Critical Control Point decision tree (adapted from Codex Alimentarius, 1993, and Mortimore and Wallace, 1998).

*Principle 6: Establishment of verification procedures*

Verification means checking that the completed HACCP plan actually *works*. This will involve checking the entire system against all of the identified hazards to ensure that the CCPs in place are performing correctly. Any problems found at this stage are then corrected and re-verified. It may be necessary to add entirely new CCPs into the system, or perhaps amend the critical limits assigned to existing CCPs.

*Principle 7: Documentation and record keeping*

As with all procedurally based systems, documentation forms an extremely

important element. All stages in the formulation of the plan will be documented, including records of the PFD, hazard identification, severity and probability (risk), critical limits and verification procedures. Possibly more important will be documents that describe monitoring procedures and corrective actions, as these will form the basis of training documents and operating instructions for relevant personnel. Documentation will also include routine record keeping of operating parameters, particularly those related to monitoring of CCPs.

### **7.2.3 HACCP and pre-requisite programmes**

In food storage and manufacture, HACCP normally operates within the framework of a Total Quality Management (TQM) system such as ISO 9000. Integrated into this will be pre-requisite programmes such as Good Manufacturing Practice (GMP), and Good Storage Practice (GSP). These codes of practice are concerned with the design of facilities, control of operations, maintenance, sanitation and training of personnel.

Good Agricultural Practice (GAP), is collectively a large set of codes of practice which feature as part of the Codex Code of General Principles on Food Hygiene (1997). These codes are concerned with all aspects of primary food production, including environmental protection and sustainability, economics, food safety, food quality and health security. In the UK some aspects of GAP, together with other codes of practice, are published by DEFRA and the Health and Safety Executive. GAP includes a related code, Good Hygienic Practice (GHP), which is concerned with the handling of the harvested commodity, and sometimes also GSP, where a commodity is stored on-farm.

All of these codes of practice are generally considered to be complementary to HACCP, helping to simplify and 'streamline' the plan by reducing the number of CCPs necessary. Indeed, the Codex Code of General Principles on Food Hygiene (1997) in particular advocates the integration of HACCP with codes of practice in the development of manageable HACCP plans, and the Food and Agriculture Organization (FAO) of the United Nations is, at the time of writing, reviewing and developing the GAP approach to primary food production.

In some instances, however, there does seem to be a danger associated with the reliance of pre-requisite programmes within HACCP. A large proportion of instances of food poisoning outbreaks emanating from food processing plants have been shown to be associated with failures not in HACCP schemes directly, but in aspects such as cleaning and hygiene, which fall within the remit of pre-requisite schemes. In fact the HACCP and pre-requisite schemes are very different, since the former identifies critical steps in a system, while the latter assumes all aspects are of equal importance. This difference in philosophy could result in failure to identify CCPs, due to over-reliance on pre-requisite programmes, and failure to direct resources to crucial points in the system. We will explore the relationship between HACCP and codes of practice again later in the chapter, when we discuss aspects of GAP relating to the cereal pre-harvest situation, which is not generally associated with HACCP at present.

**Table 7.1** Summary of some HACCP schemes developed for mycotoxin control in various crop types at the early stages of commodity supply.

Location	Crop/use	Mycotoxin	Reference
South-East Asia	Yellow maize, animal feed	Aflatoxin B1	FAO/IEAE (2001)
South-East Asia	Coconut for copra cake and meal (animal feeds following extraction of oil)	Aflatoxin B1	FAO/IEAE (2001)
Southern Africa	Groundnuts, peanut butter manufacture	Aflatoxin	FAO/IEAE (2001)
South America	Apple, juice	Patulin	FAO/IEAE (2001)
West Asia	Pistachio nuts	Aflatoxin B1	FAO/IEAE (2001)
Tropics and subtropics	Coffee	OTA	Frank (1999)

### 7.3 Applying HACCP systems to mycotoxin control

In one sense, the application of HACCP to the control of mycotoxins is already well established. If we consider the latter end of food production, i.e. where materials enter a food processing plant and a finished product results, here mycotoxins are typically well understood (for relevant industry sectors) and are treated as one of the hazards to be considered in the development and maintenance of a HACCP plan. There will typically be ‘approved supplier’ schemes in place for incoming materials, including permissible limits for mycotoxin content, backed up by documentation. In many cases, the materials entering such facilities have been subject to some degree of prior processing and are quite distinct from the raw materials from which they were originally derived. This chapter is mainly devoted to a consideration of the application of HACCP type principles to the earliest stages of food production; to the crop in the field, at harvest and the stages which follow on, including primary storage and primary processing. In fact we will be taking a holistic view ‘from field to table’ or ‘from plough to plate’ and we will consider the potential for HACCP to play a wider role in food production – beyond the areas where it has become an industry standard. Table 7.1 shows a summary of examples of HACCP schemes that have been devised for mycotoxin hazards in agricultural products during the early stages of production.

#### 7.3.1 Why HACCP for mycotoxin control?

As we have already seen, HACCP takes a proactive approach to hazard control, an approach of ‘prevention is better than cure’, and this is a particularly appropriate attitude to take with mycotoxins. The presence of mycotoxins in a finished product is often the result of events and circumstances affecting commodities much earlier in the production chain. A preventative approach spanning the entirety of the

commodity supply chain could therefore represent a very effective strategy. There are two other reasons for taking a proactive approach to mycotoxin control.

- Mycotoxins tend to be stable compounds that are difficult to remove once formed; in particular they tend to survive many of the processing stages involved in food manufacture.
- Mycotoxin analysis is typically complicated, expensive and time-consuming. These factors mean that a QC approach to mycotoxin control, involving extensive end-point testing, is not currently economically feasible or practical.

### 7.3.2 Mycotoxins as hazards

In order to develop HACCP plans for mycotoxin control, we must first establish them as significant hazards within the food industry. In fact, it is relatively straightforward to identify at least some mycotoxins as important health hazards, mainly due to research that has been carried out relatively recently.

Although mycotoxins are chemical entities, they are classed as biological hazards because their presence is always as a direct result of fungal contamination at some point in the system. This is in spite of the fact that, in some instances, mycotoxins may be present in a commodity where no trace of the fungus responsible remains. This can occur because, as we have previously observed, mycotoxins are typically very stable, and can survive processes that will eliminate the fungi that originally produced them.

There is a relatively large number of compounds produced by fungi that are classed as mycotoxins, and of these around ten are currently considered to pose a significant threat to human health. All of these are associated with specific food groups, and are produced under certain circumstances, and sometimes at specific stages in commodity production. For example, the trichothecene mycotoxins including nivalenol (NIV) deoxynivalenol (DON) and T-2 toxin are produced in growing cereal crops by fungi belonging to the genus *Fusarium*. Contamination is therefore a field event. However, the mycotoxin ochratoxin A (OTA), produced by the fungus *Penicillium verrucosum*, again occurs on cereals, but generally only during storage of the harvested grain. This reflects certain physiological differences between the producing fungi and the ecological niches they occupy in nature. Some mycotoxins are associated with certain geographical locations. This is generally the case for mycotoxins that are formed in growing crops and may reflect the requirements of the producing fungus, the host plant, or both. However, mould growth and toxin production may also occur on food products (bread, fruit jams, cheese, etc.). The fungi growing on the processed product may differ considerably from those occurring on the raw material, due to changes in the composition of the substrate and the requirements of the producing fungi. In other words, moulds that grow on bread are not necessarily the same as those that grow on unprocessed cereals and may produce mycotoxins that are not expected in cereals.

There is generally a growing understanding of the importance of mycotoxins,

both in terms of acute and chronic disease, to the extent that in many countries permissible limits for relevant food groups are either planned or already in place. There is currently a large body of data available which details the mycotoxin hazards associated with a wide range of commodities. However, for the purposes of developing a HACCP plan, where published data is lacking, mycotoxin analysis and surveillance of the product and the relevant supply chain will become necessary.

### **7.3.3 The HACCP team**

As has already been described, one of the most important preparatory stages for the development of a successful HACCP plan is the appointment of an effective team of people to carry out the work. In the case of a plan to control mycotoxin hazards in all stages of commodity production, a very diverse assemblage of experts may be required. The team will clearly need a microbiologist or a mycologist with a knowledge of mycotoxins themselves. The team is also likely to include experts in farming, storage, distribution and trading, to cover the early stages in commodity flow. Latter stages in production, e.g. primary processing and final processing, are then likely to be covered additionally by process engineers and others with specific knowledge in these areas.

### **7.3.4 The commodity flow diagram**

In consideration of a holistic approach to mycotoxin control, the PFD concept has to be extended to describe the whole commodity chain. This is then called a 'commodity flow diagram' (CFD). CFDs that are sufficiently detailed and accurate to be used as the basis for a HACCP assessment are likely to be complex documents. They are also key to the success of the final HACCP plan. A typical CFD will cover all aspects of primary production, i.e. the crop development stage, including land preparation, crop development and harvesting. It will then be concerned with drying, storage, transport and, finally, processing steps. The CFD is made more complex by the nature of modern commodity supply, where products typically pass between different groups of 'owners' such as farmers, traders, transporters and processors.

It is likely that the CFD produced will be very specific, not only to the commodity type, but also to the final product type, climatic zone and country of production. Local differences will always be encountered, even for products that may appear to be identical, and the development of the HACCP plan will need to be approached from 'first principles', rather than relying on 'generic' plans developed for similar products. When developing plans in unfamiliar locations it is essential to take into consideration the local social and economic situation and the constraints which may be in place in that area. This is particularly true in some developing countries where, for example, expensive chemical treatments such as pesticides may not be available for the treatment of crops. Under such circumstances additional skills may be required within the HACCP team, such as

personnel with local knowledge of the socio-economic factors operating within a commodity system.

## 7.4 Mycotoxin risks in wheat

Wheat is arguably the most important crop grown in Europe. It represents the 'starchy staple' in the diet of the vast majority of Europeans. Significant mycotoxin contamination of the wheat supply could therefore represent a very important threat to human health. Unquestionably widescale poisoning has occurred in the past. In fact it is thought that early use of wheat as a replacement for rye during the thirteenth century caused a massive depopulation of western Europe, due to the presence of *Fusarium* toxins (FAO/IAEA, 2001). As recently as the Second World War (1939–1945) thousands of people died in Siberia from a condition known as 'alimentary toxic aleukia' (ATA) caused by the consumption of grain grossly contaminated by *Fusarium* toxins. (Sarkisov *et al.*, 1944; Joffe, 1978). Today, it is normal to detect mycotoxins in wheat samples, but usually at levels that are not currently considered to present an acute health hazard. However, it must be acknowledged that globally the potential for problems certainly exists within the production system. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) recognized that DON can cause outbreaks of acute illness in humans (JECFA, 2001). Furthermore, JECFA noted that the provisional maximum tolerable daily intake (PMTDI) for DON may be exceeded in four of the five regional diets, although there were several uncertainties in the values used in the assessments. Although the following sections are concerned primarily with wheat, much of the discussion will be relevant to related cereals such as rye, triticale and barley.

In the modern European production system, there are two main mycotoxin hazards associated with wheat.

- Mycotoxins produced predominantly by fungi of the genus *Fusarium* (e.g. *F. culmorum*, *F. graminearum*, *F. sporotrichioides*). Toxins include NIV, DON, T-2 toxin and zearalenone (ZEA). The first three are trichothecenes, while ZEA is unrelated chemically, but may be produced by some of the same fungal species that produce trichothecenes (e.g. *F. culmorum*). DON, or vomitoxin, is probably the most important of the trichothecenes in terms of amounts produced in wheat. All of these mycotoxins contaminate the growing wheat crop pre-harvest, as a result of infection of the living plants by the relevant *Fusarium* species. In fact a number of these species are important plant pathogens causing a disease called *Fusarium* ear blight (FEB) or *Fusarium* head blight (FHB). This disease is important economically as it can significantly affect crop yield and quality. Although *Fusarium* infection is generally considered to be a pre-harvest event, there is also potential for further growth and toxin production to occur post-harvest, if grain is stored with an abnormally high moisture content.
- Ochratoxin A produced by the fungus *Penicillium verrucosum*. OTA is in fact the most important of a related group of toxins produced by *P. verrucosum* in

temperate regions. OTA is also produced by *Aspergillus ochraceus*, but this species appears to be rare in grain (Miller, 1994). The main importance of OTA is its powerful acute and chronic toxicity to the kidneys. In contrast to the situation with *Fusarium toxins*, OTA contamination is normally a post-harvest event. This is because *P. verrucosum* is adapted to grow in relatively dry conditions (it is described as 'xerophilic'), and can therefore develop in the storage environment if the moisture content is only slightly above the critical limits. As we shall see later in the chapter, bulk grain storage presents a number of technological challenges, and scope certainly exists for spoilage and contamination to occur at this stage in production.

#### 7.4.1 FEB and mycotoxin production

As discussed above, growth of fungi belonging to the genus *Fusarium* can lead to the development of both FEB and mycotoxin contamination in the growing wheat crop. FEB is in fact a disease *complex*, meaning that it can be caused by a number of individual *Fusarium* species alone or by a combination of these species. These fungi are found in virtually every continent in the world and cause a number of disease symptoms such as head blights, seedling blights and foot rots to cereals (both wild and cultivated), and a number of other plant groups (Meekes and Kohl, 2002). In Northern Europe the dominant species implicated in FEB of wheat is *F. culmorum*, while in Southern Europe this is replaced by *F. graminearum*. However, the situation is dynamic, and at the time of writing *F. graminearum* is spreading northwards and replacing *F. culmorum* as the dominant species. It should be noted that both of these fungal species are also known producers of mycotoxins including DON and NIV, and it may be that mycotoxin production is associated with FEB; in some cases a link has been established between aggressive onset of FEB and high mycotoxin levels (Mesterhazy, 2002), and it is known that DON is toxic to plants. Other species associated with FEB are *F. avenaceum*, and *F. poae* (also toxin producers) and closely related *Microdochium* species such as *M. nivale* varieties (not known to produce toxins) (Meekes and Kohl, 2002).

One of the most important aspects of the *Fusarium* disease cycle is the overwintering of the pathogens between growing seasons. All of the fungal species responsible for disease, with the exception of *F. poae*, are able to survive saprophytically (i.e. grow on dead material), and therefore persist in crop residues remaining in the field following harvest (Meekes and Kohl, 2002). This has important implications for field management practices necessary for control of FEB and mycotoxin development as will be discussed in Section 7.5.1 below.

The main difficulty with FEB is that control measures such as resistant wheat cultivars and fungicides ideally need to be equally active against the whole range of disease-causing organisms to be effective. In practice this is not attainable so that combination strategies have to be adopted involving, for example, partially resistant cultivars, fungicides and field management strategies. In fact, such an integrated type of approach lends itself to HACCP type management, where the overall situation is carefully analysed and decisions made on the important steps to

be taken in controlling the hazard(s). However, there is an additional difficulty which makes the situation even more complex: the control measures mentioned above are in place to tackle the problem of FEB, and not necessarily the problem of mycotoxin production. There are obvious reasons for this; FEB is an important and well established disease in wheat with a potential to cause significant economic losses in crop yield and quality. Mycotoxins, however, are far more obscure entities and, until recently at least, their importance has probably been underestimated. There is also still much to learn about their production in the field. What is becoming clear, however, is that a very complex relationship exists between FEB and mycotoxin production, and some treatment strategies which appear to control FEB may exacerbate the mycotoxin problem. Examples of this will be discussed below.

#### 7.4.2 Studies on environmental conditions and development of *Fusarium* mycotoxins

Environmental conditions such as relative humidity and temperature are the most critical factors controlling the development of FEB. In particular, moisture levels at anthesis are critical in ear infection, and Lacey *et al.* (1999) have shown that high levels of infection at this time are clearly related to wet weather. In addition, warm and relatively dry conditions early in the growing season have been shown to encourage *Fusarium* foot rot and the build-up of infective material on plant stem bases (Meekes and Kohl, 2002). Until recently, however, little has been known about how these critical factors influence mycotoxin production by *Fusarium* species.

Hope and Magan (2003) carried out *in vitro* studies on DON and NIV production by a strain of *F. culmorum*. They looked at the influence of water availability (measured as water activity\*) and temperature, and found that the conditions under which the toxins were produced were narrower than the conditions that permitted growth of the fungus. In particular, toxin production only occurred under the relatively high moisture conditions that supported the fastest growth of the fungus. The moisture range giving optimal production of both toxins was 25–30 %, which

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\*The most relevant parameter when considering moisture in relation to fungal growth is not the water content of a substrate but the water activity ( $a_w$ ). Water activity is the proportion of the water present in a substance that is *free*, i.e. it is not bound to the substance either chemically or physically, and is therefore immediately available to the fungus to support its growth. Fungal growth is therefore directly related to water activity rather than water content *per se*. Water activity is equivalent to per cent equilibrium relative humidity (ERH) of a hygroscopic substance (i.e. the proportion of water associated with the air around the substance at equilibrium), and can therefore be measured using an electronic hygrometer, which actually measures ERH. Water activity is measured on a scale from 1.0 (pure water) to 0.0. (water absent). The growth limit for the fungi that are most resistant to dry conditions ('storage' fungi) is around 0.7 on this scale. The relationship between water activity and water content for a given substance depends on the physical and chemical make-up of the substance. This relationship can be determined by plotting these two parameters in a graph known as a 'moisture sorption isotherm'. The importance of determining the relationship is that 'safe' water activities can be translated into 'safe' moisture contents, as it is moisture content that is normally measured in the grain commodity supply situation.

is within the normal range for harvested grain in wet years. The optimum temperature for toxin production was 25°C. This study therefore demonstrated a clear potential for high levels of contamination in relatively warm, wet years. Information such as this helps to predict when mycotoxin risk will be greatest, based on monitoring of weather conditions.

This idea has been further developed by Hooker and Schaafsma (2003) who correlated weather records and variables relating to cultivars and field management history, with DON data for around 400 Canadian wheat fields between 1996 and 2000. They produced a predictive model called 'DONcast' which could be used to advise growers when it is necessary to treat crops with fungicides, and they suggested the initiation of a grower-interactive test model for this purpose. The model identified three periods when weather conditions can critically influence DON levels in harvested crops. These periods were 4–7 days before heading (development of ears), 3–6 days after heading and 7–10 days after heading. These periods probably correspond to stages in development where the plant is most susceptible to fungal invasion. The use of such a predictive model can be integrated into a HACCP plan for mycotoxin control, by identifying high risk conditions and allowing critical controls to be implemented.

## 7.5 Pre-harvest mycotoxin control strategies

In this section, control strategies possible at the pre-harvest stage will be discussed. These strategies will be considered in the context of a holistic HACCP scheme for mycotoxin control in wheat production.

### 7.5.1 Field preparation and management

Appropriate field management is particularly important for control of both disease and mycotoxin production by *Fusarium* species. Strategies include the following.

#### *Deep ploughing*

This removes residual material from the previous crop and buries it below the surface. This takes away the fungal inoculum along with the debris and helps to break the 'chain' of infection. In general 'no tillage' methods are not appropriate for effective *Fusarium* control because decomposition of the previous crop residue is slower when it remains on the surface and the fungal inoculum present on the residue remains viable between crops.

#### *Crop rotation*

This is designed to prevent the build-up of infection. Appropriate crop rotations involve wheat/legumes/brassicas/potato etc. Maize is not useful in this instance as it is also host to *Fusarium* disease, and its residue is particularly recalcitrant, thus harboring high levels of infective material. (Meekes and Kohl, 2002). The use of maize may, in fact, cause higher disease levels than wheat monocropping.

### *Use of fertilizers*

This is related to plant vigour in general, and the ability to resist infection. In particular, adequate levels of nitrogen may be important in disease resistance.

### *Weed control*

*Fusarium* species have an extremely wide host range, both monocotyl and dicotyl species, so weed control may be important in disease control. However, the contribution made by weed species towards disease occurrence is currently unknown.

## **7.5.2 Crop management**

Control measures relevant to the crop are listed below.

### *The use of disease resistant cultivars*

In fact, at present, no durable, fully FEB resistant varieties of wheat exist, partly because of the complex nature of the disease. However, the use of partially resistant varieties is of immense importance in an integrated approach to disease and toxin control: it has been shown that wheat cultivars demonstrating resistance to highly pathogenic, and high DON producing strains of *F. culmorum* and *F. graminearum* are able to reduce both FEB and DON production (Mesterhazy, 2002). This suggests a link between pathogenicity and toxin production, although pathogenicity is not believed to depend entirely on toxin production: toxins are thought to contribute to aggressiveness, i.e. the extent of infection, but not to the ability to cause infection. In fact, the whole area of plant resistance is extremely complex, and a number of specific resistance strategies are known, including

- (i) resistance to initial infection;
- (ii) resistance to kernel infection;
- (iii) resistance to spreading;
- (iv) tolerance to infection and
- (v) resistance to mycotoxin accumulation (Mesterhazy, 2002).

Resistance to mycotoxin accumulation is of particular interest here, as this specifically targets toxin production. However, the mechanisms behind this (and the other types of resistance listed) are not well understood at present. In the future, breeding or genetic engineering of plants that demonstrate resistance to mycotoxin production and/or accumulation, or perhaps carry detoxification genes, could provide specific control measures for mycotoxins, and therefore could act as a CCP in HACCP type approaches. Critical limits attached to such a CCP would relate to the selection of certified seed. Conversely, resistance strategies that are not directly concerned with toxin production could, in theory, induce pathogen responses leading to increased toxin production, so caution would need to be exercised in the development of resistant cultivars of this type. This is particularly important since a number of different toxin producing fungi may be present, and each could behave differently with respect to host resistance. In general, much research concerning

host/pathogen interactions, the effects of environmental parameters, and the underlying mechanisms of resistance still needs to be carried out.

#### *Appropriate irrigation methods*

Irrigation itself can be viewed as an important control measure by preventing drought stress occurring during periods of dry weather (drought stress is associated with increased disease susceptibility). However, the question of irrigation has to be approached with care: there is evidence that *Fusarium* infection occurs primarily by initial infection of wheat flowers (i.e. at anthesis). Water splash is thought to be an important means by which the fungal spores reach the flowers, so irrigation methods that minimize splashing, e.g. the use of rills rather than sprinklers, may be an important means of control. However, the use of alternative irrigation methods is not always possible, and a more practical solution may be to avoid irrigation during anthesis, when the plants are most susceptible. In fact, since a link has been established between wet weather at anthesis and high levels of infection (Meekes and Kohl, 2002), there would appear to be good reasons for curtailing irrigation during this period.

#### *The use of biological control agents (BCAs)*

This does not represent a practical control measure at present, but involves applying harmless fungal species to the crop that are able to compete with and inhibit the pathogenic and toxigenic species. It is fair to say that this approach is still in its infancy, particularly for application to cereals, and much research needs to be done before commercial products can be made available. However, BCA technology has the potential to reduce the input of fungicides into the production system, which is currently a major goal in global agriculture. A recent EC Project under Framework Programme 5 (QLK1-CT-1999-00996; acronym: 'Control Mycotox Food') has made important progress with this approach. The work involved both glasshouse and field trials, and resulted in the identification of a range of candidate antagonistic fungal species which showed the ability to inhibit both development of FEB and toxin (DON) production significantly. The BCA approach shows particular promise because there is only a narrow window of around 5–10 days (i.e. at anthesis) when the crop is susceptible, and targeted spraying at this time could provide significant protection. Dawson *et al.* (2002a, b) have demonstrated the potential for application of antagonists to cereal stubble, decreasing the level of inoculum for infection of the subsequent crop. Some results to date suggest that BCAs may be more effective at reducing mycotoxin production than at eliminating FEB. Therefore, in principle, BCAs could be used to specifically target mycotoxin production, and their application would then represent a CCP in future HACCP plans.

#### *The use of fungicides*

Fungicide use probably represents the most important method used to control FEB, and a number of products have been developed by major agrochemical companies specifically to address this particular disease. Their use is made relatively simple

due to the short period of time when the crop is at greatest risk of infection (at anthesis), during which fungicide protection may be required. Therefore a modern, integrated approach to disease control will almost certainly continue to involve the use of fungicides. However, the situation becomes far more complex when the production of mycotoxins is considered. This is because recent evidence suggests that the use of some fungicides, under certain conditions, can actually stimulate and increase the production of mycotoxins. In the worst cases, this could occur with an apparent reduction in the incidence of disease, so that grain contaminated with mycotoxins, but which appears (visually) reasonably healthy, may be produced. At present, specific mycotoxin analysis does not occur frequently in the supply chain, and grain affected in this way would not be readily detected by visual inspection for disease.

The main reason for this fungicide effect seems to be the nature of the disease itself. As we have previously observed, FEB is a disease complex, with a number of fungal species implicated, and the fungicides that are currently available are not equally effective against all of the fungi that may be present. For example, certain products have been shown to have differential effects against toxin forming *Fusarium* species in comparison to non-toxin forming *Microdochium nivale* varieties (Simpson *et al.*, 2001). The outcome of the use of a particular fungicide will depend on the mixture of disease causing species present, and their relative susceptibilities to that fungicide. For example, in a study carried out in the UK by Nicholson *et al.* (2003) for the Home Grown Cereal Authority (HGCA), the use of azoxystrobin showed a significant reduction in FEB, while increasing the level of DON in the grain. In this case the primary fungal species present were *F. culmorum* and *M. nivale*, both competing for the plant resources. It is known that azoxystrobin is very effective at inhibiting *M. nivale*, so it seems likely that this species was selectively inhibited by the fungicide, allowing development of the toxigenic *F. culmorum* with concomitant increase in toxin levels. Overall disease levels were probably reduced because one of the disease causing species had been eliminated. In the same study fungicides including tebuconazole and metconazole were shown to work in the opposite way, by selectively inhibiting *F. culmorum* and having a less pronounced effect on *M. nivale*. These results suggest that it is necessary to determine which pathogens are present to make an informed choice on fungicide use. However, currently this is unlikely to be possible in most practical situations. Other fungicides that, under certain conditions, have been shown to stimulate toxin production include tridemorph (which stimulated T-2 toxin production by *F. sporotrichoides*), tubiconazole (which stimulated production of monoacetyl deoxynivalenol) and difenoconazole (which stimulated production of monoacetyl deoxynivalenol) (Magan *et al.*, 2002).

Work carried out by Nicholson *et al.*, (2003) has looked at the effect of using fungicides at application rates lower than those recommended by the manufacturer. This is because it has been suggested that the use of sub-optimal levels may induce a stress effect in the fungi resulting in increased production of toxins, perhaps above levels found where no fungicide is employed. In fact, little evidence to date supports this view: fungicides such as tebuconazole and metconazole were

able to reduce toxin production at levels that were insufficient to eliminate FEB. This is an important finding since it appears to indicate that dosage imprecision is not a major risk factor for enhanced mycotoxin production. However, this does not mean that sub-optimal fungicide levels can be employed: generally any reduction in rate away from the recommended levels will reduce overall efficiency in terms of both FEB and mycotoxin control.

The situation regarding the use and efficacy of fungicides to combat both FEB and mycotoxin production is therefore complex and suggests that a far more detailed understanding of the nature of the field situation is required. Although fungicides will certainly continue to represent the most important tool in the control of FEB, there are a number of important risk factors identified in relation to mycotoxin production, relating in particular to the mix of fungal species present in a given situation. In addition, given the range of species of fungi present and the ability of these fungi to develop resistance, it is unlikely that fungicides alone will ever represent a complete control solution. In general, fungicides with main activity against *Fusarium* species are most likely to be effective at preventing mycotoxin production in the field. Future control strategies for FEB and mycotoxin production will probably be in the form of combinations of products, incorporating *Fusarium* active agents for specific mycotoxin control. In a HACCP type approach, this type of fungicide use could represent a CCP.

### **7.5.3 The relationship between GAP and HACCP at the pre-harvest stage**

The most important pre-requisite programme which could be used in support of HACCP at the pre-harvest stage is GAP, which also invokes GHP immediately after harvest. Within the UK, most crop production is carried out within some form of crop proficiency scheme, e.g. the Assured Combinable Crops Scheme (England and Wales) and Scottish Quality Cereals (Scotland). Implicit in these crop proficiency schemes is the adherence to GAP principles.

The vast majority of the control factors discussed above may be considered to be part of GAP, and can be expected to be in place in the majority of wheat production. GAP principles are designed to produce a product that is sound, healthy and fit for purpose. In terms of *Fusarium* infection, the emphasis has been on the prevention of FEB rather than the control of mycotoxin production. However, in most instances it follows that crops free from fungal infection are also free from mycotoxin contamination (although one important exception, concerned with fungicide use, is discussed above), and it is probably fair to say that to date, mycotoxin control has been achieved almost by serendipity, given the general state of ignorance that has prevailed regarding these toxins. In considering control strategies for mycotoxin production that use the HACCP principle, particular attention needs to be paid to some of the aspects that may be considered as GAP for the purposes of FEB control, and these aspects may then be elevated to the status of CCPs within a HACCP scheme. An obvious example would be the use of fungicides: under certain environmental conditions their use is essential to prevent disease development, and we have shown in Section 7.5.2. that specific types of

fungicides need to be employed to control mycotoxins themselves. Deployment of such fungicides to specifically combat mycotoxin development would represent a CCP in a HACCP plan, and the critical limits would be represented by the recommended dosages of such fungicides. Similar arguments could be extended to the use of disease and mycotoxin resistant cultivars, and perhaps even biological control agents.

#### 7.5.4 The development of a HACCP plan at the pre-harvest stage

A hypothetical HACCP plan worksheet for the pre-harvest (and harvest) stage is shown in Table 7.2, and a simplified CFD for the pre-harvest stage is shown in the first two boxes of Fig. 7.2. In the table, column 1 identifies specific stages and hazards in the commodity chain, column 2 identifies the control type, column 3 identifies control methods (including monitoring methods where appropriate) together with corrective actions when control is lost. Column 4 identifies the critical limit(s). The decision tree shown in Fig. 7.1 has been used to guide these decisions. Few of the controls in place can truly qualify as CCPs in terms of the 'rules' we have established. None can guarantee the elimination of mycotoxins, or even their reduction to acceptable limits. Fungicide use has been designated as a CCP due to its central role in disease and toxin control and its potential for more targeted use in the future, specifically to address mycotoxin production. The other CCPs suggested are tentative choices which will depend largely on future research.

From the analysis shown in Table 7.2, it may seem that the role of HACCP in the pre-harvest stage is somewhat insignificant, and it is appropriate at this point to consider whether such an approach has value at this stage of production: we have discussed the complexity of the pre-harvest situation, in terms of the nature of FEB itself, and the variety of factors concerned with environmental conditions and farming practices that can influence the development of disease and mycotoxin production. Most experts believe that, at present, it is impossible to completely eliminate pre-harvest mycotoxin production. Given this, it is reasonable to ask whether it is appropriate, or even advisable, to attempt a HACCP type approach at this stage in wheat production.

We must remember that HACCP was originally developed for food manufacturing/processing type situations, where definitive control measures can be devised and implemented. The same levels of control cannot be expected in the field situation. In addition, farming is currently subject to intense economic pressure, and practices such as field management, cropping systems and chemical input may reflect these pressures more than best practice for disease/mycotoxin control. These arguments are certainly valid, and HACCP should not be expected to represent a panacea for mycotoxin control wherever the problems may be encountered. At the same time, we know that the pre-harvest stage is crucial in terms of the potential for mycotoxin development: we have already described how the development of *Fusarium* toxins is usually exclusively a pre-harvest event, and, as we shall see, no later stages in production are currently available (or desirable) that can

eliminate toxins produced at this stage. It is therefore wholly appropriate to apply the HACCP approach to this stage, and the detailed analysis that HACCP demands is probably the most efficient way to tackle such a complex situation. In an integrated HACCP approach to the pre-harvest stage, all the controls discussed will ideally work in a cumulative way to maintain the product within acceptable mycotoxin levels.

## **7.6 Mycotoxin control during harvest and post-harvest handling of wheat**

### **7.6.1 Harvest**

Harvest is the first stage in the production chain where moisture content becomes the most important parameter in terms of the management and protection of the crop. It also marks a shift from problems caused by plant pathogenic fungi, i.e. the *Fusaria*, to problems caused by storage fungi, especially *P. verrucosum*. Ideally, grain will always be harvested after a spell of dry weather when it is at a 'safe' moisture content, so that immediate drying is not necessary. However, this is not always possible, especially in northern Europe during wet years, and harvested grain immediately susceptible to fungal spoilage may result. Dealing with this type of eventuality requires accurate and reliable moisture content determination on-site, as well as an infrastructure in place for prompt and efficient bulk movement and drying of the commodity. Another important control measure at harvest will be visual examination of the grain for symptoms of disease, and the segregation of diseased batches from healthy grain. Recent research has highlighted the potential presence of *P. verrucosum* in residues left in combining equipment (Frisvad and Lund, 2003) so GHP at harvest will represent an important control step.

### **7.6.2 Post-harvest movement of grain**

The post-harvest stages in wheat production are those stages following harvest and leading up to primary processing of the grain, e.g. milling. This will typically involve drying (if required), storage and transportation steps. As we have previously observed, post-harvest movement of the commodity can be complex, passing as it may between a number of intermediaries such as traders and dryers, who may be situated at different geographical locations. In the simplest case, it may remain on-farm in store or buffer storage for a short period of time before being passed directly onto the processor. In more complex cases it may pass through the hands of grain merchants or third party drying facilities (if harvested wet) and held in storage for periods of time before finally arriving at the processor. At all times the grain can become susceptible to fungal contamination and mycotoxin production if the storage conditions are not strictly controlled. The

**Table 7.2** Summary HACCP worksheet for the pre-harvest and harvest stages in wheat production.

Stage/Hazard	Control type	Control measure	Critical limit(s)
<i>Field preparation:</i>			
Mono-cropping, allowing build-up of disease	GAP	Crop rotation that does not include maize	Adherence to GAP
Crop residue left in field allowing over-wintering of pathogens	GAP	Deep ploughing (tillage) to remove pathogens from the surface	Adherence to GAP
Lack of soil fertilization leading to sub-optimal plant health	GAP	Use of appropriate soil fertilizers	Adherence to GAP
<i>Crop development:</i>			
Selection of cultivar susceptible to FEB and mycotoxin production	GAP/CCP*	Selection of appropriate resistant cultivar. Future research may lead to cultivars specifically resistant to mycotoxin formation	Seed certification?
Warm/wet environmental conditions promoting fungal infection during critical periods	GAP/CCP	Monitoring weather/crop. Use of appropriate fungicides during critical periods. Application of recent research relating to fungicides and mycotoxins could lead to more targeted fungicide use	Adherence to GAP Use of recommended dosage of fungicide
	CCP*	Research work on alternative fungicides – biological control agents (BCAs) currently being undertaken but not yet available	Use of recommended dosage of BCA
Irrigation methods causing water transfer (splash) infection during critical periods	GAP	Irrigation methods preventing water splash, or cessation of irrigation during critical periods	Adherence to GAP
Lack of irrigation	GAP	Monitoring of crop. Irrigation as required	Adherence to GAP

Table 7.2 cont'd

Stage/Hazard	Control type	Control measure	Critical limit(s)
<i>Harvest:</i>			
Grain is harvested 'wet'	CCP	Moisture determination. Infrastructure in place for prompt transport, storage and drying. Use of new information (e.g. mathematical models) may allow better prediction of safe buffer storage periods relating to mycotoxin production	Moisture content >18 % requires immediate drying. Moisture content <18 % may permit buffer storage before drying depending on critical limits of moisture content, temperature and time
Grain becomes contaminated with spores of <i>P. verrucosum</i> from combining and transportation equipment	GHP	Inspection of equipment. Appropriate inspection, sanitation and maintenance of combining equipment. Training of personnel	Adherence to GHP
Grain showing signs of fungal disease above acceptable limit	GAP/GSP	Inspection and segregation	Adherence to GAP/GSP

Note 1: CCP = Critical Control Point; GAP = Good Agricultural Practice; GHP = Good Hygienic Practice; GSP = Good Storage Practice.

Note 2: CCP\* refers to possible future control measure.

range of possible movement of grain post-harvest is illustrated in the latter part of Fig. 7.2, which represents a 'composite' CFD for the wheat production chain.

### 7.6.3 Stored grain as an ecosystem

The post-harvest situation is normally characterized by activity of the 'storage' fungi, typically *Aspergillus* and *Penicillium* species that are able to grow in relatively dry conditions. As described in Section 7.4, the most important post-harvest mycotoxin in wheat production is OTA, produced (in temperate climates) by *P. verrucosum* (Lund and Frisvad, 2003). The importance of this organism was recently emphasized in a project sponsored by the EC under Framework Programme 5, which specifically looked at the problem of OTA in European cereal production (QLK1-CT-1999-00433: 'Prevention of Ochratoxin A in Cereals'; acronym: 'OTA PREV'). This project involved screening cereal samples across Europe for the occurrence of OTA producing fungi. Samples were taken at-harvest and at various points post-harvest, using traditional isolation and identification techniques as well as modern molecular 'fingerprinting' (amplified fragment

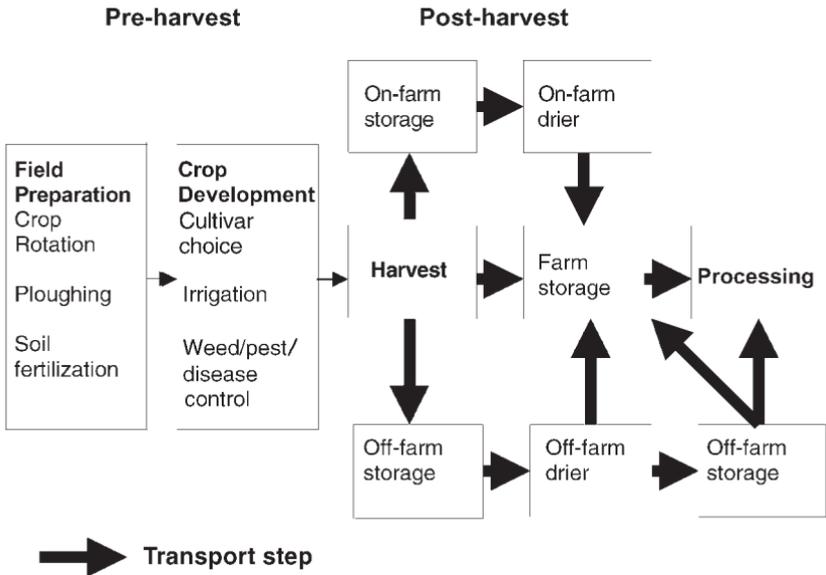


Fig. 7.2 Simplified diagram showing typical commodity flow possibilities for wheat.

length polymorphism or AFLP) methods (Frisvad *et al.*, 2003). Sample screening revealed that the sole OTA producing fungus involved in wheat production in Europe was *P. verrucosum*. Various strains of this fungus producing OTA were known in the UK, Germany, Austria, Norway, Sweden and Denmark, but the project revealed producer strains in Italy, Spain, France and Portugal for the first time. Work carried out in the UK, Sweden and Denmark has shown that contamination occurs post-harvest, and that harvesting, processing and storage equipment (combines, dryers and silos) are mainly implicated. This is a very important finding in terms of the development of HACCP plans since it enables a far more accurate hazard identification to be carried out in terms of the presence of the fungus within certain parts of the commodity chain. Also, by reference to its geographical distribution, high and low risk areas can be identified. It should, however, be borne in mind that the geographical location of a fungus is likely to change with time, especially under the current conditions of apparent global climate change.

Although *P. verrucosum* is clearly the most important fungus present post-harvest in terms of mycotoxin production, it will be present with other species, and interactions with other fungi may have an effect on growth and mycotoxin production. In fact, recent work has found that stored grain represents a dynamic, complex ecosystem where interactions occur depending on the fungal species present and the prevailing environmental conditions. Most importantly, the relative dominance of species may shift as a result of changing conditions, e.g. changes in water availability. Cairns *et al.* (2003) looked at competition for resources between *P. verrucosum* and a number of other spoilage fungi, and found that the system was in a state of flux, with the fungi tending to occupy separate niches,

based on utilization of the resources available, as conditions became dryer. *P. verrucosum* tended to become dominant in dry conditions, especially when tested against *Aspergillus ochraceus*, and was found to be relatively insensitive to high atmospheric CO<sub>2</sub> levels. These findings generally indicate that *P. verrucosum* is likely to be a dominant species in the stored grain ecosystem. Other work carried out by the same group (Magan *et al.*, 2003) looked at the effect of interaction and environmental conditions on OTA production by *P. verrucosum*. This work indicated that OTA production was strongly affected by competition between fungal species. Perhaps surprisingly, levels of OTA were shown to decrease in response to the presence of *Fusarium culmorum* and *F. poae* (this is surprising because some experts believe that mycotoxins have a role in competition between fungal species, and may therefore be expected to increase in the presence of other fungi). It should be noted, however, that these two species would not normally be present post-harvest, as they require higher moisture conditions, so this result may not be particularly relevant to a real situation.

Where insect storage pests are also present the situation becomes even more complex. Insect respiration can increase moisture conditions in the grain which may promote fungal growth, and insect damage to the grain can render it more susceptible to fungal invasion. Insects are also likely to interact directly with fungi, either by disseminating them throughout the resource or by feeding on them (Lacey and Magan, 1991). It is even possible that the presence of insects may stimulate the production of mycotoxins, although no work has been done in this area to date.

#### **7.6.4 The use of pre-requisite codes of practice at the post-harvest stage**

As discussed in Section 7.2.3, the use of a HACCP type approach at the post-harvest stage will invoke a number of codes of practice as pre-requisite programmes, supporting the overall plan. These are likely to include GAP, GSP and GHP, with GSP probably being the most important. The following factors are normally considered to be part of GSP.

- Appropriate condition of grain entering storage, including moisture content and disease levels.
- The soundness and suitability of storage buildings, including weatherproofing and the use of impermeable moisture barriers on floors.
- Sanitation of storage buildings and other equipment used for transportation and handling, including removal of previous crop residues, cleaning and insecticide application before use, where appropriate.
- Prevention of infestation by invertebrates and entry of rodents and birds into storage facilities, including insect trapping prior to use.
- Maintenance and monitoring of storage conditions, including moisture content, temperature and the presence of pests. This will require access to reliable, accurate and frequently calibrated moisture determination equipment.

- Infrastructure to identify and remedy problems promptly, including drying, segregating and transporting material as required.
- Appropriate record keeping and sample retention.

Clearly, these codes of practice are not in place specifically to control mycotoxin production, but if followed effectively will have this effect by helping to control fungal contamination. We will discuss the relationship between HACCP and post-harvest codes of practice such as GSP later in the chapter.

### 7.6.5 Major post-harvest control strategies in current use

Control at the post-harvest stage is dominated by one parameter – moisture content. Quite simply, if the moisture content is maintained at a ‘safe’ level, then no fungal growth and mycotoxin production can occur. ‘Safe’ water contents vary between commodity types. This is a reflection of their chemical make-up, which in turn dictates the relationship between water content and water activity. Any grain stored at a water activity of less than 0.7 will not support fungal growth. For wheat, this corresponds to a water content of around 14.5 %. Wheat grain is often harvested at moisture contents far in excess of this and will then need to be dried promptly following harvest, and maintained at an appropriate moisture content during all the storage and transportation steps that may follow.

Temperature monitoring is an extremely important factor during grain storage. Storage problems including pest infestation and fungal contamination will result in a rise in temperature. This can be detected at an early stage (and remedial action implemented) when effective monitoring is carried out.

Ideally, grain should be stored in conditions where temperature is controlled (i.e. cooled storage). Maintaining the grain at a low temperature has a number of beneficial effects, such as limiting the respiration of the grain (grain respiration generates water) and limiting the activity of insects and fungal contaminants. Temperature-controlled grain is also less likely to suffer from condensation due to ambient temperature changes, and is less likely to develop internal temperature differentials (‘hot spots’).

Quality control checks at the post-harvest stage include visual assessment of grain for evidence of fungal disease/contamination and pest damage. For grain entering storage after harvest, an important indication of *Fusarium* disease will be the presence of characteristic pink, shrivelled grains. During storage, visible signs of fungal contamination will only be evident when spoilage is at a very advanced stage. However, fungal growth may be detected early, indirectly, by temperature monitoring. Inspection for fungal disease and contamination is an indirect control for mycotoxins but, as we have noted earlier, it is possible to have apparently healthy looking grain that harbours significant levels of mycotoxins. Equally, highly diseased grain may contain little or no mycotoxins.

There are still a number of technological challenges associated with the bulk storage of grain that can hinder the maintenance of grain quality. These are

concerned with the development of heterogeneous conditions within the store which can lead to localized spoilage. There is also a fundamental difficulty in obtaining truly representative samples from bulk stores, which are needed to effectively monitor the quality of the grain. Many of the problems currently encountered in storage can probably be remedied by further research on storage facility design and storage practices. The recent EU project 'OTA PREV' surveyed conditions in out-door silos over a two year period, and made the following observations and recommendations.

1. Leakage of water into the silo is a surprisingly common problem, often leading to fungal growth and mycotoxin production.
2. The type of top ventilation used seems to be very important. In general, ventilation hoods, mounted in a sheltered position, have been shown to be most effective.
3. Fungal growth and mycotoxin production appeared to occur mainly in the upper layer of the grain. It was recommended that this layer should be inspected and affected material removed before unloading the silo.
4. It was recommended that silos are equipped with temperature monitoring and aeration systems to help to control temperature and reduce condensation.

Work within the 'OTA PREV' project has developed a mathematical model to predict 'safe' storage times for grain under differing conditions of moisture and temperature. The 'safe' time before detectable growth of *P. verrucosum* and OTA production was assessed. Results indicated that grain at a high moisture content (above 20 %) can only be safely buffer stored for a few days, and ideally should never be buffer stored at a moisture content above 18 %. The risk of OTA contamination was shown to be clearly related to the amount of *P. verrucosum* present in the grain (OTA levels increased significantly in experiments where greater than 1000 colony forming units were present per gram of grain). These results represent critical limits that could be applied to controls in a HACCP approach. The model is still unpublished (Jonsson *et al.*), but information concerning its publication will be presented on the project's web page (<http://www.slv.se/OTAPREV>).

#### 7.6.6 Use of preservatives

There are no chemical preservatives in common use in the wheat commodity chain at present. Exceptionally, modified atmosphere gases may be used to preserve grain. These include the use of elevated levels of CO<sub>2</sub> and N<sub>2</sub> and decreased levels of O<sub>2</sub> in sealed stores, although this has mainly been used to control insect pests and would not be likely to prevent fungal growth. Sulphur dioxide (SO<sub>2</sub>) and ammonia (NH<sub>3</sub>) have also been used on occasions to inhibit fungal growth in low temperature stores (Lacey and Magan, 1991).

A number of chemical substances, including commercial fungicides in use for other applications, have been evaluated for possible use as preservatives for wet

grain. However, their use has generally been restricted due to poor performance and possibly also cost considerations: only propionic acid (a preservative used in bakery products) has been used in farm storage (Lacey and Magan, 1991). More recent work carried out within the EU Projects 'Control Mycotox Food' and 'OTA PREV' has taken an alternative view of chemical preservation by looking at the potential of using antioxidants, essential oils from plants, extracts from bacteria, fungi and lactic acid bacteria for this purpose. Amongst many candidate materials screened, butylhydroxyanisole (BHA), propyl paraben (PP), resveratrol (all antioxidants), cinnamon oil and lactic acid bacteria demonstrated greater than 90 % reduction in mycotoxin contamination on wheat grain in laboratory experiments (Vanne *et al.*, 2001; Fanelli *et al.*, 2003) There are, however, many technical and economic obstacles preventing the widescale use of preservatives in bulk storage of grain at present, and this is unlikely to become a key control strategy in anything other than exceptional circumstances.

### 7.6.7 Mycotoxin analysis

At present, specific mycotoxin analysis is not a common monitoring tool in the post-harvest stage. Mycotoxin analysis only occurs at limited points in the chain; for example, grain traders may need to supply analyses for specific mycotoxin hazards at mill intake in support of miller's approved suppliers schemes. Ideally, mycotoxin analysis should figure far more importantly as a routine check during commodity supply. However, at present there are a number of difficulties that prevent this: suitable on-site analytical tests may be unavailable, too expensive or too time-consuming, especially for an industry which runs on very tight deadlines. In particular, if mycotoxin analysis is to be used as a monitoring tool for control points in a HACCP type approach, it needs to provide reliable and accurate results in a 'real-time' sense. At present the analytical tools that fulfil these requirements are few, and most only offer potential for verification purposes.

In fact rapid screening methods do exist for both DON and OTA (both immunoassay methods), but there is likely to be a continued need for cheaper and simpler methods for 'field' analysis. To this end, recent work within the EC Project 'OTA PREV' has been concerned with the development of new rapid monitoring methods for OTA. This work has resulted in a new enzyme-linked immunoassay (ELISA) technique with a working range well below the regulatory limits, which could fulfil the need for a practical monitoring technique within a HACCP scheme. Progress has also been made with the development of a quantitative lateral flow device (LFD) which could provide a rapid and sensitive method for field use (Danks *et al.*, 2003). There are clearly many points in the post-harvest (and pre-harvest) supply chain where such analytical methods could be used to advantage, and they could represent key tools in a HACCP type approach to mycotoxin control, either as monitoring or verification methods.

### 7.6.8 The development of a HACCP plan at the post-harvest stage

As we have already observed, the post-harvest situation is characterized by a complex chain of transfer of the commodity between locations. Although the control parameters in place may be relatively few, they need to be monitored almost continuously as the commodity is passed between 'owners'. This effectively means that the commodity is only as safe as the most careless member of the chain permits, and it can therefore be extremely vulnerable at this stage. The post-harvest controls can be summarized as follows:

1. Adherence to codes of practice such as GAP, GSP and GHP in all aspects of storage, drying and transportation.
2. The ability to recognize and segregate diseased and mycotoxin contaminated grain.
3. To operate at all times within the requirements of quality assurance (QA) schemes such as crop assurance, approved supplier and relevant trade associations (e.g. for haulage contractors).

Although complex, the post-harvest stages lend themselves far more readily to a HACCP type approach than the pre-harvest stages. This is because, unlike pre-harvest, these latter stages allow for the application of more definitive control measures, the setting of critical limits and initiation of monitoring procedures. In this respect, the post-harvest stages are similar to the food processing industries, where HACCP is currently firmly established. It could be argued that few (if any) of the control measures that would apply in a post-harvest HACCP approach would constitute CCPs, since most of the controls in place are not specifically there to control mycotoxins *per se*, and are normally considered to be within the remit of existing codes of practice. However, drying must be considered a CCP in the post-harvest system due to its central importance and the direct link between fungal growth and mycotoxin production. During buffer storage before and during drying (especially using near ambient drying) there are critical limits (moisture contents, time and temperature) to be considered. In the 'OTA PREV' project, a mathematical model for safe storage time before onset of significant growth of *P. verrucosum* and OTA production has been developed, which describes the effect of water activity and temperature on the rate of growth of *P. verrucosum*. Such models can be used to predict the safe buffer storage time or drying time. However, existing models need to be validated for other varieties and species of grain cultivated under different climatic conditions. Another possible example of a CCP at the post-harvest stage is supplier compliance schemes which require named mycotoxins to be within stated limits. This can be treated as a CCP because it addresses the mycotoxin problem directly. A hypothetical HACCP plan worksheet for the post-harvest stage is shown in Table 7.3. Table 7.3 follows on from, and adopts the same format as, Table 7.2.

**Table 7.3** Summary HACCP worksheet for the post-harvest stages in wheat production.

Stage/Hazard	Control type	Control measure	Critical limit(s)
<i>Drying:</i>			
Delays in drying lead to fungal growth and mycotoxin production	CCP	Infrastructure in place to allow prompt and efficient drying of wet grain. Includes stock control	Time Final moisture content < 14 %
Poor drying practice leads to improperly dried grain and subsequent spoilage	CCP	Monitoring of moisture and temperature with prompt remedial action. Use of appropriate drying equipment. Correct operation and maintenance of equipment. Training of personnel and full record keeping	Moisture content, temperature
<i>Storage: on-farm or off-site (3rd party)</i>			
<i>Intake:</i>			
Grain entering storage is above 'safe' moisture level	CCP/GSP	Moisture determination. Rejection/segregation/drying of affected material (Preservatives used under exceptional circumstances)	Moisture content < 14 %
Grain showing signs of fungal disease above acceptable limit	GSP	Inspection and segregation/rejection	Adherence to GSP
Grain is contaminated with mycotoxins	GSP/CCP	Mycotoxin analysis and segregation/rejection	Mycotoxins levels < legislative limits
<i>During storage:</i>			
Poor storage practice/facilities leading to moisture and temperature increase		All risks related to receipt of grain by 3rd parties minimized by trading only with growers operating within a quality assurance (QA) scheme, and by operating a 'supplier approval' system	
Poor storage practice/facilities leading to fungal growth and mycotoxin production	CCP/GSP	Moisture and temperature determination and segregation/drying	Moisture content, temperature

Table 7.3 *cont'd*

Stage/Hazard	Control type	Control measure	Critical limit(s)
Poor storage practice/facilities leading to mixing with mycotoxin contaminated batches or residues	GSP/CCP	Inspection/mycotoxin analysis and segregation/rejection. Training of personnel and full record keeping.  Use of new information (e.g. mathematical models) may allow better prediction of safe storage periods relating to mycotoxin production	Adherence to GSP Mycotoxins levels < legislative limits
<i>Transportation:</i> Inappropriate transportation causing moisture/temperature increase	GHP/GSP/CCP	Inspection/moisture and temperature determination. Use of appropriate transportation equipment. Correct operation and maintenance	Moisture content < 14 % Adherence to GSP/GHP
Mixing of grain with contaminated batches or residues (includes insect infestation)	GHP/GSP	Identification and segregation/rejection. Correct operation and maintenance. Stock control and record keeping. Where 3rd party transporters are used, risks can be minimized by using a contractor who operates in accordance with a recognized QA scheme, by carrying out audits and by maintaining an approved haulier list	Adherence to GSP/GHP

Note: CCP = Critical Control Point; GSP = Good Storage Practice; GHP = Good Hygienic Practice.

## 7.7 Mycotoxin control during primary and secondary processing of wheat

### 7.7.1 Primary processing stages

As in the previous post-harvest stage, there is potential for misuse of the product leading to spoilage (including during transportation and storage), but some of the specific activities involved in processing can affect mycotoxin levels, in certain cases offering the potential to reduce mycotoxin levels specifically. This has been an area of EU funded research within the 'OTA PREV', which looked at the effects on OTA levels of flour milling and extrusion processes (e.g. pasta making) for

**Table 7.4** Summary HACCP worksheet for processing stages in wheat production relating to bread making and consumption.

Stage/Hazard	Control type	Control measure	Critical limit(s)
<i>Flour milling</i>			
<i>Intake:</i>			
Grain showing signs of fungal disease above acceptable limit	GSP/GMP CCP*	Identification and rejection. In the future, routine determination of fungal contamination may be possible	Adherence to GSP/GMP (fungal levels below guideline limits)
Grain is contaminated with mycotoxins	CCP/GMP Verification	Determination of mycotoxins. Identification (supplier certificates of analysis) and rejection. New mycotoxin analysis methods currently in development may help to encourage routine mycotoxin analysis for verification by millers	Mycotoxin levels below legislative limits  Adherence to GMP
Grain moisture level is too high	CCP/GMP	Determination of moisture content. Identification and rejection. All risks related to procurement of grain from 3rd parties minimized by trading only with suppliers operating within a quality assurance (QA) scheme, and by operating a 'supplier approved' system including auditing suppliers	Moisture content below internal specifications (usually < 14 %)
<i>Storage (if any):</i>			
As for post-harvest storage	GMP	As for post-harvest storage	Adherence to GMP
<i>Screening (removal of foreign material and defective grain including possibly Fusarium contaminated grain):</i>			
Screening ineffective at removal of mycotoxin contaminated grain	GMP	Identification, shut-down of operation and segregation/rejection. Correct use and maintenance of appropriate equipment, including training and full record keeping (QA scheme)	Adherence to GMP

Table 7.4 cont'd

Stage/Hazard	Control type	Control measure	Critical limit(s)
<i>Conditioning:</i>			
Operational problems cause delays leading to fungal growth and mycotoxin production	CCP/GMP	Control conditioning time. Identification, shut-down of operation and segregation/rejection. Correct operation and preventive maintenance of equipment including training and full record keeping (QA system)	Temperature, moisture content, time  Adherence to GMP
<i>Milling:</i>			
Milling operations can result in a decrease in OTA. DON levels in white flour. However, there is no change in wholemeal flour and an increase of OTA/DON in bran fraction	GMP/CCP Milling is unlikely to be used as a CCP for reduction in mycotoxin levels	Only high quality grain used for bran production intended for human consumption	Mycotoxin level below legislative limits in end-product
<i>Bread baking:</i>			
No mycotoxin risks appear to be associated directly with the modern bread baking processes. Packaging processes could lead to unacceptable fungal contamination of finished product	GMP	Identification and shut-down of operation, rejection of affected material. Correction of any defective procedures. Correct operation and preventive maintenance of equipment including training and full record keeping (QA) system	Adherence to GMP
<i>Consumer use:</i>			
Fungal spoilage of bread can occur. Spoiled bread is likely to be rejected by the consumer	GMP (supply of storage and shelf-life information on packaging)	Inspection and rejection. Attention to 'use-by date' and appropriate storage. ('Use-by date' on bread packaging is based on likely mould-free shelf-life)	Adherence to GMP

Note 1: CCP = Critical Control Point; GSP = Good Storage Practice; GMP = Good Manufacturing Practice.

Note 2: CCP\* refers to possible future use as control measure.

wheat (Scudamore *et al.*, 2003a, 2003b) and malting and beer making for barley. The results can be summarized as follows.

1. Baking results in only a small decrease in concentrations of ochratoxin A; however, an overall reduction (from grain to bread) of about 80 % is achievable for white bread with scouring included and up to 35 % similarly for wholemeal bread (Scudamore *et al.*, 2003a).
2. Extrusion processes can result in a small reduction in OTA, depending mainly on extrusion temperature.
3. Malting can be a high risk process. The germination and kilning processes can potentially lead to the production of high levels of OTA. This seems to be related to process temperature, which varies considerably within the malting industry.
4. Significant reductions in OTA levels can occur during beer brewing. In the studies performed, there was a reduction of approximately 20 % in the final beer compared to the malt.

It can be seen from the above that a number of activities appear to be able to reduce mycotoxin content. They therefore provide specific control for the OTA hazard and, if we applied the decision tree shown in Fig. 7.1 strictly, we may conclude that these steps constitute CCPs for control of this mycotoxin. This may suggest that we can allow the commodity to become contaminated, and rely on a later decontamination step. However, the idea that decontaminated material is fit for human consumption is likely to be highly controversial, and it should be noted that the effects of these stages are to reduce, and not eliminate, the mycotoxin, so the final result will depend entirely on the initial levels present. Further, research so far has only looked at the effects on OTA, and other mycotoxins may be present which could respond quite differently to these processing steps. The real value of this knowledge is likely to be its use in an integrated approach, where CCPs are used cumulatively, including those concerned with the pre-harvest stage, to arrive at a final consumer product which contains an acceptable level of mycotoxin. In fact, this approach is taken with control of patulin in apple products, where a series of control steps are used to maintain the product within acceptable limits during production (FAO/IAEA, 2001). This does not imply that there should be a lack of control during production which is 'recovered' by a later decontamination step. A hypothetical HACCP plan worksheet for the flour milling stage is shown in Table 7.4. (This is extended into bread baking and consumer use, for completeness.)

### 7.7.2 Secondary processing and consumer use

Following milling, wheat flour finds an enormous number of applications in foods other than the obvious bakery products, and in particular bread, for which it is the major component. In considering the use of wheat flour, we are in fact concerned with the food processing industry, where flour is just one of a number of ingredients leading to final consumer products. This is the area where HACCP is already well established, and will therefore not form part of the present discussion

(a useful account of the conventional application of HACCP is given in Mortimore and Wallace, 1998).

As far as bread baking itself is concerned, modern rapid processes such as the Chorleywood Bread Making Process (CBMP) do not represent a mycotoxin risk. Since there is no evidence that baking decreases levels of mycotoxins significantly, this stage could not be used as a control step (and similar ethical considerations would apply as for any other decontamination steps during processing). Preservatives such as propionates and sorbates are routinely added to bread to extend the shelf-life, and this is principally due to the problems of fungal spoilage. However, there are, to our knowledge, no known cases of mycotoxin poisoning occurring due to the consumption of spoiled bread, although research is lacking in this area. Dich *et al.*, (1979) found that aflatoxin may be produced in spontaneously mouldy bread and that the toxin was well distributed in the bread and could be detected at a distance of 7 cm from the fungal colony on the surface.

If we are to take a holistic view of the wheat commodity 'from field to table', we must look finally at the consumer – what role does HACCP play at the 'table' end of the chain? The honest answer to this question is probably that HACCP can have little influence at the 'user' end of supply. The HACCP principle is based on control, and such control is lost as soon as the commodity is in the hands of the consumer. The manufacturer can do little more than supply guidelines on storage and use, by means of product labeling, but the chain of control which could be provided by a HACCP approach seems destined to be lost at this final stage. It should, however, be an important issue for the national food authorities to give advice to the consumer on how to avoid mould growth in food in the home, and how to act in cases where this occurs.

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# 8

## Environmental conditions affecting mycotoxins

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### 8.1 Introduction

Spoilage fungi are ubiquitous contaminants of raw food materials, pre- and post-harvest. Their activity and colonization levels are determined by the prevailing environmental conditions and the nutritional components of the food matrix (Magan *et al.*, 2003). These fungi can produce a wide range of secondary metabolites under environmental conditions which are conducive to growth. Some of these secondary metabolites are toxic and can have a significant impact if they enter the production and animal food chains.

Prevention of mycotoxin contamination of food raw materials is now considered more important than subsequent cure. Thus Hazard Analysis Critical Control Point (HACCP) approaches are being developed to examine the critical control points at which mycotoxigenic moulds and mycotoxins may enter a range of food chains. The key environmental determinants pre- and post-harvest are water availability and temperature (Sinha, 1995; Magan *et al.*, 2003). Accurate information is therefore needed on the impact of and relationship between these key factors, and which are marginal and which are optimum for germination, growth and toxin production. There have been only a few studies where attempts have been made to integrate the available information on these limits in relation to different raw materials for food processing, especially cereals. The relationship between water availability and moisture content of different cereals is given in Table 8.1 (adapted from Multon, 1988). This is important as information presented

**Table 8.1** Relationship between moisture content (wet weight basis, %) and water activity ( $a_w$ ) for some key cereals at 25 °C.

$a_w$	Moisture content				
	Maize	Wheat	Sorghum	Rice	Groundnuts
0.98	30–32	30–34	31–32	26–28	16–17
0.95	26–27	26–28	26–27	23–24	14.5–15
0.90	23–24	21–22	22.5–23	20–21	12.5–13.5
0.80	16–17	16–17	18–19	17–18	9–10
0.70	15–16	14–14.5	16–17	14–14.5	7–8

in this chapter will be based on water availability (water activity,  $a_w$ ) and can be interpolated to approximate moisture contents. This chapter will examine the available information on key groups of mycotoxigenic food spoilage fungi.

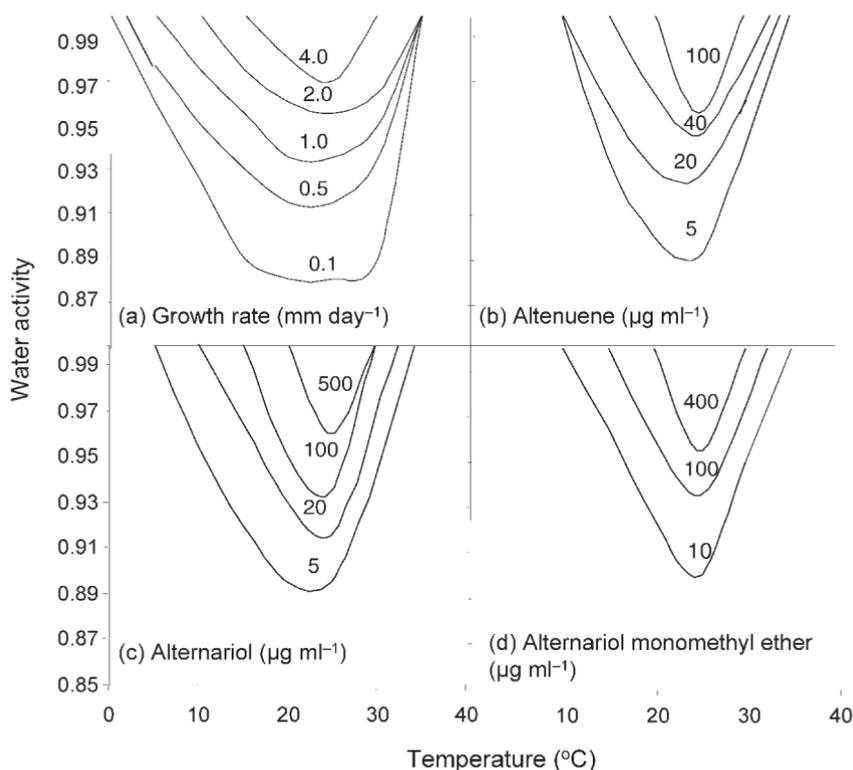
## 8.2 Key environmental conditions affecting production of *Alternaria* toxins and aflatoxin

### 8.2.1 *Alternaria* toxins

The genus *Alternaria* is widely distributed in both soil and aerial plant surfaces, with many known to be plant pathogens. Species are known to grow at low temperatures and have been predominantly associated with spoilage of fruit and vegetables during cooled transport and storage. *A. alternata* is a common spore found in the airspora (Lacey, 1986), especially in areas under arable crop production. Ripening cereal grain is colonized and it is rapidly becoming the most common sub-epidermal fungus in wheat grain (Hyde and Gallegymore, 1951). *A. alternata* and species such as *A. triticina* can also cause black or brown colouration of the wheat kernels, called black point disease, which can affect yield and grain quality. Other *Alternaria* species are important pathogens of tomato, carrots and brassicas.

The most important secondary metabolites with mammalian toxicity produced by *Alternaria* species are the dibenzo- $\alpha$ -pyrones altenuene (AE), alternariol (AOH), alternariol monomethyl ether (AME) and a derivative of tetramic acid, tenuazonic acid (Meronuck *et al.*, 1972; Pero *et al.*, 1973; Harvan and Pero, 1976). Some or all of these mycotoxins have been demonstrated to be produced by *Alternaria* species on wheat (Magan and Lacey, 1985); tomato (Harwig *et al.*, 1979); sorghum (Sauer *et al.*, 1978; Magan and Baxter, 1994), pecans (Schroeder and Cole, 1977) and on cotton (Young *et al.*, 1980). However, few of the studies have attempted to build two-dimensional profiles for germination, growth and mycotoxin production by *Alternaria* species (Young *et al.*, 1980; Magan and Lacey, 1984a,b, 1985). The available information is for *A. alternata* and *A. tenuissima* only.

Figure 8.1 shows that the water activity  $\times$  temperature limits for *A. alternata*

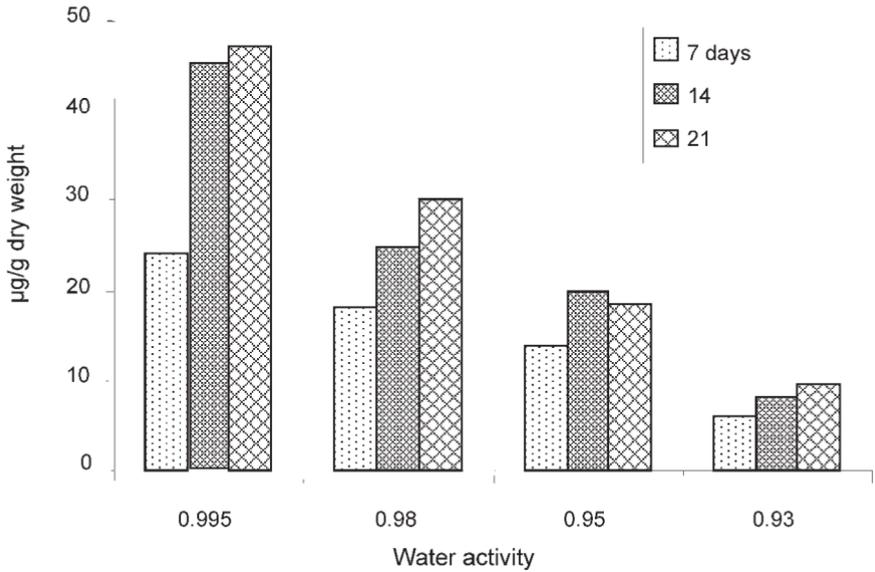


**Fig. 8.1** Comparison of profiles for growth and different mycotoxins by *Alternaria alternata* on wheat grain (adapted from Magan and Lacey, 1985).

germination are lower than those for growth and for the production of AE, AME and AOH. Furthermore, comparisons between growth and mycotoxin production show that the temperature range is narrower for AE and AME than for AOH. Absolute  $a_w$  limit for germination is about 0.86  $a_w$ , and for growth and mycotoxin production about 0.88–0.89 for all three mycotoxins. Optimum production was at about 25 °C and  $> 0.97 a_w$  for all three mycotoxins.

Some ecological studies have been carried out on tenuazonic acid (TA) production by *A. alternata* and *A. tenuissima* species on sorghum and cottonseed (Young *et al.*, 1980; Magan and Baxter, 1994). Figure 8.2 shows that both water availability and time affect TA production by *A. alternata* on sorghum grain. Minimum water availability conditions for production were found to be about 0.93–0.90  $a_w$  *in vitro* on sorghum-based media.

On cottonseed the environmental conditions optimum for TA production by *A. tenuissima* were 20 °C/37.5 % mc (= freely available water (1.00  $a_w$ )). The absolute limiting condition of water availability was 14.9 % (= 0.85  $a_w$ ). However,  $a_w$  was not accurately controlled in these studies. At intermediate moisture levels



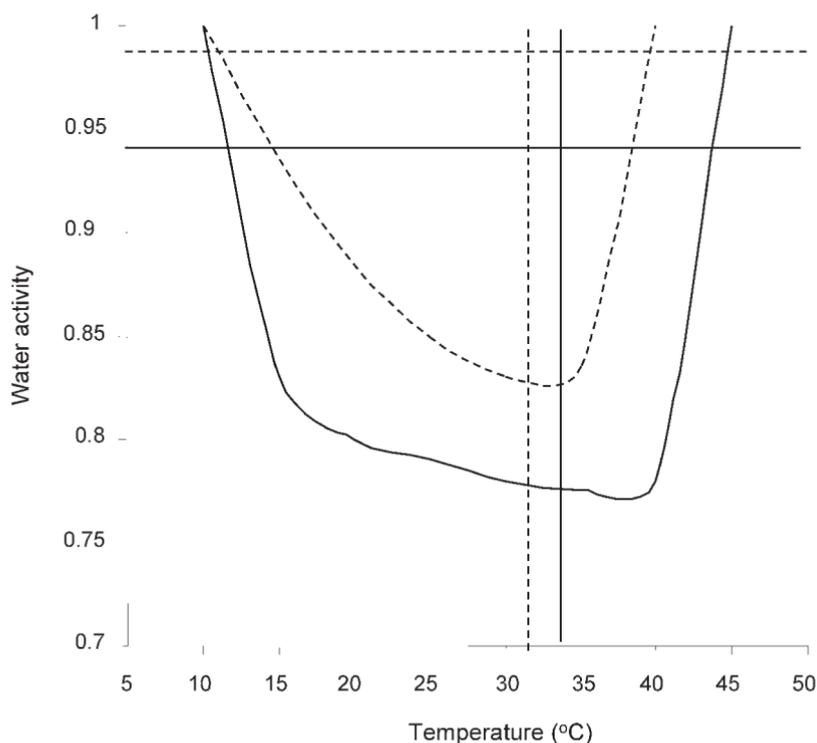
**Fig. 8.2** Tenuazonic acid by *Alternaria alternata* on sorghum grain (adapted from Magan and Baxter, 1993).

(=0.95  $a_w$ ) a 50 % reduction was recorded. They suggested that >0.90  $a_w$  and 20 °C were required for TA production. Different temperatures favour biosynthesis of these different mycotoxins by *Alternaria* spp. Visconti *et al.* (1992) found similar TA production levels for *Alternaria* from rapeseed, but no temperature or water availability aspects were considered in detail.

### 8.2.2 *Aspergillus flavus* group and aflatoxin production

In warm and humid subtropical and tropical conditions maize ears are ideal conditions for colonization and dominance of *A. flavus/parasiticus* species, resulting in the formation of aflatoxins. The aflatoxin group is carcinogenic and thus contamination results in significant economic impact. In groundnuts the presence of drought stress can lead to cracking of the pods and ingress by *A. flavus* and *A. parasiticus* resulting in significant aflatoxin accumulation.

The conditions conducive to germination, growth and aflatoxin production by *A. flavus* and *A. parasiticus* on *in vitro* media are shown in Fig. 8.3. This shows again that germination occurs over a wider range than that for growth, with the aflatoxin production range narrower than that for growth. Optimum conditions for aflatoxin production by these two species is at 33 °C and 0.99  $a_w$ ; while that for growth is 35 °C and 0.95  $a_w$ . Recent work by Pitt and Miscamble (1995) showed that the impact of environmental factors on growth of *A. flavus*, *A. parasiticus* and *A. oryzae* was similar with minima of 0.82  $a_w$  at 25, 0.81 at 30 and 37 °C. However, this study did not include comparison of aflatoxin production by *A. flavus* and *A. parasiticus*.



**Fig. 8.3** Comparison of profiles for growth and aflatoxin production by *Aspergillus flavus* and *A. parasiticus* (adapted from Hill *et al.*, 1985). Optima for growth (—) and for aflatoxin production (---) are indicated by straight lines.

It is worthwhile noting that in maize often the predominant species isolated was *A. flavus* with only about 10 % of isolates *A. parasiticus*. In groundnuts, the majority of isolates are usually *A. flavus* with *A. parasiticus* isolates about 30 % of the strains. Interestingly, studies in the USA suggest that *A. flavus* isolates almost always produce aflatoxin B1 and sometimes B2 in *in vitro* culture. However, *A. parasiticus* produces B1, B2, G1 and G2 under the same conditions (Dorner *et al.*, 1984). Furthermore, some *A. flavus* isolates also produced cyclopiazonic acid, but *A. parasiticus* isolates did not.

## 8.3 Key environmental conditions affecting production of *Fusarium* toxins, ochratoxins and patulin

### 8.3.1 *Fusarium* toxins

#### *Fumonisin*s

Fumonisin has received particular attention because of their association with cancer-promoting activity (IARC, 1993) and the induction of leuco-

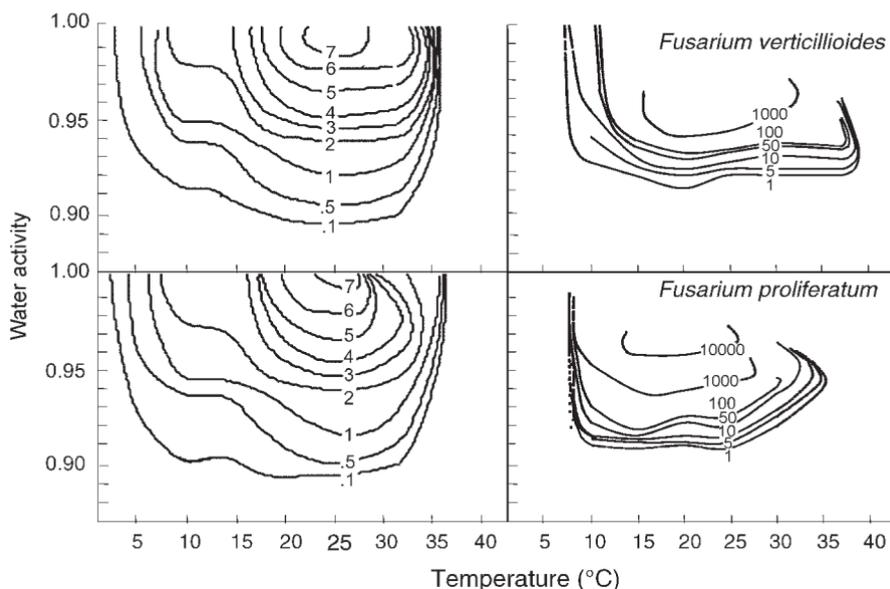
encephalomalacia (LEM) in horses (Gelderblom *et al.*, 1988; Marasas *et al.*, 1988). Maize grown in temperate regions is an appropriate substrate for *Fusarium* Liseola section species to colonize and produce fumonisins. *F. verticillioides* and *F. proliferatum* are the two main species of this section with the capacity to produce these mycotoxins (Chulze *et al.*, 1996). These species have been demonstrated to be important contaminants of maize in southern European countries and in North and South America (Sydenham *et al.*, 1993; Doko and Visconti, 1994; Sanchis *et al.*, 1995). They can colonize the maize ears and produce fumonisins in the field and, if the maize grain is harvested at a high moisture content, conducive to fungal growth and mycotoxin production, they can be accumulated in the grain before achieving an  $a_w$  low enough to control the activity of these species. Moreover, corn is a very important component in the food and feed chain and fumonisins have been commonly found in a wide range of corn-based foods in different parts of the world (Doko and Visconti, 1994; Sanchis *et al.*, 1994; Velluti *et al.*, 2001).

Most *F. verticillioides* isolates produce primarily FB<sub>1</sub> and lower amounts of FB<sub>2</sub>, FB<sub>3</sub> and FB<sub>4</sub>. Only a few strains have been isolated that do not produce any measurable fumonisins when grown on corn in the laboratory. Figure 8.4 shows the water activity  $\times$  temperature limits for growth and fumonisin production of *F. verticillioides* and *F. proliferatum*. As shown for other mycotoxins (Northolt and Bullerman, 1982) the range of  $a_w \times$  temperature conditions conducive to growth was much wider than that for fumonisin B<sub>1</sub> production. Growth occurred at 4–37 °C with an optimum at about 30 °C (Marin *et al.*, 1999a), while FB<sub>1</sub> accumulation took place at 10–37 °C, with 15–30 °C being the optimum temperature levels, depending on the isolates (Marin *et al.*, 1999b). Referring to  $a_w$ , both growth and fumonisin B<sub>1</sub> accumulation increased with increasing  $a_w$  levels, with 0.90 being the minimum  $a_w$  for growth and 0.93 the minimum for FB<sub>1</sub> production (Marin *et al.*, 1999a,b). Fumonisin production has been tested in other cereals, such as wheat and barley; however, negligible amounts of fumonisins were accumulated, suggesting that substrate composition affects fumonisin biosynthesis and that this is the reason for the low natural incidence in those cereals (Marín *et al.*, 1999a).

#### *Deoxynivalenol, nivalenol and zearalenone production*

In recent years much attention has been focussed on understanding the ecophysiology of *F. culmorum* and *F. graminearum* in relation to infection of ripening cereals (*Fusarium* head blight) and the impact of fungicides on both disease control and trichothecene production (Magan *et al.*, 2002). Contamination of cereal grain with deoxynivalenol (DON) and nivalenol (NIV) has been a particular problem for the flour milling and baking industry and, to some extent, for the brewing industry.

Early studies by Magan and Lacey (1984a) established comparative contour maps of water and temperature relations for growth of *F. culmorum*, *F. poae*, *F. avenaceum* and *F. tricinctum*. This showed that growth optima was at 20–25 °C, with marginal conditions at 5–10 and 35 °C. The water availability optimum was close to 0.98–0.995  $a_w$ , with minima around 0.90–0.91  $a_w$  over the optimal temperature range. Germination thresholds were about 0.88 for these *Fusarium*

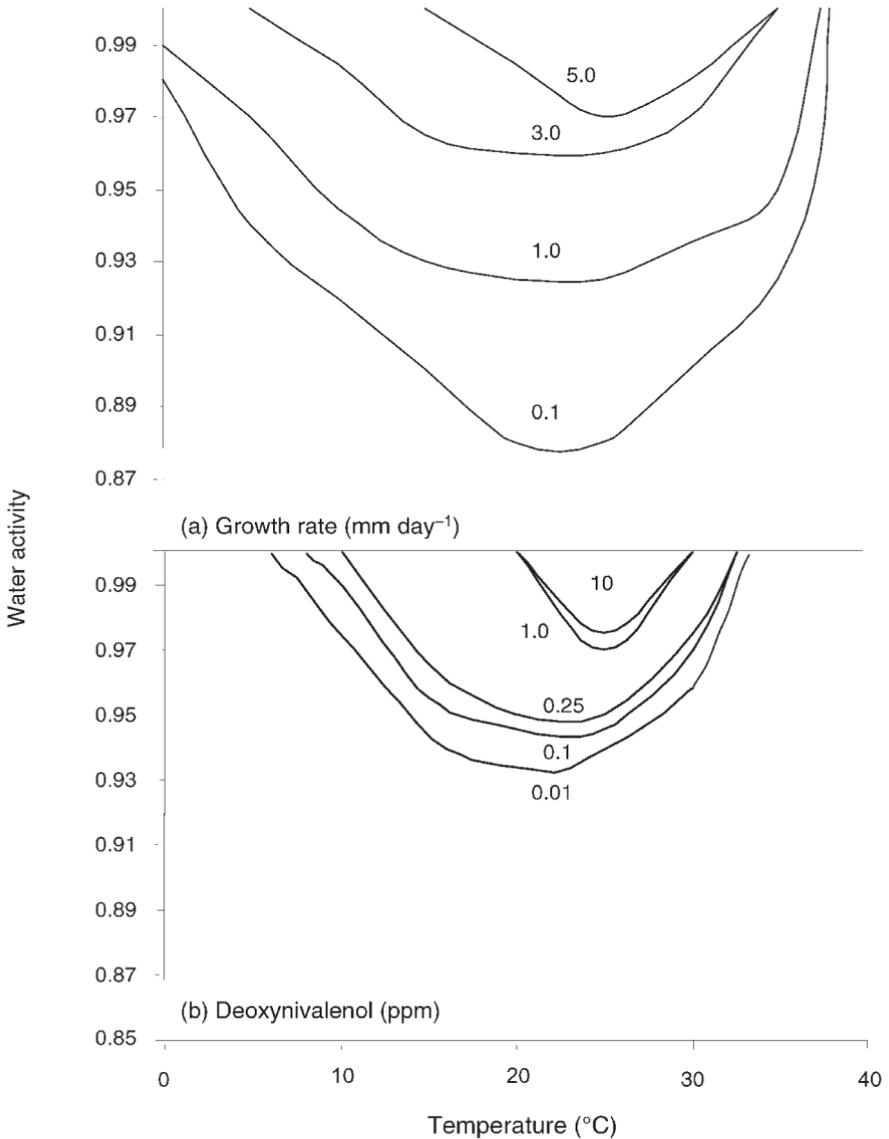


**Fig. 8.4** Comparison of growth ( $\text{mm day}^{-1}$ ) and fumonisin production ( $\mu\text{g g}^{-1}$ ) on maize grain by *Fusarium verticillioides* and *F. proliferatum* (from Marin *et al.*, 1999b).

species (Magan and Lacey, 1984b). Versonder *et al.* (1982) demonstrated that a *F. graminearum* and so-called *F. roseum* (= *F. culmorum*) strains produced DON optimally at 29–30 and 25–26 °C, respectively on cracked moist maize (30 % water content = 0.99  $a_w$ ). Minimum temperatures for production of DON were about 11 °C, dependent on time of incubation for both strains. These contour maps were very useful, but excluded interaction with water availability. Cycling of temperature can have a significant impact on both DON and NIV production. Studies by Ryu and Bullerman (1999) using rice cultures showed that temperature cycling of 15 and 30 °C over a six week period resulted in the highest biomass. However, steady-state incubation at 25 °C for two weeks resulted in the highest DON and ZEA production. There was also a correlation between DON and zearalenone (ZEA) production, but none between fungal biomass and the production of either. This suggests that environmental stress has an important influence on toxin production, often unrelated to total fungal biomass.

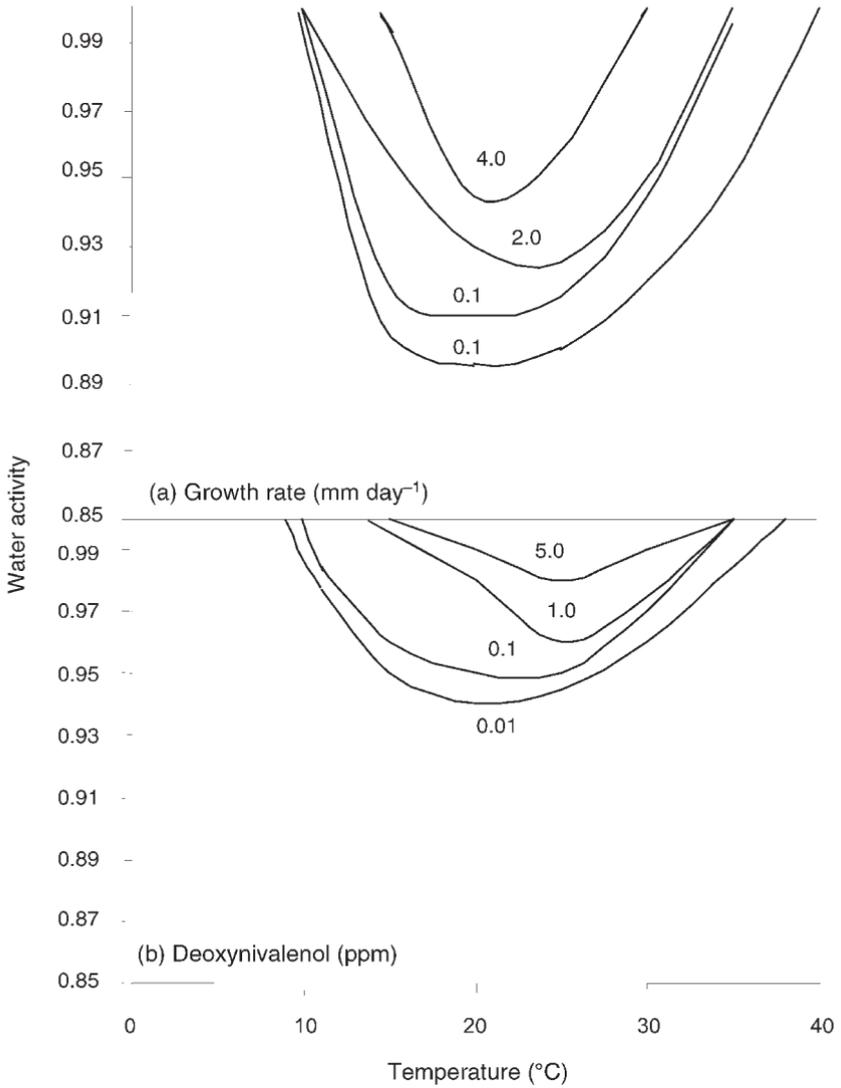
More recently, Lacey *et al.* (1999) showed that wet periods at anthesis critically influenced the amount of DON and other related mycotoxins produced in ripening wheat. Hope and Magan (2003) developed two-dimensional temporal production profiles for DON and NIV by *F. culmorum* which showed that 0.995  $a_w$  was optimum for growth on wheat-based media, with 0.90 being the limit at both 15 and 25 °C. Production of both DON and NIV was over a much narrower range than for growth. Comparison of the profiles for growth and DON production for isolates of *F. culmorum* and *F. graminearum* are shown in Figs 8.5 and 8.6.

Less information is available on the ecological profiles of production of ZEA.



**Fig. 8.5** Comparison of profiles for growth ( $\text{mm day}^{-1}$ ) and DON ( $\mu\text{g g}^{-1}$ ) production by *Fusarium culmorum* on wheat grain.

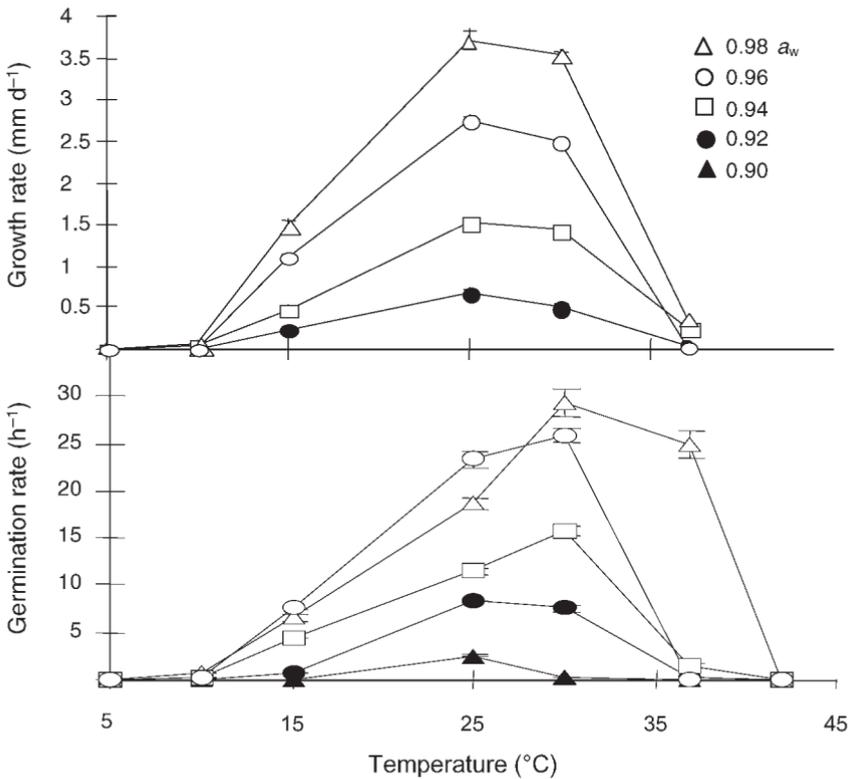
Recently, Vellutti and Sanchis (unpublished data) examined growth by *F. graminearum* on maize grain and found that the limits of production were 0.90–0.91  $a_w$  over a 12-week incubation period at 25–30 °C. Optimum conditions for production were at  $> 0.98 a_w$  at optimum temperature for growth.



**Fig. 8.6** Comparison of profiles for growth (mm day<sup>-1</sup>) and DON (µg g<sup>-1</sup>) production by *Fusarium graminearum* on wheat grain.

### 8.3.2 Ochratoxins

Ochratoxin A (OTA) is a fungal secondary metabolite that has received particular attention because of its association with cancer-promoting activity (IARC, 1993). It has also been described as a potent nephrotoxin, teratogen, with immunotoxic properties in rats and possibly in humans. OTA has been extensively documented as a global contaminant of a wide variety of foods including cereal products, nuts,



**Fig. 8.7** Comparison of profiles for germination and growth of *Aspergillus ochraceus* in relation to water activity and temperature (Ramos *et al.*, 1998).

spices, coffee, raisins and wine (Majerus and Otteneder, 1996; Zimmerli and Dick, 1996; Urbano *et al.*, 2001). Ochratoxin B (OTB) has been found very occasionally in some foods (Visconti and Bottalico, 1983) and is less toxic than OTA.

OTA has been shown to be produced by several species in the *A. ochraceus* group (Ciegler, 1972) and by *A. alliaceus*, *A. albertensis* (in section *Circumdati*), *A. niger*, *A. carbonarius* (in section *Nigri*) (Abarca *et al.*, 1994; Varga *et al.*, 1996) and *Penicillium verrucosum* (Northolt *et al.*, 1978). They contaminate different crops with a different distribution depending on climatic conditions. *Aspergillus* predominates in warm and temperate regions while *Penicillium* isolates are frequent in colder regions (Sweeney and Dobson, 1998).

#### *Aspergillus ochraceus*

*Aspergillus ochraceus* has been reported in a wide range of foods around the world, mainly those having an intermediate water availability, including cereals, beans, spices, dried fruits, nuts and oilseeds (Mislivec and Tuite, 1970; Sanchis *et al.*, 1986; Shrivastava and Jain, 1992; Cvetnic, 1994). In *A. ochraceus*, as shown

for other mycotoxins (Northolt and Bullerman, 1982) the range of  $a_w \times$  temperature conditions conducive to growth was much wider than that for OTA production. Growth occurred over the temperature range 8–37 °C (ICMSF, 1996) with an optimum of about 30 °C on barley grains (Ramos *et al.*, 1998; Fig. 8.7). The highest amounts of OTA were obtained at 0.98  $a_w$ , regardless of the temperature level, with maximum OTA production optimum between 25–30 °C, depending on the isolates. Referring to  $a_w$ , both growth and OTA accumulation increased with increasing  $a_w$  levels until 0.96–0.98, with 0.80 (at 25–30 °C) being the minimum  $a_w$  for growth on maize-based media (Marin *et al.*, 1998) and 0.83–0.87 the minimum for OTA production (Northolt *et al.*, 1979). The food matrix and nutritional status is very important in determining OTA production (Madhyastha *et al.*, 1990).

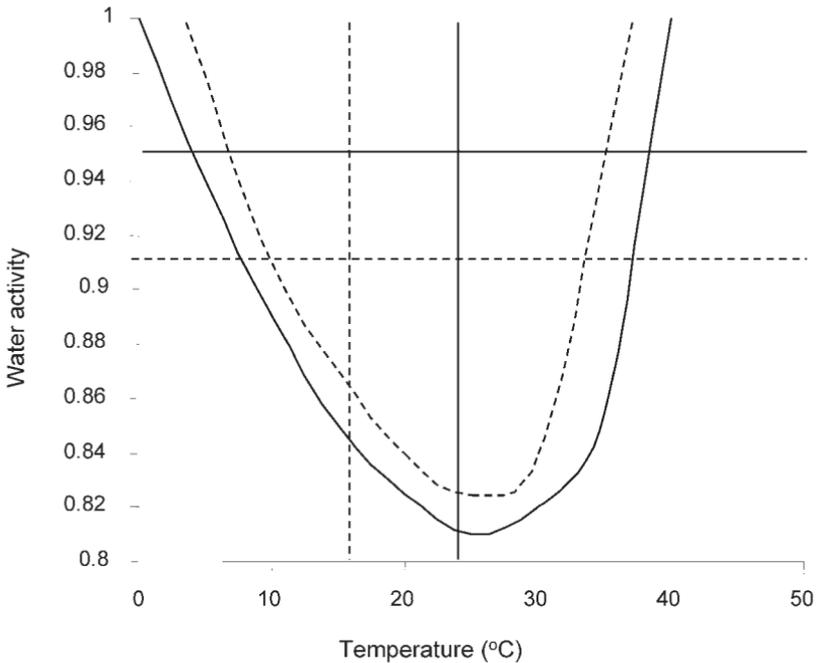
### *Penicillium verrucosum*

*P. verrucosum* effectively colonizes food matrices, particularly in cooler climatic regions of the world. It is a particular problem in cool, damp climatic regions of northern Europe in wheat and barley, especially those not efficiently dried to safe moisture contents of about 14.5 % (= 0.70  $a_w$ ). Where high-temperature drying systems are used, grain can be effectively dried and the risk from contamination is low. However, short-term buffer storage on-farm and ambient air drying can lead to moulding and contamination with *P. verrucosum* and OTA.

*P. verrucosum* can grow over a wide range of temperatures (0–35 °C). While 25 °C is optimum for OTA production on grain, at 5–10 °C it is produced during long-term storage under conducive moisture conditions. Interestingly, optimum colonization rates of stored grain are at 0.95  $a_w$ . However, optimum OTA production is at between 0.90 and 0.95  $a_w$  and amounts increase with time. The minimum  $a_w$  for OTA production is about 0.83–0.85  $a_w$  and dependent on time of storage (Cairns *et al.*, 2003; Fig. 8.8).

### *Aspergillus carbonarius*

Recently, much interest has been focussed on the source of contamination of grapes, vine fruits and wine with OTA. Studies have shown that in the section Nigri, *A. carbonarius* is predominantly responsible for this contamination (Battilani and Pietri, 2002; Cabañes *et al.*, 2002). Very little detailed information has been available on the impact of environmental parameters on germination, growth and OTA production by *A. carbonarius*. Recently, Mitchell *et al.* (2003) and Battilani *et al.* (2003) demonstrated that optimum growth occurred at 35 °C for strains from a range of southern European countries, with no growth occurring at < 15 °C and > 45 °C. The optimum  $a_w$  for growth varied between 0.93 and 0.987  $a_w$ , with the widest  $a_w$  tolerance at 25 °C on an artificial grape juice medium. On synthetic grape juice medium, temporal OTA production increased with time over periods of 56 days with more OTA produced at marginal temperatures and  $a_w$  levels than for growth (15–20 °C; 0.85–0.90  $a_w$ ). While more information is necessary on this important species, it appears that the profiles for the optimum and the range for growth differ significantly from those for OTA production.



**Fig. 8.8** Comparison of limits for growth ( $0.1 \text{ mm day}^{-1}$ ; —) and ochratoxin A production (10 ppb; ----) by a strain of *P. verrucosum* grown on irradiated wheat grain for 35 days. Straight lines indicate optimum conditions of temperature and water activity.

### 8.3.3 Patulin

Patulin is produced by a large number of species belonging to *Penicillium*, *Aspergillus*, *Paecilomyces*, *Byssosclamyces* and *Eupenillium* genera. It has been found frequently in apples decayed by *Penicillium expansum*, especially those that have fallen on the soil surface. Use of poor quality apples can lead to contamination of apple juice by patulin. Northolt *et al.* (1978) examined the impact of environmental factors on *in vitro* production of patulin by *P. expansum*, *P. patulum* and *A. clavatus*. The temperature range for growth and production was 0–24, 4–31 and 12–24 °C, respectively. Minimum  $a_w$  for patulin production by these three species were 0.99, 0.95 and 0.99, respectively. On apples the minimum conditions for patulin production by *P. expansum* were 1–4 °C depending on apple variety. Interestingly, *P. patulum* was unable to invade apple tissue (Northolt *et al.*, 1978).

## 8.4 Conclusions

For control measures to be effective it is essential that we have the relevant information on the ecophysiological influences of abiotic and biotic stress on mycotoxin production by key spoilage fungi in the relevant food chains. There is

now a significant body of information on fungi and mycotoxins which have or are being legislated for. This information is critical in accurately focussing and monitoring key critical control points in the food chain to optimize prevention strategies at all stages in the food chain.

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# 9

## **Control of mycotoxins in storage and techniques for their decontamination**

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### **9.1 Introduction: mycotoxin control**

Mycotoxins are toxic, structurally diverse, secondary metabolites produced by a wide range of moulds, and they infect food, feed and agricultural commodities. They may be produced in the field as well as during food storage, and a variety of environmental factors determine the production of each particular mycotoxin. The interplay between environmental factors, and the possibility of finding more than one mycotoxin producer in a particular defined niche make this a unique challenge for food safety. The ideal solution to the health hazards that mycotoxins pose is the prevention of mycotoxin contamination in the field. However, despite the efforts devoted in that direction, we still face a high incidence of mycotoxin presence in many countries throughout the world. The problem is intensified by the existence of global free markets and by the relatively new threat of bioterrorism.

According to 'extensive analytical results and very detailed information on the distribution of fungi in staple crops', there are only five agriculturally important fungal toxins: deoxynivalenol (DON), zearalenone, ochratoxin A, fumonisin and aflatoxin (Miller, 1995). Decontamination/detoxification procedures are useful for restoring mycotoxin-contaminated commodities. Although certain treatments have been found to reduce the levels of specific mycotoxins, no single method has been developed that is equally effective against the wide variety of mycotoxins which may occur together in various commodities. The ideal decontamination procedure should:

1. completely inactivate, destroy, or remove the toxin, or reduce its concentration to acceptable levels;
2. not produce or leave toxic residues in the food/feed;
3. preserve the nutritive value of the food/feed;
4. not alter the acceptability or the technological properties of the product;
5. destroy fungal spores and mycelia so as to prevent revival and toxin production;
6. be integrated, if possible, into the regular food-processing and preparation steps;
7. be cost-effective;
8. be easy to use;
9. not destroy or damage equipment or pose a health hazard to workers;
10. be approved by regulatory agencies.

Several strategies have been reported for the management of mycotoxins already present in produce and food (Fig. 9.1).

Reviews dealing with the elimination of mycotoxins (not only aflatoxin) include, among others, those published by: Goldblatt and Dollear, 1977; Doyle *et al.*, 1982; Scott, 1984, 1991; Park, 1993; Basappa and Shantha, 1996; Scott, 1998; Sinha, 1998; Charmley and Prelusky, 1994; Karlovsky, 1999; Dänicke, 2002. This chapter focuses on chemical removal, detoxification and biodegradation techniques.

## 9.2 Fungi in stored grain: occurrence, damage and control

Numerous publications have dealt with the subject of storage fungi. This section is based on the following reviews which contain comprehensive information on the subject: Moreau, 1979; Christensen and Sauer, 1982; Christensen and Meronuck, 1986; Lacey and Magan, 1991.

Fungi can attack grains in the field before harvest, or at the post-harvest stage during storage. In general, fungi that invade kernels in the field are referred to as field fungi, and those that proliferate during storage as storage fungi. The division is based on the ecology of the fungi, namely growth conditions, of which the water requirement seems to be the most important factor. All field fungi require a moisture content (MC) which is in equilibrium with relative humidities (RHs) above 90 %, whereas storage fungi can grow at MCs which are in equilibrium with RHs of 70–90 %. Species of *Alternaria*, *Cladosporium*, *Fusarium* and *Helminthosporium* are common among the field fungi; the major storage fungi comprise species of *Aspergillus* and *Eurothium* and, to a lesser extent, *Penicillium*, which grow mainly in grains with high MC that are stored at low temperatures. The survey presented here deals only with the storage fungi.

Fungi are considered to be a component of the grain-bulk ecosystem and, along with insects and the grains themselves, they are the biotic factors of the system (Sinha and Muir, 1973). The abiotic factors are: dockage; intergranular air; water vapour; temperature; and the storage structure. All the components interact and affect each other to cause or to prevent damage to stored grains.

	REMOVAL		DETOXIFICATION		BIODEGRADATION
	<i>Physical</i>	<i>Chemical</i>	<i>Physical</i>	<i>Chemical</i>	
Produce	separation		irradiation	chemicals	micro-organisms
↓			heat		plants
↓	milling	extraction	adsorption		
Food			irradiation	chemicals	micro-organisms
					plants

**Fig. 9.1** Strategies for the management of mycotoxins in agricultural produce and food.

Parameters affecting growth of storage fungi are: water activity ( $a_w$ ); temperature; grain condition (e.g. soundness, the state of the covering tissue, presence of mechanical or insect damage); nutrient composition in the grain and its availability; the gas composition of the intergranular air; competition between the species present (microbial interactions); and inhibiting materials (types and quantity).

According to their moisture requirement, the storage fungi are divided into three main groups: hydrophytes, which grow at an RH of 90–100 % (bacteria and many yeasts); mesophytes, with an RH range of 80–90 % (*A. candidus*, *A. flavus*); and xerophytes, which have a lower RH limit of 70 % (members of the *Eurothium* group and *A. restrictus*). When the  $a_w$  at a given spot in the grain bulk starts to rise, the xerophilic species will be the first to develop. As a result of their metabolic activity, the moisture increases first at that spot and later in other parts of the bulk also (as a result of moisture migration), thus creating favourable conditions for the appearance of mesophytes and hydrophytes. Therefore, the safest means of preventing fungal growth on grain is to reduce the  $a_w$  to below 0.70 equilibrium RH (ERH) – and to maintain that level throughout the storage period. It is worth mentioning that, numerically, ERH and  $a_w$  are the same. However,  $a_w$  is a commonly used measure of the water available for microbial activity.

According to the temperature needed for the storage fungi to proliferate, they are generally also classified into three main groups: psychrophiles, mesophiles and thermophiles. The temperature requirements (minimum, optimum and maximum, in °C) for these groups are: psychrophiles: –5 to 0, 10–20, 25–30; mesophiles: 5–10, 20–40, 40–45; thermophiles: 25–40, 50–60, 70–80. Of all the conditions known to affect fungal growth or composition, the effects of  $a_w$ , temperature and intergranular air composition are regarded as the most crucial. Note, however, that all the factors interact, and that the range of each of these parameters is closely related to the values of the other parameters involved in growth. For example, as the moisture level rises, the range of temperature over which the fungi will develop becomes broader.

Fungi growing on stored grain can cause major damage that leads to quality reduction and, as a consequence, huge economic losses. The major types of damage caused by fungi are: initiation of heating processes in the bulk; reductions in technological qualities (e.g. suitability for milling or baking); losses in quality (e.g. discoloration, high levels of free fatty acids, mustiness and caking); reductions in germination ability; nutritional losses; production of toxic secondary

metabolites – i.e. mycotoxins. The parameters leading to mycotoxin production in storage will be described in the next section.

Strategies for the management of fungi in storage include: the use of chemicals; physical means; biological control; the use of natural products; and an integrated approach in which two or more different methods are combined.

### 9.2.1 Control of storage fungi with chemical fungistats

Fungistatic agents, mostly low-molecular-weight organic acids (e.g. propionic, acetic and formic acids) and their salts have been used to preserve grains and animal feeds. However, there are many disadvantages to the use of these acids, including: these agents are fungistats and their efficacy is time-dependent; they are corrosive; treated grain is not welcome in commercial channels; acid treatments destroy seed viability; and, above all, special care must be taken by the user to avoid inhalation or contact. In a few cases, mycotoxin production increased in acid-treated grain, probably because inadequate treatment led to the presence of small amounts of the agents in the grain, and it has been suggested that at sub-lethal doses, some fungi may utilize the acids as a carbon source. These limitations, along with the worldwide trend to minimize, or even ban, the use of pesticides with agricultural commodities, give urgency to the search for alternative methods of grain preservation which can be easily and safely implemented.

### 9.2.2 The use of physical means

Of the physical methods commonly applied in grain storage, modified atmospheres (MAs) and gamma irradiation (GI) should be mentioned. The major advantages of physical means lie in the elimination of chemicals or a reduction in their use. However, many practical factors, technical as well as biological, continue to restrict the use of MAs and GI. When studying the effects of an MA, it is of the utmost importance to define the other prevailing environmental conditions clearly. These mainly comprise temperature, RH, MC, type of substrate and competing micro-organisms, all of which have been clearly shown to impair the response of each physiological stage of a mould to some MA, as a consequence of the interrelationships of these ecological factors with each tested MA. The effect of an MA on mould growth should be analysed separately for each morphological stage of the life-cycle, and should be classified in the light of its effects on sporulation, mycelial growth, and germination of resting bodies – such as sclerotia and chlamydo spores. Moulds vary in their reactions to MAs and different strains of a given species may respond differently to the same MA. Although moulds are considered to be obligate aerobes, it is difficult to generalize among them with respect to their responses to low O<sub>2</sub> and high CO<sub>2</sub> levels. The optimum amount of oxygen needed for each developmental stage may vary among strains and in some species, growth can occur even at extremely low O<sub>2</sub> levels (< 1 %). Some moulds can also tolerate high levels of CO<sub>2</sub> (> 50 %). However, the ratio between the atmospheric gases under each set of experimental conditions is an important factor,

since synergism between the gases may determine the tolerance of a mould to low O<sub>2</sub> or high CO<sub>2</sub>.

### 9.2.3 Effect of GI

The sensitivity of a fungus to radiation is governed by several factors, among which are genetic attributes and cellular composition. For example, a diploid may exhibit greater resistance than a haploid because of the presence of a second set of chromosomes. While considering the effect of GI on a fungus, several factors should be taken into account – for example, the morphological structures of the fungi (e.g. spores, mycelia, sclerotia) differ in their sensitivity to irradiation. Since all of these structures may be present in produce and/or food, studies to determine the lethal doses required to eradicate a specific fungus should cover the different structures that characterize the various stages of the fungus life-cycle. The physiological stage (e.g. dormant or germinating spores or sclerotia) can affect the sensitivity to radiation. Different strains belonging to the same species may differ in their responses to a given dose. The condition of the irradiated material (e.g. spores in a dry or wet state) may also make a difference. The sensitivity of any cell depends upon the environmental conditions (temperature, pH) under which the irradiation is carried out. Generally, the dosages needed to eradicate fungi in produce and food (mainly *Aspergillus*, *Penicillium* and *Fusarium* species) are 10–12 kGy. The influence of irradiation on issues related to mycotoxins and its relevance to food preservation are discussed below.

### 9.2.4 Biological control

Numerous fungi, especially yeasts, and bacteria have been shown to be effective in controlling post-harvest diseases of fresh agricultural produce. However, the biological control of fungi present on dry agricultural commodities has been studied only to a limited extent. The studies have focused mainly on the addition of antagonists to grain systems rather than on exploring the mechanism(s) involved in the antagonistic interactions. Among the difficulties which restrict further investigations into this field are: that antagonistic micro-organisms may have adverse effects on the quality of the produce, especially grains; difficulties involved in applying the agent to dry products; and the possible stimulation of mycotoxin production in competitive environments. The mechanisms by which fungal growth and mycotoxin production may be inhibited in a competitive environment include: competition for nutrients and space; induction of host defence mechanisms; hyperparasitic interactions; and antibiosis. Although biological control does not yet appear to be feasible in grains and food, a major benefit which might emerge from studies on antagonistic fungi would be the discovery of compounds inhibitory to storage fungi and/or mycotoxin production.

### 9.2.5 The use of natural products

Constituents of numerous plants have been found to exhibit anti-fungal activity

against storage fungi. Such plants include oregano, clove, cinnamon, garlic and thyme. However, contradictory results have been reported in many cases with respect to the activity of particular plant constituents, since many parameters need to be considered when these results are being compared. Aspects of tests which can influence the results include: the assay technique (e.g. poisoned food techniques, in which the material is dissolved in the medium, or 'agar diffusion' techniques, in which agar discs, for example, are used as a reservoir containing the materials to be tested); the culture medium, in cases where the activity of the vapour phase is assayed (constituents of the medium may react with essential oil components and the pH may also influence the activity of the test material); the botanical source of the plant material or the time of harvesting (which might affect the composition of the material); the isolation techniques (steam-distillation, extraction, etc.); and the fungal isolate being tested. There are differences in sensitivity to a given plant constituent, between different isolates belonging to the same species and between the various fungal structures of the same isolate: spores, sclerotia and mycelial fragments.

The use of plant constituents as food preservatives is not yet feasible, since the method still suffers from many disadvantages (e.g. the presence of aroma and taste residues and the large amounts needed) which make this means expensive and cast doubts on its 'generally regarded as safe' (GRAS) status.

### **9.2.6 The integrated approach**

The concept underlying this strategy is the achievement of a synergistic effect (expressed as better fungistatic activity) by the combination of different control methods, each at a lower level than if it were used alone. Examples of integrated approaches include application of fungistats + MA + GI, fungistats + natural products and fungistats + bacteriocins.

Although this approach could become important, in practice, because each component is employed at only a relatively low level, it still involves known disadvantages (those of natural products or fungistats), limitations (those of fungistats) or technical constraints (those applied to MA and GI) which have to be dealt with.

## **9.3 Factors affecting mycotoxin production during storage**

The conditions leading to fungal growth do not always correspond to those required for mycotoxin production; therefore fungi may grow and proliferate on grains without producing mycotoxins. On the other hand, the absence of toxigenic fungi does not guarantee that a commodity is free of mycotoxins, since the toxins may persist long after the moulds have disappeared. However, there are effects which arise from interaction between all the parameters involved, and it is of the utmost importance that those effects be elucidated. The relatively large number of parameters known to affect mycotoxin production, as well as the abundance of

possible combinations, complicate the planning of a predictive model for mycotoxin production in storage. It is necessary, therefore, to study mycotoxin formation in actual storage environments. The main parameters affecting mycotoxin production in storage are  $a_w$ , temperature, the intergranular gas composition, grain constituents (fats, proteins), grain condition, and the microbial interactions (suppressive or stimulating effects). However, as in the case of parameters affecting fungal growth, there are also interactions among all the parameters that control mycotoxin production. In addition, the fungi differ in their thresholds, above or below which their mycotoxin production is affected by each of the parameters. Therefore, the system of 'fungal growth vs mycotoxin production', as related to ecological conditions, should be studied for each fungal species (see Sanchis and Magan, Chapter 8, this book).

## 9.4 Physical decontamination of mycotoxins: heat, adsorption and irradiation

### 9.4.1 Heat

Many food-processing operations (e.g. roasting, cooking, frying, baking) include heat treatment, therefore, the thermal inactivation of mycotoxins has been thoroughly studied. Since mycotoxins differ in their stability under heat treatments, the rate of destruction of any specific mycotoxin must be determined primarily under the thermal conditions that prevail during the particular process to which it is to be subjected. Also, mycotoxin decomposition is affected by some additional parameters, such as the MC of the product, the level of the toxin, the type of food matrix, and the presence of additives (Scott, 1984).

Aflatoxins are relatively resistant to thermal inactivation and are destroyed only at temperatures of around 250°C. Therefore, processes which include heating should reach that temperature range. Under any given type of heat processing, mycotoxin destruction depends, basically, on the temperature and the time of exposure to it. For example, roasting of green coffee beans at 200°C for 12 min caused a reduction of 79 % in aflatoxin content, whereas after 15 min, 94 % of the toxin had disappeared (Levi, 1980).

Reduction of aflatoxin during heat processing also depends on the MC of the product. In cottonseed meal with 30 % MC, an 85 % reduction in aflatoxin was recorded following heat treatment of 100°C for 2 h but, with the same temperature–time combination, only 50 % of the aflatoxins were destroyed when the meal MC was 6.6 % (Mann *et al.*, 1967). In the light of the data accumulated from several studies of the effect of MC on aflatoxin degradation, it can be concluded that degradation is enhanced at high moisture content.

Results of studies in which aflatoxin-contaminated corn grains were heated (160–180°C), indicated a reduction in aflatoxin content (from 383 to 60 ppb) and also in those of the two limiting amino acids: lysine and methionine. Also, heating corn to a high temperature reduced the digestion and absorbence of crude fibre and

nitrogen (Hale and Wilson, 1979). It should be ensured that the effects of heating grains to destroy aflatoxin (as well as other mycotoxins) are concomitant with the findings of studies on the effect of feeding the heated grains to animals.

The destruction and detoxification of citrinin is also dependent on temperature. Under dry conditions, the toxin is decomposed at 175°C, whereas under moist condition it was destroyed at 160°C (Kitabatake *et al.*, 1991). Heating citrinin-contaminated corn to 105°C for 16 min eliminated the diuretic effect of the mycotoxin (Kirby *et al.*, 1987). Ochratoxin A is highly stable to heat treatment and it is not destroyed even at 200°C (Trenk *et al.*, 1971; Trivedi *et al.*, 1992). However, heating in the presence of NaOH resulted in decomposition and detoxification of the toxin (by using HeLa cells) (Trivedi *et al.*, 1992). Considerable destruction of ochratoxin A (approximately 80 %) occurred (in sterile coffee beans inoculated with *A. ochraceus*) during a heat treatment which simulated the roasting of coffee beans (190–227°C for 5–20 min) (Levi *et al.*, 1974). Recently, a comprehensive study of ochratoxin reduction during coffee roasting under different conditions conducted by nine different laboratories reported a relevant range of ochratoxin A reduction of between 69 and 96 % (van der Stegen *et al.*, 2001). In addition, several reports have shown a correlation between roasting time and temperature and the reduction in ochratoxin concentration (Castelo *et al.*, 1998; Viani, 2002). However, although roasting significantly and efficiently reduced ochratoxin concentration, when the raw material was heavily contaminated, a significant concentration of ochratoxin was still detected, almost all of which was subsequently infused into the coffee decoction when the roasted samples were ground and extracted with boiling water. Therefore, the reduction of ochratoxin concentration in contaminated coffee beans by roasting is sometimes insufficient (Studer-Rohr *et al.*, 1995). The thermal stability of ochratoxin in grains depends on the moisture conditions. The presence of water increased the decomposition of the toxin. However, complete destruction of ochratoxin, in either dry or wet grains, was not achieved even at 200°C or at 250°C in dry grains (Boudra *et al.*, 1995).

Most of the *Fusarium* mycotoxins are relatively resistant to heat. Zearalenone was not destroyed in corn, even after 44 h, at 150°C (Bennett *et al.*, 1980), and fumonisin B<sub>1</sub> required a high temperature (150–200°C) to achieve 87–100 % destruction in corn cultures. Canned, whole-kernel corn showed a significant decrease in fumonisins at an average rate of 15 %. Canned, cream-style corn and baked corn bread showed significant decreases in fumonisin levels at average rates of 9 and 48 %, respectively. Corn-muffin mix artificially contaminated with 5 µg of fumonisin B<sub>1</sub> per g and naturally contaminated corn-muffin mix showed no significant losses of fumonisins upon baking. Roasting cornmeal samples artificially contaminated with 5 µg of fumonisin B<sub>1</sub> per g and naturally contaminated cornmeal samples subjected to 218°C for 15 min exhibited almost complete loss of fumonisins (Castelo *et al.*, 1998).

Destruction of DON during baking of various products by means of several baking technologies (baking temperatures and times) was studied for a range of amounts of the toxin in the dough and in the presence of additives (such as yeasts)

(El-Banna *et al.*, 1983; Scott *et al.*, 1983; Young *et al.*, 1984; Seitz *et al.*, 1986; Tanaka *et al.*, 1986). It is difficult to compare the results achieved with the different baking technologies since numerous 'combinations' of temperature, time and toxin level have been reported. However, DON was not usually completely destroyed during baking – if any reduction in the amount of DON occurred, it was not more than 50 %. In the presence of yeasts, however, the DON concentration increased. For the given process, a higher rate of DON reduction was noted when small amounts of the toxin were present than with large.

Extrusion cooking (with three extrusion variables: flour moisture, extrusion temperature and sodium metabisulphite addition) was effective in the inactivation of DON (reduction of more than 95 %) but showed limited success in aflatoxin B<sub>1</sub> removal (10–25 % reduction) (Cazzaniga *et al.*, 2001). Extrusion with 0.3 % lime and 1.5 % hydrogen peroxide was the most effective process for detoxifying aflatoxins in corn tortillas, but a high level of those reagents negatively affected the taste and aroma of the product (Elias-Orozco *et al.*, 2002). Heat treatments were effective in reducing patulin in apple juice – the reduction was greater at 100 °C than at 90 °C and in the presence of activated charcoal (Kadalkal *et al.*, 2002; Kadalkal and Nas, 2003).

#### 9.4.2 Microwaves

When a rapidly oscillating radio frequency or microwave field (500 MHz–10 GHz) is applied, the water molecules reorient with each change in field direction, creating intermolecular friction and generating heat. Aflatoxin (pure or in a food model) is destroyed when exposed to microwaves, but the rate of destruction depends on the microwave power and exposure time. A reduction of at least 95 % in aflatoxin content in peanuts occurred following a 16 min treatment at a microwave power level of 1.6 kW or a 5 min treatment at a power level of 3.2 kW (Luter *et al.*, 1982). The correlation among destruction rate, microwave power and exposure time was presented also by Pluyer *et al.* (1987) and Farag *et al.* (1996). Microwave treatment was only partially successful in lowering deoxynivalenol levels but it was most effective at the highest temperatures (Young, 1986). There is no additional evidence of the destruction of other mycotoxins by microwaves. However, one might expect that the more heat-tolerant the mycotoxin, the harder it will be to affect its molecules; nevertheless, specific electromagnetic effects, which can cause ion shifts, must be considered.

Because the degradation of mycotoxins during food processing is a desirable goal, a traditional processing method that is also an effective decontamination procedure should be the first choice for the management of a particular product (López-García and Park, 1998). However, many parameters which relate to the food matrix, the nature of the process and the toxin itself are crucial for achieving partial or, preferably, full decomposition of any particular mycotoxin. Therefore it is almost impossible to establish a protocol for 'mycotoxins destruction' during an industrial process. Nevertheless, understanding the link between those parameters and toxin destruction (e.g. the direct correlation between MC and the degradation

rates of several mycotoxins; the efficacy of the time–temperature matrix; the protective effects of additives) may contribute to the better planning of whether or how to process material contaminated with mycotoxins. With any food-processing procedure, the nutritional and technological properties must be retained, and any toxic residues must be eliminated.

### 9.4.3 Irradiation

GI is a physical means which has been studied for its efficacy in destroying mycotoxins in agricultural produce and processed foods. It has attracted interest because it can be used as a multi-purpose method for the eradication of all the biotic factors responsible for food deterioration (insects, moulds, mites). In addition, radiation inhibits sprouting and delays ripening, and serves as an alternative to chemical control (Sharma, 1998). However, radiation users should take into account several important criteria that apply to toxicological hazard removal (no toxic residues should remain in the product); food acceptability (the technological characteristics or nutritional value should remain unaltered); and microbiological aspects of the application of non-lethal doses (e.g. development of radiation-resistant micro-organisms, increased pathogenicity).

Results accumulated from studies of the effects of radiation on mycotoxins have revealed several points of concern.

- The dosages required to degrade a pure mycotoxin vary, depending on the state of the toxin being tested (e.g. powder vs dissolved, type of solvent) and the concentration.
- The dosages required to destroy a pure mycotoxin are not the same as those needed for its detoxification in foods.
- The dosages needed to degrade any particular toxin might depend on the produce MC. Therefore, *in vivo* studies with the specific ‘mycotoxin–product system’ should be carried out as a preliminary step in the use of irradiation as a means of destroying mycotoxins. These studies should involve several mycotoxin levels and several product MCs.
- Irradiated fungal inocula may produce large amounts of toxins [in the case of aflatoxins (Applegate and Chipley, 1974) and ochratoxin (Applegate and Chipley, 1976; Paster *et al.*, 1985) but not patulin (Bullerman and Hartung, 1975)].
- Mycotoxin production on irradiated grains is sometimes significantly higher than on non-irradiated material (Priyadarshini and Tulpule, 1976; Niles, 1978). However, the findings on this question are controversial (Sharma, 1998).

The possibilities that irradiation may increase food susceptibility to mycotoxin production and that, following irradiation (at sub-lethal dosages), fungi may produce increased levels of mycotoxins, should be regarded as limitations on the use of GI as a means of preservation. The effects of GI on mycotoxins (either pure or in produce) have been studied with several mycotoxins. Ochratoxin (dissolved

in methyl alcohol) was found to be stable, even under GI dose of 75 kGy (Paster *et al.*, 1985). Aflatoxin in aqueous solution was degraded following radiation doses above 10 kGy but in benzene:acetonitrile solution the toxin was only partially destroyed, even at 50 kGy (van Dyck *et al.*, 1982; Reddy, 1987), and in a dry state, only 10 % of it was destroyed after treatment at 182 kGy (Aibara and Miyaki, 1970). Irradiation doses of up to 20 kGy did not affect aflatoxin B<sub>1</sub> in corn, wheat or soybeans (Hooshmand and Klopfenstein, 1995), and dosages of 50–100 kGy were needed to remove the toxic and mutagenic properties of peanut meal contaminated with this toxin (Temcharoen and Thilly, 1982). The doses required to detoxify aflatoxin (and other mycotoxins) in foods are not within the permissible dose range for application to foodstuffs, therefore, studies were carried out aimed at reducing the dose by exploiting a possible synergistic effect when radiation was combined with other means. In the presence of hydrogen peroxide, low doses were required to inactivate aflatoxin B<sub>1</sub> (using the Ames mutagenicity test) (Patel *et al.*, 1989).

Aflatoxins in contaminated diets were completely degraded by 5 % H<sub>2</sub>O<sub>2</sub> combined with radiation at 5 kGy, but the decontaminated diet induced adverse effects in animals, probably because of the formation of other toxic compounds when using H<sub>2</sub>O<sub>2</sub> and radiation (Soliman *et al.*, 2001).

In corn, wheat or soybeans, significant reductions occurred in DON and zearalenone concentrations at doses of 10 or 20 kGy and in T-2 concentrations at 7.5 kGy (Hooshmand and Klopfenstein, 1995). DON, T-2 and zearalenone were eliminated from samples of wheat and flour at 8 kGy (Aziz *et al.*, 1997). DON and 3-A DON in aqueous solution were completely destroyed by 50 kGy but, when the same dose was applied to the toxin on corn (with a moisture content of 12–13 %), 80–90 % of the toxin remained. Both toxins were stable at that dose when irradiated under dry conditions (O'Neill *et al.*, 1993). Discrepancies in the literature concerning the doses required to eliminate given mycotoxins in grains and food (DON for example) may be attributed to differing experimental conditions (e.g. moisture content).

Exposure of corn to 15 kGy reduced the concentration of fumonisins B<sub>1</sub> and B<sub>2</sub> by 20 % (Visconti *et al.*, 1996). Irradiation of apple juice concentrate spiked with 2 ppb patulin resulted in the disappearance of the toxin, and the titration acidity, reducing sugars content, carbonyl content and amino acid composition of the concentrate were all unchanged (Zegota *et al.*, 1988).

It can be concluded that, despite the advantages associated with the use of irradiation, and apart from the difficulties involved (special equipment and safety issues), the dosages required to degrade most mycotoxins are well above those permitted for use in food preservation (up to 10 kGy). Therefore, at the present time, GI cannot be regarded as a practical means for mycotoxin elimination. However, studies which will address the safety of the process (at doses relevant to the destruction of mycotoxins) along with the application of the Hazard Analysis Critical Control Point (HACCP) system in a food irradiation plant, might pave the way towards implementing this means for eliminating mycotoxins (Sharma, 1998).

#### 9.4.4 Sorption of mycotoxins

A novel approach to the prevention of aflatoxin intoxication in some animals is the inclusion in their diet of aflatoxin-selective clays that tightly bind these poisons in the gastrointestinal tract, thus significantly decreasing their bio-availability and associated toxicity (Phillips *et al.*, 1994). These methods aim at preventing the deleterious effects of mycotoxins by sequestering them to various sorbent materials in the gastrointestinal tract, thereby altering their uptake into the blood and deposition in target organs. The efficacy of various adsorbents in binding mycotoxins was first described in *in vitro* studies during the 1980s (Smith, 1980, 1984; Davidson *et al.*, 1987; Phillips *et al.*, 1988). In pioneering studies, a phyllosilicate clay which had been commonly used to reduce caking in animal feeds (NovaSil or hydrated sodium calcium aluminosilicate [HSCAS] clay) was reported: to adsorb aflatoxin B<sub>1</sub> with high affinity and high capacity in aqueous solutions (including milk); markedly to reduce the bioavailability of radiolabelled aflatoxins in poultry; significantly to diminish the effects of aflatoxins in young animals such as rats, chicks, turkey poults, lambs and pigs; and to decrease the level of aflatoxin M<sub>1</sub> in milk from lactating dairy cattle and goats.

A variety of binding agents (e.g. activated charcoal, zeolite, HSCAS, bentonite, kaoline, montmorillonite) are purported to adsorb aflatoxins, as well as other chemically diverse mycotoxins, such as T-2 toxin, ochratoxin, DON, zearalenone and fumonisins (Smith, 1980; Rotter *et al.*, 1989; Ramos *et al.*, 1996; Ramos and Hernandez, 1996; Lemke *et al.*, 1998). The most important binders tested *in vitro* and *in vivo* (in feeding trials) for the adsorption of various mycotoxins were listed by Huwig *et al.* (2001). Incorporation of HSCAS into contaminated diets had no effect on the toxicity of T-2 toxin in poultry (Kubena *et al.*, 1990). Superactivated charcoal had little effect in alleviating mycotoxicosis when T-2 toxin was fed to broiler chicks (Edrington *et al.*, 1997). Three of the inorganic sorbents tested by Bailey *et al.* (1998) gave no protection against the effects of T-2 toxin. HSCAS clay in the diet did not alter the hyperoestrogenic effects of zearalenone (Lemke *et al.*, 1998).

It is, therefore, possible that these agents are non-selective in their action and pose significant hidden risks which arise from their interactions with, for instance, critical nutrients. In addition, *in vitro* (test-tube) evidence indicates that some of these binders provide little (if any) protection from aflatoxins or other mycotoxins. Clay and zeolitic minerals comprise a broad family of functionally diverse silico-aluminosilicates. However, natural and modified clay minerals (e.g. bentonite, zeolite, diatomite) showed little or no binding to DON but extensive binding to aflatoxin B<sub>1</sub> (Thimm *et al.*, 2000). Despite the promising effect in binding aflatoxin, there may be significant risks associated with the inclusion of non-selective clays (or other adsorbents) in the diet. Aflatoxin adsorbents should be rigorously tested, with particular attention to their effectiveness and safety in aflatoxin-sensitive animals and their potential for interaction with nutrients. *In vitro* studies showed the ability of certain activated carbons (ACs) to bind fumonisin B<sub>1</sub>, but further studies are needed to demonstrate the ability of these materials to prevent fumonisin B<sub>1</sub> damage *in vivo* (Galvano *et al.*, 1997).

A few other compounds have also been tested for their ability to adsorb mycotoxins. A new approach to reducing aflatoxin M<sub>1</sub> contamination in milk uses oltipraz, a substituted dithiolthione that inhibits aflatoxin B<sub>1</sub> metabolism by inhibiting the activity of several cytochrome P450 enzyme activities. When aflatoxin B<sub>1</sub> and oltipraz were incubated with bovine hepatocytes, aflatoxin M<sub>1</sub> was not formed, which indicated that oltipraz is highly effective in inhibiting aflatoxin M<sub>1</sub> contamination of milk from dairy cows exposed to aflatoxin B<sub>1</sub>-contaminated feeds (Kuilman *et al.*, 2000). However, the authors concluded that 'in case of oltipraz treatment, unmetabolized aflatoxin B<sub>1</sub> can reach systemic circulation. The impact of alternative pathways of biotransformation and possibly bio-activation of the parent aflatoxin B<sub>1</sub> molecule cannot be predicted and thus *in vivo* experiments in bovines are needed to confirm the protective value of oltipraz'.

Granulated activated carbon (GAC) has also been studied for its ability to bind aflatoxins, both *in vivo* and *in vitro*. Results from studies with GAC varied widely according to the type of activated carbon used. Activated carbon has also proven effective in reducing patulin in naturally contaminated fruit juices (Sands *et al.*, 1976; Decker and Corby 1980; Leggott *et al.*, 2001). Feuerstein *et al.* (1988) demonstrated that the administration of monoclonal antibodies specific to T-2 toxin neutralized the inhibitory effects of the toxin on protein synthesis *in vitro*. Recently, Freimund *et al.* (2003) showed that chemically modified 1,3-β-D-glucan, derived from baker's yeast, yielded compounds which were highly efficient in adsorbing T-2 and zearalenone. The adsorption capacity of some of the compounds was greater than those described so far. Although more *in vivo* studies are needed, this approach seems promising as regards binding of *Fusarium* toxins. The approach of adsorbing (or 'binding') mycotoxins can be applied to the food matrix by developing extraction columns with binding properties for the mycotoxins, in a solid-phase extraction system (Tozzi *et al.*, 2003).

## 9.5 Mycotoxin removal by solvent extraction

Solvent extraction is one of the most practical means for mycotoxin removal since it can be integrated into food-processing lines. It is also highly effective: in the case of aflatoxins, 80–95 % of the toxin can be removed (depending on the extraction system used) during processing operations (Ellis *et al.*, 1991). However, solvent extraction is associated with possible disadvantages and limitations, including the extraction of vital nutritional components, the introduction of off-flavours, additional costs, and the possible need for specialized equipment and safe procedures for the disposal of the mycotoxin extracts.

The selection of an adequate separation system is based on the solubility of the chosen mycotoxin in the extraction agents. Aflatoxin is not soluble in water, therefore this toxin is extracted in organic solvents (single solvent, mixtures of organic agents or organics mixed with water). Significant removal of aflatoxin from peanut meal was achieved with aqueous isopropanol (Rayner and Dollear, 1968), hexane–acetone–water (Goldblatt and Robertson, 1970), aqueous acetone

(Goldblat, 1971) and methanol–water or acetonitrile–water (Cole and Dorner, 1994). The solvent-to-sample ratio (at a given aflatoxin concentration) is one factor affecting extraction efficiency (Cole and Dorner, 1994).

Aflatoxins can be removed from oils during conventional refining processes and in the presence of sodium chloride almost all of the toxin is removed (Parker and Melnick, 1966; Shantha and Sreenivasa Murthy, 1975). The amount of fumonisin B<sub>1</sub> in corn can be significantly reduced by steeping the corn in water, which is a step in wet milling (Canela *et al.*, 1996). Water was also successful in extracting fumonisins B<sub>1</sub> and B<sub>2</sub> from ground samples of several corn products but not from rice (Lawrence *et al.*, 2000). However, SO<sub>2</sub> could delay toxin extraction from the contaminated grain. Steeping corn in a 0.2 % solution (containing fumonisin B<sub>1</sub>) significantly decreased the amount of toxin in that solution, indicating that corn may contain fumonisin-binding constituents that are released into solution (Pujol *et al.*, 1999). Shetty and Bhat (1999) reported that corn contaminated with fumonisin B<sub>1</sub> had a low density and that 86 % of the toxin could be removed in the buoyant fraction after treatment with NaCl solution. A clear influence of temperature and solvent composition on the recovery of fumonisins B<sub>1</sub> and B<sub>2</sub> from some matrixes was demonstrated by Lawrence *et al.* (2000). With acetonitrile–methanol–water, the quantity of fumonisins extracted from naturally contaminated taco shells or nacho chips almost tripled when the temperature was increased from 23 to 80 °C, and increased by another 30 % when ethanol–water was used as the extraction solvent at 80 °C. The ethanol–water extraction solvent was described by Lawrence *et al.* (2000) as the cheapest, least toxic and most environmentally friendly one for organic residue analysis, and at 80 °C, the extraction capacity of this mixture was equal to or better than that of the methanol–water or acetonitrile–water–methanol combinations which are commonly used for fumonisin extraction.

## 9.6 Chemical decontamination of mycotoxins

The application of a chemical method should meet the following criteria: at the end of the process, the treated product should not contain any toxic residues; the nutritional value and/or the technological properties of the treated material should not be altered. It is only when these criteria have been met that economic considerations should be taken into account.

Numerous chemicals have been studied for their ability to detoxify mycotoxins; they include alkalis, acids, oxidizing agents, chlorinating agents, and reducing agents. However, their detoxification ability (in any ‘chemical-mycotoxin destruction’ system) depends on parameters related to the contaminated product (e.g. its MC), parameters associated with the process (such as temperature and pressure), the incubation time and the level of the mycotoxin in the product.

Chemicals which are useful for large-scale aflatoxin detoxification are: methylamine, sodium hydroxide and formaldehyde (Mann *et al.*, 1970); hydrogen peroxide (Sreenivasamurthy *et al.*, 1967; Chakrabarti, 1981); chlorine gas (Sen *et*

*al.*, 1988); sodium hypochlorite (Trager and Stoloff, 1967; Natarajan *et al.*, 1975); bisulfite (Doyle and Marth, 1978a; Hagler *et al.*, 1982, Scott, 1998); and ammonia [Mann *et al.*, 1970; Park *et al.*, 1988; Park, 1993 (using the high-temperature/high-pressure treatment)]. In the case of sodium bisulfite, which is a common feed additive, the main product of the reaction with aflatoxin B<sub>1</sub> is a sulfonate, aflatoxin B<sub>1</sub>-S, formed by addition of bisulfite to the furan ring (present in aflatoxins B<sub>1</sub> and G<sub>1</sub>). Sodium bisulfite can destroy aflatoxin B<sub>1</sub> in corn (Moerck *et al.*, 1980) and figs (Altuğ *et al.*, 1990).

Nixtamalization (alkaline cooking and heating), a technology used for preparing traditional foods from corn (tortillas, for example), significantly reduced the concentration of aflatoxin. However, much of the original aflatoxin was re-formed when the product was acidified (Ulloa-Sosa and Shroeder, 1969; Price and Jorgensen, 1985; Elias-Orozco *et al.*, 2002).

Chemicals which are useful for the decontamination of *Fusarium* mycotoxins are: calcium hydroxide monomethylamine [Ca(OH)<sub>2</sub>-MMA] for T-2, diacetoxyscirpanol (DAS) and zearalenone (Bauer *et al.*, 1987); sodium bisulfite for DON (Swanson *et al.*, 1984; Young, 1986; Young *et al.*, 1987); formaldehyde vapours (for zearalenone, Bennett *et al.*, 1980); and ammonia [for deoxynivalenol (after 18 h exposure)(Young, 1986) and for fumonisin (Park *et al.*, 1992)]. In the case of [Ca(OH)<sub>2</sub>-MMA], increasing the MC of the feedstuffs led to considerably improved destruction of the toxins which was concomitant with partial or complete elimination of the toxic effect (Bauer *et al.*, 1987). The fate of fumonisin during nixtamalization was also studied. Hydrolyzed fumonisin B<sub>1</sub> was found to be the main toxic product formed, but the formation of other toxic products could not be ruled out (Hendrich *et al.*, 1993; Sydenham *et al.*, 1995; Murphy *et al.*, 1996). Park *et al.* (1996) found that exposure of corn contaminated with fumonisin B<sub>1</sub> to a modified nixtamalization procedure resulted in the elimination of the toxin, and also of the mutagenic potential of the corn extracts. Another approach to the detoxification of fumonisin B<sub>1</sub> involves a reaction with fructose to block the amine group, which is critical for its toxicity (Lu *et al.*, 1997).

However, in some cases, although the suggested technology successfully decomposed the studied mycotoxin, it did not meet the criteria for commercial implementation. For example, aqueous sodium bisulfite significantly reduced the DON levels in wheat grain but the treatment had a detrimental effects on the rheological properties of the dough (Young *et al.*, 1986).

Of the many chemicals used for mycotoxin decontamination, ammonia, which alters the chemical structure of aflatoxins and thus reduces their toxic and mutagenic potential, is the most efficient and yields the most reliably safe product (Park, 1993). The two main products formed by ammoniation of aflatoxin B<sub>1</sub> were described by Lee *et al.* (1974), Cucullu *et al.* (1976) and Lee and Cucullu (1978). The major one, which was termed aflatoxin D<sub>1</sub>, is a non-fluorescent phenol of molecular weight 286 that retains the dihydrofurano group but lacks the lactone carbonyl moiety characteristic of aflatoxin B<sub>1</sub>. The compound derives from the opening of the lactone ring during ammoniation and subsequent decarboxilation of the resulting β-keto acid. A second compound that was isolated was identified as

a non-fluorescent phenol of molecular weight 206, similar in structure to aflatoxin D<sub>1</sub>, but lacking the cyclopentenone ring.

Aflatoxin-ammonia reaction products that were isolated in maize and cottonseed or in human foods derived from animals fed with ammonia-treated aflatoxin-contaminated feeds exhibited significantly reduced toxic and mutagenic potential. No tumours or neoplastic lesions were observed in trout fed rations containing milk obtained from lactating dairy cows that had eaten ammonia-treated aflatoxin-contaminated feed. The high mutagenic activity in milk from cows exposed to aflatoxin B<sub>1</sub> was eliminated or significantly reduced by treating their feed with ammonia (Jorgensen *et al.*, 1990). Although some decontaminated reaction products are somewhat toxic, the effects (mutagenicity, DNA or protein covalent binding potential) are many orders of magnitude less severe than those of the parent aflatoxin B<sub>1</sub> (Lawlor *et al.*, 1985). These compounds, however, usually represent < 1 % of the original aflatoxin concentration in the feed matrix. In all of the feeding studies carried out with ammonia-treated feeds, no toxic effects related to the ammoniation procedure have been detected (Park *et al.*, 1988). It should be noted that the effect of ammonia on feed composition and by-products is usually increased concentrations of total and non-protein nitrogen, crude protein, ash, and soluble solids, with reduced concentrations of sulphur-containing amino acids, available lysine, and sugars. Production parameters such as milk and egg quality have been shown to be improved, or at least not adversely affected, by ammoniation. The use of ammonia has gained acceptance in many countries, including Brazil, France, Mexico, South Africa and some states in the USA (Henry *et al.*, 2001).

There are several commercial ammoniation processes, of which the most commonly used are the high-pressure/high-temperature (HP/HT) method used in feed mills, and an atmospheric pressure/ambient temperature (AP/AT) procedure that can be used on the farm (Park, 1993). However, when two ammonia-based processes for the decontamination of aflatoxin in peanut meal were compared, clear differences between the meals were found in the growth rates of rats (Neal *et al.*, 2001). Since the two commercial-scale processes used were equally efficient in reducing the aflatoxin contents, it was concluded that the differences could be attributed to differences between the nutritive properties of the two meals. Thus, even when similar ammoniation processes are involved, which result in similar reductions in aflatoxin content, differing responses can be elicited *in vivo* by feeding these meals to animals.

As to the effect of ammonia on some other mycotoxins: an ammoniation process which significantly lowered aflatoxin levels had no effect on zearalenone contamination in yellow corn (Bennett *et al.*, 1980). Ammoniation, applied under the conditions used to destroy aflatoxin, reduced the level of fumonisin in corn, but the toxicity of the toxin was not decreased (Norred *et al.*, 1991). Ammonia inactivated ochratoxin A, citrinin and penicillic acid, but not sterigmatocystin (Chelkowski *et al.*, 1981).

The application of ozone (O<sub>3</sub>) as a strong anti-microbial agent is well known (Kim *et al.*, 1999) and its use for mycotoxin destruction has been studied. Aflatoxin can be degraded (by more than 90 %) by ozone without any effect on animals fed

with the treated material (McKenzie *et al.*, 1998; Prudente and King, 2002). Degradation of DON by ozone has also been reported with the presence of moisture being critical for degradation of the toxin (Young, 1986). Ozone was also effective in degrading patulin, ochratoxin, cyclopiazonic acid, fumonisin, aflatoxin B<sub>2</sub>, aflatoxin G<sub>2</sub> and zearalenone. However, the rate of destruction for several of these mycotoxins was dependent upon the level of ozone and the duration of application. In the case of fumonisin, degradation did not correlate with detoxification (McKenzie *et al.*, 1997).

## 9.7 Biological decontamination of mycotoxins

As a means of overcoming the disadvantages of physical and chemical methods, the biological approach seems attractive. 'Biological detoxification' of mycotoxins works mainly *via* two major processes, sorption and enzymatic degradation, both of which can be achieved by biological systems.

Live micro-organisms can absorb either by attaching the mycotoxin to their cell wall components or by active internalization and accumulation. Dead micro-organisms too can absorb mycotoxins, and this phenomenon can be exploited in the creation of biofilters for fluid decontamination or probiotics (which have proven binding capacity) to bind and remove the mycotoxin from the intestine. Enzymatic degradation can be performed by either extra- or intra-cellular enzymes. The degradation can be complete, the final product being CO<sub>2</sub> and water. Alternatively, enzymatic modification can alter, reduce or completely eradicate toxicity.

Micro-organism detoxification can be performed in many different ways:

- The entire organism can be used as a starter culture, as in the fermentation of beer, wine and cider, or in lactic acid fermentation of vegetables, milk and meat.
- The purified enzyme can be used in soluble or immobilized (biofilter) forms.
- The gene encoding the enzymatic activity can be transferred and over-expressed in a heterologous system; interesting candidates for this application include yeasts, probiotics and plants.

### 9.7.1 Binding

A few strains of lactic acid bacteria (LAB) have been reported to bind aflatoxins B<sub>1</sub> or M<sub>1</sub> in contaminated media or in a food model (El-Nezami *et al.*, 1998a; Pierides *et al.*, 2000; Haskard *et al.*, 2001). Several studies have suggested that the anti-mutagenic and anti-carcinogenic properties of probiotic bacteria can be attributed to their ability to non-covalently bind hazardous chemical compounds such as aflatoxins in the colon (Zhang and Ohta, 1993; El-Nezami *et al.*, 1998b; Turbic *et al.*, 2002). Both viable and non-viable forms of the probiotic bacterium *Lactobacillus rhamnosus* GG effectively removed aflatoxin B<sub>1</sub> from an aqueous solution (El-Nezami *et al.*, 1998b). Since metabolic activation is not necessary, binding can

be attributed to weak, non-covalent, physical interactions, such as association to hydrophobic pockets on the bacterial surface (Haskard *et al.*, 2000). The aflatoxin B<sub>1</sub>-binding affinities of various strains of the probiotic bifidobacteria, as well as of *Staphylococcus aureus* and *Escherichia coli*, have been quantified and the data suggested that there are differences in binding capacity between strains, and differences among heat-killed bacteria in their ability to bind aflatoxin B<sub>1</sub> efficiently (Oatley *et al.*, 2000). El-Nezami *et al.* (2000) using an *in vivo* system were able to demonstrate reduced aflatoxin B<sub>1</sub> uptake by the chicken duodenum in the presence of *Lactobacillus* and *Propionibacterium* strains. Considerable amounts of the *Fusarium* mycotoxins zearalenone and its derivative  $\alpha$ -zearalenol, were bound *in vitro* to the probiotic bacteria *L. rhamnosus* GG and *L. rhamnosus* LC705. Both heat-treated and acid-treated bacteria were capable of removing the toxins, indicating that binding, not metabolism is the mechanism by which the toxins are removed from the media (El-Nezami *et al.*, 2002).

### 9.7.2 Detoxification

Another approach to the biological decontamination of mycotoxins involves their degradation by selected micro-organisms. This biodegradation may enable the removal of mycotoxins under mild conditions, without using harmful chemicals and without significant impairment of the nutritive value or palatability of the detoxified food or feed (Bata and Lasztity, 1999). Two critical reviews on biological detoxification by Bhatnagar *et al.* (1991) and Karlovsky (1999) summarized different and interesting aspects of the biological detoxification of mycotoxins. Detoxification by enzymatic conversion can occur in many biological niches. Mycotoxins can be detoxified by the plant during microbial fermentation (alcoholic or lactic) or by probiotic or other symbiotic micro-organisms; the producer might degrade its own toxin; and soil or water micro-organisms (mixed or pure culture) can be very effective in degradation. Biotechnological and genetic engineering techniques enable the enzymes and genes involved in detoxification to be isolated and utilized to generate decontamination systems, such as immobilized enzymes, genetically modified recombinant plants, or probiotic expressing/over-expressing detoxifying enzymes (Karlovsky and Wolf-Gerhard, 1993; Dalboge, 1997). As the first mycotoxins to be discovered, aflatoxins were also the first target in screening for microbial degradation. Several examples of the detoxification of the most common and important mycotoxins are reviewed.

Almost 40 years ago, several species of micro-organisms – including yeasts, moulds, bacteria, actinomycetes and algae – were screened for detoxification activity; only one isolate was found, *Flavobacterium auranotiacum*, which significantly removed aflatoxin from a liquid medium (Ciegler *et al.*, 1966). It was later shown that this bacterium can remove aflatoxin M<sub>1</sub> from milk (Lillehoj *et al.*, 1971). The detoxification was not dependent on the culture nutrients and at least part of the aflatoxin was metabolized by living flavobacteria (Line and Brackett, 1995). The ability of *F. auranotiacum* to detoxify peanut milk was also demonstrated (Hao and Brackett, 1988). Organisms that have been demonstrated

to detoxify aflatoxin include the bacterium *Corynebacterium rubrum*, the yeast *Candida lipolytica*, and several fungal species, *Aspergillus niger*, *Trichoderma viride* and *Mucor ambiguous* (Mann and Rehm, 1976). In addition, several *Rhizopus* species (Bol and Smith, 1989; Nout, 1989; Faraj *et al.*, 1993), *A. niger* (Faraj *et al.*, 1993) and *Neurospora* species (Nout, 1989) were found to reduce aflatoxin levels. A number of fungal species have been shown to prevent aflatoxin biosynthesis in culture, as well as to degrade the toxin. Among these, a *Phoma* species was the most efficient, destroying about 99 % of the aflatoxin (Shantha, 1999).

Natural and industrial fermentation are traditional techniques for food and feed preservation. Aflatoxin decontamination during fermentation was reported in several cases. About 50 % reduction in aflatoxins B<sub>1</sub> and G<sub>1</sub> has been reported during an early stage of miso fermentation. It was attributed to the degradation of the toxin by micro-organisms (Manabe and Matsuura, 1972). Significant losses of aflatoxin B<sub>1</sub> and ochratoxin A were observed during beer brewing (Chu *et al.*, 1975). Detoxification of aflatoxin B<sub>1</sub> occurred during the fermentation of milk by LAB (Megalla and Mohran, 1984) and in dough fermentation during bread-making (El-Banna and Scott, 1993).

Digestive tract micro-organisms are able to reduce mycotoxin levels not only by binding and removal but also by detoxification. Significant amounts of aflatoxin B<sub>1</sub> were degraded in bovine ruminal fluid (cited in Karlovsky, 1999). When incubated with broiler or turkey faeces, aflatoxin B<sub>1</sub> disappeared after several weeks (Jones *et al.*, 1996).

Under certain conditions, some of the mycotoxin-producing moulds are able to degrade their own toxin. Aflatoxin was degraded by *A. parasiticus* (Shih and Marth, 1975; Doyle and Marth, 1978a) and *A. flavus* (Hamid and Smith, 1987). The degradation involved lactoperoxidase (Doyle and Marth, 1978b,c), peroxidases (Doyle and Marth, 1979) and P450 monooxygenases (Hamid and Smith, 1987). An atoxigenic strain of *A. flavus* has been found to degrade the aflatoxin produced by a toxigenic strain (Cotty, 1994).

Cell-free enzymatic preparations that exhibit mycotoxin-degrading activity can be used as food or feed additives, biological filters or food-processing additives. The advantage of detoxifying food with a purified enzyme lies in its specificity, and this approach avoids the drawback of using the micro-organism, which may, in addition to its degradation activity, exhibit other activities, such as flavour changes and impairment of the nutritional value and acceptability of the product. The fungus *Armillariella tabescens* has been reported to exhibit detoxifying activities against aflatoxins, and the isolation, purification and characterization of its 51.8-kDa protein has been reported (Liu *et al.*, 2001).

Mycotoxin degradation by plant enzymes has also been reported: aflatoxin degradation by maize was suggested (Mertz *et al.*, 1980), but the reduction in toxin level may have been due to conjugation or absorption and there is no clear evidence of aflatoxin-degrading enzymes in plants (Karlovsky, 1999).

Several micro-organisms have been found that can degrade DON and T-2. Jesenska and Sajbidorova (1991) identified several genera of fungi that were able

to detoxify T-2. Acetylation and deacetylation of T-2, without attacking the trichothecene skeleton, by *A. niger* and *Mucor mucedo* were reported by El-Sharkawy and Abbas, (1991). Several soil cultures metabolizing T-2 but not DON were described (Beeton and Bull, 1989). A soil bacterium from DON-enriched soil oxidized the toxin to 3-keto-4-DON, which exhibited remarkably decreased toxicity relative to DON. On the basis of morphological and phylogenetic studies, the degrader strain was classified as a bacterium belonging to the *Agrobacterium Rhizobium* group (Shima *et al.*, 1997). A new genus of  $\alpha$ -proteobacteria was selected and found to oxidize DON and then to convert it into a second product (Völkl *et al.*, 1997).

DON levels did not change during beer malting (Scott *et al.*, 1992; Schwarz *et al.*, 1995) and the amount of trichothecene did not change during wine alcoholic fermentation (Schwenk *et al.*, 1989). In contrast, trichothecene and iso-trichothecin were decomposed during alcoholic fermentation of grape juice. It was suggested that the yeast epihydroxylase might be involved (cited in Karlovsky, 1999). Transformation of DON by ruminal gut microflora of cows, sheep, rats, chickens and other animals has been demonstrated but not attributed to a particular micro-organism (Kiessling *et al.*, 1984; Cote *et al.*, 1986; Swanson *et al.*, 1988; Westlake *et al.*, 1989; Worrell *et al.*, 1989; He *et al.*, 1992).

Detoxification of DON by acetylation of the hydroxyl on carbon 3 by *Fusarium nivale* was reported (Yoshizawa and Morooka, 1975). *Fusarium graminearum* and *Colonectria nivalis* modified T-2 in the same reaction (Yoshizawa *et al.*, 1980). The gene coding for this activity, 3-O-acetyltransferase, was cloned from *F. graminearum* and the detoxification role of the enzyme in protecting the producing strain was shown (Kimura *et al.*, 1998a,b).

The DON concentration on corn ears was hypothesized to be reduced by the metabolism of corn enzymes (Miller *et al.*, 1983; Miller and Young, 1985). A decrease in DON during storage, even at sub-zero temperatures, was reported (Gilbert, 1995). However, more research is needed to determine the factors involved in this phenomenon. Recently, a gene from *Arabidopsis thaliana*, encoding a UDP-glycosyltransferase that is able to detoxify DON, was isolated and characterized; the enzyme, previously assigned the identifier UGT73C5, catalyzes the transfer of glucose from UDP-glucose to the hydroxyl group at carbon 3 of DON (Poppenberger *et al.*, 2003).

Enzymatic transformation by the producing fungi was observed when a *Fusarium moniliforme* strain reduced fumonisin B<sub>1</sub> concentrations in liquid culture (Alberts *et al.*, 1990). The black yeasts *Exophiala spinifera* and *Rhinochloidiella atrovirensa* hydrolyzed ester bonds of fumonisin B<sub>1</sub> (Duvick, 1994), and the hydrolyzed fumonisin exhibited cytotoxicity to rat hepatocytes (Gelderblom *et al.*, 1993) and human colonic cells (Schmelz *et al.*, 1998). Fungal and bacterial isolates metabolized fumonisin B<sub>1</sub> completely and released CO<sub>2</sub> (Duvick *et al.*, 1998). Oxidative deamination of hydrolyzed fumonisin B<sub>1</sub> by cultures of *Exophiala spinifera* was achieved, and work is in progress to clone the corresponding genes and express them in transgenic maize (Blackwell *et al.*, 1999). According to Karlovsky (1999), maize transgenes expressing fumonisin-

degrading enzymes have been produced and tested. Moreover, genetically modified ruminal micro-organisms have been generated (Duvick *et al.*, 1998) and pigs were fed with transgenic mycotoxin-degrading maize (Duvick and Rood, 1998).

Degradation of ochratoxin A to the less toxic compound ochratoxin  $\alpha$  by *Actinobacter calcoaceticus* has been reported (Bata and Lasztity, 1999). Detoxification of ochratoxin A by *Aspergillus fumigatus* and *A. niger* in culture media has been studied, and *A. niger* was found effectively to eliminate ochratoxin A from the medium and to metabolize the degradation product, ochratoxin  $\alpha$  to an unknown product (Varga *et al.*, 2000). Moulds isolated from grapes were able to degrade ochratoxin A; the predominant group of degraders belong to the genus *Aspergillus* (Abrunhosa *et al.*, 2002). Ochratoxin A was converted into ochratoxin  $\alpha$  during barley malting (Krogh *et al.*, 1974) and beer brewing (Chu *et al.*, 1975), by peptidase activity (Nip *et al.*, 1975). Scott *et al.*, (1995) reported the uptake of ochratoxin A, but not of fumonisin, by yeast during beer fermentation. Hult *et al.* (1976) found that hydrolytic activity of the peptide bond of ochratoxin A by symbiotic microorganisms in the stomach of ruminants accounted for these animals' low sensitivity towards this toxin.

Cell cultures of wheat, maize, tomato and other plants completely transformed ochratoxin A into a number of products (Ruhland *et al.*, 1994, 1996a,b). Sweet potato tubers converted ochratoxin A *in vitro* into at least five products, but their toxicity was not assessed (Fujita and Yoshizawa, 1989).

Patulin can be degraded by pure cultures and protoplasts of species of the mould *Paecilomyces* (Anderson *et al.*, 1979). Patulin concentrations were significantly reduced during alcoholic fermentation of apple juice (Harwig *et al.*, 1973; Stinson *et al.*, 1978, 1979). The major degradation product was identified as ascladiol E and Z forms (Moss, 1998; Moss and Long, 2002). Patulin was also degraded by yeasts during incubation of rye silage (Dutton *et al.*, 1984), and upon incubation with sterile ground grains (Harwig *et al.*, 1977). In screenings for patulin-detoxifying bacteria, our group isolated a bacterium from fermented sausage; it was identified as *Lactobacillus plantarum*, and it significantly reduced patulin levels via an intracellular enzyme (R. Shapira, unpublished results).

Zearalenone is transformed by *Thamnidium elegans*, *Mucor bainieri* and *Rhizopus* spp. to zearalenone-4-O- $\beta$ -glucoside (Kamimura, 1986; El-Sharkawy and Abul-Hajj, 1987a). Additional transformation of zearalenone to non-toxic substances was performed by *Streptomyces runosus*, *Cunninghamella bainieri* (El-Sharkawy and Abul-Hajj, 1987b, 1988). The specificity of zearalenone esterase of *Giberrela roseum* was studied (Völkl *et al.*, 1997). Zearalenone was also degraded by a mixed bacterial culture (Megharaj *et al.*, 1997). A few other microbial activities that transform zearalenone have been published but are protected by patents. Transformation of zearalenone by its producing organism has been described by Steele *et al.* (1977). Engelhardt *et al.* (1988) described its transformation via glycosylation by plant tissue, and transformation of zearalenone by farm animals and humans is well-documented (Mirocha *et al.*, 1981; Gareis *et al.*, 1990; Kennedy *et al.*, 1998).

## 9.8 Conclusions

Mycotoxins pose a serious threat to animal and human health, and efforts continue to be devoted, worldwide, to preventing or eliminating them. Research aimed at coping with the problem in the pre-harvest stages addresses, for example, genetic improvement to develop resistance to either fungal invasion or mycotoxin formation, the use of biocompetitors in the field, and improvement of cultural practices. However, more work needs to be devoted to the control of mycotoxins in the post-harvest phases, or during processing. Although mycotoxins may be produced in the field, before harvest, their formation during storage is also a clear danger; therefore, the first step towards preventing mycotoxin formation is to control or prevent the growth of storage fungi. To this end, the low-molecular-weight organic acids (acetic, propionic, formic) and their salts have traditionally been, and still are, used. Although these chemicals are efficient at controlling mould growth, their use presents many disadvantages. For example: their activity is fungistatic, not fungicidal; the acids are corrosive; they cause skin irritation and therefore caution needs to be exercised during their application. Moreover, the use of chemicals, especially for food preservation, does not conform with the worldwide trends towards replacing chemicals, for ecological and safety reasons. Thus, more research is needed to find alternatives to these acids. In fact, work on this subject has not been neglected and, on the contrary, numerous studies have been conducted in areas such as: the use of physical means (MAs, GI); application of natural products, biological control of storage fungi; and the use of integrated systems which combine two or more means, each at reduced level, to yield a synergistic effect which will result in better protection. However, more studies are needed in these areas. Also, more studies are needed, particularly in grain, to gain a better understanding of the interrelationships between the biotic and abiotic factors of the bulk and the effects of these interactions on mycotoxin production. An example of such an interaction is the role of storage pests in fungal dispersion and the resulting potential enhancement of mycotoxin contamination.

When dealing with contaminated material, separation by physical means (not covered in this chapter) appears to be a suitable approach, primarily since there is no fear of any changes occurring in the nutritional or technological properties of the produce. However, the possibility of sorting the produce to remove mycotoxin-containing material is limited, and applying the system to large-scale operations is not always feasible.

Detoxification of mycotoxins, during food processing, has also attracted a lot of interest but, unfortunately, procedures suitable for general use on an industrial scale for the destruction of multiple mycotoxins during processing are still lacking. Indeed, the differences among mycotoxin characteristics and the multitude of processes used in industry, along with the effects of the many parameters on the efficacy of the results, make it almost impossible to establish a protocol for a physical or chemical treatment that would be useful for the destruction of several mycotoxins simultaneously during any particular industrial process.

Thus, the best approach appears to be destruction of mycotoxins in raw

materials. However, here also there are unsolved problems and, out of the means tested, only ammoniation meets the criteria for a safe procedure. Radiation, for instance, which is regarded as having many advantages, has not been proven to be effective in the complete destruction of mycotoxins. Also, the public's distrust of irradiated foods, although without scientific foundation, has created wide opposition to the use of this method and has made it, at least for now, unacceptable for food preservation. Thus more research should be undertaken to find materials, such as ammonia, which will destroy mycotoxins in the raw material efficiently and will be generally regarded as safe (GRAS). Specifically with respect to ammoniation, more research is needed to calibrate and optimize the exact conditions for maximum destruction. A better understanding of the mechanisms involved in mycotoxin destruction could enable the efforts to be invested in the most appropriate 'mycotoxin-destructive materials'. Mycotoxin adsorption is another field of activity that could be explored in the future, since several adsorbents have been found to be highly efficient in alleviating the toxic effects of various mycotoxins. So far, however, no single adsorbent is known that efficiently binds more than one mycotoxin. The mechanism of binding is based on the physical structure of the adsorbent and the properties of the targeted mycotoxin. A better understanding of potential mycotoxin binder(s) and of the physical properties of mycotoxins, could lead to the discovery or development of highly efficient adsorbents which could potentially serve as multi-mycotoxin binders.

As an additional approach, the biological decontamination of mycotoxins offers many benefits. Ultimately, the mycotoxins could be neutralized in the field by transgenic plants and, therefore would never reach animal feed and human food. By using molecular biology techniques, one can generate a multi-barrier approach to the exclusion of mycotoxins. Such an approach necessitates basic and solid research in enzyme and gene isolation, the development of efficient transformation systems, and access to more information on the chemistry and toxicology of mycotoxin-degrading compounds. Recombinant plants, derivative enzymes added during food processing, genetically modified starter cultures and probiotic bacteria are expected to be major players in the offensive against mycotoxins.

As noted, despite the efforts devoted throughout the years to the elimination of mycotoxins, and the numerous technologies and means tested, we still do not have an 'ultimate' solution to the mycotoxin problem. Therefore, it has been suggested that the HACCP system for mycotoxin prevention and control should be applied in parallel to the on-going studies aimed at finding practical means for prevention or destruction of mycotoxins (López-García and Park, 1998; Anon., 2001). The HACCP concept has been successfully applied in the control of quality as well as safety in many food production operations, and it seems to be an effective programme for preventing and controlling risks associated with mycotoxins. The manual prepared by the FAO (Anon., 2001) provides guidance for the application of the HACCP approach specifically for mycotoxin control. It seems likely that data accumulated by various authorities (e.g. countries, food industries, storage plants) as a result of using the HACCP programme will be collected and will serve as a database for analysing the success of the programme in controlling mycotoxins.

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# 10

## Control of mycotoxins: secondary processing

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### 10.1 Introduction

Throughout the world there is increasing consumer demand for high-quality food and drink products with the lowest possible level of contaminants. As a result, the food industry in the developed world demands raw ingredients of the best quality and that conform to statutory limits where these have been set for contaminants such as mycotoxins. Ideally, the formation of any mycotoxins should be prevented, but this is not always possible so it is important to know how concentrations of mycotoxins present in raw materials change through the food chain. Hazard analysis critical control point or HACCP-like assessment of the food chain is now often applied to key commodities to determine the critical points at which mycotoxins can be controlled or eliminated. This approach enables strategies for minimizing consumer exposure to be developed through appropriate management of the products, including safe disposal of any contaminated waste material. In some instances, different maximum allowed limits may be set for the raw material and the finished food product. When this is done, the relationship between the different limits must be realistic and based on sound data.

Processing of raw food commodities can be considered as the application of any combination of chemical, biological or physical methods used to produce the final consumer food or animal feed. For those commodities such as cereals that undergo significant processing, the concentration of a mycotoxin in a food item that reaches the consumer may be considerably lower than that in the raw harvested crop,

although this is not always the case. If this relationship can be established and understood, it may be possible to set limits at a higher level for the raw product than for the processed food without compromising human safety. Food traders worldwide need to remain competitive so this type of assessment should help to protect the consumer without unnecessarily penalizing industry.

There are many factors that influence the changes in the amounts of mycotoxins present during processing, and understanding these factors will assist in minimizing mycotoxins formation, maximizing their elimination and ensuring that, as far as possible, no toxic reaction products are produced. Because there are many different food processes and conditions used in commercial practice, it is not intended to review these in detail but to provide published information about the fate of mycotoxins in key procedures. Important factors are discussed and current knowledge of the fate of the key mycotoxins in major food commodities is reviewed. The change in concentrations of mycotoxins during processing has been reviewed and discussed earlier by a number of authors including Patey and Gilbert (1989); Scott (1991) looked at processing and decontamination procedures on *Fusarium* toxins in grains; and Sinha (1998) studied reduction or elimination of mycotoxins present in cereal grains. Recent literature will be used to complement the work already carried out by these and other workers. It is not intended to review this earlier work except where this is essential to provide a complete picture or to review specific procedures.

## 10.2 Factors affecting mycotoxins during food processing

Most mycotoxins are quite stable at room temperature and under neutral conditions. However, many factors must be considered during processing because temperature, pH, the presence of other constituents or enzymic action may cause reaction and breakdown to occur. Temperature and moisture content are particularly important, but these must be considered along with the other factors which may markedly affect the outcome. For example, Samarajeewa *et al.* (1990) reviewed the degradation of aflatoxins in foods under different heat treatment conditions and showed losses ranging from none to almost complete destruction, while Tabata *et al.* (1992) and Price and Jorgensen (1985) studied the fate of aflatoxins during cooking processes and the effects of food components on their stability.

The general trend associated with processing is to reduce the mycotoxin concentration and the toxicity of the products present. However, there are at least three specific circumstances when this may not be true. Firstly, in cereal milling, it is usual for a mycotoxin to be unequally distributed among the milled fractions, such that in some parts, e.g. commonly bran, a concentration of mycotoxin may occur. A similar principle applies to wet-milling processes. Secondly, chemical reaction may occur so that, while concentrations of the mycotoxin may be reduced, a toxic reaction product might be formed. In general this possibility has been much less studied, although a hydrolyzed toxic product has been reported from fumonisin

(Hopkins and Murphy, 1993) and toxic products from citrinin (Trivedi *et al.*, 1993). Thirdly, the presence of moulds in the commodity may result in further mycotoxin formation during processing under specific conditions.

## 10.3 Controlling mycotoxins during cereals processing

### 10.3.1 Important cereal processes

The processing of cereals or other commodities from harvest to the point where food is eaten by the human or animal consumer involves a complex chain of actions. In the developing countries this may be as simple as hand grinding maize and boiling to obtain a product such as 'kenkey', while in the industrialized world very large-scale, semi-automated mills, bakeries, extrusion plants and breweries that involve many process steps may be necessary to produce the retail product as purchased by the consumer. A general guide to the technology of cereals is provided in a publication such as that by Kent and Evers (1994).

At each stage in these processes the concentration of a mycotoxin may decrease, increase or remain unaffected. This has been briefly summarized by Scudamore and Banks, 2003. The first stage is usually to clean whole grain to remove dust, broken grains and other foreign material. This action can significantly reduce mycotoxin concentrations. Whole grains are then milled to produce flour and other fractions, and these are the materials used to prepare the final products, such as bread, biscuits and cakes by baking, breakfast cereals and snack products by extrusion, or porridge, other breakfast cereals, polenta and similar products by boiling or steaming. Maize in particular is also wet-milled so that different products such as corn starch and sugars are produced for human foods while the residues such as gluten and germ are principally used for animal feed. Barley is used for malt and beer, but other grains may also be used in brewing.

#### *Cleaning, dehusking, scouring and polishing*

Physical procedures generate little heat other than that caused by the operation of the machinery for cleaning, dehusking and abrasion so that no significant thermal breakdown of mycotoxins would be expected at this stage. However, moulds and mycotoxins are often concentrated in dust and broken grains that are more susceptible to fungal infection and toxin contamination, or in the outer seed coat of the grains. The removal of this waste material can thus result in a considerable lowering of the mean mycotoxin concentration, although reports of the extent to which this occurs show the degree of loss to be very variable. This may depend on the quality of the grain on receipt or how the mycotoxin is distributed within individual grains.

Kutit and Merko (1991) studied aflatoxin B<sub>1</sub> and B<sub>2</sub> levels in experimentally contaminated soft and hard wheats during preparation for milling and in the resulting flours and brans. After 60 days storage, B<sub>1</sub> and B<sub>2</sub> levels in hard wheat were twice those found in soft wheat. In hard wheat, aflatoxins were reduced by mechanical surface cleaning, washing and drying and steaming. Reductions of

aflatoxin B<sub>1</sub> were 35 %, 47–85 % and 90–93 % respectively. Less aflatoxin was removed from soft wheat. Milling to flour further reduced aflatoxin levels. Lee *et al.* (1992) examined the effect of a polisher in removing nivalenol, deoxynivalenol and zearalenone from dehusked or unhusked Korean barley. Only nivalenol was detected in the pearled barley, although all three mycotoxins were found in the bran fractions. Scudamore and Patel (2000) studied the effect of cleaning on the levels of aflatoxins, zearalenone and fumonisins in maize in a large commercial mill. Aflatoxin and fumonisin concentrations were reduced by about 30–40 %, but little reduction was observed in the levels of zearalenone. However, no significant reduction of aflatoxin was observed earlier by Brekke *et al.* (1975a). Cleaning appears to be relatively ineffective for deoxynivalenol (Scott *et al.*, 1984; Abbas *et al.*, 1985; Seitz *et al.*, 1985).

### *Dry-milling*

Cleaned cereal is usually milled into component fractions, the detailed specification of which depends on the cereal and the process. However, most mycotoxins tend to concentrate in the bran fractions or outer layers of the grains so that other parts of the cereal structure, that produce fractions such as white flour or maize grits are usually contaminated with lower concentrations of mycotoxin than are found in the original whole grain.

Brekke *et al.* (1975b) studied the dry-milling of corn and found that it generally resulted in an increase in aflatoxin B<sub>1</sub> concentrations in the germ, hull and degermer fines fractions, although distribution varied with the original aflatoxin concentrations in the corn. The grits, low fat meal and low fat flour contained lower aflatoxin levels and together contained only 7–10 % of the original amount of aflatoxin B<sub>1</sub>.

Osborne *et al.* (1996) and Scudamore *et al.* (2003a) showed that white flour fractions contained lower concentrations of ochratoxin A while bran and offal fractions contained higher levels than in the original whole wheat when a Buhler mill was used. Alldrick (1996) reviewed the effects of processing on the occurrence of ochratoxin A in cereals.

Chelkowski and Perkowski (1992) and Trigostockli *et al.* (1996) also studied the distribution of deoxynivalenol and zearalenone in wheat using a Buhler mill. Levels of both mycotoxins were highest in bran and lowest in the flour. Patey and Gilbert (1989) reviewed the distribution of deoxynivalenol, nivalenol and zearalenone in wheat and maize during dry-milling. In general, the mycotoxins were distributed in all fractions obtained from the wheat but were usually concentrated in the bran, shorts and germ. In studies with maize, highest levels of these mycotoxins were found in the germ and bran fractions with least in the grits and flour.

Broggi *et al.* (2002) collected corn samples and different dry-milled fractions from an industrial mill in Argentina and analysed these for fumonisins. The average concentrations for fumonisins are shown in Table 10.1. These data show a similar distribution to that found by Scudamore (unpublished data) in samples volunteered by large commercial mills in the UK. They are also consistent with the

**Table 10.1** Fumonisin concentrations in milled corn fractions.

Corn fraction	Fumonisin concentration, µg/kg		
	B <sub>1</sub>	B <sub>2</sub>	B <sub>3</sub>
Whole corn	1540	716	152
Grits	135	39	10
'C' grade flour	358	122	46
Corn meal	148	52	28
Germ + bran	4210	2010	447

findings of Saunders *et al.* (2001) who showed that fumonisin levels were not detectable or quite low in dry flaking grits and corn flour, higher in corn germ and highest in corn bran.

#### *Wet-milling*

The fate of aflatoxins and zearalenone in corn was studied in a wet-milling plant (Bennett and Anderson, 1978). The starch produced contained extremely low levels of mycotoxins. Aflatoxins were lost in the steep water and were also concentrated in the germ, fibre or gluten fractions. Lauren and Ringrose (1997) followed the fate of zearalenone, deoxynivalenol and nivalenol through a commercial wet-milling plant and obtained similar results for the latter two mycotoxins as the highly soluble deoxynivalenol and nivalenol were found at high concentrations in the steep liquor fractions but only at low levels in the germ, fibre and gluten. The opposite situation was found for the relatively non-polar zearalenone so that low levels occurred in the steep liquor and much higher concentrations in the solid fractions. As animal feed formulations can be prepared from both steep liquor and the solid fractions, all three mycotoxins may occur in these products, sometimes in high concentrations, as was found in commercial samples (Scudamore *et al.*, 1998).

Tabata *et al.* (1999) investigated a manufacturing process in which corn is soaked in aqueous sodium hydrogen sulphite during the production of starch and showed that the breakdown product from aflatoxin B<sub>1</sub> is water soluble so that detoxification would be expected. Similarly, Pujol *et al.* (1999) found that a 0.2 % solution of SO<sub>2</sub> at 60 °C for six hours was the most effective treatment to decrease the amount of fumonisin B<sub>1</sub>. Saunders *et al.* (2001) reported that residues of fumonisins were not detectable in corn starch, the starting material for high-fructose corn syrup and most wet moiled ingredients, after wet-milling corn contaminated with these toxins.

#### *Bread, biscuits, cakes, roasting and frying*

Aflatoxins have been shown to partially degrade in cereals when heated and are much less stable in alkaline conditions (El Banna and Scott, 1983). Scott (1991) reviewed the literature on the extent of breakdown under a range of conditions and only a few studies with aflatoxins appear to have been reported since. In summary,

approximately 50 % of aflatoxins B<sub>1</sub> and G<sub>1</sub> could be destroyed during the fermentation of wheat dough, although the presence of additives such as bromates reduced the losses. Losses of aflatoxins during the baking of bread from wheat (e.g. Reiss, 1978) or corn flour were similarly variable with 0–25 % loss, although this was increased in the presence of bromates, and this is similar to the destruction of aflatoxin when corn meal was made into muffins in which 87 % of the aflatoxins survived (Stoloff and Trucksess, 1981). Almost complete destruction of total aflatoxin was obtained in producing a fried snack product from yellow dent corn naturally contaminated with 1600 µg/kg total aflatoxins (Camoou-Arriola and Price, 1989). Corn was first treated with 3 % sodium hydroxide before autoclaving at 121 °C and finally frying for 15 minutes at 196 °C.

The fate of ochratoxin A during bread making has been reviewed (Subirade, 1996). Separate studies by Osborne (1979), Osborne *et al.* (1996) and Chelkowski *et al.* (1981) showed that ochratoxin A was redistributed on milling into the fractions with highest levels in the bran and lowest concentrations in the white flour fractions. However, there was little loss on baking into bread. This result has since been confirmed by Scudamore *et al.* (2003a). Their study showed that the relationship between the concentration in whole wheat and bread was about 100:70 for wholemeal bread and 100:25 for white bread when 1–3 % by weight of the outer layers of the whole wheat grains were first removed by abrasive scouring.

Samar *et al.* (2001) found that fermentation reduced naturally occurring deoxynivalenol in Argentinean bread processing technology using a pilot scale plant. French bread and Vienna bread were prepared from wheat flour naturally contaminated with deoxynivalenol at 150 µg/kg in which dough was fermented at 30–50 °C according to standard procedures used in Argentina. The maximum reduction obtained in dough at 50 °C was 46 % for the Vienna bread and 41 % for French bread. This agreed with a study (Neira *et al.*, 1997) in which eight types of product were prepared in a low technology bakery that showed a significant reduction of deoxynivalenol during the bread-making process. Gilbert *et al.* (1984) showed that 80 % of deoxynivalenol survived in both spiked and naturally contaminated wheat, although Abbas *et al.* (1985) obtained losses varying between 19 and 69 %. Baking at 170 °C did not degrade nivalenol and zearalenone (Tanaka *et al.*, 1986).

Boyacioglu *et al.* (1993) examined the effect of additives such as potassium bromate, L-ascorbic acid, sodium bisulphite, L-cysteine and ammonium phosphate at varying levels on deoxynivalenol during baking. The reduction was about 7 % in flour without additives. Potassium bromate and L-ascorbic acid had no effect, but sodium bisulphite, L-cysteine and ammonium phosphate resulted in about 40 % reduction.

Jackson *et al.* (1997) examined the effects of baking and frying on fumonisins in corn-based foods. Losses in baking corn muffins baked for 20 minutes were between 16 and 28 % at 175 °C and 200 °C respectively. No significant loss of fumonisin B<sub>1</sub> occurred in spiked corn masa fried at 140–170 °C although degradation started to occur above 180 °C. These studies suggested that fumonisins are quite heat stable in many commercial processes. However, frying corn chips for

15 minutes at 190 °C caused a loss of 67 % of the fumonisin. Castelo *et al.* (1998) showed that bread baking resulted in a 48 % decrease in fumonisins in artificially or naturally contaminated corn, although corn muffin mix showed no decrease in fumonisin levels. However, muffins containing added glucose had lower fumonisin concentrations than the samples containing sucrose, fructose or no sugar, although the reason for this is unclear (Castelo *et al.*, 2001). Fusarin C was completely destroyed on producing corn muffins at 230 °C for 20 minutes (Scott *et al.*, 1986).

### *Cooking and canning*

Aqueous processes such as steaming and boiling may cause loss of mycotoxins by degradation of the toxin in the cereal matrix or by extraction into the cooking liquid. Scott (1991) comprehensively reviewed the literature on stability of aflatoxins in cereal products during cooking processes. Aflatoxins are relatively stable under dry conditions but, in the presence of moisture, variable losses can occur. In cooking rice, for example, loss of aflatoxin B<sub>1</sub> has been reported as being anything between 6 and 88 %, depending on the ratio of water to rice used or whether cooking under pressure. A similar range of loss was reported for aflatoxin-contaminated pasta, boiled buckwheat and for corn flour and corn grits. In village processing of corn flour, Njapau *et al.* (1998) showed that if corn kernels were dehulled and soaked for 24 hours before grinding into corn flour this reduced aflatoxins B<sub>1</sub> and G<sub>1</sub> by 85–90 %. However, the preparation of 'Nshima' by boiling a thick paste of this meal did not result in any further substantial reduction.

The presence of other ingredients can be important. Aflatoxins B<sub>1</sub> and B<sub>2</sub> were reduced by 40 and 28 % respectively in tortilla production incorporating calcium hydroxide (Abbas *et al.*, 1988). Torres *et al.* (2001) investigated the traditional alkaline process (nixtamalization) that involves cooking and steeping corn and showed this to remove 52 %, 84 % and 79 % of aflatoxins in tortilla, tortilla chips and corn chips respectively. Generally similar results were obtained by de Arrola *et al.*, 1988.

Ochratoxin A in neutral conditions is relatively stable during boiling processes, although citrinin, which is often present together with ochratoxin A, readily decomposes (Jackson and Ciegler, 1978; Madsen *et al.*, 1983). However, a toxic breakdown product from citrinin has been reported (Trivedi *et al.*, 1993) while a major decomposition product, citrinin H<sub>2</sub>, occurred on heating in the presence of moisture (Hirota, *et al.*, 2002).

Nivalenol and deoxynivalenol were shown to be relatively stable in buffer solutions over the pH range 1–10 (Lauren and Smith, 2001). Conditions of pH 12, high salt concentration and 80 °C together with long exposure were necessary for substantial breakdown. Nivalenol and deoxynivalenol in ground maize were both reduced by 60–100 % on treatment with aqueous bicarbonate at 10, 20 or 50 % by weight and subsequent heating at 80 or 110 °C for two and 12 days. By contrast, zearalenone was not reduced even by 12 days heating at 110 °C after treatment with a bicarbonate solution. Trichothecene levels in Japanese noodles fell by about 30 % for T-2 toxin and diacetoxyscirpenol, 40 % for deoxynivalenol and nivalenol, and 60 % for fusarenon-X and neosolaniol when boiled at 98 °C for ten minutes

(Kamimura *et al.*, 1980). The authors speculated that loss might be due to extraction into the cooking liquid. Variable results were obtained after boiling in water when uncooked foods were spiked with deoxynivalenol (Isohata *et al.* 1986). Loss for boiled rice was 23 % and up to 97 % for noodles while losses were 20–30 % for noodles in a similar study for nivalenol, although up to 98 % on one occasion.

Cooking and canning generally had little effect on fumonisin content (Saunders *et al.*, 2001). Shephard *et al.* (2002) showed that about 23 % of fumonisin content was lost during production of a typical South African corn porridge. In the masa process, measurable fumonisin was reduced following the cooking, soaking and washing steps, with little conversion of fumonisin to the hydrolyzed form. Castelo *et al.* (1998) studied the effect of canning on artificially and naturally fumonisin contaminated corn-based foods and showed that canning of whole cereal resulted in a decrease of 11–15 % in fumonisins. However, when alkaline steeping of corn contaminated with fumonisins was used in producing tortillas (nixtamalization) their concentration was reduced by about 90 %, although some hydrolyzed fumonisin B<sub>1</sub> was produced (Dombrink-Kurtzman *et al.*, 2000). Similar results showing up to 80 % loss were obtained by Voss *et al.* (2001). Partially hydrolyzed and fully hydrolyzed fumonisin B<sub>1</sub> were both produced. The partially hydrolyzed product was predominately in the steeping liquid and solid waste. Further studies on hydrolyzed fumonisins were reported (Eun-Kyung Kim *et al.*, 2003) that showed their presence in corn flakes and recommended that this should be taken into account by food safety authorities in estimates of human exposure to protein bound fumonisin.

Fusarin C is a mycotoxin that has not been studied extensively because it is considered unstable during processing. This was confirmed by Zhu and Jeffrey (1992) who examined its stability in wotou, small steamed corn-based cakes made in China, and showed that most fusarin C was destroyed under normal conditions. However, pickled vegetables are added in some processes and most of the fusarin C remained in the cakes after 30 minutes steaming under these high pH conditions.

### *Extruded cereal products*

Extrusion cooking is a system in which raw materials are passed through continuous processing machinery within which they are compressed and sheared at elevated temperatures and pressures. The technique has been comprehensively described (e.g. Riaz, 2000; Guy, 2001). It is used for producing a range of products including breakfast cereals, snack products, confectionery, pet foods and animal feeds. Products from the extruder may be dried or toasted to form the final product. Although temperature and pressure within the extruder may be quite high, residence time is short so that most mycotoxins survive extrusion processes to some extent. Results reported for the effects of extrusion are quite variable, and breakdown can be significantly affected by the presence of other substances, especially those alkaline in nature.

Approximately 15 % of aflatoxins B<sub>1</sub> and B<sub>2</sub> survived extrusion at 150 °C when spiked corn dough was treated (Martinez and Monsalve, 1989). However, in a

study by Cazzaniga, *et al.* (2001) in which the effects of flour moisture content, temperature and addition of sodium metabisulphite were examined, the reduction of aflatoxin B<sub>1</sub> was only between 10 and 25 % for samples of corn flour experimentally contaminated with aflatoxin B<sub>1</sub> at 50 µg/kg. In contrast, in the same study with deoxynivalenol at 5 mg/kg in the corn flour, the process was very effective for the reduction of deoxynivalenol content under all the conditions assessed, with higher than 95 % loss. During extrusion of rice flour of 17–20 % moisture content at temperatures between 140 and 200 °C and a screw speed of 130 rpm, 75 % of aflatoxins B<sub>1</sub> and B<sub>2</sub> were lost (Camargo *et al.*, 1989).

Boudra *et al.* (1995) studied the decomposition of ochratoxin A under different moisture content and temperature. Their results showed that ochratoxin A could be partially decomposed so that, in the presence of 50 % water, loss in comparison to drier grain is increased at 100 and 150 but is decreased at 200 °C. Scudamore *et al.* (2004) showed that ochratoxin A was relatively stable during extrusion of whole-meal wheat over a range of different conditions, although the loss increased with temperatures up to 200 °C and with a longer residence time in the extruder.

Reports vary on the stability of deoxynivalenol during extrusion. Wolf-Hall *et al.* (1999) showed deoxynivalenol to be quite stable in heat-treated foods when the effects of high-temperature and high-pressure processing of foods spiked with deoxynivalenol were examined. There was no significant reduction in deoxynivalenol in extruded corn grits, extruded dry dog food and autoclaved moist dog food after processing. Autoclaved cream style corn showed a reduction in deoxynivalenol of only 12 %. However, Accerbi *et al.* (1999) showed that soaking wheat highly and naturally contaminated with deoxynivalenol in sodium bisulphite solution reduced levels from 7.3 to 0.8 mg/kg and then, after further extrusion, to 0.3 mg/kg.

Ryu *et al.* (1999) studied the reduction in zearalenone during extrusion to make corn grits. Between 65 and 83 % reduction of spiked zearalenone occurred, although this varied slightly with screw configuration and was not affected by the moisture content of the grits. Losses were higher at 120 °C than at 160 °C.

Many studies have been carried out on the stability of the fumonisins during processing since their identification and the recognition that they can occur in very high levels. The grits fraction from maize is used extensively for production of cornflakes and other products. Saunders *et al.* (2001) showed that extrusion of dry-milled products reduced fumonisin concentrations by 30–90 % for mixing-type extruders and 20–50 % for non-mixing extruders. The effects of extrusion cooking, gelatinization and corn flaking on the stability of fumonisins in artificially contaminated maize grits spiked with fumonisins B<sub>1</sub> and B<sub>2</sub> at levels of 2 and 0.6 mg/kg, respectively, were investigated by Meuster (2001). All the samples showed significant decreases in the fumonisin levels. Cooking, extrusion and gelatinization reduced fumonisin levels to approximately 30–55 %, cooking the grits for flaking to approximately 20–65 %, and roasting the flakes to approximately 6–35 % (depending on the technological parameters selected).

Katta *et al.* (1998) examined the stability of fumonisin B<sub>1</sub> during the extrusion of corn grits using different temperatures and extruder screw speeds. The barrel

temperature and the screw speed both affected the loss of fumonisin that ranged from 34–95 % in all products but 46–76 % in those that gave commercially acceptable expansion and colour. Cortez-Rocha *et al.* (2002) also showed that extrusion parameters such as die configuration could affect losses of fumonisins during processing.

Degirolamo *et al.* (2001) investigated the stability of fumonisins B<sub>1</sub> and B<sub>2</sub> during the production of corn flakes from raw corn flour by extrusion and roasting. Loss through the entire process was about 60–70 %, but that during the extrusion step was less than 30 %. Extrusion processing of grits containing either sucrose or glucose destroyed more fumonisins than in the control, but glucose was again the most effective so that, when the conditions were optimized, 92 % loss of fumonisin B<sub>1</sub> occurred (Castelo *et al.*, 2001).

### *Malting, brewing and beer*

There are two main processes in the production of beer; malting and brewing. In malting, the cereal (usually barley) is steeped to obtain the correct moisture content, allowed to germinate and kilned to produce the malt. The malt, to which other cereal-based adjuncts may be added, is soaked in a brewing liquor (mashing) and the temperature raised to produce the wort that is then separated from the spent grains. Hops are added to the wort that is then boiled, cooled and fermented with yeast to produce the beer.

Scott (1996) showed that by adding ochratoxin A, aflatoxin B<sub>1</sub>, zearalenone, deoxynivalenol and fumonisins B<sub>1</sub> and B<sub>2</sub> at various stages during the brewing process all these mycotoxins could be transmitted into beer to some extent. About 2–13 % of ochratoxin A is destroyed during the fermentation stage (Scott *et al.*, 1995), although citrinin did not survive the mashing stage. Earlier Scott *et al.* (1992) showed that deoxynivalenol was stable when wort was fermented with three different strains of *Saccharomyces cerevisiae* to produce beer, while zearalenone was metabolized to form 69 %  $\beta$ -zearalenol and 8 %  $\alpha$ -zearalenol. However, when mouldy corn was used for beer production in Nigeria, the carry-over of zearalenone from starting product to beer was as high as 50 % (Okoye, 1987). Scott and Lawrence (1994) detected fumonisins (fumonisin B<sub>1</sub> above 2 ng/ml in four out of 41 samples) in Canadian and imported beer in a small survey.

The fate of ochratoxin A during malting and brewing has been reviewed (Baxter, 1996). Malt containing ochratoxin A at about 50  $\mu$ g/kg produced by inoculation with *Penicillium verrucosum* was used in a pilot plant to determine the fate of ochratoxin A (Baxter *et al.*, 2001). Overall between 13 and 32 % of the ochratoxin A survived in the beer. Up to 40 % was lost during mashing and another 16 % remained in the spent grains.

### *Other mycotoxins in cereals*

Limited studies have been reported on the fate of other mycotoxins during cereal processing. Scott (1991) reviewed the limited information for sterigmatocystin, patulin, penicillic acid, rubratoxin B, *Alternaria* mycotoxins, ergot alkaloids and moniliformin. A 100 % reduction of moniliformin occurred during alkaline pilot-

scale cooking of naturally contaminated corn during tortilla manufacture although, in a laboratory-scale study with a cultured corn sample, only 71 % reduction occurred (Pineda-Valdes *et al.*, 2002).

## 10.4 Controlling mycotoxins during oil seed, beverage, dairy and other food processing

Groundnuts are highly susceptible to aflatoxin contamination so that derived products such as peanut butter are also found to be contaminated (Gilbert and Shepherd, 1985). Dowell *et al.* (1990) examined 17 loads of farmers' stock peanuts during screening over a belt cleaner. Removal of loose-shelled peanuts and small pods reduced aflatoxin levels by 35 %. Further removal of damaged kernels and other loose-shelled nuts reduced levels from 111 to 3.8  $\mu\text{g}/\text{kg}$  in sound mature kernels. Aflatoxin concentrations are usually higher in poor quality nuts such as groundnuts and pistachios (Schatzki and Pan, 1996), so that processing pistachios for high-quality nuts involves separating them into a number of process streams. It was concluded that 90 % of aflatoxin was concentrated in about 5 % of low-quality product so that removing this would reduce the mean concentration from 1.2 to 0.12  $\mu\text{g}/\text{kg}$ . Roasting peanuts and other nuts destroys variable amounts of aflatoxins (Marth and Doyle, 1979). Abalaka and Elegbede (1982) showed from studies in an oil-extracting plant that between about 10 and 15 % of aflatoxins remained in crude oil after crushing groundnuts and cotton seed, although this was reduced further by refining. Studies have shown that the aflatoxins are moderately stable on heating peanut oil, corn oil and coconut oil (Peers and Linsell, 1975). In village processing techniques boiling peanut meal yielded a moderate reduction in aflatoxins B<sub>1</sub> and G<sub>1</sub> whereas roasting of whole peanut kernel reduced levels of these aflatoxins by about 80 % (Njapau *et al.*, 1998).

Whole cottonseed was extruded to determine if the temperature and dwell time (multiple stages of processing) associated with the process affected aflatoxin levels (Buser and Abbas, 2002). Total estimated reductions of 55 % (three stages of processing at 104 °C), 50 % (two stages of processing at 132 °C) and 47 % (one stage of processing at 160 °C) were obtained from the combined equations. If the extreme conditions (four stages of processing at 160 °C) were applied the resulting aflatoxin reduction would be 76 %.

### 10.4.1 Beverages

Patulin is produced by *Penicillium expansum* in apples and some other fruits; it can thus occur in apple juice which may be produced directly through crushing to a pulp. Harrison (1989) reviewed the occurrence of patulin in apple juice, its stability during fermentation and removal from cider. In brief, the processing of apples usually involves removal of decayed apples, initial washing, crushing/pulping, clarification and pasteurization. Acar *et al.* (1998) showed that washing and handling were critical steps in reducing patulin in apples since up to 54 % could be

removed by high-pressure water spraying. In the industry-scale production of apple juice concentrate, conventional clarification using a rotary vacuum pre-coat filter was more effective than ultra-filtration – losses were respectively 39 % and 25 %. Bissessur *et al.* (2001) examined clarification in more detail using apple pulp spiked with patulin. This was pressed and clarified using four different processes; fining with bentonite, pectinase treatment, paper filtration and centrifugation. Reduction of patulin was shown to be due to binding to solid substrates. Pressing followed by centrifugation proved the most effective, removing 89 % of the mycotoxin. Leggott *et al.* (2000) also found that a complex clarification procedure could reduce patulin levels by about 40 %. Other stages of the procedure were studied so that, with a starting level of 2010 µg/kg, an initial washing stage reduced this to 440 µg/kg, and subsequent removal of rotten and damaged apples to 200 µg/kg. However, during a pasteurization stage, patulin increased from 105 to 165 µg/kg, probably due to the raised temperature during this process.

Kadakal *et al.* (2002) followed the patulin, fumaric acid and hydroxymethylfurfural concentrations during commercial processing of apples for juice production. Heat treatment and charcoal were effective in achieving some reduction of patulin concentrations, but there was no difference in fumaric acid levels between different treatments.

### *Cider*

A number of studies have shown that patulin is generally unstable during fermentation so that products such as cider are usually free of patulin. It is likely that when patulin is reported in cider this is the result of the addition of apple juice to produce 'sweet cider'. Lipowska and Goszcz (1990) showed complete destruction of patulin by fermentation for 48 hours or by treatment with 0.125 % sulphur dioxide. Moss and Long (2002) also showed that patulin was unstable in a study of the fate of [<sup>14</sup>C]-labelled patulin during the alcoholic fermentation of apple juice with *Saccharomyces cerevisiae*. High-performance liquid chromatography (HPLC) analysis of the fermentations showed the appearance of two major metabolites, probably E- and Z-ascladiol.

### *Coffee*

Ochratoxin A often occurs in green coffee beans so considerable research has been carried out to determine its fate during processing. Green coffee is cleaned, roasted, typically for 20 minutes at about 200 °C, ground, and then extracted with hot water, concentrated and spray-dried. The fate of ochratoxin A during processing of coffee was reviewed by Viani (1996). The extent of loss reported in the literature varies considerably. Micco *et al.* (1992) examined the fate of aflatoxins when green coffee beans were roasted by gas and electricity heating for different times. Toxin loss was 93 % for lightly roasted beans and 99–100 % for dark roasted coffee. Studer-Rohr *et al.* (1994) found ochratoxin A in 13 out of 25 samples of green beans analysed, and the mycotoxin level was only slightly reduced after roasting, with most of the mycotoxin then eluting into the brew. van der Stegen *et al.* (2001) also studied the effect of roasting conditions on reduction

of ochratoxin A in coffee. A commercial lot of green coffee, naturally contaminated with ochratoxin A, was roasted under various conditions and the effects on its final ochratoxin A content were determined. Roasting time varied from 2.5 to 10 minutes and the roast colour varied from light medium to dark. The reduction was about 69 % over the combined results. Three different explanations are proposed for this reduction: physical removal of ochratoxin A with chaff, isomerization at the C-3 position into another diastereomer, and thermal degradation with possible involvement of moisture. The authors suggest that all three possibilities may play a role in ochratoxin A reduction during coffee roasting.

Blanc *et al.* (1998) investigated the fate of ochratoxin A along an industrial coffee manufacturing line. In naturally contaminated green Robusta coffee beans, the ochratoxin A levels were drastically reduced during soluble coffee production. Some ochratoxin A was removed by initial cleaning of the beans, but the main reduction occurred during roasting. The roast and the ground coffee contained only 16 % of the starting concentration. A further 3 % loss occurred in producing soluble coffee powder. Leoni *et al.* (2000) showed that between about 50 and 100 % of ochratoxin A could be transferred from roast and ground coffee into the coffee brew.

#### 10.4.2 Milk and dairy products

Aflatoxin B<sub>1</sub> in dairy rations is metabolized to aflatoxin M<sub>1</sub> in milk in the ratio of approximately 100:1. This metabolite can then contaminate subsequent dairy products. Lopez *et al.* (2001) showed that when milk artificially spiked with aflatoxin M<sub>1</sub> was used in the small-scale manufacture of cheese, 60 % was detected in whey while 40 % remained in the cheese. Prasongsidh *et al.* (1999a) examined Cheddar cheese prepared from milk spiked with aflatoxin M<sub>1</sub>; this showed an average three-fold increase in the curd and the enrichment factor in cheese was about 2.3–3.4 times. The distribution and stability of aflatoxin M<sub>1</sub> during processing, ripening and storage of Telemes cheese was studied by Govaris *et al.* (2001). Milk artificially spiked with aflatoxin M<sub>1</sub> was used to produce Telemes cheeses that were allowed to ripen for two months and stored for an additional four months to simulate commercial production. Concentrations of aflatoxin M<sub>1</sub> in the curds produced were 3.9–4.4 times higher than in the milk, while concentrations in the whey were lower than in milk and curds. However, M<sub>1</sub> concentrations fell in the cheese during the ripening process. Bakiri (2001) also showed that levels of aflatoxin M<sub>1</sub> in certain cheeses in Turkey could be higher than in bulk milk whereas levels in cream and butter could be reduced. Govaris *et al.* (2002) showed that aflatoxin M<sub>1</sub> concentrations fell between 13 and 22 % when cows' milk was fermented to produce yoghurt and by 16 and 34 % after storage for yoghurts of pH 4.6 and 4.0 respectively.

Prasongsidh *et al.* (1999b) examined the partition behaviour of cyclopiazonic acid (CPA) in cream and butter. Butter was prepared from whole milk artificially contaminated with cyclopiazonic acid at 1 µg/L. Carry-over of cyclopiazonic acid into butterfat was 4.8 % with more than 92 % remaining in buttermilk. This finding

suggests that cyclopiazonic acid (CPA) contamination of butter would be likely following manufacture with contaminated milk, should this occur in practice.

### 10.4.3 Miscellaneous

Virtually any crop subject to mould attack is at risk from mycotoxin formation, either pre- or post-harvest. Many commodities have been subject to surveillance for mycotoxins, but few other than those reviewed above that require processing before consumption have been studied in depth. A few examples described in the literature are given below.

#### *Dried fruit*

Bahar and Altug (1998) studied the transfer of aflatoxins from contaminated figs to fig molasses and found that there was a 65 % reduction of aflatoxin B<sub>1</sub>, but smaller losses for aflatoxins B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>.

#### *Beans*

Milanez and Leitao (1996) showed up to 84 % loss of ochratoxin A in processing beans (*Phaseolus vulgaris* L.), although smaller losses of about 53 % were reported previously after bleaching, salting and heat processing (Harwig *et al.*, 1974). Greater losses were observed when beans were soaked in water for 12 hours before cooking under pressure at 115 °C for 45 minutes.

#### *Animal feedstuffs*

The effects of processing on ochratoxin A to produce animal feedstuffs have been reviewed and appraised (Scudamore, 1996), although the number of reports of experimental studies is very limited.

## 10.5 Future trends

The study of the fate of mycotoxins during processing has been in general a neglected area. Most research effort has concentrated on the means for prevention of mycotoxin formation, and this must remain the best defence for protecting the consumer. However, prevention is not always possible, especially for those mycotoxins formed under field conditions. Introduction of further legislation for a wider range of mycotoxins in more food commodities means that there is a much greater need to determine how mycotoxins survive processing so that this can be taken into account when setting statutory or guideline limits. Achieving the correct limit minimizes any unnecessary restriction on the use of valuable food commodities without compromising human health. It is thus expected that there will be a trend towards further study of the fate of those mycotoxins that pose the greatest potential risk for humans. In some instances it may then be possible to introduce modifications to commercial processes that result in a significant reduction of mycotoxin content in the retail product.

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# 11

## **Risk assessment and management in practice: ochratoxin in grapes and wine**

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### **11.1 Introduction**

A Swiss survey on ochratoxin A (OTA) in human serum samples, carried out to estimate the exposure of the population to the toxin (Zimmerli and Dick, 1995), revealed that Swiss males living south of the Alps (Ticino) had higher OTA concentrations in their sera than women and people living in the rest of the country; a possible explanation was that wine could be a source of OTA because males living south of the Alps consume more than those living north of the Alps. The authors analysed some wine samples and demonstrated for the first time the presence of OTA in wine.

Several surveys followed and the worldwide occurrence of OTA detected to the present time is summarized in this paper. Fungi responsible for OTA contamination in grapes and wine, their dynamics in the vineyard and factors which influence their growth and metabolism are described. The safe management of wine production is analysed according to a Hazard Analysis Critical Control Point (HACCP) approach supported by a Decision Support System (DSS). Critical control points (CCPs) are suggested and possible preventive and corrective actions described. This is based on available knowledge, but future needs and trends in research are also taken into account. Finally, possible sources of further information and advice are given.

## 11.2 Sources and concentration of OTA

After OTA was first detected in wine, several surveys were carried out, mainly in Europe. The results of a total of 1706 wine samples analysed are summarized in Table 11.1, where the regions of provenance, the total number of samples, the median and/or the mean value (in brackets) of white, rosé, red and dessert wines analysed, the maximum level detected and the authors of the papers published are reported.

A gradient in OTA concentration in wines, red > rosé > white, was pointed out by several authors (Zimmerli and Dick, 1996; Visconti *et al.*, 1999; Rosari *et al.*, 2000; Filali *et al.*, 2001), while Stefanaki *et al.* (2003) showed that OTA concentration in dry red wines was not significantly different from that found in white and rosé wines in Greece. The amount of OTA was dependent on the latitude of the production region: the lower the latitude, the more frequent the occurrence and the greater the concentration. The considerable climatic differences, related to geographic regions, influenced mould contamination and OTA production in a conclusive manner (Zimmerli and Dick, 1996; Otteneder and Majerus, 2000; Rosari *et al.*, 2000; Pietri *et al.*, 2001; Eder *et al.*, 2002; Stefanaki *et al.*, 2003). This tendency was not very evident for white wines, but was distinct with red wines (Otteneder and Majerus, 2000). Differences in OTA level between samples collected in the same regions but in different years, probably due to different weather conditions, were also reported by Pietri *et al.* (2001) and Lopez de Cerain *et al.* (2002).

OTA can contaminate not only wine but also other grape products. Apart from wine samples, Zimmerli and Dick (1996) also analysed OTA in eight red and three white commercial grape juices; the white grape juices contained less than 5 ng/l, but the red ones ranged from <3 to 311 ng/l, with a median (mean) of 235 (188) ng/l. Burdaspal and Legarda (1999) analysed 10 samples of grape juice and found OTA levels in the range 15–102 ng/l. Twenty samples of grape juice were analysed by MAFF (1999); one sample contained no OTA and the others ranged from < 20 to 2050 ng/l, with a mean of 480 ng/l. Between 1995 and 1998, many samples of grape juice were analysed in Germany (Wolff *et al.*, 2000). Seven out of 38 white grape juices contained less than 10 ng/l of OTA, while in the rest of the samples the amount of OTA ranged from < 10 to 1300 ng/l. As regards red grape juices, eight out of the 73 samples analysed were free of OTA, while the rest ranged from < 10 to 5300 ng/l.

Vinegar too can contain OTA. Majerus *et al.* (2000) found OTA in 19 out of 38 wine vinegar samples (range 10–1900 ng/l, 90<sup>th</sup> percentile 220 ng/l); balsamic vinegar samples (29 samples analysed) were the most contaminated (range 10–4350 ng/l, 90<sup>th</sup> percentile 3110 ng/l). Markaki *et al.* (2001) analysed 15 vinegar samples and found that the most contaminated were three samples of balsamic vinegar (range 102–252 ng/l), while the other 12 samples ranged between 8 and 46 ng/l.

Dried vine fruit can be a further important dietary source of OTA. For example, MacDonald *et al.* (1999) determined OTA in 60 samples of retail dried vine fruits

**Table 11.1** Results of surveys carried out from 1996 for ochratoxin A content in white, rosé, red and dessert wines.

Regions monitored	No. of samples	White	Rosé	Red	Dessert	Maximum	Authors
Mediterranean basin	133	<3 (11)	19 (25)	13 (39)	337(250)	388	Zimmerli and Dick, 1996
Europe	267	(20)	(31)	(54)	(1048)	603	Burdaspal and Legarda, 1999
South Italy	56	80 (290)	670 (720)	760 (1240)		7630	Visconti <i>et al.</i> , 1999
World	420	10 (108)	10 (119)	20 (201)		7000	Otteneder and Majerus, 2000
Portugal	64					80	Festas <i>et al.</i> , 2000
France	138					1700	Rosari <i>et al.</i> , 2000
Italy	31					3800	Tateo <i>et al.</i> , 2000
Morocco	30	55 (73)	90 (223)	785 (912)		3240	Filali <i>et al.</i> , 2001
South Europe	31					3400	Markaki <i>et al.</i> , 2001
Italy	111			90 (419)	8 (736)	3856	Pietri <i>et al.</i> , 2001
Austria	117					20	Eder <i>et al.</i> , 2002
North Spain	40					316	Lopez de Cerain <i>et al.</i> , 2002
Greece	268	60 (250)		90 (340)		2690	Stefanaki <i>et al.</i> , 2003

*Note:* the values represent the median (mean) of OTA levels found in each survey. Both values are in ng/l.

purchased in the UK. OTA was found in excess of 0.2 µg/kg in 19 of 20 currant, 17 of 20 sultana and 17 of 20 raisin samples examined, with average (maximum) levels of 9.19 (53.6), 4.86 (18.1) and 2.79 (20.0) µg/kg, respectively. In 1999, a further survey was carried out in the UK (MAFF, 1999); OTA was determined in 301 retail products of dried vine fruits and was found in excess of 0.2 µg/kg in 96 of 100 currant, 92 of 100 sultana and 98 of 101 raisin samples examined, with average (maximum) levels of 4.97 (40.8), 3.42 (25.1) and 2.87 (29.8) µg/kg, respectively.

Important surveys were carried out also in the USA (FAO, 2001). OTA was present in 50 of 69, 76 of 114 and 90 of 133 samples of raisin analysed in 1997, 1998 and 1998–99, respectively; the average (maximum) levels were 0.42 (3.1), 0.82 (8.1) and 1.27 (29.0) µg/kg, respectively. Stefanaki *et al.* (2003) also conducted a survey for the presence of OTA on 81 samples of Greek dried vine fruits collected between 1998 and 2000. OTA was found in excess of 0.5 µg/kg in 43 of 54 currant and 17 of 27 sultana samples examined, with average (maximum) levels of 2.8 (13.8) and 2.1 (13.2) µg/kg, respectively. It is evident that dried vine fruit (currants, raisins and sultanas) can be highly contaminated. Dried vine fruit can be an important dietary source of OTA for people with high levels of consumption, in particular children. Dried vine fruits are commonly used in breakfast cereals and could represent an important route for entry into the human food chain. Due to this, the Commission of the European Communities (2002) fixed a limit for OTA in dried vine fruit of 10 µg/kg.

## 11.3 Epidemiology of OTA producing fungi

### 11.3.1 Definition of fungi responsible for OTA presence in grapes and wine

*Aspergillus* and *Penicillium* are the fungal genera reported to be capable of producing OTA; they are common saprophytes and can be present in different environments, fields and warehouses. Among the aspergilli, it is the section *Circumdati* (the yellow aspergilli), which are well known as OTA producers (Krogh, 1987). They have been studied especially on cereals, where *Aspergillus ochraceus* (formerly known as *A. alutaceus*) and *Penicillium verrucosum* are considered the major cause of toxin presence. *P. verrucosum*, belonging to the terverticillata penicillia, is the only species reported for OTA production on plant products (Frisvad and Filtenborg, 1989). Until 1998, *A. ochraceus* and *P. verrucosum* were also believed to be the relevant fungi for OTA presence in grapes (Ospital *et al.*, 1998), but OTA producing *A. carbonarius* and *A. niger* were identified in dried vine fruits in 1999 (Codex Alimentarius Commission, 1999).

Aspergilli section *Nigri* (the black aspergilli) were first described as OTA producers in 1994 (Abarca *et al.*, 1994) and confirmed in further studies (Teren *et al.*, 1996; Abarca *et al.*, 2001), even if not related to grapes. Indeed, black aspergilli are commonly present in vineyards and have the ability to cause berry rot, known as *Aspergillus* rot or black mould, both on white and red varieties (Snowdon,

1990). All studies published after 1999 confirmed that the relevant OTA producing fungi in grapes are different from those affecting cereals. During the 1997–98 harvests, grape samples were collected from Malbec and Chardonnay varieties, both in Argentina and Brazil. Aspergilli and penicillia were isolated in both countries; *A. ochraceus* was found only in Brazil, with a very low incidence and *P. verrucosum* was never identified. All the aspergilli section *Nigri* collected (131 strains) were checked for OTA production and 25 % tested positive. *A. carbonarius* was isolated only in Brazil and 25 % of the isolates were shown to be ochratoxigenic (Da Rocha *et al.*, 2002). The relevance of *A.* section *Nigri* on different grape varieties was confirmed by Magnoli *et al.* (2003). They found 70 % of samples with black aspergilli belonging to different species, *A. carbonarius* excluded, and 41 % of the isolates were able to produce OTA. Comparable results were obtained in France where samples of grapes and musts used in red table wine making were investigated. Several aspergilli and penicillia were identified, but *A. ochraceus* and *P. verrucosum* were found to be absent. Only *A. carbonarius* was tested for OTA production and all the isolates (14) were positive (Sage *et al.*, 2002).

During 1999 and 2000, an extensive survey was carried out in Italy, where nine vineyards were sampled; 508 fungal isolates were collected, 477 belonging to *Aspergillus* spp. and 31 to *Penicillium* spp.; *P. verrucosum* was never identified. Among the aspergilli, species from sections *Fumigati*, *Circumdati* and *Nigri* were identified, with section *Nigri* (464 isolates) largely dominant; section *Circumdati*, which includes *A. ochraceus*, was isolated only occasionally. Eighty-six isolates of section *Nigri* were identified as *A. carbonarius* and they represented 19 % of the black aspergilli collected in both years (Battilani *et al.*, 2003a). Studies carried out on trans-European populations of fungi on grapes indicate that *Aspergillus* species in general, and section *Nigri* in particular, are the dominant OTA producers, with *P. verrucosum* never isolated and *A. ochraceus* only occasionally (Sage *et al.*, 2002; Battilani *et al.*, 2003a; Kozakiewicz *et al.*, 2004). These results concur with those obtained by Da Rocha *et al.* (2000) for South America.

The species belonging to the *Nigri* section of aspergilli generally exhibit low percentages of OTA producing isolates (5–10 %); *A. carbonarius*, as reported in the literature, is the exception, since it includes a higher percentage of positive strains, ranging from 41.7 to 100 % (Teren *et al.*, 1996; Heenan *et al.*, 1998; Cabañes *et al.*, 2002; Abarca *et al.*, 2003; Battilani *et al.*, 2003a). In addition, *A. carbonarius* shows the ability to colonize and produce OTA in artificially inoculated grape berries *in vitro* (Battilani *et al.*, 2001).

Studies carried out so far to identify fungi responsible for OTA presence in grapes, lead to a common conclusion: black aspergilli\* are the key fungi and

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\**A.* section *Nigri* are commonly called black aspergilli because of the colour of their aerial mould, that is black when the fungus develops both on vegetables and on synthetic media. The identification of section *Nigri* is consequently easy, while the identification of species within section *Nigri*, based on morphology, is notoriously difficult. Black aspergilli can be separated into two groups, namely those with uniseriate conidial heads and those with biseriate heads. The main members of uniseriate are *A. japonicus*, *A. foetidus* and *A. aculeatus*. Among the biseriate only *A. carbonarius* is quite clearly distinguishable from other species because of big conidial heads and spores (Battilani *et al.*, 2003a). Most of the remaining species from biseriates are included in the *A. niger* aggregate. Kusters van Someren *et*

among these *A. carbonarius* is the most probable species responsible for OTA production in grapes (Pitt, 2000; Cabañes *et al.*, 2002; Abarca *et al.*, 2003). In fact, even if other *Nigri* are isolated more frequently than *A. carbonarius* from grapes, more isolates of the latter are producers and synthesize higher amounts of OTA *in vitro* (Battilani *et al.*, 2003a).

### 11.3.2 The dynamics of black aspergilli in vineyards

The dynamics of black aspergilli in vineyards is poorly studied. This is not surprising as black aspergilli are not very relevant as grape pathogens and were only recently identified as responsible for OTA production in grapes. As shown in field surveys carried out in 2000–2002 in Australia, incidence of black aspergilli is high in vineyard soil, with the highest counts of viable spores from surface soil and a decrease with soil depth (Kazi *et al.*, 2003). The population decreases on vine trash on soil and old vine bark and it becomes close to zero if other green or dead parts, both in the vine canopy and on the vineyard floor, are analysed. Spores of *A. carbonarius* and *A. niger* are common in the air of vineyards and the frequency is high, up to 100 cm above soil level.

Indeed, data from a field survey carried out in Italian vineyards managed in a traditional farming system, including the use of fungicidal sprays, highlighted that aspergilli section *Nigri* are present on bunches early in the growing season and that their frequency increases during later grape growth stages. Black aspergilli can be present on grapes starting from setting, albeit with a low prevalence and incidence (Battilani *et al.*, 2003b). One month later, with the growing berries pea-sized, these fungi are quite regularly isolated in all vineyards. The number of colonized samples (berries or pieces of rachis) is low (4–10 %), during early growth stages, but it increases to 70 % at ripening. At early veraison and ripening, the incidence of colonized berries is comparable and the differences seem to be related more to the year than to the growth stage, but they are not related to visible symptoms; in fact, it is normal to isolate fungi from berries without visible mould.

The height of bunches above ground level cannot be considered relevant for fungal presence, according to surveys carried out in Italy (Battilani *et al.*, 2003b), although in Australia soil was demonstrated to be the main source of inoculum for black aspergilli (Kazi *et al.*, 2003). In fact, almost the same number of moulded samples was observed, regardless of the position of bunches above the soil. In contrast, the substrate had a more important role, with the percentage of colonized berries being higher with respect to the parts of rachis (Battilani *et al.*, 2003b). These results on fungal dynamics are relevant, both because aspergilli are usually considered to be post-harvest moulds and because they were all isolated from symptomless berries.

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*al.* (1991), using RFLP techniques (restriction fragment length polymorphism), suggested a division of the *A. niger* aggregate into two morphologically indistinguishable species, *A. niger* and *A. tubingensis*. Studies based on a molecular approach followed, substantially confirming these results and the diversity of *A. carbonarius*, also from the molecular point of view (Mégnezneau *et al.*, 1993; Varga *et al.*, 1993, 1994; Pernicova *et al.*, 1997; Accensi *et al.*, 1999; Logrieco *et al.*, 2002)

Vineyard and year significantly influence the incidence of inoculum and of samples colonized by black aspergilli. Regarding the inoculum, in Australia the incidence of *A. carbonarius* was higher in 2000–2001 than in the following year in almost all potential sources examined (dead one year old canes, vine trash and soil; Kazi *et al.*, 2003). In Italy, taking into account the dynamics of berries colonized by black aspergilli at early veraison and ripening, fungal incidence was higher in 2000 than in 1999, especially at ripening. Most samples were colonized by *A. niger* aggregate, in both years and growth stages, but in addition *A. carbonarius* was relevant at early veraison in 1999 and at ripening in 2000. In both years, the same three vineyards were the most colonized.

The role of vineyards is confirmed by a survey carried out in southern France, where samples from different vineyards showed a different fungal contamination and OTA content (Sage *et al.*, 2002). The geographic distribution of strains has been considered in Italy; it is evident that uniseriate strains are more frequent in the North, while a large number of biseriata strains were collected in the South. The incidence of *A. carbonarius* in southern Italy was higher at early veraison in 1999 and at ripening in 2000. In the northern area, the fungal contamination was always low, especially at harvesting. The mean percentage of OTA producing fungi was very similar in both the geographic areas considered, being 6 % and 8 % respectively of the total number of black aspergilli collected in the survey. *A. carbonarius* was more frequent in the South but, both in the South and in the North, the proportion of OTA positive isolates remained around 60 % (Battilani *et al.*, 2003a). Several vineyards were free from OTA producing fungi, especially in northern Italy (Battilani *et al.*, 2003b). Relevant geographic differences were also found in South America, where the incidence of *A. niger* differed between Argentina and Brazil, being higher in the latter (Da Rocha *et al.*, 2002). Even if little data is available, geographic region seems to play a clear role, with a North–South gradient in Europe.

### 11.3.3 Role of farming system and meteorology on OTA presence in grapes

Surveys carried out on the occurrence of black aspergilli in vineyards show that these fungi are quite normally present (Da Rocha *et al.*, 2002; Sage *et al.*, 2002), but OTA is not always detected in berries, even if colonized by ochratoxigenic fungi (Battilani *et al.*, 2003b). Similarly, OTA producing fungi are not necessarily isolated at harvesting when OTA is present in berries (Sage *et al.*, 2002).

A relationship between OTA producing fungi isolated at early veraison or at harvesting and OTA content was found in Italy in 1999, but fungal presence does not mean OTA synthesis. In fact, in 2000 no OTA was detected in the same vineyards in spite of the presence of *A. carbonarius* (Battilani *et al.*, 2003b). Therefore, other factors play a relevant role. All published papers regarding surveys on black aspergilli as potential OTA producing fungi in grapes confirmed the relevance of the vineyards' position and seasonal weather conditions. As a consequence, farming methods used and climate of the considered areas play a role

in the observed differences of fungal incidence and OTA. Unfortunately, little data is available and only preliminary comments can be made at this time.

Six grape varieties were included in a study carried out in Italy (Battilani *et al.*, 2003b); Negroamaro and Trebbiano were cultivated in two vineyards, the former situated in the South of the country and the latter in the North, while Sangiovese was present in both grape-growing areas. The training system was variable, as was the height of bunches from the soil. Chemicals were normally sprayed in all the vineyards, but the total number of sprays was higher in the North, with a mean of 15 in each cropping season, compared to 10 in the South. The active ingredients used in vineyards generally differed, especially if North and South are compared, but copper and sulphur were always distributed. No active ingredient sprayed is known to be effective against *Aspergillus niger* (Khatri and Shekhawat, 1989; Suryawanshi and Deokar, 2001; Leroux *et al.*, 2002); however, very little data is available on this issue.

Vineyards located in the same farm and managed using the same annual practices showed relevant differences in OTA contamination. In 1999, OTA was practically absent in Malvasia nera, but was detected at concentrations of 1.51 and 13.08 µg/kg, in two vineyards of Negroamaro trained with different systems, bilateral spur pruned cordon and head-trained spur pruned respectively. In the second, the canopy is closer and the bunches are closer to the soil level. This is the only evidence of a role played by the training system.

'Sangiovese' was cultivated in two vineyards, one in northern and one in southern Italy. In 1999, the former was practically free from OTA, while in the latter 1.78 µg/kg of OTA was detected on bunches.

The most remarkable difference was observed between years; in fact, the same vineyards, managed following the same cropping system, showed remarkable or absent OTA contamination in 1999 and 2000, respectively. Because of the significant difference in weather conditions between years and grape-growing areas, a considerable role for these factors can be hypothesized (Battilani *et al.*, 2003b).

In this study, the meteorological data showed specific differences, both between places and the years considered. In northern Italy, the mean daily temperature ranged between 13 and 23 °C in both years and the summation in degree-days from 1<sup>st</sup> April to 30<sup>th</sup> September, the period of crop activity, was 3650. In southern Italy, the temperature was higher, with mean daily values varying between 15 and 30 °C in 1999 and between 19 and 30 °C in 2000, with a summation in degree-days of 4500 and 4800, respectively. The two grape-growing areas were also quite different regarding rainfall; in fact, the summations of April–September rainfall in the northern and southern areas were 330 and 305 mm in 1999, and 182 and 62 mm in 2000, respectively. August and September were rainy months in 1999, with more than 70 mm/month in both areas, while they were dry in 2000, with 0 mm in August in the South (Battilani *et al.*, 2003b). These data suggest that both temperature and rain play an important role.

Few indications can be drawn from this study, because of the high number of factors involved and the limited data available. The grape-growing area plays the

main role, meaning that it is unlikely that OTA is found in grapes in certain geographic areas. Regarding Europe, only the southern areas are touched by OTA problems, as shown by surveys carried out on wine; but 'southern' has to be better defined. Risk areas alone do not mean OTA occurrence, because weather conditions too are a decisive factor. In particular, rainfall during ripening seems necessary, maybe as a factor that increases environmental humidity. Grape variety also plays a role, and this was also demonstrated by *in vitro* trials (Battilani *et al.*, 2004). The cropping system involves a variety of factors, and it is very difficult to ascertain the effect of each on a response variable. A potential impact on black aspergilli growth and metabolism is played by the training system. However, in the near future, the most relevant factor could be 'fungicides'; no active ingredient is reported to be able to reduce OTA synthesis in grapes, even if *in vitro* trials have recently underlined the relevance of active ingredients on OTA synthesis by black aspergilli (Battilani *et al.*, 2003c).

## 11.4 Managing wine production: safety issues

### 11.4.1 Critical Control Points in grape production and processing: definition of CCPs based on available information

OTA is a problem that originates in the vineyard. The inoculum of black aspergilli is always present in vineyards and fungi can be isolated from bunches starting from the early stages of the berries' development, even if their incidence is relevant from early veraison. The detection of *A. carbonarius* is crucial, because it is considered to be the major cause of OTA presence in berries, except in Argentina where this fungus has never been identified. Damaged berries, by abiotic and/or biotic causes, are a favourable substrate; in fact, the efficiency of black aspergilli in producing OTA increases when wounds favour grape invasion. OTA is normally detected in symptomless bunches, but mouldy berries are associated with higher toxin levels. At early veraison, a low content/absence of OTA has been also detected in the case of relevant contamination at harvesting. Consequently, the period between early veraison and harvesting can be considered as crucial and factors able to influence fungal growth and OTA synthesis, in particular meteorological conditions, need to be monitored carefully during this time.

Regarding wine making, it seems that OTA is not completely released with the crushing of the berries; in particular, maceration can cause an increase in OTA content estimated at around 20 % (Battilani *et al.*, 2003d), while fermentation results in a decrease of OTA content. OTA synthesis during wine making was never observed probably because alcohol inhibits fungal growth (Delage *et al.*, 2002). The solid-liquid separation after fermentation is the main step responsible for the removal of OTA during vinification (Fernandes *et al.*, 2003).

Chemical adjuvants, especially if charcoal-based, can reduce OTA by up to 90 %. Their use in red wine production is not easy because of the negative effect on colour intensity and anthocyanins level. The use of charcoal-based products for must clarification, in the fermentation off-skins, is probably responsible for the

**Table 11.2** Critical control points during grape production and wine making.

CCPs	Berries status	Fungi incidence	OTA content
<i>Field</i>			
Early veraison	◆	◆	
Ripening	◆	◆	◆
<i>Wine making</i>			
Crushing			◆
Maceration			◆
Fermentation			◆

low content/absence of OTA normally observed in white wine. Lactic acid bacteria can induce around 50 % of OTA reduction during malolactic fermentation in wines. Besides, the addition of yeast cell walls allows the application of a lower dosage of chemical adjuvants with a lighter effect on wine colour (Silva *et al.*, 2003). In fact, yeast cells, both dead and alive, are able to adsorb OTA rapidly (Bejaoui *et al.*, 2003). This summary of available information gives some indications for defining CCPs (Table 11.2), but no limits for CCPs can be defined at this stage of the research, on this problem.

#### 11.4.2 Data input, structure and data output of a DSS for the safe management of grape processing

Decision Support Systems (DSS) integrate and organize all types of information required for production management. In a pathosystem, this includes relationships between pathogen, crop and control measures to support tactical and operational decision-making in crop protection. DSS are commonly planned with the final aim of reducing the use of chemicals, optimizing the positive effect of alternative methods of pests and disease control, maintaining yield quality and quantity. DSS implementation follows the step-by-step approach and four main steps can be listed (Rossi *et al.*, 1997):

- (1) problem definition;
- (2) model development;
- (3) validation of model output;
- (4) model improvement.

In problem definition, support can be obtained by growers, technicians, advisors, consumers, researchers, or any other actor in the agro-food production who knows the phytopathological problems in the considered crop. Problems can be related to yield losses, efficacy of control measures, quality and safety of products or any deviation from optimal production caused by pathogens. In most cases, fungi are studied because they severely damage the product, with significant yield reduction: a definition of conducive condition is necessary, which takes into account the whole farming system together with control measures – both preventive and corrective. When the pathogen is a mycotoxin producing fungus, the problem is

more complicated. In fact, in several cases, the toxin is not related to visible symptoms and it follows that problem definition is more difficult.

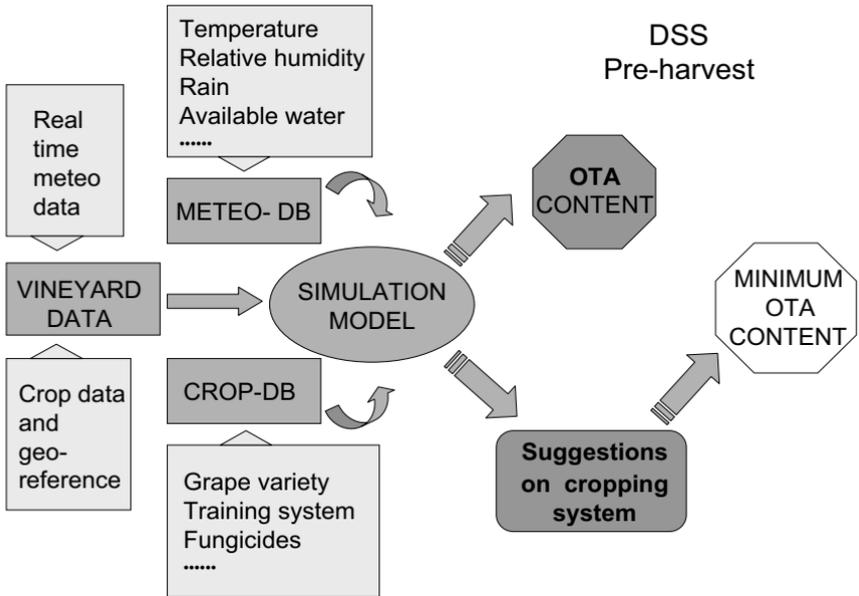
The second step consists of model design and verification. A model is a simplified representation of a system, which is a limited part of reality and contains interrelated elements. During World War II a rational approach was developed in order to study a system in detail: system analysis. System analysis was developed basically as a tool to consider military options with the objective of deciding a course of actions by systematically examining available options and any limiting factors which included cost, effectiveness risk, alternative offensive and defensive strategies (Quade, 1966). System analysis was demonstrated to be useful in different disciplines where a system is studied by distinguishing its major components, characterizing their changes, and the interconnecting elements (Leffelaar, 1993).

The system structure in plant pathology includes pathogen, host, environment, human actions and their relationships (De Wit, 1993). A simple way to represent a complicated system, like a pathosystem, is a relational diagram (Leffelaar, 1993) that shows the status of the system at a certain moment and its dynamics over time, based on the assumptions that the state of the system at any moment can be quantified and that changes in the state can be described by rates. The relational diagram is the best way to organize the available knowledge, point out relevant elements, their relationships and feedback, such as lack of information. Collection of information from different sources (step 1) is the basis of 'system analysis' that starts with drawing a relational diagram which, in the second step of DSS implementation, is translated into quantitative relationships that allow the quantification of states. Putting together all mathematical functions, a simulation model able to predict fungal development is finally obtained. Model verification is then necessary to make sure that each mathematical model represents the relationship under study reasonably well (Van Keulen, 1976), thus ensuring that model translation is an accurate phase (Teng, 1981). The provisional model is the result of the second step, generally in a computerized form.

The third step consists of model validation and evaluation. The objective of this step is to build up a final model, by testing the agreement between the model outputs and the real system (Rossi *et al.*, 1997). Validation can be done by comparing model output with data from previous experiments, with data obtained by proper trials and with data from trials at farm level, where epidemiological conditions are as representative as possible of the wide range of potential epidemics.

During the last step, improvement and enlargement of the model, feedback from the application of the model in decision-making is collected. Final users are encouraged to report back on model performance and to suggest new needs to the modeller. Unsatisfactory performances and/or new circumstances mean going back to previous steps to update the model and obtain a new version: model development is an ongoing process (Teng *et al.*, 1980).

In summary, with regard to the 'OTA in grapes and wine' problem, the core of a DSS is a simulation model which is able to predict the behaviour of a pathogen,



**Fig. 11.1** Scheme of a Decision Support System for the management of grape production with minimum ochratoxin A content.

in terms of its effect on the host plant, and which is a function of the factors identified as relevant, mainly ecological parameters and the cropping system. A 'meteorological' database and a 'crop' database support the simulation model. These contain quantitative information on the effect of relevant factors on fungal development, both natural and farmer-dependent. Information on geographic area and vineyard, as well as real-time meteorological data, is the starting point for a simulation model and results in suggestions for the cropping system; all these are necessary inputs. Data output consists of a prediction of the pathogen's effect on yield, in relation to natural conditions, and proposals for possible yield improvement, in this case especially in terms of quality, obtainable with the optimization of the cropping system (Figure 11.1).

This part of the DSS considers pre-harvest factors. Indeed, fungal activity and OTA synthesis during vinification has never been demonstrated. The post-harvest part of the DSS starts from OTA content at the beginning of the grape processing phase. Quantitative data on the effect of unit operation (unit operation database) and adjuvant addition support the simulation model of the fate of OTA during vinification. Data output consists of a prediction on the effect of unit operation on final OTA content. Although certain operations must be carried out during vinification, the DSS is nevertheless useful because it predicts the probable effect in advance, both of obligatory and possible operations.

### 11.4.3 Data available for the development of a DSS

OTA in grapes and wine is a 'recent' problem and knowledge is still limited; it is important to remember that black aspergilli were defined as relevant fungi for the first time in 1999 and *A. carbonarius* was suggested as the major cause, at least in Europe, more recently. Rot caused by aspergilli is not mentioned among main grape diseases and it was not studied in detail before OTA detection. Besides, rot is not necessarily related to toxin content. As a consequence, the main factors able to influence fungal development and its metabolism have been identified, or only hypothesized, up to now, but no quantitative relationships have yet been established.

As an example, meteorological conditions play an important role; this is confirmed by the North–South gradient observed in the OTA content of wines produced in Europe (see Section 11.2) or in different years (Battilani *et al.*, 2003b). Cause–effect functions have not yet been elaborated, but the first ecological studies published have pointed out that optimal growth of black aspergilli is at 35 °C, while no growth was observed below 15 °C. Water activity is confirmed as a relevant factor and fungi are more tolerant to unfavourable conditions at 25 °C (Mitchell *et al.*, 2003). Regarding OTA production, the optimal temperature is probably related to fungal strain; in fact, 15 °C and 25 °C were reported respectively for Italian (Battilani *et al.*, 2003e) and Portuguese (Mitchell *et al.*, 2003) strains of *A. carbonarius*. Regarding cropping system, several parameters are deemed to be relevant, such as grape variety, training system or fungicides, but until now only preliminary *in vitro* data have been available with respect to the effect of fungicides on OTA production (Battilani *et al.*, 2003c). Although only limited data is available at present, because of the great interest of grape-growing regions in this problem and the pressure of several organizations to define as soon as possible a threshold value for OTA content in grape processed products, it is reasonable to predict that sufficient data to prepare a DSS will be available in the near future.

## 11.5 Future trends

The state of the art regarding 'OTA in grapes and wine' is satisfactory, considering the recent approach to the problem. Knowledge is available as

- (a) OTA occurrence in wine and grape processed products, at least in Europe;
- (b) fungi responsible have been identified;
- (c) grape production, in particular ripening, is defined as the relevant period for OTA synthesis;
- (d) many aspects of the cropping system and meteorological conditions as relevant factors for OTA production are under study, strengthened by several recently published papers;
- (e) attention is being paid to the role of ecological conditions on fungal growth and OTA production, confirmed by *in vitro* trials and;
- (f) wine making is being considered for its effect on the fate of OTA.

Looking to the future, studies will surely continue in these same areas, to fill the gaps in knowledge and to establish quantitative relationships between the parameters involved, but further important areas also need to be considered.

One relevant point is surely fungal identification. Black aspergilli, as has been explained, are very difficult to identify at a species level and the process is time-consuming. Fungal detection with rapid methods, mainly following a molecular approach, will be useful, to monitor geographic areas already under study or new areas, where species different from *A. carbonarius* could possibly contribute to OTA synthesis.

Biological control of black aspergilli is also a good area for future research, especially from an integrated production viewpoint. In the case of positive results, a biological approach could also be hypothesized, supported by good results in post-harvest OTA degradation demonstrated by micro-organisms.

A geostatistical elaboration of data regarding fungal and OTA occurrence could produce interesting and useful results. In fact, most of the on-going studies will furnish data that, elaborated with this approach, can produce the assessment of risk related to different geographic areas and years, including areas not yet studied, if meteorological and crop data are available. These risk data, or risk maps, could be really useful as a basis for different uses, for example:

- (a) to define areas where OTA has to be monitored and preventive measures applied and;
- (b) as a basis for DSS applied not at a farm but at a regional level.

## 11.6 Sources of further information and advice

OTA in grapes and its processed products is a relevant problem for many countries and several studies are ongoing aimed at better defining CCPs, their limits and relevant factors for OTA production. In Europe, many research projects are supported at national level by Ministries, Cooperatives of grapes-wine production, Universities, Institutes of applied research, and a major effort is being made by the European Commission. In the V<sup>th</sup> FP, included in the Mycotoxin Prevention Cluster ([www.mycotoxin-prevention.com](http://www.mycotoxin-prevention.com)), a specific project was supported: 'Risk assessment and integrated ochratoxin A (OTA) management in grapes and wine' (WINE-OCHRA RISK, Contract n. QLK1-2001-01761) with the overall objective to assess the risk of OTA presence in grapes and wine in Europe and protection of the consumers' health by decreasing the amount of toxin with the aid of integrated management of production and processing. Six countries involved with grape and wine production are studying the problem in a coordinated way.

Further, included in the same cluster, another project was supported: 'Mechanism of ochratoxin A induced carcinogenicity as a basis for an improved risk assessment (Ochratoxin A-risk assessment, Contract n. QLK1-2001-01614). This project will generate data on the mechanisms of tumour induction by OTA. This

should provide a better basis for the assessment of human tumour risks and a better justification for tolerable levels of OTA in food, set on a scientific base and justifiable if challenged by anybody or in relation to restrictions on international trade.

Papers produced by all research projects and recent developments can be obtained via the websites ([www.ochra-wine.com](http://www.ochra-wine.com); [www.uni-wuerzburg.de/toxicologie/eu/ota/ochratoxina.html](http://www.uni-wuerzburg.de/toxicologie/eu/ota/ochratoxina.html)) and meetings organized at national and international level, will guarantee a rapid spread of information and advice. Further, in order to enhance dissemination of news regarding not only OTA in grapes and wine, there is another EC funded project 'European Mycotoxin Awareness Network' ([www.mycotoxins.org](http://www.mycotoxins.org)), where fourteen leading mycotoxin institutions in Europe are involved and provide the most up-to-date information on all areas of mycotoxins.

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# 12

## Controlling mycotoxins in animal feed

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### 12.1 Introduction

Moulds can grow and produce mycotoxins in plant material during the whole chain from field to table. Some crops, preservation systems and feedstuffs are more susceptible to mould growth and mycotoxin production than others. There are numerous mycotoxins, some of which are very toxic to farm animals and may cause acute mycotoxicoses. It is, however, more common for mycotoxins to be consumed by animals in lower quantities over a longer time period, causing chronic or more diffuse toxicoses. They are often not recognized as mycotoxicoses by farmers or veterinarians. Both acute and chronic mycotoxicoses will reduce animal production and increase costs. When farm animals are exposed either to high levels of mycotoxins or to lower levels over a longer period of time, there is also a risk that significant amounts of the mycotoxins will be carried over into animal products such as milk, eggs and meat. Control of mycotoxins in animal feed is thus of great importance.

Feeds are produced both at the farm and commercially at feed mills. Both home-produced and low-cost raw feed materials are used and they can be of low quality and may use degraded food material. The requirement for low feed costs makes the control of mycotoxins too expensive and it is thus often low or even absent. There is a need for new, simple, fast and cheap methods to control mycotoxins in animal feed both at farms and in feed mills.

### 12.2 Production of animal feed

Different mycotoxins are more commonly found in or associated with certain

feedstuffs. Some develop in the growing crop due to its being susceptible to certain toxigenic fungi, while infection and toxin production by others is facilitated by the preservation and storage system used if insufficient care is taken to prevent this. Some mycotoxins are associated only with crops from a certain region, due to the climatic or ecological conditions being suitable for the mycotoxin producing fungi. Feedstuffs and their often-associated mycotoxins are presented in Table 12.1.

Most feeds are produced from crops at the farm and consumed by the animals some time later. However, some straight feeds and particularly mixed feeds are also produced and sold by feed mills. There are additional circumstances surrounding the commercial production of feed, and these will be covered in this chapter.

Feed raw materials are often divided into

- (1) cereals and by-products;
- (2) oilseed by-products;
- (3) leguminous seeds;
- (4) roots and tubers;
- (5) animal by-products;
- (6) green crops/pasture;
- (7) silages;
- (8) hays;
- (9) straws.

They can be used straight, single or in combination in the feeding regime. Component types 1–5 are often combined and used as mixed feed and concentrates. The other raw material groups (6–9) are combined as roughage and mainly used for ruminants and horses. Mixed feed and concentrates are mainly produced at feed mills and, to a lesser extent, at farms.

### 12.2.1 Cereals and by-products

These are one of the main feed resources in developed countries and are used mainly for pigs, cattle and poultry.

#### *Cereals*

Cereals are normally grown, harvested, preserved and stored at the livestock farm. The culturing and harvesting of feed cereals is carried out in the same or a similar way to that for food cereals. Barley and oats are more commonly used as feed cereals, but wheat and maize are also important. During the culturing and especially the flowering period, *Fusarium* fungi may infect the cereals. The fungi produce the fusarium toxins deoxynivalenol, nivalenol, zearalenone, fumonisins, HT-2 and T-2 toxins, which are commonly found in cereals. Diagnosis and control of *Fusarium* in cereal crops are very important for lowering the risk of fusarium toxins getting into the feed. This is described in Chapter 8. Maize grown in the subtropical and tropical areas can, after drought stress or insect damage, also be infected by *Aspergillus flavus/parasiticus*, with accompanying aflatoxin formation.

**Table 12.1** Feedstuffs and their associated mycotoxins. Toxins printed in bold types are relatively more common in the feedstuff.

Feed raw material	Mycotoxins produced	
	In field during culturing	Preservation and storage
<i>Cereals and by-products</i>		
Barley	<b>DON</b> , NIV, Zea, HT-2, T-2	<b>OTA</b> , <b>Afla</b> , <b>Cit</b>
Maize	<b>DON</b> , <b>Fum</b> , <b>Zea</b> ,	<b>Zea</b> , <b>Afla</b> ,
Maize gluten	<b>DON</b> , <b>Fum</b> , <b>Zea</b> ,	<b>Zea</b> , <b>Afla</b> ,
Millet		
Oats	<b>DON</b> , <b>NIV</b> , <b>HT-2</b> , <b>T-2</b>	OTA, Cit
Rice		Afla, Sterig, OTA
Rye	<b>Ergot</b>	<b>OTA</b>
Sorghum		Afla
Wheat	<b>DON</b> , <b>NIV</b> , Zea, ergot	<b>OTA</b> , Afla, Cit
Wheat bran	<b>DON</b> , <b>NIV</b> ,	<b>OTA</b>
<i>Oilseed by-products</i>		
Coconut meal		<b>Afla</b>
Cottonseed meal	<b>Afla</b> , <b>Tenua</b>	<b>Afla</b>
Groundnut meal	<b>Afla</b> , Zea	<b>Afla</b> , OTA, Cit
Linseed meal		
Palm kernel meal		<b>Afla</b>
Rapeseed meal		
Soya bean meal		Afla
Sunflower meal		
<i>Leguminous seeds</i>		
Beans		OTA
Peas		OTA
Roots and tubers		
Cassava	Zea	Afla
Fodder beet		
Sugar beet pulp		
<i>Silages</i>		
Grass		<b>Roq</b> , <b>Pat</b> , <b>PR</b> , <b>Zea</b>
Leguminous		<b>Roq</b> , <b>Pat</b> , <b>PR</b> , <b>Zea</b>
Maize	Afla	<b>Roq</b> , <b>Pat</b> , <b>PR</b> , <b>Zea</b>
Hay		
Straw	DON	Zea, Sat
<i>Pasture</i>		
Grass	Zea, DON, Ergot	
Rye grass	Spor, Lol, Pasp	
Tall fescue	Ergot	
Red clover	Slaf	

*Note:* Afla = aflatoxins; Cit = citrinin; DON = deoxynivalenol; Ergot = ergotamin; HT-2 = HT-2 toxin; Lol = lolitrem; NIV = nivalenol; OTA = ochratoxin A; Pasp = paspalitrem; Pat = patulin; Phom = phomopsin; PR = PR-toxin; Roq = Roquefortin; Sat = satratoxin; Slaf = slaframine; Spor = sporidesmin; Sterig = sterigmatocystin; Tenua = tenuazoic acid; Zea = zearalenone.

The preservation and storage at the farm of cereals intended for animal feed are often carried out using slightly different means to those used for cereals intended for human consumption. In the northern temperate zone, the small cereals are normally harvested with moisture content above the level considered safe (13–15 %). Maize is also commonly harvested at high moisture content. In many cases, some form of preservation is needed if the grain is to be stored. Drying with heated air, as for food cereals, is often preferred, but it is expensive for high-moisture grains. Many animal farmers prefer to dry to a moisture content around 15 %, because milling conditions and utilization in the animals are better at this moisture level. Drying with ambient air for longer times is still in use, although it has been shown to increase the risk of ochratoxin A formation in the cereals by *Penicillium verrucosum* (Holmberg *et al.*, 1990; Jonsson and Pettersson, 1999).

Careful control of time and final moisture content is important in all drying processes in order to avoid ochratoxin formation. Ochratoxin may be formed during drying, if the drying time continues for some days, and during storage, if the final moisture content is > 15 %. Acid preservation and airtight storage are also used for moist feed cereals. Careful control of acid dosage and application is very important as unsuccessful acid preservation may result in aflatoxin or zearalenone formation. Propionic acid is normally used, while formic acid has been shown to promote aflatoxin formation. More than 50 % formic acid may, therefore, not be used in acid mixtures for cereal preservation according to EC regulations. With airtight storage there is a risk of air-leakage during emptying of the silos. *Penicillium roqueforti* can grow in low oxygen conditions and is often found in airtight stored cereals. Toxins have seldom been looked for, but roquefortin C has been found in at least one case.

#### *Maize gluten feed*

This consists of gluten, bran and germ from the wet-milling of maize. Studies on the fate of mycotoxins during wet-milling of maize have shown that the mycotoxins are concentrated in the bran and germ fractions (Bennett and Anderson, 1978; Lauren and Ringrose, 1997). Analysis of maize gluten feed has also revealed a high occurrence of mycotoxins, such as aflatoxin, zearalenone and deoxynivalenol. The feedstuff must, therefore, be considered as a high-risk product for mycotoxin contamination. Maize gluten feed is mostly used in concentrates for dairy cattle and in mixed feeds for poultry.

#### *Wheat bran and feed meal*

These are produced in the mills as by-products during milling of wheat to produce baking flour. They are popular feedstuffs mainly used in mixed feeds for pigs and poultry. Studies on the fate of mycotoxins during milling have shown that they are concentrated in these milling fractions (Chelkowski *et al.*, 1981; Seitz *et al.*, 1985, 1986; Lee *et al.*, 1987). Such feedstuffs made from wheat contaminated with even relatively low mycotoxin concentrations have to be considered as risky products.

### *Oat feed*

This is a by-product of processing oats and consists of oat hulls and dust. Dehusking studies of trichothecene-contaminated oats and analyses of hulls and kernels or groats have shown that most of the mycotoxin is found in the hull (Pettersson, 2004). Even food-grade oats are very often found to be contaminated with trichothecenes, including nivalenol, HT-2 and T-2 toxins (Langseth and Rundberget, 1999). The feedstuff must, therefore, be considered as a product at risk from mycotoxin contamination.

### *Cereal grain screenings*

Grain screenings are the residues from the cleaning of cereals and comprise broken pieces of grain, small grains and the dust from the outer layers of the grain. Mycotoxin concentrations are often found to be much higher in the small kernels and broken grain and in the outer layers of the grain (Chelkowski *et al.*, 1981; Trenholm *et al.*, 1991; Lee *et al.*, 1992). Cereal grain screenings have thus also been found to contain high mycotoxin levels (Ross *et al.*, 1991; Murphy *et al.*, 1993). The screenings are often diluted with more sound grain, but must be considered as a feedstuff at high risk for mycotoxin contamination. They are cheap and mostly used for ruminants, but their use as a feedstuff should be avoided.

### *Druff*

This consists of the insoluble grain material from the brewer's or distiller's malt after processing. Mycotoxins may be found in the grain used for malting or can be produced during the malting process. They may then end up in both druff and beer and are relatively common in both products (Baxter, 1996). Druff is used for dairy cows and pigs.

## **12.2.2 Oilseed by-products**

Oilseed cakes and meals are the residues remaining after removal of the greater parts of the oil from oilseeds. The residues are rich in protein and most are valuable feedstuffs for all farm animals. Cakes and meals produced from soya bean, groundnut, cottonseed, rapeseed, sunflower, coconut, palm kernel, linseed and sesame seed are used. The oilseeds produced in tropical and subtropical areas are often infected by *Aspergillus flavus* or *A. parasiticus* during both plant growth and storage and are consequently contaminated with aflatoxins. The high water activity in oilseeds in relation to moisture content may also promote growth of fungi and the production of aflatoxins. Oilseed cakes and meals are often transported by ship from the production place to the consumption country and, during transport, they may easily become moist, leading to continued fungal growth and toxin production. Cakes and meals of groundnut, cottonseed, copra and palm kernel are therefore often reported to be contaminated with aflatoxin (Scudamore *et al.*, 1997; Pittet, 1998). These oilseed cakes and meals are often used in concentrates for dairy cattle and such use is often responsible for the occurrence of aflatoxin M1 in milk.

### 12.2.3 Pasture/green crop

Pasture is a quantitatively important feed source used by grazing animals. It can be natural or more or less cultivated. Cultivated pastures often consist of different grass and legume species grown together. Fungi may be already present in the seed or the pasture can become infected during growing and especially at the over-ripe stage. Several mycotoxins can be formed in the plants, and these have been shown to cause mycotoxicoses in the grazing animals. Good pasture management is important if mycotoxins are to be avoided. Species and varieties with low susceptibility to the toxigenic fungi should be used. Seeds from certain regions and of certain varieties, e.g. tall fescue or rye grass, may contain endophytic fungi (*Acremonium* spp.), which will continue to grow and produce mycotoxins (ergovaline, lolitrems) in the plant at the pasture. It is possible to reduce or avoid this by selecting sound seeds. Legumes, e.g. red clover, can, in certain regions, be infected by fungi producing slaframine and causing slobbers.

### 12.2.4 Silages

#### *Pasture grass/clover silage*

The green crop from the pasture grass or grass/clover is also preserved through ensiling. The crop is crushed, sometimes with addition of formic acid, molasses or bacterial cultures to promote ensiling, slightly dried, and packed and stored anaerobically in silos, clamps or plastic-coated big bales. Fresh pasture is used, but early infection and occurrence of fungi and mycotoxins from the crop may result in contamination. The anaerobic condition is important for the ensiling process. If air is leaking into the silo or through the plastic of the big bale, the silage will become mouldy and deteriorate. *Fusarium* mould will often start to grow and produce toxins in the wet crop material at a surface air leakage. If the crop is not tightly packed, air will be trapped in between the crop material. This is common in the middle of big bales produced by certain big bale presses. *Penicillium roqueforti*, which is rather acid-tolerant and micro-aerophilic, will invade the middle of the big bale or the badly packed area. The fungi can produce several toxins, but roquefortin C is most commonly found in silage. Other toxigenic moulds found in deteriorated silage are *Aspergillus fumigatus*, *Byssoschlamys nivea* and *Paecilomyces variotti*. *A. fumigatus* can produce gliotoxin and the other two patulin.

#### *Maize silage*

To produce maize silage, the whole maize crop is chopped, packed and stored anaerobically in silos. Fungi and mycotoxins from the maize crop will enter the silage. The fungi may continue to grow and produce toxins as long as there is oxygen available. *Fusarium* and fusarium toxins are found commonly in maize silage produced in northern regions, while *Aspergillus flavus/parasiticus* and aflatoxins can also be found in silage produced in southern areas. Silages are mainly used for cattle, but are also given to sheep and horses.

### 12.2.5 Hay and straw

#### *Hay*

The green crop from the pasture can also be dried and pressed as bales. Nowadays it is mainly dried in strings at the ley before pressing. It is turned over during the drying and is easily contaminated with soil and rainfall during the drying process, thus starting mould growth. Pressing of bales from still moist hay can create bales with a moist and mould-contaminated core. During wet autumns and winters water can condense on the surface of the bales if they are not covered correctly. The bales will then get a mouldy outer layer. *Aspergillus fumigatus* is very common in mould-deteriorated hay, while *Fusarium* moulds may have grown in the wet crop. Mycotoxins are, however, seldom found or analysed for. Zearalenone has sometimes been found and is believed to have caused hyperestrogenism in cattle.

#### *Straw*

Straw from cereals is collected and pressed as bales after harvest. It is used as bedding for pigs, horses and cattle. Pigs and horses may consume a large quantity of the bedding straw. Straw from scab-affected cereals should not be used for animals, because zearalenone and deoxynivalenol can be relatively high in such material. The straw will sometimes remain in the field and be remoistened by rain. The bales are often stored without protection from the rain. Straw is handled less carefully than hay. Re-wetted straw may be infected by *Stachybotrys atra*, which can produce satratoxin. Stachybotryotoxicosis has mainly been reported from eastern Europe and France (Rodricks and Eppley, 1974; Hintikka, 1977; Le Bars and Le Bars, 1993), but may also occur in other countries.

### 12.2.6 Mixed feed and commercial feed production

Mixed feed and concentrates are mostly produced at feed mills, but some big farms may also have the knowledge and equipment to produce them. The concentrates are often mixed with cereals (milled or crushed) and fed to pigs and poultry or to ruminants together with forage. They are normally produced by mixing feedstuffs with high protein and fat content to get a mixture with energy and digestible protein suitable for use, together with cereals and forage, for specified animals. Oil-seed meals, mainly soya and rapeseed but also cottonseed, groundnut, palm kernel and copra, are often used. Leguminous seeds (beans, peas and lupin seeds), animal protein (fish meal, blood meal, meat by-product, milk protein) and fat of vegetable and animal origin can also be included. Maize and maize gluten meal can also be included, but this is more common in mixed feed intended for poultry and pigs. Cereals and cereal by-products are otherwise included to make up the final mixed feed. Amino acids, phosphates, minerals, trace elements and vitamins are also added to ensure the nutritional value of the mixed feed. The composition of the concentrates and mixed feeds is dependent on the nutritional requirements of the animal and the price and availability of the individual single feedstuff. Computer

programs are used by the feed manufacturers to optimize the price and composition. Low-cost and associated low-quality feedstuffs will tend to be promoted when this computerized optimization is used in the feed mills.

Commercial mixed feeds and concentrates are often extruded and pelleted. Propionic or formic acids can also be added to reduce the risk of mould and salmonella growth in the feed. Such processing of the feeds will kill or reduce fungi and bacteria. This means that conventional methods of microbiological examination of such processed feed can no longer reveal if the feed and its ingredients are of poor hygienic quality prior to processing. The mycotoxins are, however, stable and can be analysed. Many of the individual feedstuffs used in concentrate and mixed feed are products at risk from mycotoxin contamination.

### 12.2.7 Mycotoxicoses

Feedstuffs containing mycotoxins may cause toxic effects in animals fed on them. The diseases seen in the animals are called mycotoxicoses. The toxic effects and signs in the animals are dependent on the type of toxins and their concentrations, and often several toxins are involved in toxicoses. Several known diseases in animals are clear mycotoxicoses, and examples of such well-known mycotoxicoses are given in Table 12.2. However, the signs of mycotoxicoses are not always easily recognized and diagnosis can be difficult. Exposure at low mycotoxin levels for longer times is common but hard to detect.

The mycotoxicoses may be divided into three general forms originally described by Pier *et al.* (1980).

- (1) Acute primary mycotoxicoses are those which develop when high to moderate amounts of mycotoxins are consumed. Specific symptoms and signs of the toxic effect of the mycotoxins can be seen.
- (2) Chronic primary mycotoxicoses result from moderate to low levels of mycotoxin intake. Often non-specific effects such as reduced weight gain and reproductive efficiency occur.
- (3) Secondary mycotoxic diseases result from lesser levels of mycotoxin intake, which do not cause overt mycotoxicoses but which predispose to infectious diseases through impairment of immunogenesis and native mechanisms of resistance.

The latter two forms are more common but harder to diagnose. In field cases, where mycotoxins are found and suspected to be the cause of problems in animal production, it is also common to find nutritional deficiencies or low water quality. A mixture of mycotoxins is often found in the feed. In addition, the concentrations of the mycotoxins are, in many cases, slightly lower than those which produce similar toxic effects in animals when pure mycotoxins are fed in controlled feeding studies. Examples of lowest concentrations of different mycotoxins which produce adverse effects in specified animals fed the toxin in controlled feeding studies are given in Table 12.3.

**Table 12.2** Mycotoxicosis in farm animals

Mycotoxicoeses	Main toxin	Main fungus	Feed	Animal species	Primary symptoms	Reference
Aflatoxicosis	Aflatoxins	<i>Aspergillus flavus</i> , <i>A. parasiticus</i>	Ground nut, cotton seed, maize	Poultry, swine, cattle, dog	Acute hepatitis, haemorrhagic disease, death, reduced weight gain	Patterson, 1977
Porcine nephropathy	ochratoxin A	<i>Penicillium verrucosum</i>	Cereals	Swine, poultry, dog	Nephritis, reduced weight gain	Krogh <i>et al.</i> , 1973, Elling <i>et al.</i> , 1975, Hamilton <i>et al.</i> , 1982, Holmberg <i>et al.</i> , 1991
Ergotism	ergot alkaloids	<i>Claviceps purpurea</i> , <i>C. paspali</i>	Cereals, grasses	Cattle, sheep, chicken	Gangrenous necrosis, nervous seizures, reproductive failure	Robbins <i>et al.</i> , 1986
Fescue toxicosis	ergot alkaloids	<i>Acremonium coenophialum</i> ,	Tall fescue pasture	Cattle, sheep, horses	Gangrenous necrosis, nervous seizures, reproductive failure	Robbins <i>et al.</i> , 1986, Bacon <i>et al.</i> , 1986
Staggers (ryegrass and paspalum)	Lolitrems, paspalitrems, paspalinine,	<i>Acremonium loliae</i> , <i>Balansia spp</i>	Ryegrass pasture, paspalum, maize	Cattle, sheep, horse	Ataxi, tremors	Cole <i>et al.</i> , 1977, Cole and Dorner, 1986
Fusariotoxicoesis, mouldy corn toxicosis	Trichothecenes	<i>Fusarium graminearum</i> , <i>F. culmorum</i> , <i>F. sporo-trichioides</i>	Cereals	Swine, poultry, cattle	Gastroenteritis, dermo-mucal necrosis, haemorrhage, reduced weight gain	Vesonder <i>et al.</i> , 1976, Forsyth <i>et al.</i> , 1977, Wyatt <i>et al.</i> , 1972
Hyperestrogenism or F-2 toxicoses	Zearalenone	<i>F. graminearum</i> , <i>F. culmorum</i>	Maize, cereals, corn-cob, maize silage, sorghum	Swine, sheep	Swelling of vulva, vulvovaginitis, enlargement of mammary glands, fertility disorder	Sydenham <i>et al.</i> , 1988

Equine leukoencephalomalacia, Blind staggers	Fumonisin	<i>F. verticilloides (moniliforme)</i>	Maize	Horse, mule	Ataxi, circling, apathy, depression, confusion, dullness, hyperexcitation, vacuolisation of brain, white matter degeneration	Ross <i>et al.</i> , 1991, 1992
Porcine pulmonary oedema	Fumonisin	<i>F. verticilloides (moniliforme)</i>	Maize screenings, maize	Swine	Respiratory distress, pulmonary oedema, liver injury	Osweiler <i>et al.</i> , 1992
Tibial dyschondroplasia	Fusarochromone	<i>Fusarium equiseti</i>	Cereals	Poultry	Leg weakness	Krogh <i>et al.</i> , 1989
Lupinosis	Phomopsis A	<i>Phomopsis leptostromiformis</i>	Stubble	Sheep	Lower appetite, condition, liver injury, tubuli disturbance	Gardiner, 1967
Facial eczema	Sporidesmin	<i>Pithomyces chartarum</i>	Rye grass	Sheep, cattle	Photosensitivity, cholangiohepatitis	Mortimer and Ronaldson, 1983
Slaframine toxicosis, slobber	Slaframine, swainsonine	<i>Rhizoctonia leguminicola</i>	Red clover pasture, hay and silage	Cattle, sheep	Salivation, diarrhoea, polyuria	Croom <i>et al.</i> , 1995, Broquist, 1986
Haemorrhagic syndrome	Wortmannin	<i>Fusarium torulosum</i>	Cereals	Swine	Haemorrhagic	Mirocha and Abbas, 1989
Stachybotryotoxicosis	Satratoxins, verrucarins, roridin	<i>Stachybotrys atra</i>	Straw, hay	Horses, swine, cattle, poultry	Dermonecrosis, gastroenteritis, haematopoietic depression	Rodricks and Eppley, 1974, Hintikka, 1977
Dendrochiotoxicosis	Roridins, verrucarins	<i>Myrothecium</i> spp.	Straw	Sheep	Dermonecrosis, gastroenteritis, haematopoietic depression	
Myrotheciotoxicosis	Roridins	<i>Myrothecium roridum</i> , <i>M. verrucaria</i>	Grass litter	Sheep	Dermonecrosis, gastroenteritis, haematopoietic depression	

**Table 12.3** Lowest toxic effect levels in different farm animals fed different mycotoxins in controlled feeding studies.

Toxin	Animal	Lowest effect level (ppm)	Toxic effect	Reference
Deoxynivalenol	Pig	> 0.6–4	Reduced feed consumption and growth	Friend <i>et al.</i> , 1982, Overnes <i>et al.</i> , 1997, Bergsjö <i>et al.</i> , 1992, Young <i>et al.</i> , 1983
	Chicken	> 15	Vomiting	Young <i>et al.</i> , 1983, Forsyth <i>et al.</i> , 1977
		> 9	Reduced liver and gizzard weight	Kubena <i>et al.</i> , 1985
		> 16	Reduced feed consumption and growth	Kubena <i>et al.</i> , 1987, 1988, 1989; Kubena and Harvey, 1988, Harvey <i>et al.</i> , 1991
		> 50	Mucosa erosions	
Nivalenol	Pig	> 2.5	Mucosa and kidney changes	Hedman <i>et al.</i> , 1997
		> 5	Reduced feed consumption, growth and leucocyte number	Hedman <i>et al.</i> , 1997, Williams <i>et al.</i> , 1994, Williams and Blaney, 1994
	Chicken	> 3	Reduced feed consumption and growth, gizzard erosions	Hedman <i>et al.</i> , 1995
T-2 toxin	Pig	> 0.5	Reduced immune defence	Rafai <i>et al.</i> , 1995b
		> 1–2	Reduced growth and feed intake	Rafai <i>et al.</i> , 1995a, Friend <i>et al.</i> , 1992
	Chicken	> 0.5–1	Mucosa erosion	Wyatt <i>et al.</i> , 1972, 1973
		> 2	Reduced feed consumption and growth	Wyatt <i>et al.</i> , 1972, 1973

Zearalenone	Pig	> 0.25–0.47	Enlarged vulva and uterus	Friend <i>et al.</i> , 1990, Bauer <i>et al.</i> , 1987
	Chicken	> 300	Increased growth rate, comb, ovary and bursa	Speers <i>et al.</i> , 1971
Ochratoxin	Pig	0.2	Enzyme inhibition in kidney	Krogh <i>et al.</i> , 1988
		> 1	Kidney damage, degenerated tubuli	Krogh <i>et al.</i> , 1988
		0.2–2	Reduced growth	Madsen <i>et al.</i> , 1982a,b, Taipa and Seawright, 1984
	Chicken	2.5	Reduced immune response	Harvey <i>et al.</i> , 1992
		0.2–0.3	Histological changes in kidney	Krogh <i>et al.</i> , 1974, Krogh <i>et al.</i> , 1976
		0.5	Reduced T-lymphocytes	Chang <i>et al.</i> , 1979, Singh <i>et al.</i> , 1990
		0.5–1	Reduced growth	Huff <i>et al.</i> , 1975, Krogh <i>et al.</i> , 1976
Hens	> 1.0	Macroscopic changes of kidneys	Krogh <i>et al.</i> , 1974, Krogh <i>et al.</i> , 1976	
	> 0.5	Reduced egg production	Prior and Sisodia, 1978, Page <i>et al.</i> , 1980	
Fumonisin	Horse	> 1	Brain damage	Wilson <i>et al.</i> , 1992
	Pig	> 15	Changed blood enzyme levels, reduced weight gain	Motelin <i>et al.</i> , 1994
	Chicken	190–280	Change sphinganine/sphingosine ratio, decreased cholesterol	Weibking <i>et al.</i> , 1993

## 12.3 The transfer of mycotoxins from feed to animal products

### 12.3.1 Aflatoxin in milk

The carry-over of aflatoxin to bovine milk is of great importance and is treated in detail below, based on a review by Pettersson (1997). The major part of aflatoxin B1 (AFB1) consumed via feedingstuffs is degraded in the rumen of the dairy cows. A minor but important part is resorbed by the cow and rapidly metabolized into aflatoxin M1 (AFM1) in the liver. AFM1 is subsequently relatively stable and circulates in the blood until it is excreted in milk, urine and bile or is further metabolized.

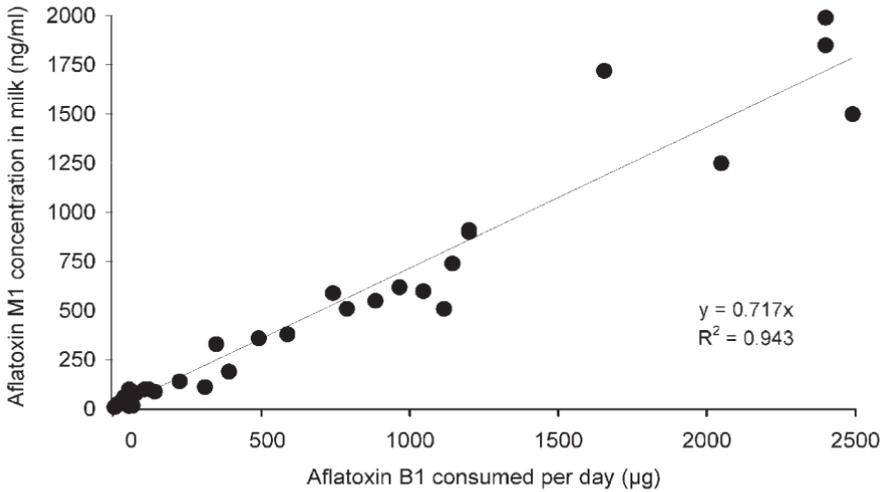
Many studies have been performed on the carry-over of aflatoxin from feedingstuffs to milk. In early studies (prior to 1985), as reviewed by Sieber and Blanc (1978), Applebaum *et al.* (1982) and Van Egmond (1989), quite variable results were obtained. The estimated carry-over of consumed quantity of AFB1 to quantity of AFM1 in the milk varied between 0.18 and 3.94 % of the quantity consumed. In these early studies, considerable quantities (in relation to the present maximum permitted level) of AFB1 were used for (single) low milk yielding dairy cows, and the chemical analytical methods for aflatoxin were often of low quality. This may explain the great variation, and the results must be deemed unreliable.

Subsequent to 1985, nine reports have been published (Price *et al.*, 1985; Frobish *et al.*, 1986; Fremy *et al.*, 1987; Munksgaard *et al.*, 1987; Pettersson *et al.*, 1989; Harvey *et al.*, 1991; Veldman *et al.*, 1992; Veldman 1992; Galvano *et al.*, 1996). Of these some are very accurate and extensive investigations, using quantities around maximum permitted level. Data from these reports are presented in Table 12.4. These more recent studies also show variations of between 0.32 and 6.2 % in the estimated carry-over of aflatoxin. The mean value for all reports is 1.81 % with a standard deviation of 1.22. If data is used exclusively from cows that have been fed a maximum of 500 µg of AFB1 per day, a carry-over of 1.63 % is obtained. A number of reasons give rise to this considerable variation. Some is a result of analytical problems and experimental techniques, but there are also considerable individual excretion differences. This individual variation has been pointed out in several of the studies, but it is outweighed, to a certain extent, by the fact that the studies have been performed on groups of cows. Differences dependent on breed may be present, but this is not clearly discernible in the studies available.

The cows' production levels are of importance to the indicated variation. The Swedish study (Pettersson *et al.*, 1989) and the Dutch (Veldman *et al.*, 1992; Veldman, 1992) with high milk producing dairy cows revealed the highest carry-over: 2.6 % and 2.7–6.2 % respectively. Veldman *et al.* (1992) demonstrate clearly that carry-over is increased in relation to increased milk production and, if data from all the studies are analysed, an increase of approximately 0.1 % per kg of milk is obtained. The mean carry-over value in high milk producing cows (> 25 kg/day) is  $2.66 \pm 1.24$  %. The major part of this carry-over is a result of the increased milk production and the resulting higher quantity of AFM1 excreted. A better and more

**Table 12.4** Carry-over of aflatoxin from feed to milk. Results from studies after 1985.

Consum. AFB <sub>1</sub> µg/day	Milk prod. kg/day	AFM <sub>1</sub> conc. ng/kg	AFM <sub>1</sub> quantity µg/day	Carry-over %	Quotient AFM <sub>1</sub> /AFB <sub>1</sub>	Reference	
39	39.5	60	2.40	6.20	1.54	Veldman <i>et al.</i> , 1992	
34	16.6	40	0.60	1.80	1.18		
7	39.4	10	0.30	3.60	1.43		
14	17.4	20	0.40	2.60	1.43		
33	39.3	30	1.20	3.80	0.91		
36	16.8	50	0.90	2.50	1.39		
57	37.0	60	2.30	4.00	1.05		
56	14.8	100	1.40	2.50	1.79		
78	24.8	80	2.08	2.70	1.02		
53	27.8	51	1.42	2.60	0.96		
108	28.6	99	2.84	2.50	0.92	Veldman, 1992	
492	31.8	360	11.45	2.33	0.73	Pettersson <i>et al.</i> , 1989	
349	14.6	330	4.82	1.38	0.95	Frobish <i>et al.</i> , 1986	
1144	32.9	740	24.35	2.13	0.65		
789	15.2	510	7.75	0.98	0.65		
2491	32.3	1500	48.45	1.94	0.60		
1655	15.5	1720	26.66	1.61	1.04		
966	30.4	620	18.85	1.95	0.64		
742	18.5	590	10.92	1.47	0.80		
1116	31.2	510	15.91	1.42	0.46		
885	20.0	550	11.00	1.33	0.62		
17	27.7	25	0.69	4.07	1.47		
122	27.7	100	2.77	2.27	0.82	Price <i>et al.</i> , 1985	
392	27.7	190	5.26	1.34	0.48		
1046	27.7	600	16.62	1.59	0.57		
2049	27.7	1250	34.63	1.69	0.61		
589	27.7	380	10.53	1.79	0.65		
56	19.4	16	0.30	0.53	0.28		Galvano <i>et al.</i> , 1996
56	19.3	16	0.31	0.54	0.28		
56	19.7	16	0.31	0.55	0.28		
67	17.6	17	0.30	0.45	0.26		
67	17.7	18	0.31	0.47	0.27		
67	17.4	18	0.32	0.45	0.27		
57	20.2	44	0.88	1.54	0.77		
142	19.9	89	1.77	1.25	0.63		
226	18.4	141	2.60	1.15	0.62		
311	20.1	112	2.24	0.72	0.36		
2400	8.2	1850	15.20	0.63	0.77	Harvey <i>et al.</i> , 1991	
2400	8.2	1990	16.32	0.68	0.83		
1200	8.2	910	7.46	0.62	0.76		
1200	8.2	900	7.38	0.62	0.75	Fremy <i>et al.</i> , 1987	
3300	21.0	510	10.71	0.32	0.15		
			Mcan	1.81	0.79		
			SD	1.22	0.38		
			Median	1.65	0.65		
			Max	6.20	1.79		
			Min	0.32	0.15		



**Fig. 12.1** The relation between aflatoxin M1 in milk and amount of aflatoxin B1 consumed by the dairy cows. Values from studies published after 1985.

natural linear relationship seems to exist between the quantity of AFB1 consumed per day and the concentration of AFM1 in the milk. This relationship is also less dependent on milk production. If all the data is analysed by regression and plotted on a graph (Fig. 12.1), the outcome is that the AFM1 concentration in the milk is increased by 0.72 ng for every µg of AFB1 consumed, with a correlation coefficient of 0.943. If the estimations are made only for such dairy cows that have been fed a maximum of 200 µg AFB1 per day, the value 0.74 ng and a correlation coefficient of 0.388 are obtained. Another way of estimating is to calculate the quotient between the number of ng of AFM1 per kg of milk and the number of µg of AFB1 consumed per day. These values are given in Table 12.4. The resulting mean value of all data is consequently  $0.79 \pm 0.38$  ng of AFM1 per µg of AFB1 consumed per day. High milk producing cows apparently have a higher excretion, as their mean value is  $0.85 \pm 0.34$ . This is assumed to be a result of a higher permeability in the cell membranes of the alveoli (Veldman *et al.*, 1992). Cows afflicted by mastitis also have a higher excretion of AFM1, which is believed to be the result of an increased permeability of the membranes (Veldman *et al.*, 1992). Feeding and milking routines may also influence the carry-over, but these have not been studied.

### 12.3.2 Ochratoxin

Animals fed feedingstuffs containing ochratoxin A take up between 40 and 66 % of the toxin. Once in the stomach, it is quickly resorbed into the blood. In the blood, ochratoxin binds to plasma proteins to varying degrees, depending on the species. A relatively high proportion of ochratoxin binds to plasma in humans, monkeys and pigs. Part of the toxin diffuses out into the tissues, is metabolized and excreted via urine and bile. Ochratoxin metabolizes fairly rapidly and is not deposited in the

**Table 12.5** Residual levels of ochratoxin A in pig's tissue after feeding a diet of naturally ochratoxin contaminated feedstuff.

Feed conc. ( $\mu\text{g}/\text{kg}$ )	Residual ochratoxin A concentrations (ng/g)						Reference
	Blood	Serum	Kidney	Muscle	Liver	Fat	
25			4	1	2	0.5	Madsen <i>et al.</i> , 1982a
50			5	1	1	0.5	Madsen <i>et al.</i> , 1982a
90	41	95	20.7	4.23	12.4	5.6	Lusky <i>et al.</i> , 1995
100			11	5	2	0.5	Madsen <i>et al.</i> , 1982a
100	20	47	16.2	2.7	7.9		Lusky <i>et al.</i> , 1994
101			1.5	0.5			Madsen <i>et al.</i> , 1982a
140	38	88	10	4	1	1	Madsen <i>et al.</i> , 1982a
140	38	88	12	4	4	3	Madsen <i>et al.</i> , 1982a
200	44	103	11	6	4	2	Madsen <i>et al.</i> , 1982a
200			5		2		Krogh <i>et al.</i> , 1974
266			17	8	10	3	Madsen <i>et al.</i> , 1982a
271			4.8	0.5			Madsen <i>et al.</i> , 1982a
400	77	179	43.6	10.3	36.7		Lusky <i>et al.</i> , 1994
880	184	429	61	32	25	17	Mortensen <i>et al.</i> , 1983b
1000			14	4	10	7	Krogh <i>et al.</i> , 1974
1380			71	38	21	29	Madsen <i>et al.</i> , 1982a
1380			62	37	22	30	Madsen <i>et al.</i> , 1982a
1380			49	44	20	59	Madsen <i>et al.</i> , 1982a
1380			45	33	20	51	Madsen <i>et al.</i> , 1982a
1400			67	37	30	8	Madsen <i>et al.</i> , 1982b
1550	360	838	36	28	15	12	Mortensen <i>et al.</i> , 1983b
1600	286	665	38	17	12	8	Madsen <i>et al.</i> , 1982a
1600	286	665	50	23	20	10	Madsen <i>et al.</i> , 1982a
1600	286	665	39	22	15	10	Madsen <i>et al.</i> , 1982a
1600	286	665	54	25	19	16	Madsen <i>et al.</i> , 1982a
1600	137	319	50	31	16	18	Madsen <i>et al.</i> , 1982a
1720			61	31	30	20	Madsen <i>et al.</i> , 1982a
1989			55.8	6.3			Madsen <i>et al.</i> , 1982a

Note: figures in italic are calculated from information given in the references.

same way as many environmental pollutants. When intake of feedstuffs containing ochratoxins is discontinued, then the ochratoxin is excreted gradually and leaves the body altogether. Problems with residues ensue when the animals have been fed feedstuffs containing ochratoxin during the period previous to slaughter. There is a considerable inter-species variation with regard to the biological half-life that affects the residues. This variation is dependent on differences between species with regard to absorption, metabolism, recirculation and, above all, the degree to which they combine with serum macromolecules. Half-life for pigs and chickens is 88–140 hours and 4.1 hours respectively (Hagelberg *et al.*, 1989).

The residues in the same animal species are affected not only by the ochratoxin

level in the feedingstuff but also by the length of the feeding period. They increase with time and, in pigs, stable levels are not attained until after a 2–3 week feeding period. The ochratoxin level is also of importance to the excretion rate, and ochratoxin is excreted more rapidly at higher doses. Natural contamination of feedstuff results in higher residues in pigs than is the case with experimental feedstuff, where chemically pure ochratoxin has been added. The reason for this is not known.

### *Pigs*

In a number of elaborate feeding experiments in Denmark and Germany, residues of ochratoxin in tissues and blood from pigs have been studied (Krogh *et al.*, 1974; Madsen *et al.*, 1982a,b; Mortensen *et al.*, 1983a,b; Lusky *et al.*, 1993, 1994, 1995). In most cases the feeding has been executed using naturally contaminated feedingstuffs with levels of ochratoxin between 25 and 1989 µg per kg. Data from these experiments are displayed in Table 12.5, and they have been recalculated and correlated with the ochratoxin level of the experimental feedingstuff. These recalculations assume a linear relation, but there are indications that the residue levels are relatively higher with lower feedingstuff concentrations. Results from these recalculations, that give a better overview, are displayed in Table 12.6. These recalculations indicate that a feedingstuff concentration corresponding to a level of 100 µg ochratoxin per kg can result in serum levels at slaughter in excess of 50 µg/litre. The level for meat/muscle lies just below this level.

### *Chickens*

Residue analyses after feeding chickens various diets have been performed by Krogh *et al.* (1976), Prior *et al.* (1980), Golinski *et al.* (1983), Niemiec *et al.* (1988) and Micco *et al.* (1987, 1988). Results are summarized in Table 12.7 and have been recalculated in relation to the feedingstuff concentration of ochratoxin. These recalculated data are displayed in Table 12.6. Most residue analyses have been performed after feeding with more than 1000 µg ochratoxin per kg of feedingstuff. In only two studies (Krogh *et al.*, 1976; Micco *et al.*, 1987) were feedingstuff levels near the maximum permitted level used. In these experiments, the residues in the liver were 2 and 11 µg/kg respectively and in muscles 2 µg/kg were indicated. When almost all diets have contained a concentration of between 1000 and 2000 µg of ochratoxin per kg, the estimate for a feedingstuff concentration of 100 µg/kg becomes highly inaccurate and probably too low. A residue of 11 µg/kg in liver from chickens having been fed 50 µg of ochratoxin per kg of feedingstuffs (Micco *et al.* 1987) also points in the same direction. A result of these estimates which is worth emphasizing is that the ochratoxin concentration in blood is clearly lower than that found in pigs. The highest levels were found in liver and kidney. Meat/muscle had relatively low levels.

### *Hens and eggs*

There are five studies into residues in hens, (Juszkiewicz *et al.*, 1982; Reichmann *et al.*, 1982; Micco *et al.*, 1987; Bauer *et al.*, 1988; Niemiec *et al.*, 1994). In these studies high feedingstuff concentrations of ochratoxin were also used. The residues have been recalculated in relation to the feedingstuff concentration and included

**Table 12.6** Residual levels of ochratoxin A in tissues from pigs, chicken and hens calculated in relation to a feedingstuff ochratoxin A concentration of 100 µg/kg.

Tissue	Pig				Chicken				Hens			
	No Obs.	Range (ng/g)	Mean (ng/g)	S.D. (ng/g)	No Obs.	Range (ng/g)	Mean (ng/g)	S.D. (ng/g)	No Obs.	Range (ng/g)	Mean (ng/g)	S.D. (ng/g)
Blood/Serum	13	20–106	51	19.8	2	0.08–0.30	0.19	0.16	7	0.01–0.90	0.39	0.33
Kidney	28	1.4–23.0	6.18	5.22	5	1.60–3.70	2.44	0.9	9	0.06–7.70	2.21	3.13
Liver	25	0.7–13.8	2.92	3.26	13	0.38–22.00	3.11	5.76	12	0.05–5.76	1.3	2.03
Muscle	27	0.2–5.0	2.31	1.25	12	0.08–0.62	0.29	0.16	5	0.01–1.60	0.68	0.72
Fat	22	0.5–6.22	1.63	1.44	1	0.09	0.09					
Egg									7	0.01–0.20	0.1	0.08

**Table 12.7** Residual levels of ochratoxin A in tissues from poultry having been fed diets containing ochratoxin A.

Feed conc. ( $\mu\text{g}/\text{kg}$ )	Residual ochratoxin A concentrations (ng/g)						Reference
	Blood/plasma	Kidney	Liver	Muscle	Fat	Egg	
<b>Hens</b>							
500		36.8	26.3	8	0		Prior & Sisodia, 1978
1000		3	1.5				Reichmann <i>et al.</i> , 1982
1000		10	2.5				Reichmann <i>et al.</i> , 1982
1000		77	57.6	12.6	0		Prior & Sisodia, 1978
1300	4.7		9.1			1.6	Bauer <i>et al.</i> , 1988
2100	13.3	8.1	3.3			4.1	Niemiec <i>et al.</i> , 1994
2500	<i>0.25</i>	3.8	3	<i>0.25</i>		<i>0.25</i>	Juszkiewicz <i>et al.</i> , 1982
2600	14.1		17.9			2.5	Bauer <i>et al.</i> , 1988
4000		106.9	72.6	20.8	0		Prior & Sisodia, 1978
4100	37	9	11.9			7.9	Niemiec <i>et al.</i> , 1994
5200	11.7		18			4	Bauer <i>et al.</i> , 1988
10000	4	5.7	5.2	2.4		1.3	Juszkiewicz <i>et al.</i> , 1982
<b>Chicken</b>							
50		0.5	5	0.5			Micco <i>et al.</i> , 1987
50		0.8	11	0.5			Micco <i>et al.</i> , 1987
324		12	2	2			Krogh <i>et al.</i> , 1976
1000			32.8	0.8			Golinski <i>et al.</i> , 1983
1000			22.8	2.8			Golinski <i>et al.</i> , 1983
1052		19	4	2			Krogh <i>et al.</i> , 1976
1052		32	7	4			Krogh <i>et al.</i> , 1976
1500	1.24		16.16	7.46			Niemiec <i>et al.</i> , 1988
1500	4.56		10.98	3.02			Niemiec <i>et al.</i> , 1988
1500			14.2	3			Golinski <i>et al.</i> , 1983
1500			39.8	5			Golinski <i>et al.</i> , 1983
2000		41	24	<i>1.75</i>	<i>1.75</i>		Prior <i>et al.</i> , 1980
2000			34.3	4.5			Golinski <i>et al.</i> , 1983
2000			58.6	8.5			Golinski <i>et al.</i> , 1983

Note: figures in italic are calculated from information given in the references.

into Table 12.6. There does not seem to be any direct relation between the feedingstuff concentration and the residues reported from the various feedings. The residues revealed vary greatly with approximately the same feeding levels. The levels are analytically low and method variation may account for the differences. Older thin-layer chromatographic as well as more recent liquid chromatographic methods have been used for the analyses.

Results of the residue estimates for ochratoxin in hens are very inconclusive. It should be emphasized that, for hens, the blood concentration is low and the highest

level was revealed in kidney. Eggs, being the most important product from hens, may, according to estimate, contain 0.11 % of the feedingstuff ochratoxin concentration. This estimation may, however, be on the low side.

### *Milk*

Transmission of ochratoxin into milk has been shown in single stomach animals such as humans, rats and pigs (Mortensen *et al.*, 1983a; Breitholtz-Emanuelsson *et al.*, 1993). Ochratoxin has also been found in bovine milk (Breitholtz-Emanuelsson *et al.*, 1993) although the toxin is rapidly metabolized to ochratoxin  $\alpha$  and phenylalanine in the rumen. Small amounts seem to be taken up and excreted into milk without being metabolized. Low concentrations (10–58 ng/L) have been found in Swedish and Norwegian milk (Breitholtz-Emanuelsson *et al.*, 1993; Skaug, 1999) but not in German milk (Valenta and Goll, 1996). Ochratoxin from bovine milk may, however, be an important part in the total intake of ochratoxin by human high milk consumers such as children (Olsen *et al.*, 1993).

### 12.3.3 Other mycotoxins

#### *Zearalenone*

Zearalenone is metabolized relatively quickly in most animals. It is primarily conjugated with glucuronic acid and already metabolized to zearalenol during uptake from the intestinal tract. Relatively low residue concentrations (59–681 ng/g) have been found in muscle and liver from pigs and chickens fed with even very high levels (40–100 mg/kg) of zearalenone in the feed (James and Smith, 1982; Mirocha *et al.*, 1982). In hens fed C14-labelled zearalenone at 10 mg/kg bw the radioactivity was equivalent to 1.95  $\mu$ g of zearalenone and its metabolites per gram in the eggs with an accumulation in the yolk (Dailey *et al.*, 1980). Excretion of zearalenone in milk from cows fed 200 mg or 1000 mg per day have been analysed and found to contain 1359 and 440 ng of zearalenone and its metabolites per ml milk (Mirocha *et al.*, 1981), with the highest concentration after the lowest administration. In another study (Prelusky *et al.*, 1990) with three lactating cows fed more normal levels (50 and 165 mg/day) during 21 days, neither zearalenone nor its metabolites were found in the milk. Zearalenone was, however, detected in milk at trace levels (max 2.5 and 4 ng/ml) when the cows were fed higher levels (545 and 1800 mg/day). Traces (3 and 5.6 ng/ml) of the zearalenol metabolites were also detected in the milk at the higher feed levels. Studies with other animals (pigs, sheep) have also shown low transmission of zearalenone to milk (Hagler *et al.*, 1980; Palyusik *et al.*, 1980). Very few surveys of zearalenone residues in animal fluid and tissues have been done and most have had negative results (Gilbert, 1989). Traces have mainly been found in bile and serum (Curtui *et al.*, 2001). Residues of zearalenone and its metabolites in bovine milk and other animal products are not considered to be of public concern (JECFA 2000; SCF2000a).

*Trichothecenes*

The occurrence of residues in the tissues and body fluids of animals fed trichothecenes (deoxynivalenol, diacetoxyscirpenol and T-2 toxin) has been reviewed by Beasley and Lambert (1990). They conclude that trichothecene residues in animal products are very low and not likely to be toxic to humans, especially if the animals have been fed trichothecenes below 1 ppm in the feed during the last four days. There is little evidence for a selective uptake of trichothecenes in specific tissues. However, higher concentrations of trichothecenes and their metabolites may be found in kidney and liver, a finding based on radioactivity from radiolabelled trichothecenes given to the animals (Chi *et al.*, 1978; Corley *et al.*, 1985). Deoxynivalenol is rapidly taken up and excreted in pigs (Coppock *et al.*, 1985; Eriksen *et al.*, 2003) and sheep (Prelusky *et al.*, 1986). It is partly conjugated and may be de-epoxidated in the lower intestine by microbes. The elimination half-life has been found to be between 1.2 and 3.65 hours. The maximal plasma or serum level of deoxynivalenol found in pigs fed 5.8 ppm deoxynivalenol (Coppock *et al.*, 1985) or 2.5 ppm acetyl-deoxynivalenol (Eriksen *et al.*, 2003) has been 100 and 65 ng/ml respectively. Analysed deoxynivalenol tissue residues have mostly been lower or non-detectable in both pigs and poultry (El-Banna *et al.*, 1983; Coppock *et al.*, 1985; Kubena *et al.*, 1985).

Nivalenol is not excreted as fast as deoxynivalenol in pigs and was found to increase with feeding days (Hedman *et al.*, 1997). The plasma levels were, however, nearly ten times lower than for deoxynivalenol. Residues of nivalenol have not been found in liver and muscles but are present in bile from the pigs fed 5 ppm nivalenol (Hedman *et al.*, 1997). In hens fed 5 ppm nivalenol traces of nivalenol have been found in liver and bile in amounts up to 11 ng/ml (Garaleviciene *et al.*, 2002).

Neither T-2 toxin nor diacetoxyscirpenol have been found as tissue residues, when chemical methods are used. This is probably because they are rapidly metabolized at least to their deacetylated metabolites. Small amounts of diacetoxyscirpenol (22 ng/ml) and its metabolites (13 and 15 ng/ml) have been found in serum of pigs fed 2 ppm diacetoxyscirpenol (Bauer *et al.*, 1985). Milk from cows fed relatively high amounts of deoxynivalenol (Prelusky *et al.*, 1984; Cote *et al.*, 1986) or T-2 toxin (Robison *et al.*, 1979; Yoshizawa *et al.*, 1981; Mirocha, 1983) has been analysed for residues. No deoxynivalenol, or only traces (3 ppb), was found, while a maximum of 160 ppb T-2 toxin were found. Trichothecene residues have been analysed in eggs from hens fed 5 ppm deoxynivalenol (El-Banna *et al.*, 1983) or 5 ppm nivalenol (Garaleviciene *et al.*, 2002), but no trichothecenes were detected with a detection limit of 10 ng/g for both toxins.

*Fumonisin*

Studies on the pharmacokinetics of ingested fumonisins in farm animals have shown low absorption from the intestinal tract. The oral bioavailability on average has been 4 % for pigs (Prelusky *et al.*, 1996) and 0.7 % in laying hens (Vudathala *et al.*, 1994). Residue levels of fumonisins in meat and eggs were considered not to be a public health concern by JECFA (2001) and SCF (2000b). In a recent study

by Meyer *et al.* (2003), however, rather high residue levels were found in pig tissues (43–1530 ng/g), where pigs were fed between 50 and 100 mg fumonisin B1 per day. The highest mean concentrations were found in kidney (1530 ng/g), spleen (1020 ng/g) and liver (379 ng/g). Some exceptionally high residue concentrations were also found in individual animals (kidney 4760 ng/g, spleen 7980 ng/g, liver 710 ng/g, lung 1150 ng/g and myocardium 838 ng/g). They conclude that feeding pigs a ration containing 2 mg fumonisin B1 per kg would lead to residues of significance for human intake. Excretion into milk has been very low, and only very low levels of fumonisins have been detected (Prelusky *et al.*, 1995; Scott *et al.*, 1994).

## 12.4 Techniques for controlling mycotoxins in animal feed

### 12.4.1 Control strategies

Four different control strategies may be distinguished:

- (1) Use of high-quality and selected feedstuffs.
- (2) Control by monitoring for selected mycotoxins in all feed ingredients or final feed, independent of quality or origin.
- (3) Combining feedstuff of normal or high-quality with feedstuff of known low cost and quality or high risk of mycotoxin contamination.
- (4) Prevention of uptake of mycotoxins by use of feed additives.

These are discussed further below.

- (1) High-quality feedstuffs from selected or contracted farms are only used. Good agricultural practice and quality control, sometimes based on Hazard Analysis Critical Control Point (HACCP), are required for the entire production process. This will minimize the risk for growth of fungi and production of mycotoxins during both the growing and the preservation of the crops to be used for feed. Feedstuffs with a high risk for mycotoxin contamination, e.g. oilseed by-products or cereal grain screenings, are avoided. Mycotoxins are normally not analysed in the feed raw material, but mycological investigations may be used for hygienic quality evaluation. This strategy is used at relatively big farms for their own feed production. Feed mills are at least partly using this strategy with contracted quality production of feedstuffs. The cost of the feeds produced in this way will be considerably higher.
- (2) The most probable mycotoxins or those that are regulated are screened for by rapid methods to check that they are not above maximum limits or recommendations. It is mainly big feed mills that use this strategy, although present and impending regulations may require control analysis of mycotoxins to be carried out by all feed producers. The quantities of raw material and produced feed need to be relatively large in order not to increase the final price of the feed too much, due to the cost of the extended mycotoxin analyses.
- (3) Normal or high-quality feedstuffs are used together with feedstuffs known to

be of low cost and quality or to have a high risk of mycotoxin contamination. The normal or high-quality feedstuffs are normally only visually investigated for hygiene. The at-risk feedstuffs are checked by mycological investigations and analysed for probable or regulated mycotoxins. This is probably the most common strategy used both by farms producing their own feed and by feed mills.

- (4) Normal, low-cost and hygienic at-risk feedstuffs are used for the feed production. They are not controlled by mycotoxin analyses, due to the relatively high cost or lack of analytical facilities. Feed additive adsorbents, antioxidants, vitamins or enzymes are added to the feed in order to prevent uptake and any eventual toxic effects of mycotoxins that may be present in the feed. This strategy seems to be becoming more and more common at big farms and feed mills, especially in Eastern Europe and in developing countries.

#### 12.4.2 Maximum limits

Several countries have introduced legal or recommended maximum limits for mycotoxins in feedingstuffs and the situation in 1995 has been summarized by the FAO (1997). There are now, October 2003, some new countries which have introduced limits for mycotoxins in feed; some countries have also introduced limits for additional toxins. Examples of legal limits and recommended maximum limits for different mycotoxins in feeds introduced by, e.g., the European Union, USA and some European countries, are presented in Table 12.8. In most cases there are no clear and documented rationales or evaluations behind those limits.

The European Commission has initiated research and asked the Panel on Contaminants in Food Chain at the European Food Safety Authority (EFSA) to give a scientific opinion on the rationales behind and necessity for maximum limits for certain mycotoxins in animal feed. The risks for both toxic effects in animals and residues in animal products will be considered. Aflatoxin B1 and ergot had earlier been in the list under 'Undesirable substances in animal feed', but they will now be reconsidered in new risk assessments. New mycotoxins to be considered are deoxynivalenol, zearalenone, ochratoxin A and fumonisins.

#### 12.4.3 Analytical control

Control analyses for mycotoxins in feed are increasing due to public demand for safe feeds and regulation by the authorities of maximum levels for mycotoxins in feed. There are two types of mycotoxin analytical control, official by the authorities and in-house by the feed producer. These two types of controls require different analytical methods.

##### *Official control*

Methods used in official control need to have low variation and exact quantification and to be accepted by other laboratories and authorities. The analytical results have to stand reanalyses by an independent laboratory and an eventual legal trial.

**Table 12.8** Maximal or advisory limits for mycotoxins in feed to farm animals. Values are given in µg/kg feed.

Toxin	Animal-feed	EU	USA	Canada	Sweden	Germany	Hungary	Austria
Aflatoxin	Dairy, complementary	5	20				20	
	Cattle mature	50	20			50	50	
	Pigs	20	100			20	30	
	Pigs, complementary	30						
	Poultry	20	100		20		30	
	Finishing beef cattle		200					
Deoxynivalenol	Pigs		5000	1000			400	500
	Sows					500–1000		
	Cattle		10000	5000			2000	1000
	Poultry		10000	5000		5000	400	1500
Fumonisin	Horses		1000					
	Pigs		10000					
	Poultry		50000					
	Cattle		30000					
Ochratoxin	Pigs						25	
	Poultry						10	
	Cattle						100	
Zearalenone	Pigs						500	50
	Poultry						500	
	Cattle						2000	
T-2 + HT-2	Pigs						300	
	Poultry			100			500	
	Cattle			100			1000	
	Dairy cattle			25				

Standardized chemical methods, which have been validated and performed by an accredited laboratory, are therefore preferred. There are standardized methods of analysis for most of the important mycotoxins in some commodities. The standardized and validated methods and their performance have recently been reviewed by Gilbert and Anklam (2002). Because they are often hard to analyse for mycotoxins, the standardized methods have generally not been studied in interlaboratory trials on mixed feeds. Good standardized methods for trichothecenes are still lacking, and there is a great need to develop and collaboratively study methods for trichothecenes and methods for analysing the other important mycotoxins in animal feed. Certified reference materials (CRM) and calibrants for mycotoxins would allow improvements in control analyses, and proficiency testing programmes for mycotoxins in animal feed would also increase the quality. CRMs are available for aflatoxins, ochratoxin and zearalenone from the Institute of Reference Materials in Geel, Belgium.

#### *In-house control*

Methods for in-house control of mycotoxins in feedstuffs and mixed feeds need to be relatively fast, cheap and sensitive enough to detect mycotoxins at levels of concern. They can, however, be semi-quantitative, indicating only levels over a certain concentration. The important thing is that they are easy to perform and that the cost of the analysis will not considerably increase the price of the feedstuff. A lot of different test kits for the most common mycotoxins, mainly based on immunological techniques, have appeared on the market and further kits will probably become available. A presentation of commercially available test kits for mycotoxins can be found on the website of the European Mycotoxin Awareness Network ([www.lfra.co.uk/eman2/kittable.asp](http://www.lfra.co.uk/eman2/kittable.asp)). Their specifications are given and, based on them, some of the kits may be suitable for mycotoxin control in feed. Both the AOAC and the Grain Inspections, Packers and Stockyards Administration (GIPSA) (<http://www.usda.gov/gipsa/tech-servsup/metheqp/testkit.htm#Mycotoxins>) have tested some rapid mycotoxin kits and verified their performance according to the company's specifications. The test kits are still rather expensive, especially for use in extensive feed control and when the feed prices have to be kept low. Rapid test kits and other detection methods for mycotoxins in grain and flour are described in Chapter 5. Emerging rapid detection methods are also described. Cheap and fast methods with minimal or no sample preparation are highly desirable for mycotoxin control in feedstuffs. Near infrared spectroscopy may become such a method, at least for deoxynivalenol in cereals (Pettersson and Åberg, 2003). No sample preparation is needed, the analysis takes just 1.5 minutes and the instruments are often already in use at elevators and feed laboratories to determine protein and water content in the cereals.

#### **12.4.4 Decontamination and detoxification of feedstuffs**

Mycotoxin-contamination of crops will still occur even if prevention through good agricultural practice during growing and preservation can reduce the incidents.

There has, therefore, been much research carried out on how to decontaminate or detoxify the mycotoxin-containing crops, and many compounds and treatments have been tested. However, only a few methods are in practical use. It is probably due to this that the requirements on the processes, which have been outlined by Park *et al.* (1988), are hard to prove or fulfil. These requirements can be summarized as follows:

- (1) The mycotoxins must be inactivated and transformed into non-toxic compounds.
- (2) Fungal spores and mycelia should be killed, so that new toxins are not formed.
- (3) The food and feed material should retain its nutritional value and remain palatable.
- (4) The physical properties of the raw material should not change significantly.
- (5) The process must be economically feasible and thus the cost must be considerably less than the value of the decontaminated crop.

Even those methods which are in practical use cannot fulfil all those requirements. Some merely reduce the mycotoxin levels and others lower the nutritional value. The decontaminated or detoxified crops are mostly considered to be of lower quality and thus fetch a lower price than a normal uncontaminated crop. This is a serious constraint on the practical use of decontamination and detoxification methods, and the processed crops are mainly used for feed production and animal feeding.

Methods for decontamination and detoxification of crops are commonly classified as physical, chemical and biological and will be briefly presented below.

#### *Physical methods*

Blending of contaminated crops with batches of good-quality material will reduce the mycotoxin concentration in the feedstuff, but this cannot be considered as a decontamination procedure. The practice has been forbidden within the EU countries, at least for food and feed containing regulated mycotoxins. It is, however, probably used at farm level for low-quality crops, which may contain mycotoxins at low or unknown levels.

Cleaning methods for cereals can be very effective in reducing mycotoxin contamination, and extensive cleaning is in practical use for lowering mycotoxin contamination of feed cereals. The efficacy partly depends on the degree of fungal growth within the kernels. The highest fungal infestation and mycotoxin concentrations of cereals are found in the husks, debris, hull and the outer layer of the kernel. *Fusarium*-damaged kernels are often also of smaller size than sound kernels.

The cleaning process often consists of scouring, aspiration, sieving, and specific gravity separation. Dust, husks, hair and loose superficial particles are blown away by scouring and aspiration. Extensive aspiration can considerably reduce the quantity of mould spores and mycelia on the kernel surface. Sieving and specific gravity separations remove small kernels, kernel debris, weed seeds and other impurities which differ in size and gravity in comparison to normal kernels.

Reduction of mycotoxin contamination by cleaning has mainly been reported for *Fusarium* toxins in cereals (Seitz *et al.*, 1985,1986; Lee *et al.*, 1987). Deoxynivalenol in wheat has been removed in varying degrees in different batches and by different cleaning methods, but is normally reduced by 50–86 %. The reduction of deoxynivalenol in wheat by the use of a specific gravity table has also been demonstrated (Tkachuk *et al.*, 1991). Ochratoxin A was, however, not reduced by cleaning in the study by Chelkowski *et al.* (1981) on contaminated wheat and barley. Washing procedures, which are sometimes used for cleaning, could, in laboratory trials, reduce the level of deoxynivalenol in barley and maize by 65–69 % (Trenholm *et al.*, 1992). By using 1 M sodium carbonate solution instead of water additional deoxynivalenol could be removed as well as 80–87 % of the zearalenone contamination.

Dehulling of wheat, barley, rye and oats has been shown to lower deoxynivalenol, zearalenone and T-2 toxin in the cereals by 40–100 % (Trenholm *et al.*, 1991; Pettersson, 2004). Polishing and perling of barley, which also removes the hull, has likewise been shown to reduce both the fusarium toxins deoxynivalenol and zearalenone (Lee *et al.*, 1992) and ochratoxin (Chelkowski *et al.*, 1981).

Normal dry milling of wheat will also reduce the mycotoxins in the flour, but higher concentrations will be found in bran and in the feed meal fractions (Chelkowski *et al.*, 1981; Seitz *et al.*, 1985, 1986; Lee *et al.*, 1987).

#### *Chemical methods*

Various chemicals (acids, bases, aldehydes, bisulphite, oxidizing agents and different gases) have been tried for detoxification of mycotoxins mainly aflatoxins in different crops. Treatments with alkali have been the most successful in destroying or lowering mycotoxins in contaminated crops.

Treatment with ammonia of aflatoxin in groundnut meal-cakes, maize and cottonseed products has been able to destroy up to 99 % of the contamination (100–4000 µg/kg). Ammonia was first used for the detoxification of aflatoxin-contaminated cottonseed meal in the USA in the late 1960s (Park *et al.*, 1988). Different processing techniques using atmospheric pressure and ambient temperature or elevated temperature and pressure have since then been applied to different contaminated crops. Industrial plants for commercial processing have also been built in some countries, e.g. France, the UK and certain states in the USA. Feeding studies using the ammoniated aflatoxin-contaminated products have shown significantly reduced toxicity compared to untreated products (Park *et al.*, 1988). The US Department of Agriculture and Food and Drug Administration also evaluated the safety of animal products from animals fed diets containing ammoniated products. The use of ammoniated aflatoxin-contaminated crops has been approved in some states of the USA as well as Mexico, Senegal, Brazil and South Africa. In some EU countries, e.g. France and the UK, use of ammoniated contaminated feed is also approved. It is, however, not allowed to be used for dairy animals, due to the risk that residual aflatoxin may become higher than the maximum permitted level. The mutagenicity of aflatoxin-contaminated groundnut meal extracts was also not completely eliminated

by ammoniation in a European safety study of the ammoniation procedure (Hoogenboom *et al.*, 2001).

Ammoniation at ambient temperature and pressure has also been studied for ochratoxin, zearalenone and fumonisins in cereals (Chelkowski *et al.*, 1982; Madsen *et al.*, 1983; Norred *et al.*, 1991). Ochratoxin and zearalenone were nearly completely degraded, while 70–55 % of fumonisin remained. In studies in which the ammoniated ochratoxin-contaminated barley was fed to pigs, some toxicity and lower nutritional value was observed (Madsen *et al.*, 1983). Residues of ochratoxin were also found in the kidneys, and it was thought to be reformed in the animals by recyclization.

Calcium hydroxide monomethylamine has been used successfully to decontaminate T-2 and HT-2 toxin in maize (Bauer, 1994). Deoxynivalenol (4.4 mg/kg) in contaminated maize has also been reduced by 85 % through treatment with sodium bisulphite solutions (Young *et al.*, 1987). The deoxynivalenol-sulphonate conjugate formed appeared to be non-toxic to pigs in the feedings.

### *Biological methods*

The degradation of mycotoxins by micro-organisms in silage and other moist feed is an attractive method for the decontamination of crops. Bacteria, fungi and yeasts have been investigated for their ability to degrade mycotoxins, but no practical working system has been presented although patents have been claimed for some (Bata and Lasztity, 1999; Karlovsky, 1999). Soon after the discovery of aflatoxin, Ciegler *et al.* (1966) screened about 1000 micro-organisms for their ability to detoxify aflatoxin and found that *Flavobacterium aurantiacum* was effective. This organism has since been studied extensively for its ability to degrade aflatoxin and its possible degradation products (Bata and Lasztity, 1999). Apart from *F. aurantiacum*, a number of bacterial and especially fungal species have been found to detoxify aflatoxin (Karlovsky, 1999). *Rhizopus* sp. has been claimed to be particularly suitable for large-scale detoxification of aflatoxin-contaminated feeds by solid-state fermentation (Knol *et al.*, 1990, as cited by Karlovsky, 1999).

Ochratoxin A is rapidly degraded by micro-organisms in the rumen to ochratoxin  $\alpha$  and phenylalanine (Hult *et al.*, 1976; Kiessling *et al.*, 1984). The responsible micro-organisms have not been isolated and the main activity was obtained through the protozoas. Cheng-An and Draughon (1994) have screened bacteria, yeast and moulds for their ability to detoxify ochratoxin and found *Acinetobacter calcoaceticus* to be able to degrade ochratoxin in an ethanol-containing medium. Different strains of *Lactobacillus*, *Bacillus* and *Saccharomyces* have also been shown to degrade ochratoxin *in vitro* to varying degrees (Böhm *et al.*, 2000). Some strains were able to degrade up to 94 %. They were also tested for degradation of trichothecenes, but with less success. Additional yeast strains have also been tested, and some were able to partly degrade ochratoxin, nivalenol, deoxynivalenol, zearalenone and fumonisins (Styriak *et al.*, 2001).

Reduction of zearalenone to  $\alpha$ - and  $\beta$ -zearalenols has been shown in ruminal fluid and for many mixed and pure cultures of bacteria, yeast and fungi, but the transformation cannot be considered as a detoxification as the zearalenols still

show estrogenic activity. Non-estrogenic products from zearalenone have been demonstrated from degradation by some micro-organisms, e.g. *Thamnidium elegans*, *Mucor baineri*, *Rhizopus* sp., *Streptomyces rimosus*, *Cunninghamella baineri*, *Gliocladium roseum* (Kamimura, 1986; El-Sharkawy and Abul-Hajj, 1987, 1988). The degradation product will, in some cases, probably regain its estrogenic potential when fed to animals.

Soil bacteria have been investigated for the degradation of trichothecenes (Binder *et al.*, 1997; Shima *et al.*, 1997; Völkl *et al.*, 1997), but only a single active culture and bacterium could be isolated. Microbial degradation of trichothecenes to their non-toxic de-epoxides has been found in ruminal fluid and intestinal contents from pigs, hens and rats (Swanson *et al.*, 1988; He *et al.*, 1992). From ruminal fluid Binder *et al.* (2001) have isolated an anaerobic bacterium belonging to the genus *Eubacterium*, which is able to degrade trichothecenes to de-epoxy metabolites (Fuchs *et al.* 2000, 2002). This bacterium has been cultured, produced and stabilized in order to be used as a feed additive. The efficacy of the additive has been tested in trials with pigs. Significant feed gain and feed conversion ratio in piglets fed 2.5 ppm deoxynivalenol have been claimed (Binder *et al.*, 2001), but the results from the study have not been published.

Ensiling of mycotoxin-contaminated crops for detoxification has been proposed as an interesting and possible method for elimination or reduction of mycotoxins. Normal ensiling has, however, only rarely been studied for its mycotoxin degrading potential. A study by Rotter *et al.* (1990) showed that ensiling of ochratoxin-contaminated barley could reduce the toxin by approximately 68 %. In feeding studies with chicken, no improvement in performance or mortality could, however, be found compared with the non-ensiled diets. Yeasts in grass silage have been found to degrade patulin in silage inoculated with *Paecilomyces* sp. to induce patulin production (Dutton *et al.*, 1984). Both bacteria and yeasts from maize silage have also been shown to be able to degrade fumonisins (Camilo *et al.*, 2000). Stimulation of mycotoxin degradation by naturally occurring micro-organisms in silage or the addition of yeasts or bacteria with known mycotoxin degradation ability to silage may in the future become practical means to detoxify mycotoxins in certain crops.

The degradation of mycotoxins during alcohol fermentation for the production of ethanol has been investigated only in a few studies, but many papers have appeared on the fate of mycotoxins during the production of beer and wine. In one study, zearalenone-contaminated maize was used for ethanol production, but the toxin was not degraded and remained in the fermentation residues (Bennett *et al.*, 1981). Mycotoxins can often be found in beer and wine, but they are partly degraded and partly transferred to beer, with part remaining in the draff during the fermentation process (Chu *et al.*, 1975; Scott *et al.*, 1992; Niessen and Donhauser, 1993; Scott and Lawrence, 1995, 1997; Baxter, 1996). The mycotoxin concentration remaining in the draff may be relatively high and so fermentation is not an effective means of detoxifying feed material.

Mycotoxin-contaminated cereals may in the future be used more often in industrial ethanol production. Enhanced degradation of mycotoxins by eventual

addition of micro-organisms with mycotoxin-degradation ability is needed if the fermentation remains are to be utilized for animal feed.

#### 12.4.5 Feed additives for prevention

The use of feed additives to prevent absorption and toxic effects from mycotoxins in farm animals is a subject of growing interest. Many companies are putting new products on the market and such feed additives are used in many countries. Some are claimed to work for multiple mycotoxins, and they are advised to be mixed in the feed even if no or only low levels of mycotoxins have been detected in the feed ingredients. Examples of trade names for such feed additives, mainly adsorbents, are MycoSorb, MycofixPlus, Antitox-Vana, Ergomix, Novasil, Mervobind, Curitox. Feed additives are, however, not yet approved within the European Community for the purpose of preventing adverse effects of mycotoxins.

The feed additives proposed, tested and launched can be classified in three different groups according to their main mechanism for prevention. Adsorbents prevent the absorption of the mycotoxins from the intestinal tract and thereby may reduce the adverse effects. Antioxidants and vitamins protect the animals against the toxic effects in tissues and cells through, e.g., reduced lipid peroxidation or by inducing or stimulating detoxifying enzyme systems in the liver and other organs. Some food components with claimed chemoprotective effects may also fall within this group. Enzymes and bacteria as feed additives are supposed to degrade the mycotoxins within the intestinal tract before absorption into the animals.

##### *Adsorbents*

Hydrated sodium calcium aluminium silicate (HSCAS) was shown by Philips *et al.* (1988) to effectively absorb and retain aflatoxin. The stability of the aflatoxin–HSCAS complexes may explain the *in vivo* effectiveness of the adsorbent in preventing the toxic effects of aflatoxin. HSCAS has been shown in feeding studies to have protective effects against aflatoxicosis in several animals and its use has been thoroughly reviewed by Ramos *et al.* (1996) and Huwig *et al.* (2001).

HSCAS is a phyllosilicate derived from natural zeolite clay. Zeolite, other aluminosilicate clays and clay products have also been studied as adsorbents for aflatoxin. They normally have less capacity than HSCAS to absorb aflatoxin *in vitro*, but efficiency *in vivo* in practical conditions is more important. Bentonite, clinoptilolite and some other aluminosilicates have been shown to be effective to some degree in feeding studies reviewed by Huwig *et al.* (2001).

The potential of HSCAS and bentonites to reduce the carry-over of aflatoxin to milk in dairy cows has been investigated in some studies (Harvey *et al.*, 1991; Veldman, 1992; Galvano *et al.*, 1996). Variable reductions in carry-over have been obtained and it will be hazardous to trust the efficiency of these adsorbents at the low EU tolerance limits for aflatoxin in feed and milk. The ability of HSCAS and other clays to bind other mycotoxins and alleviate the toxic effects has also been studied. Limited effects against zearalenone and ochratoxin have been found, and

they are more or less ineffective against trichothecenes (Ramos *et al.*, 1996; Huwig *et al.*, 2001).

Activated charcoal, which is formed by pyrolysis of organic material, is used as an antidote to poisoning. Therefore it has been investigated for its ability to adsorb mycotoxins. It has been shown to adsorb aflatoxin, ochratoxin, fumonisins and trichothecenes rather efficiently, but its effect in feeding studies has mainly been very low (Ramos *et al.*, 1996; Galvano *et al.*, 2001; Huwig *et al.*, 2001).

Colestyramine is an anion exchange resin used pharmaceutically to decrease cholesterol, and it has been shown to efficiently bind zearalenone, ochratoxin and fumonisins *in vitro*. Feeding studies with rats have shown that it can lower the uptake and toxic effects of ochratoxin, zearalenone and fumonisins (Madhyastha *et al.*, 1992; Ramos *et al.*, 1996; Kerkadi *et al.*, 1998; Solfrizzo *et al.*, 2001).

Other resins, divinylbenzene-styrene and polyvinylpyrrolidone have also been tested and found to bind zearalenone and trichothecenes. Divinylbenzene-styrene reduced the absorption and the toxic effects of zearalenone and T-2 toxin in rats (Smith, 1982; Carson and Smith, 1983), while polyvinylpyrrolidone had no effect on pigs fed deoxynivalenol-contaminated feed (Friend *et al.*, 1984).

Yeast membranes and the yeast membrane product esterified-glucomannan used in Mycosorb can *in vitro* efficiently adsorb aflatoxin and, to a lesser extent, ochratoxin and T-2 toxin (Bauer, 1994; Raju and Devegowda, 2002). Feeding studies with chicken have shown that esterified-glucomannan can reduce or eliminate many of the toxic effects from aflatoxin, ochratoxin and T-2 toxin found in the contaminated control group (Raju and Devegowda, 2000, 2002; Aravind *et al.*, 2003).

#### *Antioxidants and vitamins*

Natural and synthetic antioxidants have been investigated for their potential to alleviate toxic effects of mycotoxins [review by Galvano *et al.* (2001)]. Selenium, some vitamins (A, C, and E) and their precursors also have marked antioxidant properties and are believed to work as free radical scavengers and to protect against membrane damage by mycotoxins. Some antioxidants, selenium and vitamins can also induce or stimulate detoxifying enzyme systems in the liver and other tissues and thereby increase the metabolic detoxification of mycotoxins. Reduced toxic effects of mycotoxins by antioxidants and vitamins have been found in several studies with rats, as reviewed by Galvano *et al.* (2001). More feeding studies with antioxidants and vitamins carried out with farm animals are, however, needed for an evaluation of their practical advantages.

#### *Enzymes and bacteria*

Enzyme preparations from *Saccharomyces telluris*, which are claimed to contain activities for hydrolyzing the lactone bond of zearalenone and to destroy the epoxide group in trichothecenes, are included in Mycofix Plus (Biomin, Herzogenburg, Austria). These enzyme activities have, however, been questioned by Karlovsky (1999).

In a study by He *et al.* (1992) the intestinal microflora of chicken with a

deoxynivalenol de-epoxidation capacity was used to detoxify deoxynivalenol-contaminated maize which was fed to pigs. Feed intake, weight gain and feed efficiency were significantly improved. Eriksen *et al.* (2002) were able to transfer the trichothecene de-epoxidation capacity of some sows by placing their faeces in the pens of pigs lacking this intestinal de-epoxidation ability. Binder *et al.* (2001) have isolated from ruminal fluid an anaerobic bacterium belonging to the genus *Eubacterium*, which is able to de-epoxidate trichothecenes (Fuchs *et al.*, 2000, 2002). This bacterium has been cultured, produced and stabilized to be sold and used as a feed additive BBSH 797 by Biomin, Herzogenburg, Austria. The efficacy of the additive has been tested in trials with pigs and chickens. Significant feed gain and increased feed conversion ratio in piglets fed 2.5 ppm deoxynivalenol have been seen, and the chickens fed 10.5 ppm deoxynivalenol had reduced mortality and a positive influence on weight development as reported by Binder *et al.* (2001). The final results from the studies have, however, not yet been published.

## 12.5 Future trends

New maximal limits for additional mycotoxins in more food and feed will appear in many countries in the next few years. The European Commission is discussing or preparing new limits for ochratoxin, patulin, deoxynivalenol, T-2 and HT-2 toxins, fumonisins and zearalenone in different foodstuffs. Most of them will also be considered for animal feed in order to reduce the risk for toxic effects on farm animals and carry-over to animal products. Contaminated crops condemned as food can otherwise be diverted for use as animal feed.

The control, both official and industry in-house, of mycotoxins in food and feed will need additional and more rapid and reliable analytical methods. There will be pressure to standardize additional and new methods for official control, especially since they are lacking for trichothecenes. Rapid mycotoxin tests will be further developed and adjusted for control of the maximum limits in new food and feed. They will probably also become cheaper, and tests for common mycotoxin combinations may appear.

Mycotoxin-contaminated crops must be used in more cases for purposes other than direct food and feed and their utilization will be further investigated. Decontamination procedures will be further studied. Physical methods will be studied, but biological methods probably more so. Mycotoxin-contaminated cereals may in the future be used more often in industrial ethanol production. An enhanced degradation of mycotoxins by eventual addition of micro-organisms with mycotoxin-degradation ability is needed if the fermentation remains are to be utilized for animal feed.

Prevention and reduction of mycotoxin contamination during crop and feed production will become more important. Good agricultural practice and HACCP in the production process will become more of a requirement and the feedstuffs will be selected and bought under special purchase contracts specifying quality and absence of mycotoxins.

The use of feed additives to prevent absorption and toxic effects of mycotoxins in farm animals is of growing commercial interest and new products will appear. The effectiveness of the additives for the various mycotoxins and in the different farm animals has to be proven, e.g. by peer-verified studies. This will probably be a requirement for eventual approval within the European Union of additives intended to prevent adverse effects of mycotoxins in farm animals.

## 12.6 Sources of further information and advice

The book *Mycotoxins and Animal Food*, edited by Smith and Henderson (1991) contains several informative chapters on most aspects of mycotoxins in feedstuffs and their effects on animals. It may be a little old, but most of the information is still relevant although additional new information has been presented since it was published.

Toxic effects of trichothecenes in farm animals and risk evaluations for setting guide levels in feed are presented by Eriksen and Pettersson (2004). A toxicological background paper in support of fumonisin levels in animal feed is presented on the internet by the U.S. Food and Drug Administration ([www.cfsan.fda.gov/~dms/fumonbg4.html](http://www.cfsan.fda.gov/~dms/fumonbg4.html)).

More information on carry-over and residues of mycotoxins in animal products can be found in the reviews by Prelusky (1994), Galtier (1998) and Beasley and Lambert (1990). Decontamination and detoxification of mycotoxins in feed crops by chemical and physical methods are treated in more detail by Charmley and Prelusky (1994), Park *et al.* (1988) and Pemberton and Simpson (1991) and biological detoxification is reviewed by Karlovsky (1999) and Bata and Lasztity (1999).

Up-to-date information on standardized methods for mycotoxin analyses in food and their performance is clearly presented by Gilbert and Anklam (2002). An extensive list of mycotoxin test kits and their applicability can be found on the EMAN website.

Background information on the efficiency and use of feed additives, and especially adsorbents for prevention of toxic effects from mycotoxins, is extensively reviewed by Ramos *et al.* (1996), Galvano *et al.* (2001), Huwig *et al.* (2001). Brief information on adsorbents and decontamination procedures can be found at the EMAN website.

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## **Part III**

### **Particular mycotoxins**

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# 13

## Ochratoxin A

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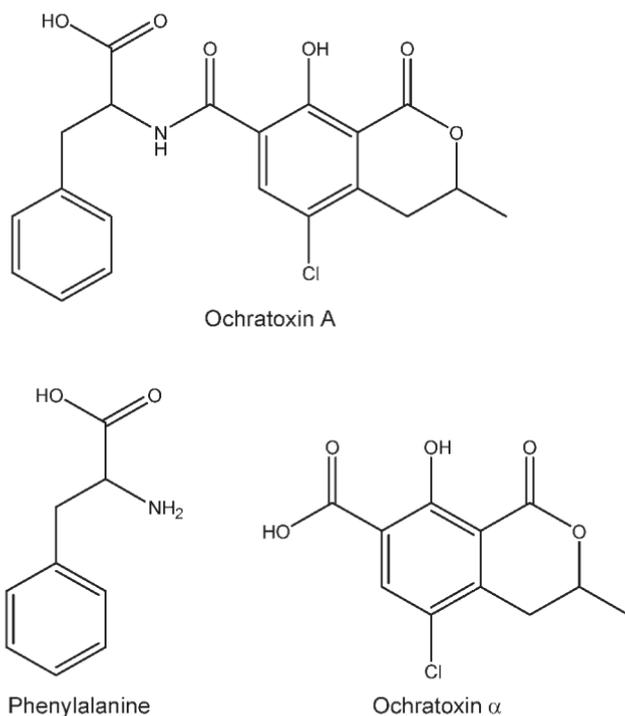
### 13.1 Introduction

Ochratoxin A (OTA) is a toxin naturally produced by several species of *Aspergillus* and *Penicillium* (see Fig. 13.1). These mould species are capable of growing in different climates and on different plants and so contamination of food crops with OTA can occur worldwide. OTA can be found in a wide range of human foods such as cereals, beer, wine, cocoa, coffee, dried vine fruit and spices, as well as in some meat products, as a result of contamination of animal feed. OTA is toxic to animals, the principal effect being nephrotoxicity. It has also been implicated in a human kidney disorder known as Balkan Endemic Nephropathy (BEN). The toxicology and human health risks of OTA have been assessed at both European and international levels by the European Commission Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA), who have established tolerable intakes of OTA from food (SCF, 1998; JECFA, 2001). These tolerable intakes have been used in many countries as a basis for regulation of OTA in some foods. This chapter reviews the experimental and human data on the toxicology of OTA, the occurrence of OTA in food and likely human exposure.

### 13.2 Absorption, distribution, metabolism and excretion of OTA

#### 13.2.1 Absorption

OTA is absorbed in appreciable quantities from the gastrointestinal (GI) tract in a



**Figure 13.1** Chemical structures of ochratoxin A, phenylalanine and ochratoxin alpha.

variety of different species, with absorption into the systemic circulation ranging from 40 % in chickens, 56 % in rabbits, to 66 % in pigs after oral administration (Galtier, 1978). There are, however, wide species variations in the rate of OTA absorption, and the time to maximum serum concentration following single oral dosing of OTA has been reported as ranging from 0.33 hours in chickens, 1 hour in rabbits (Galtier *et al.*, 1981), 2–4 hours in ruminant calves (Sreemannarayana *et al.*, 1988), 4–10 hours in rats (Suzuki *et al.*, 1977; Galtier, 1978) to 10 hours in pigs (Galtier *et al.*, 1981).

There is some disagreement about the site of absorption of OTA from the GI tract. Some studies have suggested that the major site of absorption for most species is the stomach as, under acidic conditions, OTA is uncharged and is therefore more lipid-soluble (Galtier, 1978; Roth *et al.*, 1988). This site of absorption was proposed as the main route of uptake by the authors of a study in which radiolabelled OTA was given orally to mice (Roth *et al.*, 1988). However, the results of this study are inconclusive and may be interpreted as suggestive of rapid transit of OTA from the stomach to the small intestine with absorption from the intestine rather than the stomach (Fuchs *et al.*, 1988; Roth *et al.*, 1988). The small intestine has also been shown to be the major site of absorption in studies using animals with ligated intestinal loops, where maximal absorption occurred in the proximal jejunum (Kumagai and Aibara, 1982). OTA has been shown to be

excreted efficiently in the bile, and analysis of intestinal contents and serum after administration of single doses of OTA to rats revealed secondary peaks in profiles of OTA concentration over time. These observations suggest that OTA undergoes enterohepatic circulation (Fuchs *et al.*, 1988; Roth *et al.*, 1988).

### 13.2.2 Tissue distribution and accumulation

Once absorbed, OTA readily binds to serum albumin and is distributed in the blood predominantly in bound form (Galtier *et al.*, 1980). OTA binds strongly to human serum albumin and shares a common binding site with other known anionic compounds, including warfarin, naproxen and phenylbutazone, giving rise to the possibility of OTA–drug interactions (Il'ichev *et al.*, 2002).

Generally, tissue concentrations observed in pigs and chickens are found to be highest in the kidney, followed in descending order by the liver, muscle and fat (Harwig *et al.*, 1983; Jorgensen and Petersen, 2002) or the muscle, liver and fat (Madsen *et al.*, 1982; Mortensen *et al.*, 1983b).

Transfer of OTA to milk has been demonstrated in rats, rabbits and humans. However, transfer to milk is minimal in ruminants (Ribelin *et al.*, 1978), presumably due to the extensive metabolism of OTA by the rumen microflora in these animals (Galtier and Alvinerie, 1976; Kiessling *et al.*, 1984). Hallen *et al.* (1998) reported that OTA transfer to milk was efficient, with an approximate milk:plasma concentration ratio of 0.6 in female rats exposed to OTA. Likewise, in lactating rats given a single dose of OTA, the milk:blood concentration ratio was 0.4 at 24 hours and 0.7 at 72 hours, indicating that transfer to milk is relatively rapid (Breitholtz-Emanuelsson *et al.*, 1993a). In addition, perinatal studies have shown that OTA is present in the blood and kidney of offspring of rats (Hallen *et al.*, 1998) and rabbits (Ferruffino-Guardia *et al.*, 2000) fed OTA contaminated diets. Two human studies by Micco *et al.* (1991 and 1995), suggest that OTA is also transferred to human milk. In the first study nine out of 50 samples of breast milk collected from nursing mothers were found to contain OTA concentrations that ranged from approximately 1.7–6.6 ng/ml. In the second study 22 out of 111 breast milk samples were contaminated with OTA in the range 0.1–12 ng/ml. However, as neither study reported the OTA exposure of the mothers, an estimate of the efficiency of OTA transfer to breast milk could not be derived. Nevertheless, Breitholtz-Emanuelsson *et al.* (1993b) showed that the concentration of OTA in human milk was approximately 10-fold lower than that in human blood (concentrations ranged from 10–40 ng/L and 90–940 ng/L in milk and blood respectively) in a study of samples from 40 women.

OTA has been detected in eggs following administration of high doses of OTA to hens of 10 mg/kg feed (Juszkiewicz *et al.*, 1982) and 5–20 mg/kg body weight (Piskorska-Pliszczyńska and Juszkiewicz, 1990), but not at lower doses of 1 mg/kg body weight (Piskorska-Pliszczyńska and Juszkiewicz, 1990). In hens, OTA causes a dose-related decrease in egg production, which could result in fewer eggs from exposed hens passing into the food chain. An increase in egg-shell staining has also been associated with OTA consumption by hens, resulting in reduced saleability of their eggs (Page *et al.*, 1980).

OTA has been shown to cross the placenta leading to exposure of the foetus (Fukui *et al.*, 1987). In a study of a pregnant pig fed naturally contaminated feed the blood concentration in the mother was 0.2 ng/ml, whilst the concentration in the offspring ranged from 0.075–0.12 ng/ml at birth (Barnikol and Thalmann, 1988). This limited study suggests that placental transfer is reasonably efficient. In contrast, two similar studies in pigs did not detect OTA in the offspring (Patterson *et al.*, 1976; Mortensen *et al.*, 1983a). In addition, only 0.1 % of the dose was found in the foetus of pregnant rats after subcutaneous injection of a single dose of radiolabelled OTA, with maximum OTA levels reached 2–3 days after administration (Ballinger *et al.*, 1986).

### 13.2.3 Metabolism

The common major metabolite of OTA in mammalian species is ochratoxin  $\alpha$  (see Fig. 13.1), a hydrolysis product from metabolism of OTA by the gut microflora (Suzuki *et al.* 1977; Galtier, 1978). Ochratoxin  $\alpha$  has been found in the urine of rats following both oral and intraperitoneal administration of OTA (Storen *et al.*, 1982). This suggests that OTA can be metabolized in the gut directly or after excretion of the parent compound in the bile (Moroi *et al.*, 1985). Small amounts of OTA conjugates have also been identified in rat urine following administration of a single oral dose of OTA (Zepnik *et al.*, 2003). Two further metabolites have been identified as 4-OH (4R and 4S) epimers of OTA which are produced by cytochrome P<sub>450</sub> enzymes in the liver although, by comparison with ochratoxin  $\alpha$ , these are relatively minor metabolites (Stormer *et al.*, 1981). 10-OH epimers of OTA have also been identified in a rabbit liver microsomal system (Stormer *et al.*, 1983).

There are large species differences in the half-life of OTA. Half-lives for OTA after oral administration have been reported to range from 4.2 hours in chickens (Galtier *et al.*, 1981), 55–230 hours in rats (Galtier *et al.*, 1979; Ballinger *et al.*, 1986; Hagelberg *et al.* 1989; Zepnik *et al.*, 2003), 77 hours in pre-ruminant calves (Sreemannarayana *et al.*, 1988), 89 hours in pigs (Galtier *et al.*, 1981), 510 hours in a study of one macaque monkey (Hagelberg *et al.*, 1989) to 853 hours in a human volunteer (Studer-Rohr *et al.*, 2000). The large species differences in half-life may reflect differences in the degree of absorption of OTA from the gut, differences in the types and activity of microflora in the gut, the level of binding to serum proteins to prevent metabolism or the rate of enterohepatic circulation. The large range of half-lives in rats probably reflects differences in the doses of OTA administered and experimental methodology used in the studies

### 13.2.4 Excretion

The major routes of excretion of OTA from the blood are via glomerular filtration into the urine or via the bile duct and then into the faeces. In a study in rats, OTA, ochratoxin  $\alpha$  and the 4R-OH-ochratoxin A epimer were identified in the urine and faeces following oral administration of OTA. Of the original dose, 12 % OTA and

9 % ochratoxin  $\alpha$  were found in the faeces, whilst in the urine ochratoxin  $\alpha$  and OTA represented 25–27 % and 6 % of the dose, respectively (Storen *et al.*, 1982). The extent of clearance of OTA from the blood is affected by factors such as enterohepatic circulation (Roth *et al.*, 1988; Fuchs and Hult, 1992) and serum protein binding (Hagelberg *et al.*, 1989; Il'ichev *et al.*, 2002). There may, therefore, be marked species differences in the proportion and rate of OTA clearance by the bile duct and kidney. The mechanism of OTA clearance has been investigated in two studies, which have suggested that transport of OTA is mediated by human organic anion transporter 4 (hOAT4) on the apical side of the proximal tubule (Babu *et al.*, 2002) and by the hOAT1 and 3 transporters on the basolateral side of the proximal tubule (Jung *et al.*, 2001).

### 13.3 Biochemical effects of OTA

The biochemistry of OTA results primarily from its structural similarity to the essential amino acid, phenylalanine (Phe). The principal effect appears to be inhibition of protein synthesis (Creppy *et al.*, 1984), although secondary effects such as inhibition of RNA and DNA synthesis have also been implicated in its mechanism of action. The chemical structure of OTA consists of a 5'-chlorinated-3,4-dihydro-3-methylisocoumarin moiety linked to L-Phe (see Fig. 13.1). The Phe moiety allows OTA to bind to the active site of Phe-tRNA synthetase and to competitively inhibit amino acid-acylation and halt peptide elongation. Analogues of OTA in which Phe is absent or has been replaced with other amino acids, such as tyrosine (Tyr), either do not inhibit amino acid tRNA synthetase or inhibit the respective amino acid-specific tRNA synthetase (Creppy *et al.*, 1983a,b). However, OTA is a relatively weak inhibitor of Phe-tRNA, with a binding affinity that is orders of magnitude weaker than Phe, ranging from 300-fold weaker in yeast ( $K_m = 1.3$  mmol/L for OTA compared with 3.3  $\mu$ mol/L for Phe) (Creppy *et al.*, 1983a) to 20-fold weaker in rat liver cells ( $K_m = 0.28$  mmol/L for OTA compared with 6  $\mu$ mol/L for Phe) (Röschenthaler *et al.*, 1984). OTA inhibition of the enzyme is easily reversed by the addition of Phe (Zanic-Grubisic *et al.*, 2000). The lethality of a single intraperitoneal injection of OTA was completely prevented by simultaneously injecting phenylalanine (Creppy *et al.*, 1980). However, the low relative potency of OTA compared with Phe may be offset by the greater concentrations of OTA in cells compared with Phe, as an *in vitro* study has shown that the influx of OTA into cells is efficient, with OTA concentrations 200–300-fold greater within cells compared with the culture medium (Creppy *et al.*, 1983a).

OTA is also able to interact with other enzymes that use Phe as a substrate. *In vitro* experiments showed that OTA competitively inhibits phenylalanine hydroxylase, an enzyme that catalyzes the irreversible hydroxylation of Phe to Tyr, which is a key stage in Phe catabolism. However, similar to the interactions with Phe-tRNA synthetase, the interaction with this enzyme is relatively weak with apparent inhibition constants of  $K_i = 12$   $\mu$ M for liver and  $K_i = 13$   $\mu$ M for kidney phenylalanine hydroxylase reported (Zanic-Grubisic *et al.*, 2000).

Unrelated to its structural similarity to Phe, it has been suggested that OTA may induce lipid peroxidation and superoxide and hydrogen peroxide radical formation (Baudrimont *et al.*, 1994). In support of this effect on radical formation, it has been shown experimentally that ethane exhalation, a marker of lipid peroxidation, was increased seven-fold in rats after oral administration of OTA (Rahimtula *et al.*, 1988). In addition, Meki and Hussein (2001) showed that when OTA (250 µg/kg body weight) was administered by oral gavage daily for four weeks, levels of malondialdehyde (MDA), a product of lipid peroxidation, were significantly increased in serum and in liver and kidney tissues compared with levels in control rats. Gautier *et al.* (2001b) also reported that oral administration of OTA (1 mg/kg body weight) to rats increased, five-fold, the level of haem oxygenase-1, an oxidative stress responsive protein in the kidney, although the levels of MDA in plasma, kidney or liver were unchanged.

The mechanism by which OTA may stimulate lipid peroxidation is unclear. However, it has been postulated that OTA complexes with flavoprotein-bound iron (Fe<sup>3+</sup>) to facilitate reduction to Fe<sup>2+</sup> and subsequent oxygen binding. This may lead indirectly to oxygen radical formation (Omar *et al.*, 1990). However, an alternative mechanism may involve lowering the levels of compounds or enzymes that remove reactive oxygen species. An *in vivo* study showed that glutathione (GSH), glutathione reductase, glutathione peroxidase, superoxide dismutase, catalase and glutathione-S-transferase were significantly decreased in liver and/or kidney of OTA treated animals when compared with controls (Meki and Hussein, 2001).

## 13.4 Toxic effects of OTA

### 13.4.1 Nephrotoxicity (experimental data)

The kidney is the major site of OTA-induced toxicity, where it acts principally on the middle (S2) and terminal (S3) segments of the proximal convoluted tubules (Jung and Endou, 1989). OTA has been shown to be nephrotoxic in all monogastric species tested, although there are species differences in sensitivity to nephrotoxic effects. Kuiper-Goodman and Scott (1989) suggested a rank order of species sensitivity of dog > pig > chicken > rat > mouse. In addition, studies in rats have shown that females and older animals have a greater susceptibility to the adverse effects of OTA on the kidney compared with male and younger animals (National Toxicology Program, 1989; Dortant *et al.*, 2001). For example, when OTA was administered daily to different age groups of rats for about one month, the lowest dose at which lesions in the kidney were observed in old adult females was 70 µg/kg body weight/day, but was about 5-fold higher in young adult females (Dortant *et al.*, 2001).

Clinical chemistry parameters have been used to monitor the effects of OTA on renal function. Studies have shown that OTA exposure can lead to increased urine volume, blood urea nitrogen (Hatey and Galtier, 1977), urinary glucose, and proteinuria (Berndt and Hayes, 1979) as well as to reductions in the activity of

enzymes in the kidney, such as alkaline phosphatase, leucine aminopeptidase, and  $\gamma$ -glutamyl transferase (Kane *et al.*, 1986a). OTA has also been shown to reduce the glomerular filtration rate (Stoev *et al.*, 2002) and to affect pH homeostasis in the vasa recta, proximal tubules, distal tubules and collecting ducts within the kidney (Kuramochi *et al.*, 1997a,b). In studies of rodents treated with OTA, karyomegaly, nephrosis and eosinophilia of the nephron tubules, granular and vacuolar degeneration of the tubular epithelial cells and interstitial fibrosis and thickening of the tubular basement membrane were evident on histopathological examination of the kidney (Dortant *et al.*, 2001; Stoev *et al.*, 2002).

The mechanism of action of OTA-induced nephrotoxicity is unclear. However, the finding that the nephrotoxic effects of OTA were prevented when the radical scavenging enzymes superoxide dismutase and catalase were co-administered with OTA strongly suggests that superoxide radicals and hydrogen peroxide may play a role (Baudrimont *et al.*, 1994). Alternatively, OTA may induce apoptosis in the kidney as it has been shown to activate caspase 3 and induce apoptosis in cultured canine renal collecting duct (Gekle *et al.*, 2000) and human proximal tubule cells (Schwerdt *et al.*, 1999). The mechanism of OTA-induced nephrotoxicity has recently been explored by microarray analysis of renal tissue exposed to OTA both *in vitro* and *in vivo*. Analysis of the genes expressed in these experiments showed that 215 genes were differentially expressed after both *in vivo* and *in vitro* exposure of kidney tissue to OTA. The results indicate that perhaps both of the above mechanisms may play a part in OTA-induced nephrotoxicity as the genes differentially expressed included oxidative stress response genes (e.g. hypoxia-inducible factor 1, which was upregulated, and catalase, which was repressed), genes involved in DNA damage response and apoptosis (e.g. GADD 153, GADD 45 and annexin V, which were upregulated), and genes involved in inflammatory reactions (e.g. alpha 2 macroglobulin, ceruloplasmin and cathepsin S, which were upregulated) (Luhe *et al.*, 2003).

#### 13.4.2 Nephrotoxicity (human data)

A number of epidemiological studies have identified OTA as a likely aetiological agent responsible for a fatal kidney disease primarily affecting rural populations in the central Balkan peninsula including Bosnia and Herzegovina, Bulgaria, Croatia, Romania, Serbia and Slovenia (reviewed by JECFA, 2001). The disease, referred to as Balkan endemic nephropathy (BEN), is characterized by tubular degeneration, interstitial fibrosis and hyalinization of the glomeruli and a slowly developing impairment of renal function with a progressive decrease in kidney size (Vukelic *et al.*, 1991). Proteinuria, headaches, lumbar pain, anaemia and weight loss are also reported symptoms of this condition (Pfohl-Leskowicz *et al.*, 2002). The disease affects more women than men, with fatalities occurring more frequently in women (Ceovic *et al.*, 1992). An association between BEN and urinary tract tumours has also been identified (see Section 13.4.4).

Although OTA is a contaminant of food and feed worldwide (see Section 13.5), it has been detected at high levels in a wide range of foods and in human blood

**Table 13.1** Ochratoxin A in wheat and corn from different regions of Croatia and Slovenia.\*

Sample area	Wheat		Corn	
	No. samples	Ochratoxin A (µg/kg)	No. samples	Ochratoxin A (µg/kg)
Slavonski Brod	33	39 (160)	11	20 (40)
Osijek	27	9 (32)	15	< 1 (4)
Hrvatsko-Zagorje	17	2 (5)	5	< 1 (2)
Istria	6	1 (6)	4	< 1 (2)
Celje	9	< 1 (2)	16	< 1 (6)

\*Corn and wheat samples were obtained from the surroundings of Slavonski Brod, a BEN-endemic area of Croatia. Samples were also obtained from the surroundings of the unaffected areas Osijek, Hrvatsko-Zagorje and Istria in Croatia and Celje in Slovenia. Levels of OTA were measured by thin-layer chromatography (TLC), and mean and (maximum) values (µg/kg) are presented. Limit of detection 0.02 µg/kg. Adapted from Puntaric *et al.* (2001).

samples taken from areas where BEN is endemic (Pavlovic *et al.*, 1979; Hult *et al.*, 1982; Petkova-Bocharova *et al.*, 1988; Petkova-Bocharova and Castegnaro, 1991; Peraica *et al.*, 2001; Pfohl-Leszkowicz *et al.*, 2002). Puntaric *et al.* (2001) quantified OTA in wheat and corn from the surroundings of Slavonski Brod, an area of Croatia where BEN is endemic, and from four areas in Croatia and Slovenia where BEN occurs rarely or not at all. The results are summarized in Table 13.1, and show that the amount of OTA was significantly higher in samples from Slavonski Brod compared with samples from non-endemic areas. Although the available evidence is suggestive of a causal role for OTA in BEN, it is unclear whether OTA is solely responsible, or whether synergy with other environmental toxicant(s) is required. In an analysis of OTA levels in home-grown food and feed from BEN-endemic and non-endemic villages of North-Western Bulgaria, Abouzied *et al.* (2002) suggested that OTA exposure alone is probably not sufficient to cause BEN. Furthermore, although Vrabcheva *et al.* (2000) detected higher levels of OTA in BEN-endemic Bulgarian villages than in a control village, higher levels of the nephrotoxic mycotoxin citrinin were also measured in the BEN-endemic villages.

It has also been suggested that OTA may be a factor in a similar type of renal disease found in Tunisia, termed chronic interstitial nephropathy of unknown cause (CIN). In 1994, Abid *et al.* (2003) showed that OTA levels were higher in food and in blood samples from people with CIN than in healthy people (see Section 13.5.4). However, confirmatory studies are lacking.

### 13.4.3 Carcinogenicity (experimental data)

The carcinogenicity of OTA has been established in a number of long-term studies in rodents, with the kidney being the principal site and the liver the major

secondary site of tumour formation. In a small study of male mice fed OTA at 5.6 mg/kg body weight per day for 44 weeks, followed by five weeks of an uncontaminated diet, 5/9 surviving mice had hepatic tumours but 9/9 had renal tumours. No tumours of the liver or kidney were observed in the control group (Kanisawa and Suzuki, 1978). Similar findings were reported in larger studies in mice by Kanisawa (1984), who found renal cystic adenomas in surviving mice treated with 3.5 mg/kg body weight per day after 70 days and hepatic tumours after 25 weeks in mice treated with 7 mg OTA/kg body weight per day. In a study of mice fed OTA at a concentration of 40 mg/kg in the diet for 24 months, benign and malignant renal tumours were seen at incidences of 53 % and 29 %, respectively (Bendele *et al.*, 1985a).

Similar findings have been reported in studies of rats. In a long-term study carried out as part of the National Toxicology Program (1989), the frequency of renal adenomas increased in a dose-dependent manner in all groups of male rats treated with OTA. A combined incidence of renal adenomas and carcinomas of 20/51 and 36/50 was reported at the two highest OTA doses administered (70 or 210 µg/kg body weight per day, respectively). Renal carcinomas were less common in female rats at the highest dose levels, with combined incidences of 2/50 and 8/50 reported for renal adenomas and carcinomas, respectively. However, fibroadenomas in the mammary gland were found in 45–56 % of these treated females.

#### 13.4.4 Carcinogenicity (human data)

OTA has been implicated in the development of cancers of the human urinary tract because of the higher incidence of urinary tract tumours in humans in regions where BEN is endemic (see Section 13.4.2) (Chernozemsky *et al.*, 1977; Ceovic and Miletic-Medved, 1996). For example, in Croatia's BEN endemic region, the prevalence of tumours of the pylon and ureter is 11 times greater than in the non-endemic region (Vukelic *et al.*, 1987). Results from a study of 766 patients in Serbia showed that women and young people are more commonly affected in endemic regions and the usual site of tumours is the renal pelvis and the urethra, whilst in non-endemic regions, tumours are most frequently seen in the urinary bladder at a lower incidence (Djokic *et al.*, 1999).

A role for OTA in the aetiology of testicular cancer has also been hypothesized on the basis of ecological data (Schwartz, 2002). Schwartz showed that incidence rates of testicular cancer were significantly correlated with the per capita consumption of coffee and pigmeat, two commodities known to contribute to the exposure to OTA in the diet. However, no studies have confirmed this hypothesis.

#### 13.4.5 Genotoxicity

Some studies have demonstrated that OTA is genotoxic both *in vitro* and *in vivo*, although OTA produces a negative result in most conventional tests for mutagenicity, using standard protocols. However, results have been inconsistent, as some studies have reported positive results for gene mutation in bacteria (e.g. Hennig *et*

*al.*, 1991; Obrecht-Pflumio *et al.*, 1999) whilst in mammalian cells, two gene mutation studies were negative (Umeda *et al.*, 1977; Bendele *et al.*, 1985a), and a third was positive (de Groene *et al.*, 1996).

There is some disagreement about whether OTA reacts directly with nucleic acids or acts via an indirect mechanism to disrupt DNA. Studies using a  $^{32}\text{P}$  post-labelling technique to examine whether OTA produces DNA adducts have shown that adducts are produced in the kidney, liver and spleen from mice, rats and monkeys after exposure to OTA in a number of studies (Pfohl-Leszkowicz *et al.*, 1991, 1998; Grosse *et al.*, 1995, 1997; Castegnaro *et al.*, 1998). The number of adducts was highest in kidney DNA, with the number of adducts ranging from 1–200/10<sup>9</sup> nucleotides. However, in these studies the identity of the putative OTA-induced DNA adduct could not be determined. Thus the adducts observed may not necessarily be due to a direct reaction between nucleic acid bases and OTA or its metabolites. In fact, Schlatter *et al.* (1996) found that no radioactivity was detected in liver or kidney DNA following administration of  $^3\text{H}$  labelled OTA to rats. Likewise, Gautier *et al.* (2001a) did not find covalent binding of  $^3\text{H}$  labelled OTA to DNA in the kidneys of male rats dosed 24 hours earlier by gavage (detection limit 2.7 adducts/10<sup>9</sup> DNA bases). However, using a  $^{32}\text{P}$  post-labelling method, adducts were detected at a level 3–17 times higher than the detection limit for  $^3\text{H}$  labelled OTA in this study. Taken together, the information from these studies suggests that, although OTA may induce the formation of DNA adducts, the adducts may not contain OTA.

Other types of OTA-induced DNA damage have also been reported. DNA single strand breaks have been observed consistently in mammalian cell cultures, with the number of breaks increasing in a dose-dependent manner (Lebrun and Follmann, 2002). DNA strand breaks have also been observed in *in vivo* assays in the spleen, liver and kidney cells of mice following intraperitoneal injection of OTA (Creppy *et al.*, 1985). Induction of DNA repair, as demonstrated by unscheduled DNA synthesis, has been reported in rat hepatocytes, porcine urinary bladder epithelial cells and human urothelial cells upon OTA treatment (e.g. Dorrenhaus and Follmann, 1997; Dorrenhaus *et al.*, 2000). Tests for the induction of sister chromatid exchange have proved positive in two of four *in vitro* studies, but were negative *in vivo* at a range of doses, including those that were cytotoxic (e.g. Bendele *et al.*, 1985b; National Toxicology Program, 1989; Hennig *et al.*, 1991). Chromosomal aberrations have also been induced *in vivo* in mouse cells (Bose and Sinha, 1994) and *in vitro* in human lymphocytes (Manolov *et al.*, 1991; Donmez-Altuntas *et al.*, 2003), as well as in human-derived hepatoma cells (Ehrlich *et al.*, 2002) and ovine seminal vesicle cells (Degen *et al.*, 1997). The results of some representative genotoxicity studies are summarized in Table 13.2.

#### 13.4.6 Mechanism of carcinogenicity

The mechanism by which OTA induces tumour formation remains controversial and is an area of active study. Formation of DNA adducts is thought by some researchers to be an important event in the tumorigenicity of OTA and it is

**Table 13.2** The results of selected *in vitro* and *in vivo* assays for genotoxicity with OTA.

Assay	Test cell/organism	OTA concentration	Result	Reference
<b><i>In vitro</i></b>				
Micronucleus formation	Human-derived hepatoma (HepG2) cells	0–50 µg/ml	Positive. Dose-dependent response at 5 µg/l and greater	Ehrlich <i>et al.</i> (2002)
Micronucleus formation	Cultured human lymphocytes	100 pmol/l–25 µmol/l	Positive at 25 µmol/l	Donmez-Altuntas <i>et al.</i> (2003)
DNA damage	Madin-Darby canine kidney cells	1 nmol/l–250 µmol/l	Positive (maximum damage at 100 µmol/l)	Lebrun and Follmann (2002)
Reverse mutation	<i>S.typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538, G46, G3076, D3052	0.1–100 µg/ml	Negative	Bendele <i>et al.</i> (1985b)
Reverse mutation	<i>S.typhimurium</i> TA97, TA98, TA100, TA1535	1–100 µg/plate	Negative with rat or hamster liver activation	National Toxicology Program (1989)
DNA repair	<i>E.coli</i> , SOS assay	1–2 mg/100 µl	Negative	Auffray and Boutibonnes (1986)
Gene mutation	NIH 3T3 cells transfected with human cytochrome P450	25 µg/ml	Positive	De Groene <i>et al.</i> (1996)
Unscheduled DNA synthesis	Fischer 344 rat primary hepatocytes	0.000025–500 µg/ml	Negative	Bendele <i>et al.</i> (1985b)
Unscheduled DNA synthesis	Primary human urothelial cells	10–2000 nmol/l	Positive	Dorrenhaus <i>et al.</i> (2000)
Sister chromatid exchange	Human peripheral blood lymphocytes	5–10 µg/ml	Negative	Cooray (1984)
Sister chromatid exchange	Chinese hamster ovary cells	a) 0.5–5 µg/ml b) 5–160 µg/ml	a) Negative b) Positive	National Toxicology Program (1989)
<b><i>In vivo</i></b>				
DNA damage	Wistar rat kidney and liver	290 µg/kg bwt every 48 h by gavage for 6 or 12 weeks	Positive	Kane <i>et al.</i> (1986b)
Chromosomal aberration	Mouse	1 µg/kg bwt per day in diet for 45 days	Positive	Bose and Sinha (1994)
Sister chromatid exchange	Chinese hamster bone marrow	25–400 mg/kg by gavage	Negative	Bendele <i>et al.</i> (1985b)

postulated that OTA or one of its metabolites may act through a genotoxic mechanism involving direct covalent binding to DNA. Although numerous studies have investigated the transformation of OTA into a reactive intermediate and its possible role in tumorigenesis (e.g. Obrecht-Pflumio *et al.*, 1999; El Adlouni *et al.*, 2000), these studies did not suggest a possible structure for a reactive intermediate, nor did they assess the capacity of OTA to be transformed to DNA reactive metabolite(s). In addition, a study found that neither OTA nor its metabolites bound covalently to DNA following the incubation of  $^3\text{H}$  labelled OTA with rat hepatocytes. Thus, the notion that OTA undergoes bioactivation to a metabolite that forms DNA adducts was not supported in this instance (Gross-Steinmeyer *et al.*, 2002). Hence, it is not possible to draw firm conclusions about the possible mechanism of OTA-induced tumour production from these studies.

An alternative explanation, that is perhaps more consistent with the observations from studies on DNA adduct formation, is that DNA damage is caused by an indirect mechanism related to oxidative stress leading to the induction of reactive oxygen species (see Section 13.3) that may then react with DNA to produce adducts (e.g. Bose and Sinha, 1994; Schlatter *et al.*, 1996). In support of a role for oxidative stress Grosse *et al.* (1997) found that, in mice given ascorbic acid or alpha-tocopherol, the number of adducts resulting from OTA administration was significantly decreased. Preliminary studies have also shown that administration of superoxide dismutase or catalase prior to OTA exposure reduces DNA adduct formation in the liver, kidney and testis (Pfohl-Leskowicz *et al.*, 1993). A recent *in vitro* study demonstrated that, in response to exposure to OTA, rat proximal tubule cells produced reactive oxygen species in a concentration-dependent manner together with a decrease in cellular glutathione levels and an increase in the formation of 8-oxoguanine, thus providing supporting evidence for this mechanism (Schaaf *et al.*, 2002). Furthermore, oxidative stress is known to play an important role in tumour induction in the rat kidney (e.g. Hard, 1998).

#### 13.4.7 Immunotoxicity

Data on the possible effects of OTA on the immune system are limited. However, a number of *in vivo* and *in vitro* studies suggest that OTA may affect both humoral and cell-mediated immunity, although the reported effects were generally observed at higher doses than those capable of causing nephrotoxicity.

At very high doses (0.5–80 mg/kg body weight) OTA has been shown to cause gross changes to organs of the immune system, such as reductions in thymus size in mice, rats and chickens (Boorman *et al.*, 1984; National Toxicology Program, 1989; Singh *et al.*, 1990) and necrosis of cells in the spleen and lymph nodes in rats (Kanisawa *et al.*, 1977).

At lower levels, OTA has been shown to affect a number of parameters of immune function. Thuvander *et al.* (1995) reported a reduction in the number of splenocytes in mice orally administered 2.6 mg/kg feed, equivalent to 400  $\mu\text{g}/\text{kg}$  body weight per day) for 90 days. In addition, the primary antibody response to sheep red blood cells was also suppressed and the proportion of mature CD4<sup>+</sup> and

CD8<sup>+</sup> cells decreased after OTA administration at 40 µg/kg body weight per day. However, OTA (400 µg/kg body weight per day) fed to adult mice for up to 90 days did not affect lymphoid organ weight, leukocyte count, blood or thymic T lymphocyte levels, or the antibody response to viral antigen.

Prenatal OTA exposure (200 µg/kg feed, equivalent to 30 µg/kg body weight per day) altered the absolute and relative numbers of lymphocyte subpopulations in lymphoid organs of BALB/C mice, although immune function was not suppressed (Thuvander *et al.*, 1996a). In contrast, short-term exposure of suckling pups to OTA via the milk stimulated the immune response in rats, as shown by the proliferation of lymphocytes in response to an antigen (Thuvander *et al.*, 1996b). In studies in chickens, OTA reduced the lymphoid cell population of immune organs (Dwivedi and Burns, 1984b), the plasma levels of  $\alpha$ 1-,  $\alpha$ 2-,  $\beta$ - and  $\gamma$ -globulins (Rupic *et al.*, 1978), and the levels of IgG, IgA and IgM in lymphoid tissues and serum (Dwivedi and Burns, 1984a).

The immunosuppressive effects of OTA were evaluated in a study by Stoev *et al.* (2000), where three groups of six pigs were fed feed contaminated with OTA (0–3 mg/kg diet) for up to three weeks. All animals treated with the most contaminated feed contracted salmonellosis, with *Salmonella choleraesuis* found in the faeces and liver, and died between days 15 and 17. A smaller number of pigs were affected at the lower dose and none were affected in the control group.

The mechanism of OTA-induced immunosuppression in animals is unclear although studies suggest that it may be related to the inhibitory effect of OTA on DNA and protein synthesis in lymphocytes, macrophages and other immune system cell types (Stormer and Lea, 1995; Donmez-Altuntas *et al.*, 2003; Muller *et al.*, 2003). The potential immunotoxic effects of OTA in humans have not been investigated.

### 13.4.8 Neurotoxicity

The potential neurotoxicity of OTA has been investigated in a limited number of animal and *in vitro* studies. In a small study, lesions in the ventral mesencephalon, hippocampus, striatum and cerebellum of the brain were found after four rats were administered 290 µg OTA/kg body weight by oral gavage for eight days (Belmadani *et al.*, 1998b). In a further study in rats, OTA was found to accumulate in the brain in a linear, time-dependent manner following oral administration of 290 µg/kg body weight every 48 hours for six weeks, and tissue damage was observed in the hippocampus (Belmadani *et al.*, 1998a). In a study of rats given 120 µg OTA/kg body weight per day by oral gavage for between 10 and 35 days, increases in  $\gamma$ -glutamyl transferase activity in the brain were reported (Zanic-Grubisic *et al.*, 1996). In a comparative neurotoxicity study of young and old rats, OTA was administered over four weeks by oral gavage at 0, 0.07, 0.34 or 1.68 mg/kg body weight. Lesions in the cerebellar medulla and central part of the brainstem were significantly increased at the lowest dose level versus the control in older rats, whilst a significant increase was seen only at the two highest dose levels in the younger rats. Thus, the authors suggest that old rats are more sensitive to the

neurotoxic effects of OTA than their younger counterparts (Dortant *et al.*, 2001). This could be a reflection of poorer renal clearance of OTA in the older animals.

The mechanism of neurotoxicity is unclear. However, a study that investigated the effects of OTA on neurones and astrocytes isolated from embryonic or newborn rat brain mesencephalon and cerebellum suggested that OTA inhibits protein and DNA synthesis (Belmadani *et al.*, 1999). The potential neurotoxicity of OTA in humans has not been reported.

#### 13.4.9 Effects on fertility

Some animal studies have indicated that OTA may adversely affect the male reproductive system. Administration of OTA (290 µg/kg body weight) to male rats every 48 hours by oral gavage for up to eight weeks caused a two-fold increase in the testicular testosterone levels. An accumulation of premeiotic germinal cells was also observed, as measured by increased  $\alpha$ -amylase, alkaline phosphatase and  $\gamma$ -glutamyl transpeptidase activities in testis homogenate, which are indicative of impaired spermatogenesis (Gharbi *et al.*, 1993). Biro *et al.* (2003) investigated the effects of OTA on spermatogenesis in breeding boars fed OTA (0.08 µg/kg body weight per day) for six weeks. After a nine week withdrawal period, semen analysis showed that sperm motility and viability were significantly reduced, suggesting that OTA may reduce semen quality. The possible effects of OTA on female fertility have not been investigated, nor have possible effects on human fertility.

#### 13.4.10 Developmental toxicity

OTA is able to cross the placenta and has been shown to be both teratogenic and embryotoxic in rats and mice. In one study, pregnant mice were either administered a single dose of OTA by gavage (up to 4 mg/kg body weight) on days eight or nine of gestation or administered 4 mg OTA/kg body weight two days before mating and once between days 2 and 16 of gestation. Prenatal survival of pups was reduced in the groups that received the maximum dose of 4 mg/kg body weight on days seven, eight or nine of gestation. Overt craniofacial anomalies were seen only after treatment on day eight or nine. The incidence and severity of these anomalies increased with increasing dose. Fetal malformations were seen predominantly in the central nervous system, the eye and the axial skeleton. The commonest malformation was incomplete formation of the skull, occurring in 89 % of animals treated with 4 mg/kg body weight on day nine (Arora and Frolen, 1981).

A dose-related increase in the resorption of foetuses and decreases in mean foetal and placental weight were seen in pregnant rats administered OTA by gavage on days 8 and 9 (2.5 mg/kg body weight) or days 8–10 (1.7 mg/kg body weight) of gestation (Moré and Galtier, 1974). Similarly, in a study in which pregnant rats were orally administered 1 mg OTA/kg body weight per day on days 6–15 of gestation, an increased number of resorptions were observed, as well as

decreased foetal weight. The incidence of renal malformations in the foetuses was 40 % and the incidence of skull or lung malformations was 20 % (Abdel-Wahhab *et al.*, 1999). OTA also caused dose-dependent reductions in cell protein and DNA content in cultured rat embryos (Mayura *et al.*, 1989). In this study, several different malformations were induced in the embryos, including hypoplasia, telencephalon, stunted limb bud development and reduced mandibular and maxillary bone size.

## 13.5 Distribution, levels and the detection of OTA in food

### 13.5.1 Occurrence of OTA in food

The three major species of mould that produce OTA are *Penicillium verrucosum*, *Aspergillus ochraceus* and *Aspergillus carbonarius*. All three differ in physiology and ecology, which in turn affects the types of foodstuffs in which these moulds are most commonly found. The moulds grow in atmospheres with a relatively high moisture content, and hence harvesting of crops at high moisture levels and storage of contaminated foodstuffs in damp conditions favours the production of OTA (e.g. Birzele *et al.*, 2000).

The major habitat of *P. verrucosum* is cereal crops in the cool and temperate climates of northern Europe and Canada (JECFA, 2001). Consequentially, OTA is found in cereal products, especially flour-based foods. It is also found in commodities such as cheese and meat products from animals that eat cereals as a major dietary component.

In contrast, *A. ochraceus* is most commonly found in dried and stored foods, such as smoked and salted dried fish, soya beans, chick peas, nuts, pepper and dried fruit. It has been reported infrequently in cereals and green coffee beans. *A. ochraceus* is generally present at low levels and rarely causes spoilage. Its presence may not be a good indicator of significant OTA contamination (JECFA, 2001).

*A. carbonarius* grows optimally at 32–35 °C and is resistant to sunlight. Its major habitat is dried vine fruit. It is therefore the main source of OTA in grapes and grape products, such as raisins and wine. *Aspergillus niger* is often found in association with *A. carbonarius* and is commonly isolated from nuts, fresh fruit and some vegetables in warm climates. However, only 1–2 % of *A. niger* isolates have been reported to produce OTA (Heenan *et al.*, 1998).

The results of selected recent studies on the occurrence of OTA in foodstuffs are shown in Table 13.3. These data confirm that OTA is found in a wide range of foodstuffs from many different countries and at varying concentrations. Surveys for OTA have been comprehensively reviewed by JECFA (2001). In a total diet study in the UK low concentrations of OTA were detected in all 50 samples analysed using high-performance liquid chromatography (HPLC) with fluorescence detection (see Section 13.5.5) which was capable of detecting 0.002 µg/kg (MAFF, 1999). In this study, OTA levels have been shown to vary between different commodities and also within different batches of commodities.

**Table 13.3** Occurrence of OTA in selected foodstuffs from recent studies.

Foodstuff	Country of origin	Range of concentrations detected (mean) µg/kg or µl/l	LOQ (LOD) <sup>1</sup> µg/kg or µl/l	Number of OTA-contaminated samples	Reference
<b>Cereals</b>					
Wheat	Germany 1997	0.6–0.7 (0.7)	0.4	2/14	Birzele <i>et al.</i> (2000)
Wheat	Germany 1998	0.6–0.8 (0.6)	0.4	7/29	Birzele <i>et al.</i> (2000)
Wheat	Hungary	0.12–0.5 (0.29)	(0.1)	3/36	Fazekas <i>et al.</i> (2002)
Wheat flour	Hungary	0.11–0.15 (0.13)	(0.1)	2/16	Fazekas <i>et al.</i> (2002)
Maize course meal	Hungary	0.4 (0.4)	(0.1)	1/6	Fazekas <i>et al.</i> (2002)
Oats	Norway	(0.065, maximum 0.47)	0.01	3/22	JECFA (2001)
<b>Beer</b>					
Home brewed beer	South Africa	3–2340	(1)	10/32	Odhav and Naicker (2002)
Beer	Europe	(0.025, maximum 0.121)	0.004	40/40	JECFA (2001)
<b>Coffee</b>					
Pure roasted coffee and coffee blends	Purchased in Hungary	0.17–1.3 (0.57)	(0.1)	33/50	Fazekas <i>et al.</i> (2002)
Coffee products	Europe	(0.9)	0.2–1	299/633	JECFA (2001)
<b>Cocoa and chocolate</b>					
Chocolate	UK	(0.16)	0.02	30/40	JECFA (2001)
Cocoa powder	UK	(1.67)	0.2	20/20	JECFA (2001)

<b>Wine</b>					
Red wine	Morocco	0.04–3.24 (median 0.912)	0.01	20/20	Filali <i>et al.</i> (2001)
Red wine	UK	(0.074, maximum 0.46)	0.02	28/50	JECFA (2001)
White/Rosé wine	Morocco	0.028–0.18 (median 0.117)	0.01	10/10	Filali <i>et al.</i> (2001)
White wine	Italy	(0.57, maximum 8.86)	0.01	7/21	JECFA (2001)
<b>Dried vine fruit</b>					
Raisins	UK	(2.79, maximum 20)	0.2	17/20	JECFA (2001)
Currants	UK	(4.97, maximum 40.8)	0.2	96/100	JECFA (2001)
<b>Meat and offal</b>					
Pig kidney	Romania	(0.54)	0.01	41/52	Curtui <i>et al.</i> (2001)
Pig kidney	Denmark	0–15 (0.5)	0.09	284/300	Jorgensen and Petersen (2002)
Pig meat	Romania	(0.15)	0.01	9/52	Curtui <i>et al.</i> (2001)
Pig meat	Denmark	0–2.9 (0.12)	0.06	228/300	Jorgensen and Petersen (2002)
Pig liver	Romania	(0.16)	0.01	39/52	Curtui <i>et al.</i> (2001)
<b>Herbs, spices and sweets</b>					
Chilli powder	UK	(Maximum 50.4)	0.1	?/4	JECFA (2001)
Curry powder	UK	(Maximum 21.3)	0.1	?/10	JECFA (2001)
Liquorice	Purchased in Germany	Sweet 0.4–3.0 Root 0.3–216.5	(0.3)	Sweet 18/19 Root 9/19	Bresch <i>et al.</i> (2000)

<sup>1</sup>Limit of quantitation (Limit of detection)

### 13.5.2 Geographical distribution and annual variation

OTA has been found in coffee sourced from all over the world (Bucheli *et al.*, 2000; Leoni *et al.*, 2000; Romani *et al.*, 2000; Joosten *et al.*, 2001; Fazekas *et al.*, 2002; Taniwaki *et al.*, 2003). OTA has also been found in European and North American cereal crops (Kuiper-Goodman *et al.*, 1993; Trucksess *et al.*, 1999). A high mean concentration and incidence of contamination in breast milk samples from Sierra Leone provides indirect evidence for OTA contamination of foodstuffs of African origin (Jonsyn *et al.*, 1995). Further to this, very high levels of OTA were measured in wheat, barley, mixed cereals, dried vegetables and olives collected in Tunisia from families with one or more patients with nephropathy (Maaroufi *et al.*, 1995). OTA has also been found in spices such as turmeric, coriander, ginger (Thirumala-Devi *et al.*, 2001) and chillies (Thirumala-Devi *et al.*, 2000) from the Indian subcontinent. Thus, the occurrence of OTA in foodstuffs is a global phenomenon.

Annual variation in OTA contamination has been observed in several studies. Higher mean concentrations were found in maize collected in Croatia in 1997 (57 µg/kg) than in 1996 (38 µg/kg) (Jurcevic *et al.*, 1999). In a UK survey of barley and wheat only 0.3 % of samples were contaminated in 1993, whilst in 1994, 9 % of samples were contaminated (Scudamore, 1999). In a study of OTA levels in wheat harvested in sequential years in Germany, OTA was found in 14.3 % of samples from 1997 and 24.1 % of samples from 1998 (Birzele *et al.*, 2000).

### 13.5.3 Effects of processing

The physical processes of scouring and milling decrease the levels of OTA in white flour (Osborne *et al.*, 1996). There is little effect on wholemeal flour, however, since OTA is still present in the wholemeal fractions. OTA is relatively stable to heat and will survive most food processing to some extent. However, roasting can reduce OTA levels in coffee by 69–96 % (van der Stegen *et al.*, 2001). Decaffeination of coffee leads to a similar reduction (Heilmann *et al.*, 1999). A study carried out by MAFF (1996) indicated that OTA was reduced by processing in manufactured breakfast cereals and biscuits, but not in manufactured egg noodles and pasta.

### 13.5.4 Level of human exposure

Human exposure to OTA can be estimated by quantifying levels of OTA in samples of blood, urine and breast milk. OTA has been detected in a large proportion of blood samples, often at high levels, from residents of BEN-endemic areas and from sufferers of related renal diseases. In one study performed by Abid *et al.* (2003) in 1994, OTA was detected in the blood of 100 % of subjects with CIN. The mean concentration detected was 55 ng/ml compared with a mean concentration of 2 ng/ml found in the blood of 77 % of healthy controls.

Numerous studies performed worldwide have detected OTA in biological samples from healthy people living outside of BEN-endemic areas, suggesting that

**Table 13.4** Occurrence of OTA in biological samples from healthy humans.

Country	Matrix	Positive/analysed		OTA (ng/ml)		Reference*
		No.	%	Mean	Range	
Morocco	Serum	185/309	60	0.29	0.08–6.59	Filali <i>et al.</i> (2002)
Norway/ Sweden	Serum	406/406	100	0.20	–	Thuvander <i>et al.</i> (2001)
UK	Serum	50/50	100	–	0.15–2.17	Gilbert <i>et al.</i> (2001)
UK	Urine	46/50	92	–	<0.01–0.058	Gilbert <i>et al.</i> (2001)
Italy	Urine	22/38	58	–	0.012–0.046	Pascale and Visconti (2001)
Norway	Milk	17/80	21	0.03	0.01–0.182	Skaug <i>et al.</i> (2001)

\*N.B. The studies were performed in the 1990s or in 2000.

**Table 13.5** The relative contribution of different food categories to human ochratoxin A exposure (adapted from JECFA, 2001).

Food category	Contamination ( $\mu\text{g}/\text{kg}$ )	Intake		
		(g)	$\mu\text{g}/\text{person}$ per week	ng/kg bw per week
Cereals	0.94	230	1.5	25
Wine	0.32	240	0.54	8.9
Grape juice	0.39	69	0.19	3.1
Roasted coffee	0.76	24	0.13	2.1
Pork	0.17	76	0.09	1.5
Beer	0.023	260	0.04	0.69
Dried foods	2.2	2.3	0.03	0.58
Pulses	0.19	25	0.03	0.55
Cocoa	0.55	6.3	0.02	0.40
Poultry	0.041	53	0.02	0.25
Tea	0.3	2.3	0.00	0.08

the general population may be exposed to low levels of OTA. The results of several recent surveys are summarized in Table 13.4. Intake of OTA was estimated by JECFA (2001) by combining mean consumption data for various foodstuffs with mean contamination figures. The consumption data used were mainly based on intake in Europe. The results are summarized in Table 13.5. The total mean intake of OTA was estimated to be 45 ng/kg body weight per week, assuming a body weight of 60 kg. Cereals and wine contributed about 25 and 10 ng/kg body weight per week, respectively, to the mean intake. Grape juice and coffee contributed 2–3 ng/kg body weight per week. Other food products (dried fruits, beer, tea, milk, cocoa, poultry and pulses) in total contributed less than 11 ng/kg body weight per week. The contamination level for the pig and pig meat products was mainly based on samples of pig liver and kidney, whereas the figure for consumption was based on pig meats. The resulting estimate of 1.5 ng/kg body weight per week may therefore be an over-estimate of intake.

However, the level of OTA to which humans are exposed may vary considerably with season. Peraica *et al.*, (2001) showed that the mean concentration of OTA in plasma samples from inhabitants of four Croatian cities differed between March (0.36 ng/ml), June (0.39 ng/ml), September (0.25 ng/ml) and December (0.19 ng/ml). The finding that OTA contamination was highest in June and lowest in December may reflect changes in conditions favouring mould growth and toxin production. Additionally, the period of time between harvesting and consumption of cereals may contribute to seasonal variation in OTA exposure. Cereals consumed during June will probably have been stored since the previous year, during which time the growth of OTA producing mould species can occur. In contrast, cereals consumed between September and December are likely to have been harvested more recently and are therefore a lower risk for OTA contamination (Holmberg *et al.*, 1991).

### 13.5.5 Particular problems in detection

Numerous analytical methods have been developed for the detection and measurement of OTA in foodstuffs and body fluids. Representative methods of analysis in a range of matrices are summarized in Table 13.6. The range of different food matrices, both solid and liquid, in which OTA may be present makes development of a single analytical method appropriate for all foods and for biological fluids very difficult. For this reason, methods have been developed for the measurement of OTA in a single food matrix or a group of similar matrices.

In general, the methods involve extraction of OTA from the food matrix using an organic solvent, and the extract may undergo a further clean-up stage incorporating hydrophobic or immunoaffinity solid-phase chromatography. Thereafter, separation of the components of the extracts is achieved, in most cases, using high performance liquid chromatography (HPLC). Most methods utilize the fluorescent properties of OTA for subsequent detection and quantification. However, the complex nature of the matrices from which OTA is extracted gives rise to the potential for interference with the fluorescence signal and, therefore many laboratories have used a secondary technique such as liquid chromatography with mass spectrometric detection (LC-MS) to confirm the analytical results obtained from their primary method (Scott, 2002). Some methods have used mass spectrometry (MS) or immunoassay instead of fluorescence to quantify OTA. For the most part, the recoveries reported using the various methods are above 80 or 90 % and detection limits are in the low parts per billion. Thus, low levels of OTA can be detected in most matrices. Recently, certified reference materials have become available and proficiency testing schemes (e.g. UK Food Analysis Performance Assessment Scheme) have been set up to improve the accuracy and reliability of the various analytical methods in use (Scott, 2002).

**Table 13.6** Representative examples of analytical methods for the detection of ochratoxin A and ochratoxin  $\alpha$ .

Method	Matrices	Extraction/clean-up	LOD or LOQ <sup>1</sup> (ppb)	Recovery/%	Reference
HPLC <sup>2</sup> -fluorescence	Wheat, maize, red pepper, cheese, wine	Chloroform	8	80	Aboul-Enein <i>et al.</i> (2002)
HPLC-fluorescence	Barley	Immunoaffinity column	0.2	93	Entwisle <i>et al.</i> (2000)
HPLC-fluorescence	Baby food	Immunoaffinity column	0.1	108	Burdaspal <i>et al.</i> (2001)
HPLC-fluorescence	Ham	Immunoaffinity column	0.7	83	Chiavaro <i>et al.</i> (2002)
HPLC-fluorescence	Liver pâté	Acetonitrile	0.6	86	Jimenez <i>et al.</i> (2001)
ELISA <sup>3</sup>	Spices	Methanol	0.035	>90	Thirumala-Devi <i>et al.</i> (2001)
HPLC-fluorescence	Animal feed	Immunoaffinity column	10	95	Dalcero <i>et al.</i> (2002)
HPLC-fluorescence	Coffee	Immunoaffinity column	0.2	85	Entwisle <i>et al.</i> (2001)
Enzyme immunoassay	Coffee	Methanol	4	73–87	Sibanda <i>et al.</i> (2002)
Thin layer chromatography	Green coffee	Immunoaffinity column	0.5	98–104	Santos and Vargas (2002)
HPLC-fluorescence	Wine, beer, fruit juice	Toluene	0.01	80–90	Filali <i>et al.</i> (2001)
HPLC-fluorescence	Wine	Immunoaffinity column	0.01	98	Shephard <i>et al.</i> (2003)
HPLC-fluorescence	Wine	Immunoaffinity column	20–45	90	Castellari <i>et al.</i> (2000)
GC <sup>4</sup> -photodiode array	Wine, beer	Solid-phase extraction	0.1	83–94	Soleas <i>et al.</i> (2001)
HPLC-fluorescence	Serum	Dichloromethane	100	90	Curtui <i>et al.</i> (2001)
LC-MS/MS <sup>5</sup>	Plasma	Solid-phase extraction	0.5	Not given	Lau <i>et al.</i> (2000)
HPLC-fluorescence	Human milk	Ethyl acetate	100	80–87	Miraglia <i>et al.</i> (1995)

<sup>1</sup>Limit of detection (Limit of quantitation)<sup>2</sup>High-performance liquid chromatography<sup>3</sup>Enzyme-linked immunosorbent assay<sup>4</sup>Gas chromatography<sup>5</sup>Liquid chromatography with tandem mass spectrometry

### 13.6 Conclusions and future trends

OTA is produced by a number of different species of mould which are capable of growing in a range of climates. Crops that are particularly susceptible to infestation by these moulds include cereals, some types of bean and fruits. OTA can, therefore, be present to some extent in many commonly consumed plant-based foods as well as some products from animals that have consumed contaminated feed.

Experimental studies in animals show that OTA is absorbed in appreciable quantities from the GI tract following oral administration and can undergo enterohepatic circulation. Once absorbed it binds readily to serum proteins and is transported in the blood, predominantly in bound form. In the body, the highest concentrations of OTA are found in the blood and the kidney. OTA can also be transferred to milk and can cross the placenta. The metabolism of OTA is simple and it is predominately converted to ochratoxin  $\alpha$  prior to urinary excretion.

OTA is structurally similar to the amino acid, Phe. For this reason, it has an inhibitory effect on a number of enzymes that use Phe as a substrate, in particular, Phe-tRNA synthetase, which can result in inhibition of protein synthesis. OTA may also stimulate lipid peroxidation.

The kidney is the principal target organ for OTA-related toxicity in animal studies, and in long-term studies it is the predominant site of tumour formation, with the liver as the major secondary site. The mechanism of carcinogenicity is unclear. It appears that OTA may not be directly genotoxic but may act to stimulate oxidative damage to DNA, although conclusive evidence for this mechanism is lacking. OTA has also been implicated as the causative agent in human nephropathies and cancers of the urinary tract in regions of the world where elevated levels of OTA have been identified in foodstuffs.

The toxicology and human health risks of OTA have been assessed at both European and international levels by the European Commission Scientific Committee on Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) who have established tolerable intakes of OTA from food (SCF, 1998; JECFA, 2001). In 1998, the SCF noted that tolerable daily intake (TDI) estimates derived by other bodies ranged from 1.2–14 ng/kg bodyweight. However, due to concerns about the potential genotoxicity of OTA, the SCF recommended that exposures should be kept towards the lower end of the range (e.g. below 5 ng/kg bodyweight). In 2001, JECFA established a provisional tolerable weekly intake (PTWI) of 100 ng/kg bodyweight. However, the tolerable intakes will be re-evaluated by such bodies in the future when more toxicological information becomes available. Thus, these tolerable intakes may be altered, particularly if more information on the mechanism of genotoxicity influences the basis of the threshold approach taken.

One potential future source of information is an ongoing FLAIR-FLOW 4 project on OTA toxicity (see Section 13.7 for website addresses), which is supported by the European Commission within its 5<sup>th</sup> Framework Programme. The project is a collaborative effort between a number of research groups, and has focussed on OTA metabolism and the effects of the toxin on DNA. Preliminary

results have suggested that covalent binding between OTA and liver or kidney DNA does not occur, although OTA induces DNA strand breaks. OTA was also found to induce nitric oxide production in cells, and to be more cytotoxic to human cell lines than to animal cell lines. It is anticipated that the results of the study will help to improve understanding of the mechanisms by which OTA causes DNA damage and their contribution to tumour induction. The results should serve as a basis for a reassessment of the tolerable intakes set for OTA and inform the maximum permitted levels in foodstuffs set within the European Union.

### 13.7 Sources of further information

The 2001 JECFA report and 1998 SCF opinion on ochratoxin A can be accessed at the following websites respectively:

<http://www.inchem.org/documents/jecfa/jecmono/v47je04.htm>

[http://europa.eu.int/comm/food/fs/sc/scf/out14\\_en.html](http://europa.eu.int/comm/food/fs/sc/scf/out14_en.html)

Information on the FLAIR-FLOW 4 European Commission project on OTA (FFE 621/03/SME81) can be found at:

<http://www.flair-flow.com/industry-docs/ffe62103.html>

*The opinions expressed in this chapter are personal and do not represent the views or policy of the Food Standards Agency.*

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## Patulin

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### 14.1 Introduction

Patulin (4-hydroxyl-4H-furo(3,2c)-pyran-2 (6H)-on) is a metabolite produced by a large number of fungi within several genera such as *Byssochlamys*, *Eupenicillium*, *Penicillium*, *Aspergillus* and *Paecilomyces* species in a variety of food products, e.g. apricots, grapes, grape fruit, peaches, pears, apples, olives and cereals (Thurm *et al.*, 1979; Paster *et al.*, 1995; Askar and Siliha, 1999; De Sylos and Rodrigez Amaya, 1999; Arici, 2000; Gokmen and Acar, 2000, Abrunhosa *et al.*, 2001; Laidou *et al.*, 2001; Yurdun *et al.*, 2001; Kadakal and Nas, 2002; Moreau, 2002). Several studies have shown that patulin is stable in dry cereals, and in apple and grape juice, but that it is decomposed in wet cereals and during production of cider (Pohland and Allen, 1970; Speijers and Franken, 1989; Moss and Long, 2002; Armentia *et al.*, 2000). Residues of patulin can cause particular safety issues in products such as juices derived from apples and citrus fruits. Between 1975 and 1995 many studies were performed both on the occurrence and the toxicology of patulin, including safety assessments by international bodies such as the Scientific Committee of the European Commission and the Joint Expert Committee on Food Additives (JECFA) of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO). The main interest was focussed on apple juices as intake can be considerable, particularly in children (Verger *et al.*, 1999; Beretta *et al.*, 2000). An additional reason for the interest in patulin was the incorrect assumption that patulin is carcinogenic, based on an outdated and inadequate study by Dickens and Jones (1961). Recent safety assessments have concluded that patulin is not carcinogenic and a tolerable daily intake (TDI) has been established.

Since then research on patulin has declined. In this review, a summary of the most recent safety assessment of patulin (JECFA), presented by Wouters and Speijers (1996), as well as an update on the toxicity data, intake data since 1995, will be given.

## 14.2 Absorption, distribution, metabolism, excretion and biochemical processes of patulin

Patulin administered via the diet is not representative of the human intake as most of that intake comes from drinks containing patulin. Patulin also reacts with terminal sulphhydryl groups of protein and polypeptides present in food (Wouters and Speijers, 1996; Fliege and Metzler, 1999). From a pharmacokinetic study in rats it seems that patulin is absorbed at a moderate rate. Within seven days, 49 % and 36 % of the administered dose was recovered in faeces and in urine, respectively. Most of the excretion occurred within the first 24 hours. Approximately 1–2 % of the dose was recovered as  $^{14}\text{CO}_2$ . At the end of seven days, 2–3 % of the dose was recovered in soft tissues and blood (Dailey *et al.*, 1977a; Wouters and Speijers, 1996).

Patulin has an inhibiting effect on several biochemical parameters such as AT-Pase, alkaline phosphatase, aldolase and hexokinase activity. Patulin was able to activate glycogen phosphorylase in the liver, and blood glucose levels increased by 60 %.

Patulin inhibited protein synthesis. As a result, the concentration of glycogen in liver, kidney and intestinal tissues was reduced by high intakes of patulin. The decrease in hepatic glycogen indicated glucose intolerance, which may be due to insulin insufficiency. This may be reflected in decreased concentration of insulin-dependent enzymes. On the other hand gluconeogenesis was stimulated as evidenced by increased glucose-6-phosphatase and fructose 1,6-diphosphase activity (Wouters and Speijers, 1996). It is amazing how little is known yet on the pharmacokinetic behaviour and metabolism of patulin. Nevertheless no data on these aspects have been published recently.

## 14.3 Acute, short- and long-term toxicity of patulin

### 14.3.1 Acute and short-term toxicity

Acute toxicity in mice, rats and hamsters ranged from 9–55 mg/kg bw. The toxic signs consistently reported in all studies were agitation, convulsions in some cases, dyspnea, pulmonary congestion and oedema, and ulcerations, hyperaemia and dilatation of the gastrointestinal tract.

Short-term toxicity studies showed variable effects. A number of rat studies, including a few reproduction studies and a long-term toxicity/carcinogenicity study, showed high mortality rates. This mortality seen in studies with conven-

tional rats was due to severe dilatation of the gut and/or pneumonia. These changes are most probably due to the fact that patulin acts as antibiotic against Gram-positive bacteria, thereby giving a selective advantage to pathogenic Gram-negative bacteria, causing an infection in these animals. The fact that in most studies patulin was administered by gavage might have accentuated this mortality rate (Speijers and Franken, 1989; Wouters and Speijers, 1996). The results in these conventional rodents which did not die during the study were characterized by haemorrhage, ulceration of the gastric mucosa, dilatation and exudation and epithelial desquamation in the duodenum (McKinley and Carlton, 1980a,b; McKinley *et al.*, 1982; Dailey *et al.*, 1977b; Becci *et al.*, 1981).

Studies in which specified pathogen free (SPF) Wistar rats receiving patulin via drinking water buffered with 1mM citrate for 4 weeks and 13 weeks showed a different toxicological pattern, the most striking difference being that, at comparable dose levels, no mortality was seen in the SPF Wistar rats. In fact, in these studies the real systemic toxic effects were seen at a dose level that would have caused mortality in conventional rats. In the four-week toxicity study, food and liquid intake was decreased in the mid- and high-dose groups (85 and 295 mg patulin/l drinking water). Body weights were reduced in the high-dose group. Creatinin clearance was reduced in the high-dose animals, but no morphological glomerular damage in the kidneys was observed. In the high-dose group, fundic ulcers in the stomach were observed in combination with enlarged and active pancreatico-duodenal lymph nodes, while villous hyperaemia of the duodenum was observed in mid- and high-dose groups. The no observed adverse effect level (NOAEL) was 25 mg/l drinking water (Speijers and Franken, 1989). In the 13-week study with SPF Wistar rats, effects similar to but less severe than those observed in the 4-week study were seen, except that fundic ulcers in the stomach were not observed at any dose level.

At the mid and high dose, 30 and 150 mg/l drinking water, a slight impairment of the kidney function and a villous hyperaemia in the duodenum of the rats were observed. Therefore the NOAEL in this study was 6 mg patulin/l drinking water equivalent to 0.8 mg patulin/kg bw per day (Speijers *et al.*, 1986). There has been only one very limited (only one animal/group) non-rodent mammalian study carried out which found very limited toxicity in pigtail monkeys, a finding which was of no value in assessing the safety of patulin (Garza *et al.*, 1977). Since 1995 no new data from acute and short-term toxicity studies have been published.

#### **14.3.2 Long-term toxicity/carcinogenicity studies**

Dickens and Jones (1961) performed a study in rats in which subcutaneous injections of 0.2 mg of patulin in arachis oil were administered biweekly for 61–64 weeks. It is now known that such repeated injections with many chemicals which are non-carcinogenic can in fact lead to local (fibro) sarcomas at the injection site, similar to those found by Dickens and Jones with patulin. In the light of this knowledge, such studies are useless in establishing possible carcinogenicity, particularly for oral exposure.

A more recent and better carcinogenicity study with patulin was performed by Osswald *et al.* (1978). Although this study had some limitations (limited dosing) and discrepancies (number of animals, duration of the study), it revealed no significant increases in tumour incidence. The occurrence of four fore-stomach papillomas and two glandular stomach adenomas, as compared to none in the control animals, was considered noteworthy (Wouters and Speijers, 1996).

Another carcinogenicity study (Becci *et al.*, 1981) was performed with animals from F<sub>1</sub> generation from a reproduction study with patulin. Thus the rats were already prenatally exposed. Patulin was administered three times per week by gavage in dose levels of 0, 0.1, 0.5 or 1.5 mg patulin/kg bw. Although the initial number of animals was 70, due to high mortality in the high-dose groups all the male rats were lost and only 19 % of the female rats remained. This mortality reduced the validity of the study. Its causes were discussed in the previous section. The NOAEL of 0.1 mg patulin/kg bw is based on the decreased weight gain in males. However, as patulin was administered only three times per week, the NOAEL derived from this study is 0.043 mg patulin/kg bw per day. No increases in tumour incidence were seen in the patulin treatment groups (Wouters and Speijers, 1996).

Although only two reasonably performed carcinogenicity studies, both with some limitations, have been carried out since 1995, no new data from long-term toxicity and/or carcinogenicity studies have been published.

### 14.3.3 Reproduction and developmental studies

One developmental study and one reproduction study have been performed (Wouters and Speijers, 1996). Groups of Sprague-Dawley rats (30/sex/group) received doses of 0, 1.5, 7.5, or 15.0 mg patulin/kg bw/day in citrate buffer by gavage five times per week for seven weeks before mating. The pregnant dams were gavaged daily at the same levels during gestation. Half of the dams were sacrificed on day 20 of gestation and used for teratological evaluation. The remaining dams were allowed to produce the F<sub>1</sub> generation. Some of the F<sub>0</sub> and F<sub>1</sub> males were used for a dominant lethal experiment, 23 and 15 per sex, respectively, were continued to produce an F<sub>2</sub> generation. One half of the latter generation was again used for teratological evaluation. The only lesion found at necropsy of the parent animals was gaseous distension of the gastrointestinal tract. All treated males of F<sub>0</sub> generation had a dose-related reduction in weight gain. High mortality occurred at 7.5 and 15.0 mg/kg bw per day in both males and dams. Although litter size at 7.5 mg patulin/kg bw per day was comparable to controls, survival of male progeny was severely impaired. At the 1.5 mg patulin/kg bw per day level, pup growth of both sexes was reduced, and there was increased mortality among the F<sub>2</sub> females. No significant alterations were found in the haematology and blood chemistry levels in selected animals of the F<sub>1</sub> generation (Dailey *et al.*, 1977b).

Groups of Wistar rats (50/sex per group) were exposed to 0, 0.1, 0.5 or 1.5 mg patulin/kg bw per day in citrate buffer by gavage for four weeks before mating, and pregnant females were dosed through gestation and lactation. The parent genera-

tion was sacrificed after weaning. Body weight gain was comparable among the groups. Ten female rats died in the high-dose group. Reproductive parameters, such as mating success, litter size, fertility, gestation, viability, and lactation indices, and pup weight at birth, at four days and at weaning, were not statistically different among experimental groups. Histopathological evaluation of grossly abnormal tissues of the  $F_0$  generation did not show any effects of patulin treatment. The  $F_1$  generation was used for a two-year toxicity/carcinogenicity study as described previously (Becci *et al.*, 1981).

In addition to previous studies, some special studies on development and embryotoxicity have been performed. One study was carried out with pregnant Swiss mice receiving 0 or 2 mg/kg bw per day twice daily by gavage for six days starting 14 days after mating. The mean survival time was significantly reduced in the pups of the dams treated with patulin. The number of suckling mice that died in the patulin group was 19 % in males and 20 % in females. They showed hyperaemia and bleeding in the brain, lungs and skin. Excluding the early deaths, the survival time was not affected in the animals exposed in utero. When these animals were observed for a long-term period, no evidence of carcinogenicity was seen (Osswald *et al.*, 1978). In another study, mice received orally during day 12 and 13 of gestation 0 or 3.8 mg patulin/kg bw per day or intraperitoneally (ip) 0, 1.3, 2.5 or 3.8 mg/kg bw per day. Higher dose levels caused maternal toxicity. Oral administration did not induce an effect on any developmental parameters. The effects (cleft palate, enlarged kidneys) seen after ip high dose were only noticed at dose levels causing maternal toxicity (Roll *et al.*, 1990).

In a two-generation reproductive study  $F_1$  and  $F_2$  offspring of Sprague-Dawley rats were exposed to 0 or 1.5 mg patulin/kg bw per day by gavage and examined for developmental abnormalities. Except for some effect on resorption ( $F_1$ ) and foetus weight ( $F_2$ ), no increase in skeletal or soft tissue abnormalities was observed (Dailey *et al.*, 1977b). In contrast, when patulin was also ip administered (0, 1.5 or 2.0 mg patulin/kg bw/day) to groups of pregnant Charles River CDI mice a significant decrease in average foetal body weights was observed at low dose, and at high dose implanted embryos were resorbed (Reddy *et al.*, 1978). In a study with fertilized chick eggs with injection into the air cell, embryotoxicity was seen at 24–64  $\mu\text{g}/\text{egg}$  and developmental effects at 1–2  $\mu\text{g}/\text{egg}$ . *In vitro* studies in whole rat embryo culture (60  $\mu\text{M}$  patulin in serum) caused complete mortality. At lower dose levels, all kinds of developmental effects were observed (Small *et al.*, 1992; Wouters and Speijers, 1996). Since 1995 no new data on reproductive or developmental studies have been published.

#### 14.4 Genotoxicity, cytotoxicity, immunotoxicity and neurotoxicity of patulin

In contrast to the other aspects of toxicology discussed above where the amount of data is rather limited, in the area of genotoxicity ample data were available and have been summarized by Wouters and Speijers (1996) in a table. Most studies

carried out since 1995 have been in the fields of genotoxicity and cytotoxicity. Nevertheless having ample data most toxicity data being published since 1995 consist of genotoxicity and cytotoxicity studies.

Several studies revealed that patulin was cytotoxic in different types of cells, such as protozoa (*Tetrahymena pyriformis*), rat granulosa cell line and renal cultured cells (LLC-PK<sub>1</sub> cells). The exposure ranged from 0.1 to 50  $\mu\text{M}$  patulin. The most commonly observed effects are on the ion transport over the biomembranes, influencing both Ca<sup>2+</sup> balance and intracellular Na<sup>+</sup> and K<sup>+</sup> ion concentration. In addition, intracellular electronegativity followed by a sustained depolarization is observed. With high concentrations (50  $\mu\text{M}$  patulin) lipid peroxidation, abrupt calcium influx, extensive blebbing and total lactate dehydrogenase (LDH) release occur and these appear to be implicated in the loss of structural integrity of the plasma membrane (Riley and Showker, 1991; Wouters and Speijers, 1996).

Many genotoxicity studies have been performed, both *in vitro* and *in vivo*. The *in vitro* tests were carried out with bacteria, fungi and mammalian cells. The endpoints studied were reverse mutation, chromosome aberrations, cell cycle retardation, sister chromatid exchange, DNA-breakage, DNA synthesis, unscheduled DNA synthesis (UDS), DNA-repair, host mediated assay and dominant lethal test. These studies were evaluated by JECFA (Wouters and Speijers, 1996). Although the results from the genotoxicity tests were variable, most assays carried out with mammalian cells were positive, while quite a number of assays with bacteria were negative. In addition, some studies indicated that patulin impaired DNA synthesis. These genotoxic effects might be related to its ability to react with sulfhydryl groups and thereby inhibit enzymes involved in the replication of genetic material. Nevertheless, it was concluded from available data that patulin is genotoxic. Since 1995, several studies have been published in this area.

Pfeiffer *et al.* (1998) studied the aneuploidogenic and clastogenic potential of patulin by determining inhibition of microtubule assembly under cell-free conditions and by measuring induction of mitotic arrest and micronuclei in cultured Chinese hamster V79 cells. Patulin did indeed inhibit cell-free microtubule polymerization in a concentration-dependent manner. It bound covalently to reactive thiol groups of microtubule proteins. At concentrations without gross cytotoxicity, mitotic arrest and CREST (staining)-positive micronuclei, i.e. micronuclei containing whole chromosomes/chromatids, were induced by patulin in V79 cells. The time course of micronucleus induction and positive CREST-staining indicates the aneuploidogenic potential of patulin. CREST-negative micronuclei, i.e. micronuclei containing acentric chromosomal fragments, were induced by patulin, implying a clastogenic potential. The authors claim that the aneuploidogenic and clastogenic potential may contribute to the putative carcinogenicity of patulin in long-term animal studies.

Dirheimer (1998) evaluated the variable results of genotoxicity tests and concluded that single- and double-strand breaks of DNA caused by patulin have also been reported in eucariotic cell cultures: in FM3MA cells and Hela cells.

Clastogenic activity of patulin in Chinese hamster V79-E cells was also detected. Sister chromatid exchange (SCE) and UDS tests were negative. He suggested that the clastogenicity is caused by interaction of patulin with chromosomal protein and not with DNA.

Alves *et al.* (2000) also evaluated the genotoxicity of patulin in a chromosomal aberration assay in V79 Chinese hamster cells, with a view to clarifying whether concomitant exposure to ascorbic acid with patulin modulates its clastogenicity. The results – abolition of patulin clastogenicity at 30  $\mu\text{M}$  ascorbic acid – unequivocally showed induction of DNA damage in the cell by patulin. This was supported by the effects seen in induction of micronuclei in cytokinesis-blocked human lymphocytes.

In a recent study Liu *et al.* (2003) evaluated the genotoxic risk and oxidative DNA-damage in mammalian cells exposed to patulin. This study included Chinese hamster ovary cells (CHO-K1), human peripheral blood lymphocytes, and human embryonic kidney cells (HEK 293). Patulin caused a significant dose-dependent increase in SCE frequency in both CHO-K1 cells and human lymphocytes. Patulin also elevated the levels of DNA gaps and breaks in treated CHO-K1 cells. In a single cell gel electrophoresis (SCGE)-assay, exposure of HEK 293 to concentrations above 15  $\mu\text{M}$  patulin induced DNA-strand breaks, and the tail moment values also greatly increased after post-treatment with formido-pyrimidine-DNA glycosylase. These results suggest that in human cells patulin is a potent clastogen with ability to cause oxidative damage. Fliege and Mezler (1999) demonstrated that patulin induces intermolecular protein–protein cross-links.

Schumacher *et al.* (2003) showed results which were consistent with earlier studies demonstrating DNA interstrand cross-links in cell-free DNA treated with patulin. However, concentrations of patulin leading to DNA cross-links in cultured cells were three orders of magnitude lower than those with cell-free DNA. Results from studies on the potential of patulin in cultured mammalian fibroblasts suggest that genotoxic effects of patulin occur at similar concentrations, but they can differ qualitatively and quantitatively in different mammalian cells (Lehman *et al.*, 2003).

#### 14.4.1 Special studies on immunotoxicity

##### *In vitro*

Studies in peritoneal exudate cells of mice, preincubated for two hours with 0.01–2  $\mu\text{g}$  patulin/ml, revealed a diminished phagocytosis and phagosome–lysosome fusion above 0.1  $\mu\text{g}$  patulin/ml. Lysosomal enzymes and microbiological activity were decreased above 0.5  $\mu\text{g}$  patulin/ml, whereas  $\text{O}_2$ -production was inhibited only above 2  $\mu\text{g}$  patulin/ml (Bourdiol *et al.*, 1990).

Patulin had a stimulatory effect on mouse splenocytes at lower concentration (1–10 nM) and a strong inhibitory effect on lymphocyte proliferation at higher concentration ( $\text{ID}_{50}$  ranging from 0.02–0.24  $\mu\text{M}$ , depending on the type of mitogen

used) (Paucod *et al.*, 1990). Patulin at concentrations from 0.25–1 µg/ml decreased the chemotactic index of dog neutrophilic granulocytes and stimulated opsonized zymosan. At the same concentrations, patulin favoured the migration of the cells. At the high concentration patulin inhibited the liberation of superoxide ions by neutrophils, but did not modify their ability to phagocyte *Saccharomyces cerevisiae*. The immuno-suppressive actions may be explained by binding of patulin to the sulfhydryl group present on the neutrophil membrane (Dubech *et al.*, 1993). Patulin also caused a significant increase in the mean volume of macrophages. Patulin-induced chromium release from these macrophages of Long Evans Farnhooded rats was both time- and concentration-dependent. ATP concentrations in the macrophages were inhibited. Also tritium incorporation in protein and RNA was strongly inhibited. Patulin strongly inhibited phagocytosis of sheep erythrocytes ( $> 5 \times 10^{-7}$  M) (Sorenson *et al.*, 1986).

### *In vivo*

In Balb/C mice patulin (2 and 4 mg/kg bw) reduced delayed type hypersensitivity to *Bordetella pertussis* antigen but did not reduce anti-KLM antibody product (Paucod *et al.*, 1990). In another study, mice receiving 10 mg patulin/kg bw showed enhanced resistance to ip challenge with viable *Candida albicans*. Immunoglobulin levels (IgA, IgM, and IgG) were markedly depressed (Escoula *et al.*, 1988a). In another study, using mice and rabbits, with dose levels of 0, 2.5 and 10 mg patulin/kg bw, there was no effect on neutrophil count. A significant suppression of the chemiluminescence response of peritoneal leukocytes was observed in both species. Mitogenic responses of mice splenic lymphocytes and rabbit peripheral cells were slightly suppressed by treatment on B-cell mitogen compared with T-cell mitogen. In mice and rabbits IgG, IgA and IgM levels on day five were lower with patulin (Escoula *et al.*, 1988b). Patulin inhibited DNA synthesis in peripheral lymphocytes. These effects were mitigated by cysteine, which suggested that sulfhydryl binding could be involved in patulin toxicity. In mice, an increased resistance to *Candida albicans* was observed with patulin. Concentrations of circulating immunoglobulin were also decreased. In rabbits, decreased serum immunoglobulins, reduced blastogenesis of lymphocytes and reduced chemiluminescence of peritoneal leukocytes were also observed (Sharma, 1993).

Since 1995 only a few studies on the influence of patulin on the immune system have become available. Exposure to moulds diminishes the number of T-helper type 1 cells (Th1) in the peripheral blood of children and is a risk factor for the development of allergic disease. Therefore, the influence of patulin on human peripheral blood mononuclear cells (PBMC) was investigated. CD3/CD28-stimulated PBMCs of healthy donors were incubated for 24 hours with patulin. Viability and proliferation were tested using MTT assay and T-cell function test by the expression of cytokines. Patulin (64.8 ng/ml) caused 50 % inhibition of interferon- $\gamma$ . The inhibition 50 % (ID50) value for interleukin-4 was 243.2 ng/ml patulin (Wichmann *et al.*, 2002). In the presence of patulin, anti-CD3/CD28/CD40-

stimulated PBMC showed a suppressed interferon- $\gamma$  secretion, whereas interleukin-4 was not affected or even stimulated (1–10 ng patulin/ml). The observed T-cell polarization toward the T-helper cell 2 phenotype was accompanied by upregulated IgE synthesis. In addition, patulin reduced the allergen-specific (birch pollen) expression of interferon- $\gamma$ -m RNA, while interleukin-4 mRNA expression was unaltered or enhanced. These data suggest that patulin may enhance allergic responsiveness (Wichman *et al.*, 2003).

#### 14.4.2 Special studies on neurotoxicity

There has been only one limited study in rats testing one dose-level of patulin (1.6 mg/kg bw) administered in comparison with a control group. The patulin-treated animals showed convulsions, tremors, impaired locomotion, stiffness of hindlimbs, and wagging of the head. Patulin inhibited acetylcholinesterase and NaK-ATPase in the cerebral hemisphere, cerebellum and medulla oblongata. Concomitantly, acetylcholine levels were raised in these brain segments (Devaraj *et al.*, 1982). As this is the only study claiming neurotoxic effects, further confirmation will be needed before any definitive conclusion on possible neurotoxic properties can be drawn.

#### *Intake assessment*

At present in many countries the maximum level of patulin in apple juice is regulated within the range 25–50  $\mu\text{g}$  patulin/kg juice (Rosner, 1998; Chapter 3 of this book). Fortunately, the off-flavour level corresponds with a level of patulin near 50  $\mu\text{g}$  patulin/kg juice (EC regulation No 1425/2003, 11-08-2003).

Data based on a United States Department of Agriculture (USDA) survey (Plunkett *et al.*, 1992) show a very high consumption of apples and their derivatives in the first year of life (6.4 g/kg bw per day), with intakes declining with age (children 1–6 years: 2.3 g/kg bw per day, children 7–12 years: 1.0 g/kg bw per day down to 0.4 g/kg bw per day for adults). Combining the occurrence of patulin in apple-based juices and baby foods with intake of apples/apple products leads to an extrapolation of an approximate intake of patulin by children and adults. The highest intake for babies was 6.39  $\mu\text{g}$  patulin/kg juice, which corresponds to an intake of 40.9 ng/kg bw per day. For adults the approximate intake is 0.24  $\mu\text{g}$  patulin/kg bw per day provided that the producers respect established limits (Beretta *et al.*, 2000). The SCOOP Committee of the European Commission also monitored patulin in a number of food items in different EU countries (SCOOP, 2002) and calculated an intake for high consumers 93 ng/kg bw for children and 22 ng patulin/kg bw per day for the total population. When the high consumption figures were calculated for only those children (consumers only) who actually consumed food products containing patulin the high intake levels were 199 ng/kg bw and 57 ng patulin/kg bw per day, respectively. In Sweden intake calculation revealed an intake for high consumers of 24 and 11 ng patulin/kg bw for children and adults, respectively (Thuvander *et al.*, 2001).

## 14.5 Regulation, detection and control of patulin in food

### 14.5.1 Regulation

Both within Europe and worldwide, there is an urgent need for harmonization of authorized mycotoxin maxima, including patulin levels in food, to give effective protection to consumers while avoiding unjustified penalization of producers (Rosner, 1998). The Commission Recommendation of 11 August, 2003 on the prevention and reduction of patulin contamination in apple juice and apple juice ingredients in other beverages recommended that member states:

- (1) Take the necessary measures so that the Code of Practice for the prevention and reduction of patulin contamination in apple juice and apple juice ingredients in other beverages as described in the Annex to this Recommendation, is implemented by all operators in the apple processing industry and;
- (2) Ensure that all the appropriate measures, including – if necessary – corrective actions, are taken by the operators in the apple processing industry to achieve lower levels than the maximum level of 50 µg/kg for apple juices with the aim of achieving the level of 25 µg/kg patulin as target.

In the code of practice several important issues are discussed.

- (1) The major sources of contamination are apples and apple products.
- (2) Alcohol fermentation of fruit juices destroys patulin and, therefore, fermented products, such as cider and perry will not contain patulin. However, patulin has been found in fermented products to which apple juice has been added after fermentation. Ascorbic acid has been reported to cause the disappearance of patulin from apple juice, although the optimal conditions for inactivation have not been fully established. Patulin is relatively temperature stable, particularly at acid Ph. High-temperature (150 °C) short-term treatments have been reported to result in approximately 20 % reduction in patulin concentrations. However, processing alone is not sufficient to ensure a product free of patulin;
- (3) Washing of fruit, or removal of mouldy tissue, immediately prior to pressing will not necessarily remove all the patulin present in the fruit since some may have diffused into apparently healthy tissue;
- (4) The condition of the fruit at harvest, the way in which it is handled subsequently and the extent to which storage conditions are inhibitory to the growth of moulds, will all affect the likelihood of patulin contamination of juice and other products prepared from fresh and stored fruit. The recommendations for reducing patulin contamination in apple juice in this code are based partly on Good Agricultural Practice (GAP) and partly on Good Manufacturing Practice (GMP) (EU, 2003a).

### 14.5.2 Detection and control

A collaborative trial has been conducted to validate the effectiveness of a liquid chromatographic (LC) procedure for the determination of patulin in both clear and cloudy apple juices and apple puree. From this study it can be concluded that the

detection method for levels as low as 25–50 µg patulin/kg is acceptable with regard to relative standard deviation for repeatability and reproducibility, respectively (MacDonald *et al.*, 2000). The Joint Research Centre (JRC/IRMMT) of the EU in Geel, Belgium has now included this method in the methods of sampling for official control of the levels of patulin in certain foodstuffs and in the criteria for sample preparation and for methods of analysis used in official checking of the levels of patulin in certain foodstuffs. This is presented in Annex 1 and Annex 2 of Commission Directive 2003/78/EC, laying down the sampling methods and the methods of analysis for official control of the levels in foodstuffs (EU, 2003b,c).

In EU countries the patulin concentrations in apple juice and other apple containing products are often controlled in monitoring programs. All these data are regularly collected by the SCOOP Committee of the European Commission (SCOOP, 2002), reported, and used to calculate the intake of patulin with a view to a risk assessment.

## 14.6 Conclusions

Since the safety evaluation of patulin by JECFA in 1995 (Wouters and Speijers, 1996), with the exception of a quantity of additional data on the occurrence of patulin and some papers on intake assessment, the few recent data on the toxicological properties of patulin consist almost entirely of publications in the genotoxicity area.

Although no specific studies on the mode of action of patulin have been performed, it is becoming clear that the ability of patulin to react with the sulfhydryl group under binding would explain the cytotoxicity, some of the genotoxic effects and some of the immune toxic effects. It is also the reason why patulin is less toxic when administered via the feed than when taken in a buffered drinking water. This is due to the reaction with the sulfhydryl group in dietary protein, which makes it far less bioavailable than via drinking water. Since humans are also exposed to patulin mainly by drinks, studies performed with patulin in the drinking water are the most representative. No new toxicity data on patulin have become available which would make a new safety assessment necessary. Therefore, the provisional maximum tolerable daily intake (PMTDI) set by JECFA (Wouters and Speijers, 1996) and SCF (2000) of 0.4 µg/kg bw remains valid. The PMTDI was based on a dose level of 0.043 mg/kg bw per day of patulin producing no effect in terms of decreased weight gain in males as was seen in the next higher dose group of a combined reproductive toxicity/long-term toxicity/carcinogenicity study and the application of a 100-fold safety (uncertainty) factor. Most intake situations were below the PMTDI. However, according to Verger *et al.* (1999), who considered contamination at a level of 50 µg/l apple juice, this could lead to ingestion in excess of the PMTDI of 80 ml by a 10 kg child and of 160 ml by a 20 kg child. Of course this is a worst case scenario. In general, if the producers adhere to the tolerance limits set and maintain good manufacture practice (GMP) there is no appreciable health risk from patulin.

## 14.7 References

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# 15

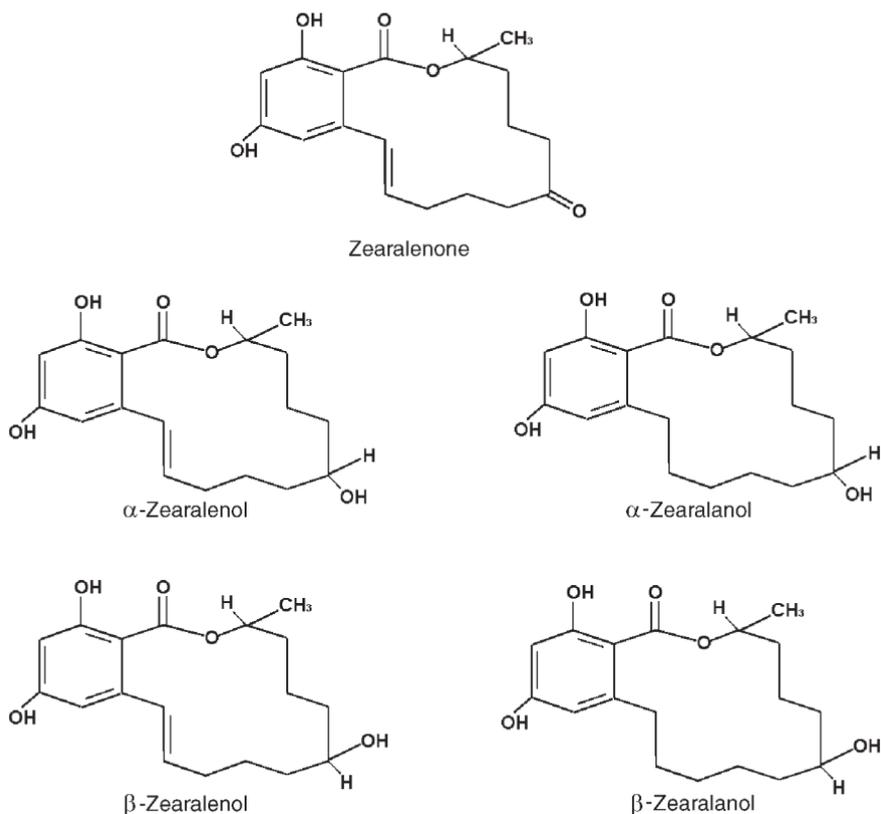
## Zearalenone

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### 15.1 Introduction: chemical composition

Zearalenone is a mycotoxin by virtue of its estrogenic effects (discussed in Section 15.3.1). The first description of the clinical symptoms associated with zearalenone was in 1927 by Buxton.<sup>1</sup> Working with pigs, he observed gilts with swelling and eversion of the vagina. Subsequently McNutt *et al.*<sup>2</sup> described this condition as vulvovaginitis and established that it was caused by mouldy maize. Further studies led to the identification of *Fusarium graminearum* as a causative organism.<sup>3</sup> Later work demonstrated that extracts of certain strains of *F. graminearum* elicited the toxic effect. The principal toxic component was named zearalenone by Urry *et al.*<sup>4</sup> who also determined the mycotoxin's chemical structure.

Zearalenone (3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-1*H*-2-benzoxycyclotetradecin-1,7(8*H*)-dione; C<sub>18</sub>H<sub>22</sub>O<sub>5</sub>; molecular weight 318.36; CAS Registry No. 17924-92-4), is a non-steroidal mycotoxin produced by certain strains of various species of the genus *Fusarium*, including: *F. culmorum*, *F. equiseti*, *F. graminearum* and *F. moniliforme* (reviewed in<sup>5</sup>). As with other mycotoxins, only certain strains of any one particular species can produce zearalenone.<sup>6</sup> The chemical structure (Fig. 15.1) of zearalenone was determined by Urry *et al.*,<sup>4</sup> while the absolute conformation of the asymmetric carbon atom C10' was elucidated by Kuo *et al.*<sup>7</sup> Initial studies using radio-labelled acetate<sup>8,9</sup> suggested that zearalenone is synthesized by the acetate–polymalonate pathway. This leads to the formation of a polyketide, which can be converted, with little



**Fig. 15.1** Chemical structures of zearalenone and some of its metabolites.

modification, to the mycotoxin.<sup>10,11</sup> Although zearalenone is the most studied estrogenic mycotoxin, it is a member of a group of compounds (at least 7<sup>12</sup>) of similar structure and pharmacological properties.

In common with other toxins produced by *Fusarium* spp., zearalenone is associated mainly with cereal crops, in particular: maize; barley; oats; wheat; rice; and sorghum, together with their related products.<sup>13</sup> Although the mycotoxin is probably most common in maize, very high levels (11–15 mg/kg) can be found in other cereals, for example barley.<sup>14</sup> In addition, it has been reported that *Fusarium* spp. isolated from bananas can be capable of producing the mycotoxin.<sup>15</sup> Zearalenone has also been shown to be a potential contaminant of both amaranth<sup>16</sup> and black pepper.<sup>17</sup> Different members of the *Fusarium* genus predominate in different climatic zones. Those capable of producing zearalenone may survive in the temperate and/or tropical zones.<sup>5</sup> Consequently, the question of zearalenone contamination is one which has to be addressed worldwide.

## 15.2 Production of zearalenone in crops

As discussed by Alldrick,<sup>18</sup> mycotoxin contamination of any food commodity is the consequence of a three-stage set of events: infection of the plant with a fungal inoculum; growth of that fungi; and eventual production of the mycotoxin. The infective cycle of *Fusarium* spp. in cereal crops has been described by others.<sup>19</sup> The principal mechanisms of infection include splash dispersal of infective material from the soil by rain during anthesis (wheat and barley) or silking (maize), together with transfer of infective material by wind. Physical damage to the developing maize crop has also been shown to promote fungal infection. Canadian studies<sup>20</sup> have shown that transmission of *F. graminearum* in maize can be increased as a consequence of physical damage, mediated by either birds or insects. They further demonstrated a positive correlation between the amount of bird damage and the level of zearalenone contamination. Others<sup>21</sup> have observed higher levels of fungal infection in crops infested with the western corn rootworm beetle (*Diabrotica virgifera*) than in those which were not. They also demonstrated that the beetle was a vector for *F. moniliforme* and *F. subglutinans* infection.

In the laboratory, *Fusarium* spp. generally grow and produce mycotoxins at an optimum water activity in the order of 0.98.<sup>22-25</sup> This is equivalent to a grain (wheat) moisture content in excess of 25 % at 25 °C.<sup>26</sup> Given this requirement for high water activities/grain moisture contents, most *Fusarium* spp. activity takes place only at certain times during kernel development. As a consequence *Fusarium* spp. mycotoxins have been generally accepted to be 'field' mycotoxins.<sup>27</sup> Early studies demonstrated that deoxynivalenol contamination occurs relatively early in kernel development in both wheat<sup>28</sup> and maize.<sup>29</sup> However, production of zearalenone in maize appears to occur later during kernel development.<sup>29,30</sup> In Canada, elevated zearalenone contamination of maize was associated with heavy rainfall during August.<sup>20</sup>

Agricultural practices are a key determinant in the occurrence and management of fusarial diseases and consequent mycotoxin contamination.<sup>31</sup> Introduction of no-tillage systems to combat soil erosion appears to exacerbate the situation with regard to the amount of plant residue remaining on the surface.<sup>32</sup> Mouldboard ploughing has been shown to lower incidences of *Fusarium* ear blight and deoxynivalenol contamination.<sup>33</sup> Stubble management is therefore important and extends beyond tillage practices. For example, studies in Austria<sup>34</sup> have shown that the practice of stubble burning may actually favour zearalenone formation in the subsequent year's crop. The same study found a correlation between high nitrogen application rates and levels of zearalenone contamination. Subsequent studies by others<sup>35</sup> have shown that stubble burning can promote *Fusarium* ear blight.

Although zearalenone is considered to be a 'field' mycotoxin, there is evidence that it can also be produced in harvested grain. This is particularly the case in countries where grain is harvested wet and drying has been delayed.<sup>36-40</sup> Orsi *et al.*<sup>41</sup> have reported high numbers of *Fusarium* spp. surviving in maize after five months in storage. This is further compounded by the fact that grain moisture distribution within a bulk is heterogeneous.<sup>42</sup> Storage at high moisture levels is

considered to be a significant problem for maize. In areas where maize is stored on the cob for long periods (e.g. when used for animal feed) either in cribs or on the plant, it has been suggested that incidences of disease related to zearalenone intoxication (discussed in Section 15.4) are more likely to be due to improper storage than pre-harvest development.<sup>43</sup>

Where it is necessary to store grain with relatively high moisture contents for prolonged periods (this applies mainly to cereals intended for animal feed), other strategies need to be applied to maintain quality. This usually involves modifying the environment to suppress fungal activity. In terms of environment modification, the most commonly used approach is to ensile the crop. Use of silage techniques makes the environment anaerobic and prevents the aerobic *Fusarium* spp. from producing mycotoxins such as zearalenone (it does not, however, lead to the degradation of the mycotoxin). However, where anaerobic conditions are compromised, e.g. due to air leaks, fungal activity can resume<sup>44</sup> and mycotoxin production can occur, as in the case of zearalenone production within ensiled maize.<sup>37</sup> Enriching the atmosphere under which the silage is stored with carbon dioxide has been shown to inhibit zearalenone production. The amount of carbon dioxide required was found to be inversely proportional to the amount of oxygen present.<sup>45</sup>

## 15.3 Metabolism and experimental toxicology of zearalenone

### 15.3.1 Biochemistry

Following oral administration, zearalenone is rapidly absorbed. The principal site of zearalenone metabolism appears to be the liver.<sup>46</sup> Here, the mycotoxin is converted to the two different isomers of zearalenol ( $\alpha$  and  $\beta$ ); as well as zearalanol (see Fig. 15.1). *In vitro* studies have also shown that biotransformation can occur in both rat erythrocytes<sup>47</sup> and the small intestinal mucosa of pigs.<sup>48</sup> Zearalenone and its metabolites can undergo further conjugation reactions to produce glucuronide conjugates.<sup>46</sup> Species-dependent differences in metabolism have been noted. In pigs and (probably) humans zearalenone is primarily found as glucuronide conjugates of either  $\alpha$ -zearalenol or the parent compound. In contrast, most of the zearalenone found in the rat is the free compound or its glucuronide conjugate.<sup>13</sup>

Similar species-dependent differences in the major excretory pathways have also been observed. Biliary excretion tends to predominate in rats and mice, while urinary excretion is the principal means in rabbits, pigs and humans.<sup>49</sup> Consequently, there are also differences in the half-lives of zearalenone and its metabolites. Their disappearance is much slower in animals (including man, e.g. 22 hours) where excretion is primarily through the urine rather than the bile.<sup>50</sup>

The ability of zearalenone and its structurally related metabolites to exert a toxic effect lies in their ability to assume a molecular conformation similar to that of 17 $\beta$ -estradiol.<sup>11</sup> They have been shown to be able to bind estrogen receptors in a number of *in vitro* tissue systems, including uterus, mammary gland and liver, from a number of different species. The mycotoxin and its metabolites exert their

pharmacologic effects by behaving like artificial estrogen agonists competitively binding for the receptor.<sup>51</sup> Zearalenone and its metabolites bind to these receptors less efficiently than estradiol (relative efficiencies of between < 0.01–0.1). Differences in binding efficiencies between zearalenone and its metabolites have also been noted. Their general order of binding to the rat uterine cytoplasmic receptor was  $\alpha$ -zearalanol >  $\alpha$ -zearalenol >  $\beta$ -zearalanol > zearalenone >  $\beta$ -zearalenol.<sup>13,50</sup>

### 15.3.2 Experimental toxicology

Extensive reviews of the toxicology of zearalenone have recently been produced by a Nordic Council of Ministers working group,<sup>50</sup> the Scientific Committee on Food of the European Commission<sup>49</sup> and the Joint FAO/WHO Expert Committee on Food Additives (JECFA).<sup>52</sup> Zearalenone has a low acute oral toxicity (LD<sub>50</sub> values of 4000 up to 20 000 mg/kg body weight).<sup>13</sup> In terms of subacute or subchronic toxicity, the effects of the mycotoxin appear to reflect its ability to bind to estrogen receptors, leading to alterations in the reproductive tract and a variety of symptoms including: decreased fertility; increased embryo/lethal resorptions; and reduced litter size.<sup>53,54</sup> Pigs appear to be more sensitive than rodents. Studies by Edwards *et al.*<sup>55</sup> in pigs and by Becci *et al.* in rats,<sup>56</sup> suggest No Observed Effect Levels (NOEL) of 40  $\mu$ g/kg body weight per day for pigs as opposed to 100  $\mu$ g/kg body weight per day for rats.

The genotoxicity of zearalenone has been assessed in a number of experimental systems and has been reviewed by others.<sup>13,46</sup> In microbial-based systems, the mycotoxin did not induce: SOS error-prone DNA repair in *Escherichia coli*; mutations in *Salmonella typhimurium* ('Ames Test'); nor mitotic cross-overs in *Saccharomyces cerevisiae*. However, it did induce differential toxicity in *Bacillus subtilis* repair-proficient and -deficient strains (*rec* assay). When assayed, using *in vitro* mammalian cell assay systems, zearalenone has been found to induce chromosomal aberrations, sister chromatid exchange (SCE) and polyploidy in Chinese Hamster Ovary (CHO) cells as well as weakly inducing SCE in cultured human lymphocytes. In terms of DNA-binding, <sup>32</sup>P-labelling experiments have shown species-related differences with adducts being found in the liver and kidneys of mice treated with zearalenone,<sup>57</sup> but not in the livers or kidneys of rats treated with the mycotoxin.<sup>58</sup>

Long-term (two-year) chronic toxicity studies have demonstrated that consumption of zearalenone (0, 50 or 100 mg/kg diet) for 103 weeks was significantly correlated with an increased incidence of hepatocellular adenomas and pituitary adenomas in mice.<sup>59</sup> The same report described a study in Fischer 334 rats (0, 25 or 50 mg/kg diet) where, although a significant increase in the incidence of pituitary tumours was seen in male rats fed the lowest dose, no significant dose-related trend was seen. A multi-generation study in rats<sup>56</sup> reported that long-term consumption of zearalenone was not associated with any increase in tumour incidence. These data were reviewed by an IARC (International Agency for Research on Cancer) working party in 1993<sup>46</sup> who concluded that there was limited evidence for zearalenone being carcinogenic in experimental animals. A subsequent

assessment, performed by the EU Scientific Committee on Food,<sup>49</sup> noted that the tumourigenic effect was observed at doses far in excess of those required for hormonal effects. It also concluded that the occurrence of the tumours was due to estrogenic effects of the mycotoxin. Based on these data the Committee proposed a provisional tolerable maximum daily intake of 0.2 µg/kg body weight per day; this contrasts with a value of 0.5 µg/kg body weight per day proposed by JECFA.<sup>52</sup>

## 15.4 Health impacts of zearalenone

### 15.4.1 Incidence and levels of contamination

An analysis of the incidence of zearalenone in human foods and the raw materials to produce them has been performed by JECFA.<sup>52</sup> It was found that the incidence and levels of contamination varied in relation to the type of raw material together with climatic, harvest and storage conditions. The raw materials of greatest concern appeared to be wheat and maize. In general, values for zearalenone contamination of barley tended to be low, those for wheat reasonably low and for maize highly variable. In terms of foods actually consumed by the general population, those made from cereals, including breads, breakfast cereals, biscuits and noodles, had levels of contamination ranging from below the level of detection up to 50 µg per kg. In contrast, levels of contamination in some beers manufactured in different parts of Africa (Lesotho, Nigeria, and Zambia) have been reported in an overall range of 90–4600 µg per litre.<sup>60–62</sup> This contrasts with beers sold in Korea and Canada where generally no zearalenone was found.<sup>63,64</sup>

In absolute terms, more cereals are eaten by livestock than by humans.<sup>65</sup> The potential for transmission of the mycotoxin in meat and other animal products has been evaluated. With regard to meat, the amount of zearalenone present appears to be a reflection on the degree of contamination of feed, the duration of exposure, persistence in the animal and species differences in metabolism. The rapid metabolism and excretion of zearalenone has formed the basis for the assumption that the mycotoxin does not usually accumulate in muscle tissue (meat).<sup>66</sup> Feeding trials (90 days) involving swine consuming grain contaminated with 250 µg zearalenone per kg feed<sup>67</sup> have substantiated this assumption. Studies of swine serum in Romania<sup>68</sup> have shown that less than 20 % of the pigs studied had circulating levels of zearalenone. Even in the cases where the mycotoxin was found, the circulating levels were low (maximum 0.96 ng/ml). Similarly, only low levels of the zearalenone are transmitted through the milk of cattle fed realistic doses of the mycotoxin.<sup>69</sup>

Nevertheless, some studies have shown the potential for the accumulation of zearalenone in both animal tissue and other products. The mycotoxin was found in the livers of pigs fed zearalenone under experimental conditions<sup>70</sup> and also in the livers and milk of pigs exhibiting mycotoxicoses following consumption of mouldy grain.<sup>71</sup> Studies with chickens fed high doses (100 mg per kg diet) of zearalenone<sup>72</sup> found that the mycotoxin was accumulated primarily in the liver,

although some accumulation in skeletal muscle was also observed. Other radio-chemical studies<sup>73</sup> demonstrated that accumulation was highest in the liver and lowest in skeletal muscle. These studies also demonstrated that, even though most (> 90 %) of the mycotoxin was excreted, there was a significant accumulation of zearalenone metabolites in the yolks of eggs laid.

## 15.4.2 Clinical significance

### *Livestock*

Reference has already been made to the initial discovery of the toxic effects of zearalenone in pigs.<sup>1-3</sup> Pigs are considered to be the most sensitive of livestock with poultry the most resistant.<sup>74</sup> However, zearalenone-mediated toxicoses have also been reported in horses,<sup>75,76</sup> poultry<sup>77</sup> and cattle.<sup>78</sup> Other outbreaks of vulvovaginitis in pigs have been reported, some associated with zearalenone production while the feed was kept in store.<sup>79</sup> While not being associated with many toxicoses in ruminants, zearalenone can have other commercial implications since it can be converted to zeranol (a growth enhancer prohibited by the EU) in the rumen.<sup>80</sup>

A key element in reducing the risk of zearalenone intoxication is ensuring that any levels of contamination are not excessive. It has been recommended that feed for pigs should contain no more than 200 µg/kg.<sup>74</sup> The animal-feed industry often incorporates by-products from food processing industries in its products. These include such materials as wheat-offal, maize gluten and brewers spent grains. In terms of potential zearalenone contamination, feed-compounders have to take care in their use. As discussed in Section 15.5.1, production of these materials leads to a concentration of mycotoxin within them. This makes them potentially more hazardous in terms of mycotoxin contamination than the original raw material.

### *Humans*

Some estimates have been made regarding exposure to zearalenone by the general public. Canadian studies<sup>13</sup> estimated that average levels of consumption were 0.19 µg per day, equivalent to an intake of 0.003 µg/kg body weight per day (assumes 60 kg body weight). Data from the Nordic Countries<sup>50</sup> indicated that average levels of consumption ranged from 0.48 µg per day in Denmark to 1.46 µg per day in Norway. JECFA,<sup>52</sup> using the five WHO GEMS/Food regional diets, estimated average consumption values of zearalenone as being: < 3.5 µg in the Middle East; < 3.3 µg in the Far East; < 2.5 µg in Africa; < 2.2 µg in Latin America; and < 1.5 µg for those eating a 'European' diet (Europe, North America and Australia).

For humans there are less data regarding the toxic effects of zearalenone. Polish studies<sup>81</sup> have reported that the mycotoxin was often detected in the endometrial tissue of women suffering from endometrial adenocarcinoma and in some cases of endometrial hyperplasia. Zearalenone was not detected in biopsies taken from healthy women.

An epidemic of precocious puberty in Puerto Rico between 1978 and 1981 was suspected to be attributable to zearalenone or zearalenol.<sup>82,83</sup> The mycotoxin or its

metabolites were detected in blood plasma. The authors also reported that homogenates of locally produced meat gave positive results in an estrogen binding assay. This observation was not supported by subsequent analyses performed by the United States Food and Drug Administration, who failed to detect any estrogen-like compounds in food.<sup>84</sup> Nevertheless, a statistically significant correlation was found between consumption of meat products and soya-based formula in about 50 % of the investigated cases.<sup>85</sup> More recently, Hungarian scientists<sup>86</sup> have reported an increased incidence of early telearche in the south-east region of their country. Zearalenone was found both in the serum of patients and in food collected from them.

## 15.5 The impact of food processing on zearalenone

Most cereals undergo further processing prior to human consumption. Generally speaking, this is a two-step process, involving cleaning, physical reduction and fractionation of the grain (milling), followed by thermal processing (cooking).

### 15.5.1 Milling

In countries, where *Fusarium* Head Blight is a perennial problem, flour mills often include gravity tables within the screen room.<sup>87,88</sup> These not only remove the lighter *Fusarium* spp. damaged grains, but also apparently reduce the mycotoxin (including zearalenone) load. Other methods, which may reduce the zearalenone-loading of small grain cereals such as wheat, barley and rye, include kibbling or the removal of the outer layers of the kernel by abrasion.<sup>89</sup>

The same may not apply to contamination of other grains. Although maize dust from elevators has been shown to be a potential source of zearalenone,<sup>90</sup> survey data for maize in the UK<sup>91</sup> revealed that, while removal of fines could contribute to a reduction in fumonisin contamination, the same was not true for zearalenone. Milling itself is essentially a reductive process, which in the production of white flour also involves the separation of the endosperm from the bran layers.<sup>92</sup> Korean studies,<sup>93</sup> investigating the distribution of *Fusarium* spp. mycotoxins within the kernel, found that while concentrated in the bran layers, deoxynivalenol and nivalenol appeared also to permeate the wheat endosperm to one degree or another. However, no contamination of the endosperm by zearalenone was found, as evidenced by its absence in white flours produced from contaminated wheat. Other reductive/extractive processes can have a similar concentrating effect. Studies on the production of starch by the wet milling of maize have shown that while it is possible to obtain zearalenone-free starch, there is a positive concentration effect in the gluten fraction.<sup>94</sup> A similar effect has been observed with brewers' spent grains.<sup>94</sup>

### 15.5.2 Food manufacture

Zearalenone is highly thermostable and it has been estimated that about 60 % of the zearalenone present survives after bread baking, and 80 % in biscuit production.<sup>95</sup> There is also some evidence that HTST (High Temperature Short Time) processes may be effective in reducing levels of zearalenone contamination in maize, with the degree of reduction being dependent on the configuration of the screws and the amount of water present.<sup>96</sup>

Probably the most interesting dichotomy is the fate of zearalenone in the beer brewing process. As already discussed (Section 15.4.1), beers sampled in a number of African states were found to have high concentrations of contaminating zearalenone compared with those sampled in Korea and Canada. This may simply reflect the fact that the cereal-base used to brew beer in Africa is far more heavily contaminated with zearalenone than that found in the Western hemisphere. A further contributing factor may be that northern beers are based on malt rather than maize as is the case in parts of Africa. Studies with contaminated malt<sup>97</sup> have shown that while deoxynivalenol was 'extracted' into the wort during the brewing process, zearalenone was not.

## 15.6 Conclusions

It has been estimated that worldwide, 25 % of food crops are contaminated with mycotoxins, with those from *Fusarium* spp. making a significant contribution.<sup>98</sup> Zearalenone is a significant mycotoxin for both humans and livestock. Consideration of JECFA data<sup>52</sup> indicates that, in some areas, intakes of zearalenone could exceed the provisional tolerable daily intake of 0.2 µg per kg body weight per day proposed by the EU Scientific Committee for food.<sup>49</sup> There is a general consensus, that the problem of mycotoxin contamination can be best managed through a philosophy of 'prevention is better than cure'.<sup>99</sup> Implicit to this is the need for appropriate management systems to be in place to ensure that, ideally, contamination to levels prejudicial to human or livestock health does not occur in the first place; or that if significant contamination does occur, contaminated material does not proceed further down the food chain. One approach to achieving this objective is the application of Hazard Analysis Critical Control Point (HACCP)<sup>100</sup> principles. In connection with cereals, this approach has been discussed both generically<sup>101</sup> and specifically, with regard to agronomic practices and the production of *Fusarium* spp. mycotoxins in cereals.<sup>18</sup> The requirement applies not only to raw materials intended for human consumption, but also to those for animal feed, a fact recognized by regulations set in some countries, e.g. the Netherlands.<sup>102</sup>

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# 16

## Fumonisin

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### 16.1 Introduction

Fumonisin are mycotoxins produced by a variety of fungi of the *Fusarium* genus. These toxins are natural contaminants of cereal grains worldwide and are mostly found in corn and products derived from corn. Fumonisin were first isolated in 1988 from cultures of *F. verticillioides* strain MRC 826 by Gelderblom and colleagues (Gelderblom *et al.*, 1988) at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) of the Medical Research Council (MRC) in South Africa (Marasas, 2001). The isolation and chemical characterization of fumonisin in 1988 came after 18 years of research into the causal agent(s) for equine leukoencephalomalacia (ELEM), a syndrome characterized by liquefactive necrotic lesions in the white matter of the cerebral hemispheres of horses and other equids (Marasas, 2001), and human esophageal cancer in some populations in the Transkei region of South Africa. In 1989, shortly after the discovery of fumonisin, there were widespread, large-scale outbreaks of ELEM and pulmonary edema (PE) in pigs in the USA resulting in the death of large numbers of horses and pigs fed fumonisin-contaminated corn. This chain of events set the stage for proliferation of research programs throughout the world aimed at

- (1) identifying and characterizing the forms of fumonisin that contaminate food and feed;
- (2) determining the factors affecting fumonisin formation;
- (3) monitoring the occurrence and levels of fumonisin in food and feed;
- (4) developing analytical methods for quantifying fumonisin;

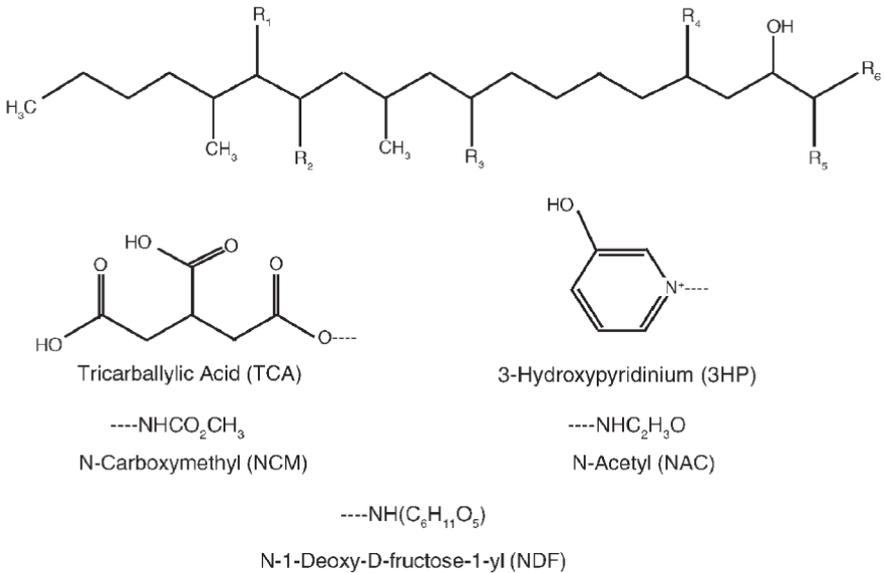
- (5) understanding the biological activity of fumonisins in a variety of animals; and
- (6) identifying methods for controlling fumonisin levels in food.

This chapter will review the research conducted in each of these areas. For more information, the reader is urged to refer to the book *Fumonisin in Food* (Jackson *et al.*, 1996a) that describes the research on fumonisins that occurred before 1996. More up-to-date reviews on the discovery of the fumonisins, the biological activity of the fumonisins, factors affecting fungal production of these mycotoxins, and methods for controlling fumonisin levels in food can be found in volume 9, supplement 2 (2001) of the journal *Environmental and Health Perspectives*. Rheeder *et al.* (2002) wrote a comprehensive review of the *Fusarium* species that produce fumonisins and the fumonisin analogues produced by each of these species. Several papers (Shephard *et al.*, 1996a; Weidenbörner, 2001) are overviews on the occurrence of fumonisin levels in corn-based food and feed. Reviews have summarized the status of analytical methods used to quantify fumonisins and other mycotoxins (Jackson, 1996; Shephard, 1998; Jackson, 1999; Gilbert, 1999).

## 16.2 Chemical and physical properties of fumonisins

Fumonisin is a family of more than ten mycotoxins produced by *Fusarium verticillioides* (formally *F. moniliforme*; teleomorph *Gibberella fujikuroi*) and related species in corn worldwide. They are polar compounds that are soluble in water and aqueous solutions of methanol and acetonitrile, but are not soluble in non-polar solvents. An in depth list of the physical/chemical properties (i.e. NMR structural data, melting points, infrared spectra, stereochemistry, etc.) of the fumonisins can be obtained from Scott (1993), Blackwell *et al.* (1995; 1996), Vesconder *et al.* (1992), Bezuidenhout *et al.* (1988), Sydenham *et al.* (1996a) and ApSimon (2001).

Unlike most mycotoxins, the fumonisins do not have a cyclic structure. They are characterized by a 19- or 20-carbon aminopolyhydroxyalkyl chain that is diesterified with propane-1,2,3-tricarboxylic acid groups (tricarballic acid) (Bezuidenhout *et al.*, 1988, Seo and Lee, 1999) (Fig. 16.1). Several chemically related groups or series (A, B, C and P) of fumonisins have been isolated from cultures of *F. verticillioides* and/or from corn-based food. Of the forms that have been characterized, fumonisin B<sub>1</sub> (FB<sub>1</sub>), fumonisin B<sub>2</sub> (FB<sub>2</sub>) and fumonisin B<sub>3</sub> (FB<sub>3</sub>) (the B series of fumonisins) are the major forms found in food (Bezuidenhout *et al.*, 1988; Cawood *et al.*, 1991; Musser and Plattner, 1997). Fumonisin B<sub>1</sub>, also known as macrofusin, is the most abundant of the fumonisin family and usually accounts for about 70–80 % of the total fumonisin content of *F. verticillioides* cultures and naturally contaminated foods (Ross *et al.*, 1992; Rheeder *et al.*, 2002). Fumonisin B<sub>2</sub> and B<sub>3</sub> usually make up 15–25 % and 3–8 %, respectively, of the fumonisin content of food. The A series of fumonisins, isolated from cultures of *F. verticillioides* and from whole corn, differs from the B series by the presence of a *N*-acetyl amide group rather than an amine group at the C-2 position (Cawood *et al.*, 1991). The C series of fumonisins, which has been isolated from moldy corn,



Fumonisin	Group						Formula	M.W.
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>6</sub>		
FA <sub>1</sub>	TCA	TCA	OH	OH	NHCOCH <sub>3</sub>	CH <sub>3</sub>	C <sub>36</sub> H <sub>61</sub> NO <sub>16</sub>	763.87
FA <sub>2</sub>	TCA	TCA	H	OH	NHCOCH <sub>3</sub>	CH <sub>3</sub>	C <sub>36</sub> H <sub>61</sub> NO <sub>15</sub>	747.87
FA <sub>3</sub>	TCA	TCA	OH	H	NHCOCH <sub>3</sub>	CH <sub>3</sub>	C <sub>36</sub> H <sub>61</sub> NO <sub>15</sub>	747.87
FAK <sub>1</sub>	=O	TCA	OH	OH	NHCOCH <sub>3</sub>	CH <sub>3</sub>	C <sub>30</sub> H <sub>63</sub> NO <sub>11</sub>	603.74
FB <sub>1</sub>	TCA	TCA	OH	OH	NH <sub>2</sub>	CH <sub>3</sub>	C <sub>34</sub> H <sub>59</sub> NO <sub>15</sub>	721.83
FB <sub>2</sub>	TCA	TCA	H	OH	NH <sub>2</sub>	CH <sub>3</sub>	C <sub>34</sub> H <sub>59</sub> NO <sub>14</sub>	705.83
FB <sub>3</sub>	TCA	TCA	OH	H	NH <sub>2</sub>	CH <sub>3</sub>	C <sub>34</sub> H <sub>59</sub> NO <sub>14</sub>	705.83
FB <sub>4</sub>	TCA	TCA	H	H	NH <sub>2</sub>	CH <sub>3</sub>	C <sub>34</sub> H <sub>59</sub> NO <sub>15</sub>	721.83
FC <sub>1</sub>	TCA	TCA	OH	OH	NH <sub>2</sub>	H	C <sub>33</sub> H <sub>57</sub> NO <sub>15</sub>	707.80
FP <sub>1</sub>	TCA	TCA	OH	OH	3HP	CH <sub>3</sub>	C <sub>39</sub> H <sub>62</sub> NO <sub>16</sub> <sup>+</sup>	800.91
FP <sub>2</sub>	TCA	TCA	H	OH	3HP	CH <sub>3</sub>	C <sub>39</sub> H <sub>62</sub> NO <sub>15</sub> <sup>+</sup>	784.91
FP <sub>3</sub>	TCA	TCA	OH	H	3HP	CH <sub>3</sub>	C <sub>39</sub> H <sub>62</sub> NO <sub>15</sub> <sup>+</sup>	784.91
PH <sub>1a</sub>	TCA	OH	OH	OH	NH <sub>2</sub>	CH <sub>3</sub>	C <sub>28</sub> H <sub>53</sub> NO <sub>10</sub>	<b>563.72</b>
PH <sub>1b</sub>	OH	TCA	OH	OH	NH <sub>2</sub>	CH <sub>3</sub>	C <sub>28</sub> H <sub>53</sub> NO <sub>10</sub>	<b>563.72</b>
AP <sub>1</sub> (HFB <sub>1</sub> )	OH	OH	OH	OH	NH <sub>2</sub>	CH <sub>3</sub>	C <sub>22</sub> H <sub>47</sub> NO <sub>5</sub>	<b>405.61</b>
NCMFB <sub>1</sub>	TCA	TCA	OH	OH	NH(C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> )	CH <sub>3</sub>	C <sub>36</sub> H <sub>61</sub> NO <sub>17</sub>	<b>779.87</b>
NDFB <sub>1</sub>	TCA	TCA	OH	OH	NH(C <sub>6</sub> H <sub>11</sub> O <sub>5</sub> )	CH <sub>3</sub>	C <sub>46</sub> H <sub>89</sub> NO <sub>20</sub>	<b>883.97</b>

Note: Forms of fumonisin in bold face are formed during processing

**Fig. 16.1** Chemical structures and molecular weights of the fumonisins. From: Musser and Plattner (1997); Howard *et al.* (1998) and Poling *et al.* (2002).

is chemically similar to the B series, except that the C-1 terminal methyl group is missing in the C series of fumonisins (Seo and Lee, 1999). Musser *et al.* (1996) isolated and characterized a new series of fumonisins, designated the P series, from cultures of *F. proliferatum* grown on corn. These compounds contain a 3-hydroxypyridinium moiety at the C-2 position in the backbone instead of the amine found in the B series of fumonisins (Musser *et al.*, 1996).

Several fumonisin-related derivatives have been found in corn or are created as a result of chemical and/or thermal processing of food (Fig. 16.1). Heating purified fumonisins or naturally contaminated corn meal in the presence of alkali, in a process known as nixtamilization, results in partial and complete hydrolysis of the tricarballic acid ester groups from the aminopolyol backbone (AP<sub>1</sub>) (Sydenham *et al.*, 1992b; Scott, 1993). Two different fumonisin derivatives, *N*-(carboxymethyl)FB<sub>1</sub> (NCMFB<sub>1</sub>) and *N*-(deoxy-D-fructos-1-yl)FB<sub>1</sub> (NDFFB<sub>1</sub>), have been isolated from thermally treated solutions of FB<sub>1</sub> and fructose or glucose, or in foods containing fumonisins and reducing sugars (Howard *et al.*, 1998; Poling *et al.*, 2002).

### 16.3 Factors affecting fumonisin production by *Fusarium* spp.

Experts at the Food and Agriculture Organization (FAO) of the United Nations have estimated that over 25 % of the world's food crops are lost each year due to mycotoxin contamination, with the *Fusarium* species contributing substantially to food contamination (Mannon and Johnson, 1985; Chelkowski, 1998). Several species of *Fusarium*, namely *F. graminearum*, *F. culmorum*, *F. verticillioides*, *F. proliferatum*, are important field or pre-harvest pathogens of corn, wheat and barley causing substantial crop damage due to root, stalk and ear rot (Julian *et al.*, 1995; Bilgrami and Choudhary, 1998). In addition to structural damage to crops, these *Fusarium* species are capable of producing a variety of fungal toxins, such as zearalenone, zearalenols, trichothecenes, fumonisins, fusarins, and moniliformin, which threaten the health of humans and animals who consume them.

*F. verticillioides* and *F. proliferatum* are considered the dominant *Fusarium* species that produce fumonisins in crops, although other species including *F. nygamai*, *F. acutatum*, *F. begoniae*, *F. brevicatenuatum*, *F. phyllophilum*, and *F. napiforme* (Nelson *et al.*, 1993; Weidenbömer, 2001; Fotso *et al.*, 2002) have been shown to produce fumonisins, but only under carefully defined conditions (Howard *et al.*, 2001). Fumonisin-producing *Fusaria* have been isolated from a wide variety of grain crops, including corn, barley, wheat, rice, oats, millet and sorghum (Scott, 1993; Bacon and Nelson, 1994; Visconti and Doko, 1994; Sala *et al.*, 1994; Desjardins *et al.*, 1997). However, fumonisins have only been reported in corn and corn-based foods and feeds with the exception of the discovery of fumonisins in 'black oats' (Sydenham *et al.*, 1992a), New Zealand forage grass (Scott, 1993), sorghum (Shetty and Bhat, 1997), beans (Tseng *et al.*, 1995; Scott *et al.*, 1999) and rice (Abbas and Riley, 1996; Kim *et al.*, 1998; Marin *et al.*, 1999). In addition to their capabilities to produce fumonisin, *F. verticillioides* and

*F. proliferatum* produce other toxic secondary metabolites, such as moniliformin, beauvericin and the fusarins.

*F. verticillioides* can be recovered from virtually all corn kernels including those that appear healthy since the fungus can cause asymptomatic infection of the growing corn plant as well as symptomatic infections that manifest themselves as ear and kernel rot (Miller, 2001). Ear and kernel rot in corn are characterized by the presence of white, pink or salmon-colored mold and brown, tan or white-streaked kernels. Although the moldy corn is more likely to contain fumonisins than sound corn, the level of the toxin is not correlated with the amount of fungal contamination (Shephard *et al.*, 1990; Sohn *et al.*, 1999). High levels of fumonisin have been reported in undamaged, healthy-appearing kernels (Rheeder *et al.*, 1995; Sohn *et al.*, 1999).

*F. verticillioides* is considered a corn endophyte since its hyphae occur systemically in the leaves, stems, roots and cobs of the corn plant (Bacon and Williamson, 1992). Kernel infection may result from internal growth of *F. verticillioides* in the plant stalks, through wounds to the kernel (i.e. from insect or bird damage), and from growth along the silks through the tip end of the ear (Bacon *et al.*, 1992; Leslie, 1996; Munkvold *et al.*, 1997). Using scanning electron microscopy, Bacon *et al.* (1992) provided evidence that the fungus enters intact kernels through the pedicel and grows into the embryo and ultimately the endosperm (Shim *et al.*, 2003).

Although it is well known that fungal and fumonisin contamination of corn occurs mainly before harvest, little is known about when the toxin is formed in the developing ear of corn in the field. Warfield and Gilchrist (1999) studied the relationship between kernel maturity and fumonisin production and found that the highest fumonisin levels were produced at the later stages of kernel development (dent stage) and the lowest were produced in the immature kernels (blister stage). They concluded that fumonisin biosynthesis is affected by kernel composition, which changes during kernel development. Although fumonisin formation is believed to occur predominately in corn pre-harvest, there are some reports that the toxin forms post-harvest, mainly when corn is inadequately stored at high temperatures and high relative humidities (Le Bars *et al.*, 1994; Cahagnier *et al.*, 1995; Marin *et al.*, 1995).

The level of contamination of agricultural commodities with fungi and mycotoxins varies with crop cultivar, climate, and agricultural practices (Humphreys *et al.*, 2001; Bakan *et al.*, 2002). Several studies have indicated that several high-lysine and supersweet corn hybrids that contain the opaque-2 and shrunken-2 endosperm mutations, respectively, are most susceptible to kernel infection by *F. verticillioides* (Warren, 1978; Headrick and Pataky, 1990; Bacon and Williamson, 1992). However, open-pollinated corn cultivars (field and sweet) are also susceptible. In addition, corn hybrids with upright cobs (Fandohan *et al.*, 2003), thin pericarp (Hoenisch and Davis, 1994), tight husks (Emerson and Hunter, 1980) and an increased propensity for split kernels are more likely to be contaminated (Odvody *et al.*, 1990). Inbreds and hybrids show variation in susceptibility from location to location, suggesting that environment affects vulnerability to fungal infection (Bacon and Williamson, 1992).

Although *F. verticillioides* and *F. proliferatum* are found in all corn-growing regions of the world, these fungi are most prolific in humid tropical and subtropical regions of the world (Bacon and Williamson, 1992; Bakan *et al.*, 2002). In general, mycotoxin production in corn is influenced by factors that stress the corn plant including insect damage, moisture content of the soil, high daytime maximum temperatures, and nutrient-deficient soils (Lew *et al.*, 1991; Humphreys *et al.*, 2001; Miller, 2001; Abbas *et al.*, 2002).

Studies on the occurrence of fumonisin in naturally and artificially infected corn demonstrate the importance of insect damage, drought and temperature stress on formation of fumonisin (Humphreys *et al.*, 2001; Miller, 2001). Insects play an important role in the infection of corn since they act as wounding agents or as the vector spreading the fungus to the corn plant (Fandohan *et al.*, 2003). Field surveys in Austria demonstrated that the incidence of the European corn borer increased *F. verticillioides* disease and fumonisin concentrations in corn (Lew *et al.*, 1991). Disease incidence was also correlated to infestation with thrips (Farrar and Davis, 1991; Miller, 2001).

Both temperature and rainfall index were closely related to the development of *F. verticillioides* and fumonisin contamination in corn grown in Brazil (Pozzi *et al.*, 1995; Almeida *et al.*, 2002) and South Africa (Marasas *et al.*, 1979). Surveys of corn grown in Iowa in 1988–1992 showed high levels of contamination over the first four years followed by a significant reduction in fumonisin levels in 1992 (Murphy *et al.*, 1993; Shephard *et al.*, 1996a). The reduced fumonisin levels were attributed to the lack of heat or moisture stress to the corn plants since the growing conditions in the Midwestern USA in 1992 were cool and damp (Murphy *et al.*, 1993; Shephard *et al.*, 1996a). Studies conducted in the USA, Africa, Italy and Croatia have shown that corn hybrids that adapt well to environmental stresses such as excessive heat and drought tend to have reduced fungal and fumonisin contamination (Shelby *et al.*, 1994; Doko *et al.*, 1995; Visconti, 1996). These studies illustrate the importance of choosing corn hybrids that are well adapted to the environment in which they are grown.

## 16.4 Toxicological effects of fumonisins

### 16.4.1 General comments

Since the late 1800s, *F. verticillioides* has been suspected of being involved in a variety of human and animal diseases (Sheldon, 1904). The fungus was first isolated and identified by Sheldon (1904) in response to widespread field outbreaks of animal diseases in the early 1900s associated with consumption of moldy corn (Nelson *et al.*, 1993). Some of the toxic effects that were reported in these outbreaks included hoof sloughing in cattle and horses, loss of bristles in pigs, loss of feathers in poultry, development of convulsions in some animals, and death in a high percentage of affected animals (Peters, 1904; Nelson *et al.*, 1993). More recent reports indicate that when cultures of *F. verticillioides* were fed to

experimental animals, some of the lesions that were observed included ELEM and toxic hepatitis in horses, PE in swine, cirrhosis and nodular hyperplasia of the liver in rats, nephrosis and hepatitis in sheep and vervet monkeys and congestive heart failure in baboons (Kriek *et al.*, 1981a,b; Kellerman *et al.*, 1990; Wilson *et al.*, 1992; Nelson *et al.*, 1993; Ross *et al.*, 1993, 1994; Gelderblom *et al.*, 2001; Voss *et al.*, 2001a). This section will discuss the toxicological effects of fumonisins as measured in whole animal studies, in human populations (epidemiological studies), and *in vitro* tests.

#### 16.4.2 Toxicity of fumonisins – whole animal studies

Since the isolation and characterization of the fumonisins in 1988, considerable efforts have been made to study the toxicological properties of purified FB<sub>1</sub>, the most abundant and toxic of the fumonisin homologues. Purified FB<sub>1</sub> was first shown to cause ELEM when the toxin was administered intravenously (Marasas *et al.*, 1988) or orally (Kellerman *et al.*, 1990) to horses. Pulmonary edema was induced in pigs by intravenous injection of FB<sub>1</sub> (Harrison *et al.*, 1990; Colvin and Harrison, 1992; Gumprecht *et al.*, 1998; Haschek *et al.*, 2001). Introducing the toxin by gavage to Syrian hamsters at doses of up to 18 mg FB<sub>1</sub>/kg body weight produced developmental toxicity marked by prenatal deaths and fetal resorptions (Floss *et al.*, 1994; Shephard *et al.*, 1996a). Feeding FB<sub>1</sub> to broiler chicks caused reduced weight gains, mortality, and lesions in several organs including the liver, kidney, heart and lung (Javed *et al.*, 1992a,b). The toxin also caused pathological changes and death in chick embryos (Javed *et al.*, 1992c). FB<sub>1</sub> promoted aflatoxin B<sub>1</sub>-initiated liver tumors in rainbow trout fed over 23 mg FB<sub>1</sub>/kg for 42 weeks (Carlson *et al.*, 2001). The mycotoxin was found to be a rodent carcinogen that caused hepatic tumors in female B6C3F<sub>1</sub> mice (Howard *et al.*, 2001) and rats (Gelderblom *et al.*, 1991) and induced renal tubule tumors in male F344 rats (Howard *et al.*, 2001). In all species tested thus far, the liver and kidney are the main target organs for FB<sub>1</sub> (Carlson *et al.*, 2001).

The literature at this point is lacking on the effects of the other fumonisin derivatives on whole animal toxicity. Howard *et al.* (2002) compared the toxicity of several forms of fumonisin (FB<sub>1</sub>, FB<sub>2</sub>, FB<sub>3</sub>, FP<sub>1</sub>, AP<sub>1</sub>, FA<sub>1</sub>, and NCMFB<sub>1</sub>) in a 28-day feeding study with female B6C3F<sub>1</sub> mice. In this study, FB<sub>1</sub> was the only fumonisin derivative to have hepatotoxic effects. Studies by Gelderblom *et al.* (1993), Norred *et al.* (1997) and Lemke *et al.* (2001) suggest that the primary amine group, found in the B and C series of fumonisins, is necessary for the fumonisins to elicit toxicity. Voss *et al.* (1996; 2002) reported that feeding male rats diets devoid of FB<sub>1</sub>, but containing FB<sub>2</sub>, FB<sub>3</sub> or hydrolyzed FB<sub>1</sub>, resulted in hepato- and nephrotoxicity. However, these compounds were less toxic than FB<sub>1</sub>. Ross *et al.* (1994) showed that *F. proliferatum* corn culture diets containing predominantly FB<sub>2</sub>, but not diets containing FB<sub>3</sub>, caused hepatotoxicity and ELEM in ponies (Ross *et al.*, 1994). Similarly, Riley *et al.* (1997) reported that feeding culture material of *F. proliferatum* to horses caused disruption of sphingolipid metabolism and induction of ELEM.

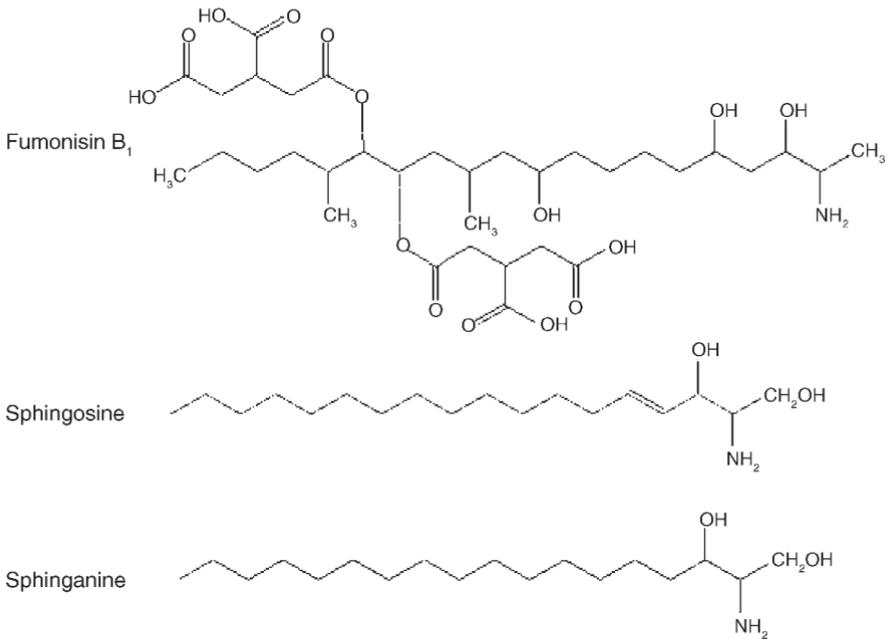
The toxicity of AP<sub>1</sub> (hydrolyzed FB<sub>1</sub>) is not as clearly defined as for FB<sub>1</sub> (Meredith *et al.*, 1999). Voss *et al.* (1996) reported that a nixtamalized culture of *Fusarium verticillioides* had reduced toxicity when compared to the untreated culture. In contrast, nixtamalized culture material as well as corn containing AP<sub>1</sub> increased the incidence of liver and kidney lesions in rats exposed to diethylnitrosamine (Hendrich *et al.*, 1993).

### 16.4.3 Effects of fumonisins on human health

Although it is fairly certain that the fumonisins cause several disease states in animals, the impact of fumonisins on human health is not clear. Consumption of food containing *F. verticillioides* and/or fumonisins has been linked epidemiologically to the high incidence of cancer and other health conditions in some areas of the world where corn is a dietary staple (Sydenham *et al.*, 1991; Rheeder *et al.*, 1992). Several reports have suggested that the high levels of esophageal cancer in the Transkei region of South Africa (Jaskiewicz *et al.*, 1987; Makaula *et al.*, 1996), in the Cixian and Linxian counties in the Republic of China (Chu and Li, 1994; Yoshizawa *et al.*, 1994; Zhang *et al.*, 1997), and in northern Italy (Franceschi *et al.*, 1990) may be due to the natural occurrence of fumonisins in corn grown in these regions. Fumonisin contamination of the corn crop was also suggested to be at least partially responsible for the high incidence of liver cancer in Haimen, China (Ueno *et al.*, 1997). While the consumption of fumonisin-contaminated corn has been correlated with high rates of esophageal and liver cancer, the simultaneous presence of other factors associated with cancer induction in these high incidence areas such as the presence of other mycotoxins, hepatitis viruses, and other contaminants confounds the issue (Chu and Li, 1994; Voss *et al.*, 2002). Exposure to fumonisin or *F. verticillioides*-contaminated corn has also been implicated as a possible risk factor for neural tube defects in South Africa, China and in the Southwestern USA (Cornell *et al.*, 1983; Ncayiyana, 1986; Sydenham *et al.*, 1990; Chu and Li, 1994; Moore *et al.*, 1997; Hendricks, 1999). Clearly, more data are needed to establish a link between consumption of fumonisins and cancer incidence, neural tube defects and possibly other disease states in human populations.

### 16.4.4 Toxicity of fumonisins – *in vitro* studies

*In vitro* studies have indicated that the fumonisins are not mutagenic compounds (Gelderblom and Snyman, 1991), nor do they induce unscheduled DNA synthesis in primary rat hepatocytes (Norred *et al.*, 1992). Although the fumonisins are not genotoxic, they are potent liver cancer promoters as shown by their ability to induce  $\gamma$ -glutamyl transpeptidase and glutathione-S-transferase positive foci in rat liver (Gelderblom *et al.*, 1988; Mehta *et al.*, 1998; Voss *et al.*, 2001a). Fumonisin B<sub>1</sub> is cytotoxic to various mammalian cell lines (Shier *et al.*, 1991; Yoo *et al.*, 1992; Abbas *et al.*, 1993; Gelderblom *et al.*, 1993). Tolleson *et al.* (1996) reported that fumonisins caused apoptosis in cultured human cells and in rat

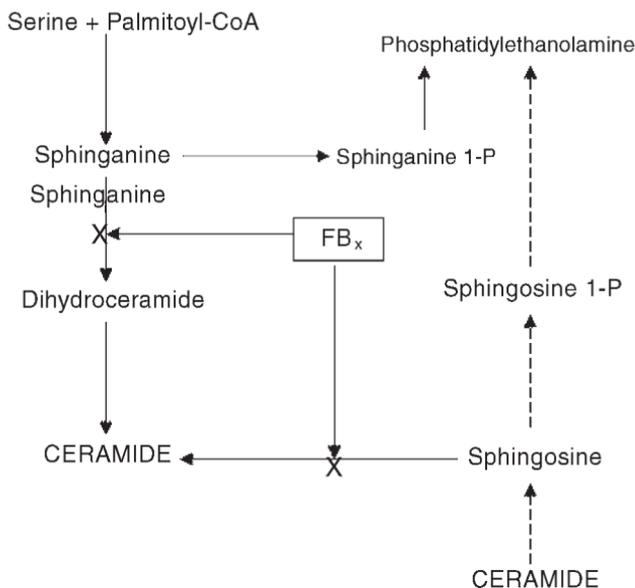


**Fig. 16.2** Structures of fumonisin B<sub>1</sub>, sphingosine and sphinganine.

kidneys. A recent study by Myburg *et al.* (2002) found FB<sub>1</sub> to be cytotoxic to human esophageal epithelial cell lines, providing evidence for the involvement of the toxin in the etiology of esophageal carcinoma. *In vitro* exposure to FB<sub>1</sub> inhibited folate receptor-mediated transport of 5-methyltetrahydrofolate in Caco-2 cells (Stevens and Tang, 1997) and caused apoptosis in African green monkey kidney cells (Wang *et al.*, 1996). The implication of the study by Stevens and Tang (1997) is that daily exposure to FB<sub>1</sub> could reduce folate uptake and cause neural tube defects. The toxic potential of AP<sub>1</sub> and AP<sub>2</sub> backbones of FB<sub>1</sub> and FB<sub>2</sub>, respectively, is not clear but in a brine shrimp bioassay, they were four to six times less toxic than the parent compounds (Hartl and Humpf, 2000). In the same study, NCMFB<sub>1</sub> had 100 fold less toxicity than FB<sub>1</sub> (Hartl and Humpf, 2000).

#### 16.4.5 Mode of action of fumonisins

As previously described, fumonisins cause a myriad of different diseases including neurotoxicity, liver and kidney toxicity and carcinogenicity, porcine PE, ELEM, immunosuppression, and others. Most of the toxicities resulting from exposure to fumonisins can be explained by the ability of the toxins to alter sphingolipid metabolism by inhibiting the enzyme ceramide synthase, an enzyme responsible for the acylation of sphinganine and sphingosine. Structurally, fumonisin resembles sphinganine and sphingosine, free sphingoid bases that play critical roles in cell communication and signal transduction (Figs 16.2 and 16.3)



**Fig. 16.3** Fumonisin ( $FB_x$ ) inhibits ceramide synthase, which catalyzes the formation of ceramide from a sphingoid base (sphinganine or sphingosine) and palmitate or another fatty acid. The consequences of ceramide synthase inhibition are increased cellular sphinganine (and to a lesser degree, sphingosine) concentrations, elevated sphingoid base breakdown products such as sphingosine-1-phosphate, and decreases in cellular complex sphingolipids. From: Voss *et al.* (2002) and Riley *et al.* (2001).

(Wang *et al.*, 1996; Desai *et al.*, 2002). The disruption of the sphingolipid biosynthetic pathway leads to increased levels of sphingolipid precursors and decreased levels of complex sphingolipids. As a result, tissue levels of sphinganine become elevated initiating a cascade of cellular alterations that may result in toxicity and carcinogenicity (Riley *et al.*, 1996; Tsunoda *et al.*, 1998; DeLongchamp and Young, 2001; Seefelder *et al.*, 2001; Merrill *et al.*, 2001; Riley *et al.*, 2001; Desai *et al.*, 2002). The mechanism(s) by which this cascade results in carcinogenesis is not known, but it had been hypothesized that sphinganine-induced apoptosis and the cell proliferation that follows may lead to increased cancer incidence (DeLongchamp and Young, 2001; Kodell *et al.*, 2001).

Since fumonisins disrupt sphingolipid biosynthesis, the resulting elevation in the sphinganine/sphingosine ratio in serum, plasma, or urine has been used as a biomarker for estimating dietary exposure to fumonisins in animals (Shephard *et al.*, 1996a,b; Shephard and van der Westhuizen, 1998; Marasas, 2001). However, use of the ratio as a biomarker for human fumonisin exposure has shown limited promise (van der Westhuizen *et al.*, 1999). Research is needed to develop a biomarker for estimating human exposure to fumonisins.

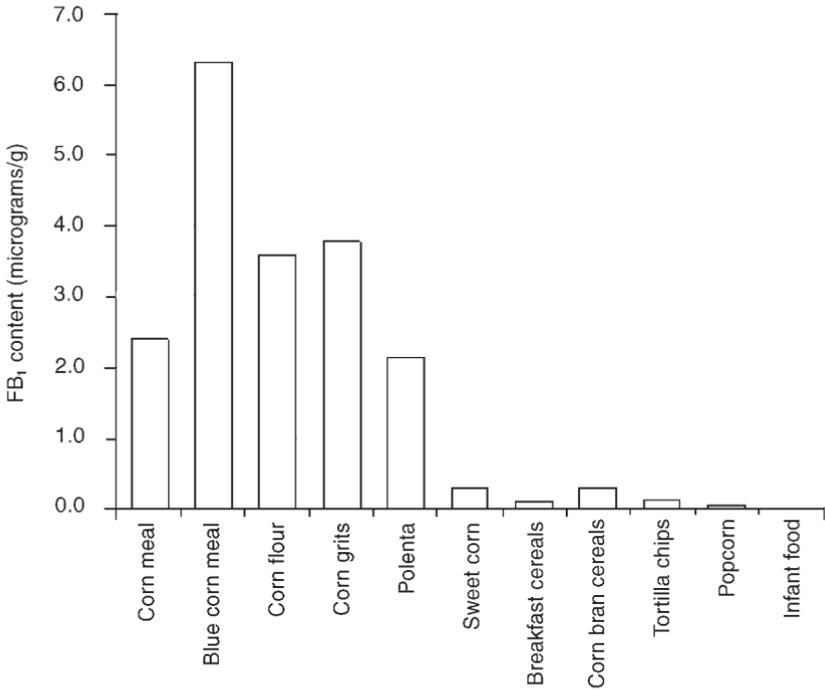
## 16.5 Fumonisin levels in foods: data collection and regulation

### 16.5.1 Data collection

Surveys have emphasized the ubiquitous nature of fumonisin contamination in animal and human corn-based products (Bullerman and Tsai, 1994; Shephard *et al.*, 1996a; Castelo *et al.*, 1998a). Fumonisin has been detected in virtually all types of corn-based foods produced or sold in most areas of the world including Argentina (Gonzalez *et al.*, 1997; Solovey *et al.*, 1999; Hennigen *et al.*, 2000), Brazil (Machinski and Soares, 2000; Hirooka *et al.*, 1996), China (Ueno *et al.*, 1993; Groves *et al.*, 1999), Denmark (Petersen and Thorup, 2001), Germany (Usleber and Martlbauer, 1998; Seefelder *et al.*, 2001), Guatemala (Meredith *et al.*, 1999), Honduras (Julian *et al.*, 1995), Hungary (Fazekas *et al.*, 1996), India (Jindal *et al.*, 1999), Iran (Shephard *et al.*, 2000), Italy (Doko and Visconti, 1994; Cirillo *et al.*, 2003), Japan (Ueno *et al.*, 1993), Kenya (Kedera *et al.*, 1999), Korea (Sohn *et al.*, 1999), Nepal (Ueno *et al.*, 1993), the Netherlands (de Nijs *et al.*, 1998), Poland (Doko *et al.*, 1995; Visconti, 1996), Sardinia (Bottalico *et al.*, 1995), South Africa (Doko *et al.*, 1996; Schlechter *et al.*, 1998; Stockenstrom *et al.*, 1998), Spain (Sanchis *et al.*, 1994), Switzerland (Pittet *et al.*, 1992), Uruguay (Pineiro *et al.*, 1997), the United Kingdom (Scudamore and Patel, 2000), the USA (Stack and Eppley, 1992; Shephard *et al.*, 1996a; Bullerman, 1996; Pohland, 1996; Castelo *et al.*, 1998a; Schlechter *et al.*, 1998) and Venezuela (Medina-Martinez and Martinez, 2000). Countries with relatively cold climates such as Canada, New Zealand and those in eastern and northern Europe (Poland, Croatia and Romania, Scandinavia) have reported lower fumonisin contamination of corn, presumably since *F. proliferatum* infection of corn does not occur extensively at colder, damper climates (Doko *et al.*, 1995; Shephard *et al.*, 1996a; Visconti, 1996). In contrast, high levels of the toxin have been reported in corn grown in countries with warmer climates: Benin, Italy, Portugal and Zambia (Doko *et al.*, 1995).

Most corn and corn-containing foods and feeds are contaminated with fumonisin at detectable levels. Sound, whole corn kernels may contain up to 10 µg/g fumonisin while as much as 63–140 µg/g fumonisin were reported in moldy corn kernels (Rheeder *et al.*, 1992). In general, the highest levels of fumonisin have been reported in corn products or fractions that are not associated with human foods, e.g. damaged corn, cob parts, stalk parts and corn screenings (Bullerman and Tsai, 1994). In particular, corn screenings tend to be highly contaminated (> 5 µg/g) and some lots containing > 20 µg/g have been implicated in field cases of ELEM and porcine PE (Ross *et al.*, 1990; Wilson *et al.*, 1990; Ross *et al.*, 1991b, 1992). Fumonisin has been detected in human food, feed for livestock, laboratory rodent feeds and in pet foods (Ross *et al.*, 1991a; Shephard *et al.*, 1996a).

Some of the highest levels of fumonisin recorded in corn for human consumption were found in moldy home-grown corn in high esophageal cancer risk areas of Cixian County, China (154.9 µg/g; Chu and Li, 1994) and the Transkei region of South Africa (117.5 µg/g; Rheeder *et al.*, 1992). However, relatively low amounts, < 1 µg/g, are generally found in commercial corn products from North



**Fig. 16.4** Ranges of (0 to highest amount) fumonisin content of corn-based foods (data from Shephard *et al.*, 1996a).

America, Europe, and southern Africa (Voss *et al.*, 2003). A wide variety of foods that have been analyzed for fumonisins include corn flour, corn grits, corn meal, polenta, sweet corn, hominy, ready-to-eat breakfast cereals, tortilla and corn chips, muffins, beer and popcorn. Concentrations of the toxin tended to be greater in foods that received little processing (corn flour, corn meal, polenta, corn grits) than those that were more highly processed (snack foods, breakfast cereals) (Fig. 16.4). Fumonisin levels in alkaline-processed foods (masa, tortilla, tortilla chips) manufactured in the USA and Canada were low ( $< 1 \mu\text{g/g}$ ) (Hopmans and Murphy, 1993; Murphy *et al.*, 1996; Scott and Lawrence, 1996; Maragos *et al.*, 1997; Stack, 1998), but fairly high levels ( $> 2 \mu\text{g/g}$ ) were found in products manufactured in Uruguay. Low levels of fumonisins have been reported in beer ( $< 1 \mu\text{g/g}$ ) (Scott and Lawrence, 1995; Torres *et al.*, 1998; Hlywka and Bullerman, 1999) manufactured with corn adjuncts.

Research has been published on the presence of fumonisin residues in animal-derived foods (e.g. milk, meat). Since fumonisins in feed are poorly absorbed by farm animals, the levels of these toxins in milk (Maragos and Richard, 1994; Scott *et al.*, 1994; Richard *et al.*, 1996), eggs (Vudathala *et al.*, 1994), and meat (Prelusky *et al.*, 1994, 1996, Smith and Thakur, 1996) are typically very low ( $< 100 \text{ ng/g}$ ).

### 16.5.2 Regulation

Corn is a staple food for millions of people throughout the world, including the USA, Mexico, Central America and some parts of China and Africa. In addition, corn is an essential component of animal feed. Data from surveys and toxicological studies using a variety of livestock and experimental animals suggest that there are possible human health risks associated with exposure to fumonisins. On the basis of the toxicological evidence, the International Agency for Research on Cancer (IARC) classified *F. verticillioides* toxins, including fumonisins, as potentially carcinogenic to humans (class 2B carcinogens) (IARC, 1993).

In 2001, the US Food and Drug Administration (FDA) issued guidance levels (FDA, 2001) for fumonisins in corn-based foods and feeds to reduce human and animal exposure to these toxins. The guidance document states that, based on the toxicological information about fumonisins, human health risks associated with exposure to fumonisins are possible. The document also states that human exposure to the toxin should not exceed levels achievable with the use of good agricultural and good manufacturing practices. Finally, the guidance document lists the maximum levels for fumonisins in corn and corn-based products used for human consumption as well as for animal feed (Table 16.1). At present, regulatory

**Table 16.1** US Food and Drug Administration guidance levels for total fumonisins in human food and animal feed (source: FDA, 2001).

Food/feed	Total fumonisin level (FB <sub>1</sub> + FB <sub>2</sub> + FB <sub>3</sub> )
<i>Human food</i>	
Degermed dry-milled corn products (e.g. flaking grits, corn meal, corn flour with fat content < 2.5 %, dry weight basis)	2 µg/g
Whole or partially degermed dry-milled corn products (e.g. flaking grits, corn meal, corn flour with fat content ≥ 2.5 %, dry weight basis)	4 µg/g
Dry-milled corn bran	4 µg/g
Clean corn intended for masa production	4 µg/g
Cleaned corn intended for popcorn	3 µg/g
<i>Animal feed</i>	
Equids and rabbits	5 µg/g (no more than 20 % of diet)*
Swine and catfish	20 µg/g (no more than 50 % of diet)*
Breeding ruminants, breeding poultry and breeding mink**	30 µg/g (no more than 50 % of diet)*
Ruminants > three months old being raised for slaughter and mink being raised for pelt production	60 µg/g (no more than 50 % of diet)*
Poultry being raised for slaughter	100 µg/g (no more than 50 % of diet)*
All other species or classes of livestock and pet animals	10 µg/g (no more than 50 % of diet)*

\* Dry weight basis

\*\* Includes lactating dairy cattle and hens laying eggs for human consumption

limits for fumonisins in food have not been set in most other countries. However, Switzerland has set a maximum permissible level of 1000  $\mu\text{g/g}$  for fumonisins ( $\text{FB}_1 + \text{FB}_2$ ) in human foods (Scudamore and Patel, 2000).

## 16.6 Methods of detecting and measuring fumonisins in animal feed and food

As evidence mounts worldwide implicating fumonisins in human and animal disease, there have been increasing efforts to develop sensitive, selective, rapid and versatile procedures for measuring fumonisin levels in foods. Method development has been helped by the commercial availability of analytical standards for  $\text{FB}_1$  and  $\text{FB}_2$  (Thiel *et al.*, 1996). Methods for detecting and quantifying fumonisins in foods include high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), thin-layer liquid chromatography (TLC), immunochemical methods and capillary zone electrophoresis (CZE). This section will describe methods used to analyze fumonisin and will discuss advantages and limitations associated with each of these methods.

### 16.6.1 High-performance liquid chromatography

HPLC methods are the most commonly described procedures for the quantitation of fumonisin in foods and feeds. Most of the published data on the occurrence of fumonisin in corn-based foods were obtained with HPLC methods that used extraction with a mixture of methanol:water or acetonitrile:water followed by purification of the toxins on  $\text{C}_{18}$  solid phase extraction (SPE) columns, strong anion exchange (SAX) SPE columns, or with immunoaffinity columns (IACs) (Scott and Trucksess, 1997; Duncan *et al.*, 1998; Lawrence *et al.*, 2000a; De Girolamo *et al.*, 2001). These extraction and purification procedures are also used for the analysis of fumonisins by TLC, GC, and CZE.

Fumonisin does not fluoresce nor do they contain UV absorbing chromophores. Consequently, most HPLC methods measure fumonisin after derivatizing (pre-column) the free amino group with fluorescent compounds. Derivatization reagents currently in use include *o*-phthalaldehyde (OPA) (Shephard *et al.*, 1990; Ross *et al.*, 1991a), 4-fluoro-7-nitrobenzofurazan (Scott and Lawrence, 1992), naphthalene-2,3-dicarboxaldehyde (NDA) (Bothast *et al.*, 1992; Scott and Lawrence, 1994), 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) (Akiyama *et al.*, 1995), and fluorecamine (Murphy *et al.*, 1996). A shortcoming of the derivatization methods is that  $\text{FA}_1$  and  $\text{FA}_2$ , the *N*-acetylated fumonisin analogs, can not be detected (Plattner *et al.*, 1996). In addition, OPA derivatives of fumonisin are not stable and consequently need to be analyzed immediately. Evaporative light scattering detectors (ELSD) have been successfully used to quantify underivatized fumonisins (Plattner *et al.*, 1996; Wilkes *et al.*, 1996). In addition Plattner (1999), Seefelder *et al.* (2001) and Lukacs *et al.* (1996) developed LC/MS and LC/MS/MS

methods (using electrospray ionization) for analyzing *N*-acetylated fumonisin analogs, NCMFB<sub>1</sub> and other fumonisin compounds that lack the free amine group.

The most widely used HPLC method was developed by Shephard *et al.* (1990). The method is based on extracting fumonisins from the food with methanol:water (3:1), then purifying them on SAX SPE columns. Fully hydrolyzed fumonisins cannot be purified by the SAX clean-up steps because they lack tricarballic acid groups (Shephard, 1998). Subtle changes in pH or ionic strength in extracts can reduce recovery of fumonisins. Sydenham *et al.* (1992b) have indicated that the pH of the extract must be > 5.8 and the elution flow rate must be < 2.0 mL/min to get good recoveries during SAX purification. After derivatization with OPA, fumonisins are separated by isocratic separation on a C<sub>18</sub> reverse phase column and measured by fluorescence detection.

The Shephard *et al.* (1990) procedure has been subjected to two inter-laboratory studies on method reproducibility and accuracy. Mean recoveries of FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub> spiked into non-contaminated corn at a range of 100–8000 ppb were over 75 %. Relative standard deviations within- and between-laboratories ranged from 5.8–26.7 % (Sydenham *et al.*, 1996c; Thiel *et al.*, 1996). Detection limits for FB<sub>1</sub> and FB<sub>2</sub> were 50 and 100 ng/g, respectively. As a result of the collaborative studies, the Shephard *et al.* (1990) method was adopted as a first action method for the determination of FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub> in whole corn (at concentrations of 0.8–12.8 µg/g) by the AOAC International (Sydenham *et al.*, 1996c).

Another commonly used HPLC method was developed by Ross *et al.* (1991a,b). The procedure involves extracting fumonisins from the food with acetonitrile/water (1:1), followed by purification on C<sub>18</sub> SPE columns. HPLC separation and quantitation is similar to that used in the Shephard *et al.* (1990) procedure. Using this method, Rice *et al.* (1995) reported greater than 80 % recoveries of FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub> spiked into corn and poultry feed. Coefficients of variation for these analyses were 3.5–11.3 % for ground corn and poultry feed at the levels tested. Detection limits for fumonisins in naturally contaminated foods are 100 ppb. Rice *et al.* (1995) and Hopmans and Murphy (1993) reported that the method of Ross *et al.* (1991a,b) could be used to identify the presence of hydrolyzed FB<sub>1</sub> in corn-containing foods. Scott and Lawrence (1996) published a method using both SAX and C<sub>18</sub> SPE columns to detect hydrolyzed FB<sub>1</sub> in alkali-processed corn foods.

IACs have been developed for purifying fumonisins from sample extracts (Scott and Trucksess, 1997). The columns are prepared by conjugating antibodies to a gel support such as silica or sepharose contained in a plastic column or cartridge (Lawrence *et al.*, 2000a). These columns are then used as clean-up columns for subsequent analyses by HPLC. The principal advantage of IACs over SAX and C<sub>18</sub> solid phase extraction columns is their ability to bind only the analyte of interest. Fumonisin is first extracted from a food with polar solvents such as methanol:water. The extract is then diluted and applied to the column. Finally, the column is washed and the purified fumonisins are eluted, derivatized with OPA or NDA, and analyzed by HPLC. Ware *et al.* (1994) and Trucksess *et al.* (1995) evaluated a commercially available IAC for fumonisins (FumoniTest, Vicam, Watertown, MA). Using these columns, Ware *et al.* (1994) reported that they could

detect levels as low as 10 and 4 ppb  $FB_1$  and  $FB_2$ , respectively, in corn. Analytical recoveries of over 85 % were obtained for  $FB_1$  and  $FB_2$  spiked into sweet or dent corn at levels of 50–1000 ng/g (Ware *et al.*, 1994). The columns were used to determine  $FB_1$  and  $FB_2$  levels in corn (Ware *et al.*, 1994; Fazekas *et al.*, 1999), milk (Scott *et al.*, 1994), beer (Scott *et al.*, 1995) and in sweet corn (Trucksess *et al.*, 1995).

DeGirolamo *et al.* (2001) compared clean-up procedures for determining fumonisin levels in a variety (corn flakes, corn flour, extruded corn, and infant formula) of foods. They found that using an IAC clean-up procedure (vs SPE procedure) produced the most robust method for the analysis of fumonisins in the matrices studied. An advantage of IACs is that they are subject to less matrix interferences than other SPE methods. Other benefits include good sensitivity and reproducibility. Although the cost of IACs is higher than SPE columns, there has been a report (Fazekas *et al.*, 1999) that IACs can be reused several times without a decrease in column performance. Care must be taken when using IACs since they have a limited capacity which should not be exceeded (Shephard, 1998).

Acceptable results are obtained when the methods described above are used to measure the fumonisin content of unprocessed corn products (whole corn, corn grits, corn flour, corn meal). However, these methods may under-estimate the fumonisin content of complex matrices such as corn bran or processed corn-based foods (breakfast cereals, tortillas, muffins, snack foods), mainly due to poor extraction of fumonisins from the matrices. Several studies have shown that there is no one optimum extraction procedure suitable for all foods (Lawrence *et al.*, 2000b). Significant differences were reported in the extraction of fumonisins with different solvent mixtures, solvent temperatures and extraction methods (blending vs shaking) (Alberts *et al.*, 1993; Bennett and Richard, 1994; Rice *et al.*, 1995; Scott and Lawrence, 1996; Visconti *et al.*, 1996; Scott *et al.*, 1999; Kulisek and Hazebroek, 2000; Lawrence *et al.*, 2000b; De Girolamo *et al.*, 2001; Kim *et al.*, 2002; Sewram *et al.*, 2003).

Several investigators have studied the effects of extraction solvents on fumonisin levels and found that acetonitrile:methanol:water gave the best recovery of fumonisin from both processed and non-processed corn-based foods (De Girolamo *et al.*, 2001). Acidified extraction solvents were found by some to improve recovery of fumonisins in tortillas (Stack, 1998), corn grits and corn meal (Lukacs *et al.*, 1996), thermally processed corn-based foods (Jackson *et al.*, 1997), and a variety of corn products (Murphy *et al.*, 1996). Other approaches reported in the literature for improving the extraction of fumonisins from corn and corn-based foods included raising the temperature of the extraction solvent (Lawrence *et al.*, 2000b), adding EDTA (Sydenham *et al.*, 1995a,b; Scott and Lawrence, 1996; Dombrink-Kurtzman and Dvorak, 1999; Kim *et al.*, 2002) and using extraction solvent mixtures at alkaline pH (Scott and Lawrence, 1994). An intercomparison study for fumonisin analysis in corn flour that was performed under the sponsorship of the European Commission Measurements and Testing Programme indicated that better recoveries were obtained by laboratories using extraction by shaking

(20 min) instead of blending (5 min), and extracting with a high solvent to corn ratio (De Girolamo *et al.*, 2001).

Surveys have shown that processed foods contain less fumonisin than those that are unprocessed, suggesting that the reduction in fumonisin levels may be due to decomposition reactions. However, another possible explanation is that fumonisins become 'hidden' and not recoverable with the currently used extraction/purification procedures (Shier, 2000; Kim *et al.*, 2003). These 'hidden' fumonisins may still be toxic if they are released into the gastrointestinal tract. Kim *et al.* (2003) found that some of the observed losses in fumonisin in heat-processed foods may be due to binding of fumonisins to proteins. They found that treating corn flakes with sodium dodecyl sulfate (SDS) and potassium hydroxide released some of the bound fumonisin from the food matrix. Using this extraction procedure, they found an average of 2.6 times more FB<sub>1</sub> in corn flakes than what was recoverable using conventional extraction methods. Clearly, more work is needed to develop an extraction/purification method which can be used to analyze all types of corn-based foods. Additional investigations are to determine the fate of bound fumonisins in the gut and establish if they become free and bioavailable during digestion (Kim *et al.*, 2003).

### 16.6.2 Gas chromatography-mass spectrometry

GC-MS has been used to a limited extent in the analysis of fumonisin, mainly for the positive identification of fumonisins (Plattner *et al.*, 1990a). Like TLC and LC procedures, fumonisins must be extracted from the food matrix and purified before they are separated by GC. One of the first GC methods developed for fumonisins was based on the quantitation of tricarballic acid formed from base hydrolysis of fumonisins (Sydenham *et al.*, 1990). Another GC-MS method uses a methylsilyl or trifluoroacetate derivative of the aminopentol backbone of the fumonisins after base hydrolysis (Plattner *et al.*, 1990a,b). The advantage of the GC-MS methods is that they combine specificity with quantitation. Disadvantages of these methods are that they are time-consuming and require expensive equipment and a highly trained technician to operate the GC-MS.

### 16.6.3 Thin-layer chromatography

Normal phase (silica) and reverse phase (C<sub>18</sub>) TLC methods have been developed for the detection and quantitation of FB<sub>1</sub> and FB<sub>2</sub> in corn and *F. verticillioides* culture material (Jackson and Bennett, 1990; Plattner *et al.*, 1990b; Ross *et al.*, 1991a; Bothast *et al.*, 1992). Both types of methods require extraction and purification of the fumonisins before they are measured by TLC. Normal phase plates can be developed with chloroform:methanol:acetic acid (6:3:1) or acetonitrile:water (85:15) while reverse phase plates are often developed with methanol:water (75:25). The separated fumonisins fluoresce under UV light after developed plates are sprayed with borate buffer, then with fluorescamine solution and finally with 0.1 M boric-acid-acetonitrile (Preis and Vargas, 2000). The

absolute amount of FB<sub>1</sub> detectable on a TLC method using the combination sprays with fluorescamine was 2 ng, giving a detection limit for the method of 0.1 µg/g (Preis and Vargas, 2000). Alternatively, fumonisins appear as purple spots after spraying developed plates with *p*-anisaldehyde. The detection limit using the *p*-anisaldehyde spray is about 500 µg FB<sub>1</sub>/plate (Shephard, 1998). TLC is well suited for screening corn for fumonisins since many test samples can be analyzed simultaneously. Another advantage is that TLC does not require expensive instrumentation such as GC or LC. However, the use of TLC to quantify fumonisins in corn or culture material sometimes can be difficult due to interfering compounds that co-migrate with fumonisins.

#### 16.6.4 Immunochemical methods

The production of monoclonal (Azcona-Oliveira *et al.*, 1992; Chu *et al.*, 1995; Elissalde *et al.*, 1995) and polyclonal (Azcona-Oliveira *et al.*, 1992; Usleber *et al.*, 1994; Sutikno *et al.*, 1996; Yu and Chu, 1996) antibodies against fumonisins have permitted the development of direct and indirect competitive enzyme-linked immunosorbent assays (ELISAs) for screening foods, feeds, animal tissues and fungal cultures for fumonisins (Minervini *et al.*, 1992; Pestka *et al.*, 1994; Tejada-Simon *et al.*, 1995; Chu, 1996; Sutikno *et al.*, 1996; Bird *et al.*, 2002). Azcona-Oliveira *et al.* (1992) developed a monoclonal antibody-based (Mab) ELISA for fumonisins. The Mab ELISA correlated with GC-MS and HPLC methods when non-contaminated corn was spiked with different levels of FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub>. However, the Mab ELISA method tended to over-estimate the fumonisin content of naturally contaminated corn when compared to GC-MS or HPLC (Sutikno *et al.*, 1996; Sydenham, *et al.*, 1996b). The over-estimation might be a result of cross reactivity of the antibodies to compounds that are structurally related to fumonisin (Sutikno *et al.*, 1996).

A commercially available polyclonal antibody-based (Pab) ELISA kit (Veratox, Neogen) is available for rapidly (< 30 min) quantifying the total fumonisin content (FB<sub>1</sub> + FB<sub>2</sub>) in agricultural commodities. This kit is based on a competitive direct ELISA whereby fumonisins in a food extract compete with enzyme-labeled fumonisin for binding to the solid-phase bound antibodies. The color intensity of wells, formed by substrate reacting with enzyme, is inversely proportional to the fumonisin concentration (Ware *et al.*, 1994). Recovery of FB<sub>1</sub> from corn spiked with different levels of FB<sub>1</sub> was > 74 % and coefficients of variation were < 8 % (Abouzied *et al.*, 1996; Bird *et al.*, 2002). Detection limits for FB<sub>1</sub> in methanol:water solutions and in corn were < 0.1 µg/mL and 0.4 µg/g, respectively (Abouzied *et al.*, 1996; Bird *et al.*, 2002). The ELISA results for naturally contaminated corn had a high correlation to values obtained by an HPLC method (Abouzied *et al.*, 1996; Sutikno *et al.*, 1996; Sydenham *et al.*, 1996b; Bird *et al.*, 2002). ELISA is well suited for rapidly screening corn for fumonisins. Other advantages of the ELISA kits are their portability and ease of use. Since ELISA has a potential for over-estimating fumonisin levels in corn, HPLC methods should be used to confirm ELISA results.

Immunosensors (biosensors) are currently being developed to detect and quantify mycotoxins in food. One such biosensor, developed by Thompson and Maragos (1996), was successful at measuring FB<sub>1</sub> levels in the range of 10–1000 ng/mL food extract. The sensor exhibited cross reactivity with FB<sub>2</sub>, but did not have interferences with hydrolyzed fumonisins or tricarballic acid. Mullett *et al.* (1998) reported the development of a surface plasmon resonance (SPR) immunosensor to quantify FB<sub>1</sub>. These authors reported that the biosensor had a detection limit of 50 ng/mL sample extract and that analysis time was less than 10 min. The development of biosensors for fumonisins as well as other natural toxins is likely to increase in the future due to the advantages of biosensors over traditional methods of analysis (minimal sample preparation, fast response and portability).

### 16.6.5 Capillary electrophoresis

The presence of two tricarballic acid groups enables the fumonisins to be separated by electrophoretic techniques. Maragos (1997) described a method for measuring fumonisin levels in corn and equine serum by capillary zone electrophoresis (CZE). The fumonisins were extracted from corn with methanol:water (75:25) and purified with immunoaffinity columns. Fluorescein isothiocyanate (FITC) derivatives of the fumonisins were prepared and separated by CZE with laser-induced fluorescence detection. Recoveries from corn spiked with 0.25–5.0 µg/g FB<sub>1</sub> averaged 89 %. The limit of detection for FB<sub>1</sub> in corn was 50 ng/g and coefficients of variation were 7.7 % when FB<sub>1</sub> levels were greater than 200 ng/g corn. CZE has several advantages over other methods for analysis of fumonisins. These include generation of substantially less hazardous waste and acceptable accuracy and precision. More work is needed to determine if CZE can be used to determine levels in more complex matrices such as processed corn-based foods.

## 16.7 Control of fumonisin levels in food and feed

Since fumonisins are natural products of *F. verticillioides*, a ubiquitous endophytic contaminant of corn, it will be difficult, if not impossible, to eliminate fumonisin from food and feed (Duvick, 2001). However, there are several strategies that have been proposed to reduce the fumonisin content of food. They include

- (1) the development of genetically resistant strains of cereal grains to infection by *Fusarium* spp.,
- (2) preventing continued fungal development after harvest, and
- (3) processing infected kernels to decrease mycotoxin content (Pujol *et al.*, 1999).

This section will discuss some of the pre- and post-harvest methods for controlling contamination of corn with *F. verticillioides* and fumonisins.

### 16.7.1 Pre-harvest control

Control, prevention and detection of the endophytic infections by *F. verticillioides* in corn are difficult, mainly because kernels may be highly contaminated, yet appear sound (Bacon *et al.*, 2001). Due to the intercellular nature of the fungal infection, the use of chemical control (fungicides) is not effective at controlling the fungus. Several pre-harvest controls have been proposed for reducing fumonisin contamination of corn. The first involves the use of biocontrol, i.e. use of micro-organisms and their metabolites to suppress *F. verticillioides*. Bacon *et al.* (2001) developed a biological control of contamination of corn in the field using the endophytic bacterium, *Bacillus subtilis*. The bacterium operates by the mechanism of competitive exclusion, inhibiting the growth of *F. verticillioides* and, consequently, reducing fumonisin production *in planta*. More work is needed to determine the practicality of the use of biocontrol to reduce mycotoxin levels in corn.

Genetic modification of corn through plant breeding or by transgenic means offers several approaches for controlling corn ear mold and mycotoxin formation. These include the development of corn hybrids that

- (1) are resistant to environmental stresses such as heat, drought and insect damage,
- (2) are resistant to fungal infection and disease,
- (3) prevent mycotoxin from being formed, and
- (4) detoxify mycotoxins (Duvick, 2001).

The potential for imparting resistance of corn to insect infestation through molecular genetic means is now being realized. Research by Bakan *et al.* (2002), Munvold *et al.* (1999) and Masoero *et al.* (1999) has shown that corn containing genes coding for insecticidal proteins from *Bacillus thuringiensis* (Bt) had significantly less damage from the European corn borer and pink stem borer. These same Bt corn hybrids grown in the USA and in Italy had reduced incidence and severity of *Fusarium* ear rot and reduced fumonisin levels, in some cases nine to 10 times lower than levels measured in non-Bt corn (Council for Biotechnology Information, 2001).

Currently, research is attempting to generate transgenic plants with increased resistance to fungal diseases such as *Fusarium* ear rot. However, transgene-enhanced disease resistance has not yet achieved the same level of success as insect resistance in corn plants, mainly due to our incomplete knowledge of how and when *Fusarium* infects the kernel and when and where fumonisins are produced in the ear (Duvick, 2001).

Although reducing fungal infection is the most desirable method to eliminate mycotoxins, strategies directed at suppressing their synthesis or degrading them to non-toxic metabolites represent alternative approaches for reducing contamination. If mycotoxin biosynthetic pathway genes are identified, molecular methods can be used to modulate the pathway. At present, it is difficult to predict how difficult this strategy will be until the key factors that turn the production of fumonisins on and off are identified (Duvick, 2001). Another transgene-based

method for reducing mycotoxin accumulation involves the use of catabolic enzymes to detoxify the mycotoxin *in situ* before it accumulates in the plant (Karlovsky, 1999; Duvick, 2001). The success of this strategy will depend partly on the extent to which the plant-produced enzymes reach the target tissue where fumonisin accumulates (Duvick, 2001). Since little is known about the cellular location of fumonisin accumulation, it is difficult to predict how successful a detoxification approach would be. In addition, fumonisins appear resistant to the activity of most known esterases and amine modifying enzymes, so novel enzymes and genes must be sought (Duvick, 2001).

### 16.7.2 Post-harvest control

Post-harvest control of fumonisins in corn-based foods and feed may be achieved through

- (1) proper storage of corn before processing,
- (2) physical treatments that cause redistribution, removal, or destruction of fumonisins,
- (3) chemical treatments that destroy or change the toxins, and
- (4) biological treatment to remove or destroy fumonisins (Bullerman *et al.*, 2001).

This section will review these post-harvest controls.

#### *Storage*

Corn is often harvested at a moisture level which is conducive to growth, colonization and mycotoxin production by a range of fungi including *Fusarium* species (Marin *et al.*, 1999). Ineffective or delayed drying of corn to the desired moisture content of 14–15 % ( $0.70\text{--}0.73a_w$ ) can result in proliferation of the fungus and substantial formation of fumonisins during storage (Marin *et al.*, 1999). In addition, inadequate storage at high temperature and high relative humidity can allow fungal growth and further mycotoxin production (Ono *et al.*, 2002). Proper storage of corn is essential for controlling fungal and fumonisin formation before processing.

#### *Sorting and cleaning*

Visual grading of corn for human consumption may not reduce fumonisin levels, since visibly sound corn (no visible signs of fungal infection) can be highly contaminated with the toxin (Rheeder *et al.*, 1995; Sohn *et al.*, 1999; Weidenbörner, 2001). However, in rural populations, where the corn used for human consumption can be of poor quality, sorting visibly diseased kernels from lots of corn is effective at reducing fumonisin levels (Desjardins *et al.*, 2000).

Cleaning treatments such as sieving fines or broken kernels from bulk shipments of corn can reduce fumonisin levels by over 50 % (Sydenham *et al.*, 1994; Saunders *et al.*, 2001). Canela *et al.* (1996) have shown that steeping corn in water before processing may reduce fumonisin levels. Fumonisin-contaminated corn kernels have a low density and up to 86 % of the toxin was removed in the buoyant

fraction when naturally contaminated corn was washed with an aqueous saturated sodium chloride solution (Shetty and Bhat, 1999).

#### *Dry and wet milling*

Dry-milling is the process of converting whole corn to corn grits, cornmeal and corn flour, the raw ingredients used to manufacture breakfast cereals, snack foods and bakery products. In the dry-milling process, corn is cleaned to remove dirt, debris and broken kernels from whole corn (Saunders *et al.*, 2001) and then the moisture level of the corn is raised by addition of water. The hull, germ and tip cap are removed leaving endosperm which is converted to flour, grits and meals through a series of mills, sifters and gravity tables (Saunders *et al.*, 2001). Katta *et al.* (1997) and Broggi *et al.* (2002) reported that dry-milling of fumonisin-contaminated corn tended to concentrate the fumonisins in the bran and germ fractions and produced grits relatively free of contamination. Since many processed foods are made from flaking grits, this would explain the relatively low levels of fumonisins in these products.

Wet-milling, the processing of converting corn into starch, is an effective method for reducing fumonisin levels. Bennett *et al.* (1996) studied the effects of a lab-scale wet-milling process on fumonisin in naturally contaminated corn. Starch from wet-milling of corn, naturally contaminated with 13.9  $\mu\text{g}$  FB<sub>1</sub>/g, was free of detectable toxin. However, fumonisin was found in the by-products of the process (gluten, fiber and germ), which are typically used for animal feed.

#### *Thermal processing*

Thermal processing is used by the food industry to convert corn into a multitude of consumer products, including muffins, breads, breakfast cereals and snack foods. Fumonisin is fairly heat stable. However, thermally processed corn-based foods generally contain lower levels of fumonisins than unprocessed foods (Castelo *et al.*, 1998b; Jackson and Bullerman, 1999). Studies focusing on the effects of thermal processing conditions indicate that when foods are heated under the same conditions encountered in boiled or retorted food (temperatures < 125 °C), little change in fumonisin level can be expected (Alberts *et al.*, 1993; Dupuy *et al.*, 1993; Maragos and Richard, 1994; Jackson *et al.*, 1996b,c,d; Pineiro *et al.*, 1999; Saunders *et al.*, 2001). In contrast, foods that reach temperatures > 150 °C (baked, extruded, fried), may have some losses of fumonisins (Pineiro *et al.*, 1999; Saunders *et al.*, 2001).

Jackson *et al.* (1996b,c,d) found that temperatures  $\geq 150$  °C were required to cause decomposition of fumonisins in aqueous solutions. Jackson *et al.* (1997) and Voss *et al.* (2003) reported that baking corn bread or muffins at temperatures of  $\leq 200$  °C for  $\leq 60$  min resulted in small losses (<27 %) for FB<sub>1</sub>. In a lab-scale study, Jackson *et al.* (1997) reported that frying corn tortillas in oil at 140–170 °C for 0–6 min resulted in no losses of fumonisin. Similarly, Voss *et al.* (2001b) found minor losses of FB<sub>1</sub> during the commercial production of fried tortilla chips. Overall, these studies indicate that fumonisin levels are not reduced significantly in fried and baked foods. These results are supported by a short-term rat feeding

study that showed that baking, pan-frying and deep-frying did not reduce the toxicity of fumonisin-contaminated cornmeal (Voss *et al.*, 2003).

Extrusion appears to be an effective method for reducing fumonisin levels in corn (Castelo *et al.*, 1998c). Extrusion is a process by which cornmeal or corn grits are subjected to high pressures, temperatures and shear forces. The extruded product can be baked or fried to produce snack foods and ready-to-eat cereals (Saunders *et al.*, 2001). Katta *et al.* (1999) reported up to 76 % losses of FB<sub>1</sub> when corn grits were extruded (twin-screw mixing-type extruder) under temperatures and extruder screw speeds that resulted in acceptable product expansion and color. In a similar study, Pineiro *et al.* (1999) found 70–90 % reduction in fumonisin levels in naturally contaminated corn flour extruded with a single-screw mixing-type extruder. Further investigations are needed to determine if extrusion reduces the toxicity of fumonisin-contaminated corn.

Although fumonisins are relatively heat stable compounds that persist during most food processing operations, they undergo reactions in food that alter their structure and potentially their toxicity (Seefelder *et al.*, 2001). Murphy *et al.* (1996) was the first to suggest that fumonisins undergo a non-enzymatic browning reaction with reducing sugars (e.g. glucose or fructose) in thermally processed food. NDFFB<sub>1</sub> and NCMFB<sub>1</sub> are two compounds that have been chemically characterized as products of this reaction (Howard *et al.*, 1998; Poling *et al.*, 2002). However, only minor amounts of either compound have been detected in processed foods (Jackson *et al.*, 1997; Seefelder *et al.*, 2001; Voss *et al.*, 2001b). It is likely that other products are formed from the reaction between fumonisins and sugars. Further investigations are necessary to determine the fate of fumonisins and their reactions in heated food (Seefelder *et al.*, 2001).

### *Chemical processing*

Very little has been reported on the effect of chemical treatments on fumonisins. Norred *et al.* (1991) found that atmospheric pressure/ambient temperature ammoniation reduced the fumonisin content of *F. verticillioides* culture material but did not reduce the toxicity of the material when fed to rats. In contrast, Park *et al.* (1992) reported 79 % reduction in fumonisin levels of corn after high-pressure/ambient temperature ammoniation followed by a low-pressure/high-temperature treatment. However, they did not measure the toxicity of the treated corn.

McKenzie *et al.* (1997) found that treating an aqueous solution of FB<sub>1</sub> with 10 % (w/w) ozone (O<sub>3</sub>) gas for 15 s resulted in the conversion of the parent compound to the 3-keto FB<sub>1</sub> (3k-FB<sub>1</sub>) derivative. In two separate toxicity tests, 3k-FB<sub>1</sub> was found to retain most of the toxicity present in the parent compound. Consequently, O<sub>3</sub> treatment is not an effective method for detoxifying FB<sub>1</sub>.

In the preparation of tortillas, corn is treated with a lime (CaOH<sub>2</sub>) solution and heat in a process known as nixtamalization. This treatment has been shown to hydrolyze the tricarballylic acid side-chains of fumonisins, resulting in the aminopentol backbone (Hendrich *et al.*, 1993; Sydenham *et al.*, 1995a,b; Meredith *et al.*, 1999) which partitions into the lime solution and rinse water. As a result, < 25 % of the original fumonisin content of the corn remains in the treated corn

(Dombrink-Kurtzman *et al.*, 2000). A survey by Sydenham *et al.* (1991) showed that corn products from South America and the USA that were treated with lime water and heat (i.e. masa and tortillas) had very low levels of fumonisins. In contrast, high levels ( $> 10 \mu\text{g/g}$ ) of total fumonisins ( $\text{FB}_1 + \text{AP}_1$ ) were detected in some tortilla products produced in Guatemala, a region where corn is a dietary staple (Meredith *et al.*, 1999). These data suggest that poor quality of corn is used in production of tortillas in some regions of the world.

Reports by Murphy *et al.* (1996), Lu *et al.* (1997) and Castelo *et al.* (2001) suggest that a combination of chemical and thermal treatment may reduce fumonisin levels in food. Murphy *et al.* (1996) and Liu *et al.* (2001) reported that in a model system, fumonisins participated in a non-enzymatic browning reaction that appeared to detoxify these compounds. Castelo *et al.* (2001) found that adding glucose rather than sucrose or fructose to corn muffin mix resulted in significant reductions ( $> 50\%$ ) in  $\text{FB}_1$  levels in the baked product. The same group also reported that extrusion cooking with glucose could be used as a means to further reduce the concentration of fumonisins in foods and feeds. When extrusion conditions were optimized, 92.1% loss of  $\text{FB}_1$  was found when grits were extruded with glucose.

Howard *et al.* (1998) reported that reaction of reducing sugars with  $\text{FB}_1$  produced NCMFB<sub>1</sub> and elucidated its structure. Seefelder *et al.* (2001) analyzed corn-based German foods and found that NCMFB<sub>1</sub> was present in low amounts in fried tortilla chips and corn flakes. Similarly, Castelo *et al.* (2001) reported that less than 1% of the fumonisin B<sub>1</sub> was detected as NCMFB<sub>1</sub> in baked corn muffins with added glucose, despite a 45–70% reduction in detectable  $\text{FB}_1$  during baking. From these data it can be concluded that NCMFB<sub>1</sub> is only a minor reaction product of fumonisin in food and that more work is needed to determine the identity and toxicity of other product(s).

### *Biological processing*

Very little is known about the effects of biological processing on fumonisins. Scott *et al.* (1995) determined the stability of  $\text{FB}_1$  and  $\text{FB}_2$  during the beer-making process. Both toxins, which were spiked into the wort, were fairly stable in the fermentation process. Similarly, little degradation of  $\text{FB}_1$  was found in a three-day yeast fermentation of corn (Bothast *et al.*, 1992).

## **16.8 Future trends**

Since the discovery of the fumonisins in 1988, considerable progress has been made at understanding the chemical properties of the fumonisins, conditions by which this group of mycotoxins are produced by *F. verticillioides*, the extent of contamination of the food and feed supply by the toxin, the biological activity of some of the major fumonisin derivatives, and the effects of processing on the stability of the toxin. However, more work is needed in all of the above areas.

Fungal growth and mycotoxin production result from a complex interaction of

many factors, and understanding each factor is essential to understanding the overall process and to predict and prevent mycotoxin production. In order to devise strategies for controlling fumonisins, there is a need to gain a clearer understanding of the nature of the asymptomatic infection of corn with *F. verticillioides*. In addition, more work is needed to determine when, how and where fumonisins are produced by the fungus in the corn plant.

Currently, there are several possible strategies for reducing fumonisin contamination of corn through genetic modification. Since fumonisin accumulation in the corn plant appears to be triggered by environmental stresses, such as drought and high temperatures, greater efforts should be directed at developing and growing corn hybrids that are less vulnerable to these stresses. Breeding corn for resistance to ear mold and accumulation of fumonisins is being now practised. However, the ability to achieve the desired resistance may be limited due to complicated genetics and/or the linkage to undesirable agronomic traits (Duvick, 2001).

Transgenic approaches for achieving reduced fumonisin levels in corn, including enhanced resistance to insect damage and fungal infection, are now feasible. There have been recent reports that indicate that it may be possible to genetically modify corn to express genes that produce fumonisin degrading enzymes in the corn plant. At present, transgene-mediated approaches for controlling fumonisin have not reached their commercial potential (Duvick, 2001).

Available epidemiological evidence has suggested a link between fumonisin exposure and esophageal cancer, liver cancer and neural tube defects in some regions of the world. However, more work is needed to verify the link between fumonisin consumption and the incidence of cancer and other chronic diseases. To obtain an accurate measure of human exposure to fumonisins, an effective biomarker for this toxin needs to be identified.

More work is needed to study post-harvest control methods of fumonisins in food and feed. In particular, research is needed to determine the effects of processing on these and other *Fusaria* toxins. Studying the effects on the stability of the fumonisins is complicated by the lack of reliable analytical methods. Fumonisin are not always uniformly extracted and recovered from different matrices, especially those that have been processed. Once methods are developed that efficiently extract the most biologically active fumonisin analogues from a variety of food, accurate and extensive surveys of the worldwide food supply are needed. Finally, more work is needed to identify and determine toxicological effects of products resulting from physical decomposition or chemical modification of the fumonisins.

## 16.9 References

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## Other mycotoxins

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### 17.1 Introduction

The mycotoxins discussed below are mainly selected from those produced by the more important genera of fungi likely to contaminate foods and food crops, i.e. *Alternaria*, *Fusarium*, *Aspergillus* and *Penicillium*. Mycotoxins from some other fungi associated with mycotoxicoses in animals, e.g. *Pithomyces chartarum*, *Myrothecium* spp., *Diplodia maydis*, or associated with sick buildings, e.g. *Stachybotrys* spp., are not covered in this chapter nor are toxins from *Trichothecium* spp., *Trichoderma* spp. and from several other genera of fungi not common in foodstuffs. In view of the considerable literature on the other fungal toxins included in this review, key and/or recent references will be included as far as possible. Mycotoxins will be described in terms of chemical structure, major toxic effects, any association with human health problems, natural occurrence in foods and food crops and analytical methods. There are no specific international regulations for any of these mycotoxins (Boutrif and Canet, 1998).

### 17.2 *Alternaria* toxins: chemical structure, toxicity, sources in foods and associated human health risks

Fungi of the genus *Alternaria* are common pathogens of food crops (Chekowsky and Visconti, 1992). They may also infect foodstuffs after harvest. *Alternaria alternata* produces a number of mycotoxins, including alternariol, alternariol monomethyl ether, altertoxin I and tenuazonic acid, so it is a species of particular

interest to mycotoxicologists. Several reviews on the *Alternaria* toxins have been published and are listed by Scott (2001). A corn flour culture of *A. alternata* was carcinogenic to rats and other culture extracts were mutagenic in various *in vitro* systems. Recently, liver and kidney damage in rats was produced by feeding *A. alternata* for 28 days (Combina *et al.*, 1999). *A. alternata* might be an etiological factor in human esophageal cancer in Linxian, China (Liu *et al.*, 1991). There are no specific regulations for any of the *Alternaria* toxins in foods.

### 17.2.1 Alternariol and related metabolites

Alternariol (3,7,9-trihydroxy-1-methyl-6*H*-dibenzo[*b,d*]pyran-6-one), alternariol monomethyl ether (3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo[*b,d*]pyran-6-one) and altenuene (2 $\alpha$ ,3 $\beta$ ,4 $\alpha\beta$ -tetrahydro-2,3,7-trihydroxy-9-methoxy-4 $\alpha$ -methyl-6*H*-dibenzo[*b,d*]pyran-6-one) are phenolic metabolites of *Alternaria alternata* (Chekowski and Visconti, 1992). They are not very acutely toxic, but alternariol and alternariol monomethyl ether are mutagenic (Schrader *et al.*, 2001). There is also some evidence of carcinogenic properties: squamous cell carcinoma were induced in mice subcutaneously inoculated with human embryo esophageal tissue that had been treated with alternariol; and NIH/3T3 cells transformed by alternariol monomethyl ether were subcutaneously tumorigenic in mice. No cancer studies of these *Alternaria* mycotoxins in animals have been carried out. However, precancerous changes were recently observed in esophageal mucosa of mice fed 50–100 mg/kg body weight (bw) per day of alternariol monomethyl ether for 10 months (Yekeler *et al.*, 2001). Additional toxicological studies are clearly needed, preferably with purified toxins but difficulty in obtaining these in large quantities is a problem.

Natural occurrence of alternariol and alternariol monomethyl ether has been reported in various fruits, including tomatoes, olives, mandarins, melons, peppers, apples and raspberries, and also in grains, sunflower seeds, oilseed rape and pecans (Scott, 2001). The occurrence of low levels of alternariol in processed fruit products – apple juice, processed tomato products, grape juice, red wine, cranberry nectar, prune nectar and raspberry juice – is possibly of human health interest; in apple juice and prune nectar, alternariol monomethyl ether has also been detected (Lau *et al.*, 2003). Surveillance of fruit juices and other fruit products for these toxins is so far limited (only single samples of some of these drinks were analyzed) and further efforts are needed to determine human exposure.

Alternariol and alternariol monomethyl ether are usually extracted from solid foods with organic solvents such as methanol, acetonitrile or ethyl acetate (Scott, 2001). Clean-up may involve treatment with sodium bicarbonate, ammonium sulphate or lead acetate solutions and silica gel chromatography. Solid phase extraction (SPE) columns or cartridges are used for both extraction and clean-up of alternariol and alternariol monomethyl ether in liquid foods, such as apple juice; C<sub>18</sub> and aminopropyl SPE columns in series were used in our laboratory for clean-up of apple juice and other fruit beverages (Lau *et al.*, 2003). Liquid chromatography (LC) has now largely replaced gas chromatography (GC) and thin-layer

chromatography (TLC) for determination of *Alternaria* toxins in food extracts; after separation by reversed phase LC, alternariol and alternariol monomethyl ether can be detected by UV, fluorescence, electrochemical detection or mass spectrometry (MS) (Scott, 2001; Lau *et al.*, 2003). Detection limits in an SPE/LC-UV method for apple juice are of the order of 1 ng/mL and are much lower by LC-MS. No immunochemical methods have yet been developed for these mycotoxins; an immunoaffinity clean-up column would be of great value as UV and fluorescence detection can be hindered by the presence of interferences.

### 17.2.2 Alttoxins

Alttoxin I {[1*S*-(1 $\alpha$ ,12 $\alpha$  $\beta$ ,12 $\beta\alpha$ )]1,2,11,12,12a,12b-hexahydro-1,4,9,12a-tetrahydroxy-3,10-perylene-1,10-dione} and related perylene derivatives alttoxins II and III are also produced by *Alternaria alternata*. Alttoxins are more potent mutagens and acute toxins to mice than alternariol and alternariol monomethyl ether (Chekowski and Visconti, 1992; Schrader *et al.*, 2001). Alttoxins I and III tested positively in a cell bioassay that detects tumor promoters (Osborne *et al.*, 1988). Natural occurrence is more limited, however. Alttoxin I has been found in infected apples, wheat and sorghum (Scott, 2001). It is moderately stable in foods (Scott and Kanhere, 2001) and development of a sensitive method for this mycotoxin is warranted. Poor recoveries of alttoxin I were obtained from a silica-based C<sub>18</sub> SPE column, but this problem could be overcome by using a polymer-based reversed phase SPE column. Reversed phase LC of alttoxin I can be carried out in the same mobile phase as alternariol and alternariol monomethyl ether; alttoxins I and II are electroactive and good sensitivity has been achieved by coulometric and amperometric techniques.

### 17.2.3 Tenuazonic acid

L-tenuazonic acid (5*S*,6*S*-3-acetyl-5-*sec*-butyl-4-hydroxypyrrolidone-2,4-dione) is another toxin produced by *Alternaria alternata*, as well as some other *Alternaria* species (Chelkowski and Visconti, 1992). It is not mutagenic in bacterial systems. However, it is toxic to several animal species; in dogs, it caused hemorrhages in several organs at daily doses of 10 mg/kg bw and in chickens sub-acute toxicity was observed with 10 mg/kg in the feed. Precancerous changes were observed in esophageal mucosa of mice fed 25 mg/kg bw per day of tenuazonic acid for 10 months (Yekeler *et al.*, 2001). Tenuazonic acid has been shown to occur in several *Alternaria* infected fruits and vegetables, accompanying alternariol and alternariol monomethyl ether, and in other foodstuffs, such as grains and sunflower seeds (Scott, 2001). The only processed foods where its occurrence has been looked for are tomato products (Scott, 2001) – up to 111  $\mu$ g/kg was found in Brazilian tomato pulp (da Motta and Soares, 2001) – and corn flakes (Aresta *et al.*, 2003). Tenuazonic acid could be determined in tomato paste at a level of 6  $\mu$ g/kg by GC-MS single ion monitoring of its trimethylsilyl ether, and this technique showed separation of L-tenuazonic acid from isomeric D-*allotenuazonic* acid. This isomer

forms from L-tenuazonic acid standard on storage, so its occurrence in a food is not unexpected; nothing is known about its toxicity.

For extraction of tenuazonic acid from a food it is preferable to have an acidic aqueous organic extraction solvent (Scott, 2001). Solid phase microextraction (SPME) was recently demonstrated (Aresta *et al.*, 2003). A commonly used clean-up procedure for tenuazonic acid is extraction into sodium bicarbonate solution followed by acidification. Since tenuazonic acid is not fluorescent, UV detection is necessary to determine it by reversed phase LC; 280 nm is the wavelength of choice and diode array detection (DAD) is a useful confirmation procedure. A metal ion chelating agent such as zinc sulfate should be added to the mobile phase to avoid peak tailing. Ion pair, anion exchange and ligand exchange LC have also been explored.

### 17.3 *Fusarium* toxins: chemical structure, toxicity, sources in foods and associated human health risks

Of the *Fusarium* toxins described below, the first six (moniliformin, wortmannin, fusaproliferin, beauvericin, fusarochromanone and fusarin C) as well as fusaric acid were recently reviewed in detail by Bryden *et al.* (2001). Zearalenone (Chapter 15), fumonisins (Chapter 16) and trichothecenes (Canady *et al.*, 2001a,b) are reviewed elsewhere.

#### 17.3.1 Moniliformin

Moniliformin is produced by many species of *Fusarium*, including *F. proliferatum* (originally misidentified as *F. moniliforme* – hence the name of the mycotoxin), *F. avenaceum* and *F. subglutinans* (Schütt *et al.*, 1998). It was characterized as the sodium or potassium salt of 3-hydroxycyclobut-3-ene-1,2-dione (Springer *et al.*, 1974). Moniliformin is toxic to several experimental animals and the main toxicological interest is its cardiotoxicity, an effect which has been shown in poultry by acute administration of purified moniliformin (Zhang and Li, 1989; Nagaraj *et al.*, 1996), in mink by intraperitoneal (i.p.) dosing of an extract of *F. fujikuroi* culture material (Morgan *et al.*, 1999), and in broiler chicks and pigs by chronic feeding of a diet containing *F. fujikuroi* culture material (Harvey *et al.*, 2001; Broomhead *et al.*, 2002). The latter diet is also immunosuppressive in chicks (Li *et al.*, 2000). While Zhang and Li (1989) considered that moniliformin might be a causal agent of Keshan Disease, an endemic heart disease occurring in humans in China, an epidemiological study by Yu *et al.* (1995) found no difference in moniliformin contamination of grains from areas with and without Keshan Disease. There have been other reports demonstrating that natural occurrence of moniliformin is widespread in other parts of the world (Sharman *et al.*, 1991); as a recent example, 15 % of maize samples analyzed in Austria contained over 50 µg/kg (Lew *et al.*, 2001). Also, in the USA, 50 % of food grade commercial maize samples were contaminated with 26–774 µg moniliformin/kg and 68 % of maize-based foods contained similar concentrations of moniliformin (Gutema *et*

*al.*, 2000). Up to 1340 µg/kg was found in maize-based foods in Switzerland (Noser *et al.*, 2001). It was stable at pH 4 when heated in aqueous buffer solution at 100–150 °C (Pineda-Valdes and Bullerman, 2000) so could survive processing into such foods as beer, which has not been surveyed for moniliformin. It also has moderate stability during baking, frying, roasting and other heat processing (Pineda-Valdes *et al.*, 2003). On the other hand, 97 % reduction in moniliformin concentration occurred during alkali cooking of maize (containing 1.4 mg/kg) in the tortilla making process (Pineda-Valdes *et al.*, 2002).

Reviews of analytical methods for moniliformin have been included in recent papers (Munimbazi and Bullerman, 1998; Kandler *et al.*, 2002). In one method, the toxin was extracted from maize with water containing 1 % tetrabutylammonium hydrogen sulfate, the ion-paired moniliformin was partitioned into dichloromethane, cleaned up on a strong anion exchange SPE column and determined by ion pair reversed phase LC with detection at 229 nm (determination limit 25 µg/kg (Munimbazi and Bullerman, 1998). Another method employed extraction with water alone, clean-up on cation exchange resin and direct injection into an ion chromatograph (limit of quantitation 120 µg/kg) (Kandler *et al.*, 2002). Ion pair LC with atmospheric pressure chemical ionization MS measured moniliformin down to 10 µg/kg maize extracted with acetonitrile–water and cleaned up on a C<sub>18</sub> SPE column (Sewram *et al.*, 1999a). These methods will allow further surveys for the occurrence of moniliformin in grain-based foods and assessments of human exposure. The latter should take into account co-occurrence of moniliformin and fumonisins (Munimbazi and Bullerman, 1998).

### 17.3.2 Fusaproliferin

Fusaproliferin (originally named proliferin) was first isolated from *Fusarium proliferatum* (Ritieni *et al.*, 1995) and later found to be produced by several other *Fusarium* species (Wu *et al.*, 2003). Structurally it is the sesquiterpene 18-[1-(acetoxymethyl)-2-methylethyl]-10,17-dihydroxy-3,7,11,15-tetramethylbicyclo[13.3.0]octadeca-*trans,trans,trans,cis*-2,6,11,17-tetraene-16-one (Santini *et al.*, 1996). It causes severe teratogenic effects in chicken embryos exposed to 5 mM and is toxic to brine shrimp larvae (*Artemia salina*) (Logrieco *et al.*, 1996, 1997; Ritieni *et al.*, 1997a) but has not so far been fed to animals. Using LC with UV detection at 261 nm, with confirmation by GC-MS, 9/22 samples of Italian maize were found to contain fusaproliferin at levels of 6–500 mg/kg (Ritieni *et al.*, 1997b). There are also reports of natural occurrence of fusaproliferin in maize grown in South Africa, Slovakia and Iowa, USA (Shephard *et al.*, 1999; Srobarova *et al.*, 2002; Wu *et al.*, 2003). Sensitive LC-MS procedures for its determination have been developed (Sewram *et al.*, 1999b); low µg/kg levels of fusaproliferin could, with no clean-up, be detected in naturally contaminated maize from Transkei by positive ion electrospray ionization LC-MS/MS (Sewram *et al.*, 1999b). Standard fusaproliferin should be stored frozen as it deacetylates at room temperature (Wu *et al.*, 2003). Deacetyl fusaproliferin is still toxic to brine shrimp larvae. A stability study of fusaproliferin in wheat at various temperatures (80–

240 °C) for 15–60 min showed that considerable amounts of fusaproliferin would survive baking (Ritieni *et al.*, 1999).

### 17.3.3 Beauvericin

Beauvericin was first isolated from *Beauveria bassiana*, which is an insect pathogen (Hamill *et al.*, 1969). It is a cyclodepsipeptide consisting of three *N*-methyl-L-phenylalanine and three D- $\alpha$ -hydroxyisovaleric acid moieties condensed by alternating *N*-methylated peptide and ester bonds to form a threefold symmetrical molecule. Like the enniatins, which are also cyclodepsipeptides, it acts as an ionophore for alkali metals. Its conformation in a polar solvent is different from that of the ion-complexed form and from its conformation in a non-polar solvent (Abu Khaled and Davies, 1982). Beauvericin was first isolated from *Fusarium* by Gupta *et al.* (1991). It is in fact produced by several *Fusarium* species (Logrieco *et al.*, 1998), including the common corn pathogens *Fusarium proliferatum* and *F. subglutinans*. Several publications relate to both beauvericin and fusaproliferin regarding production by fungi and natural occurrence (e.g. Logrieco *et al.*, 1997; Sewram *et al.*, 1999b; Shephard *et al.*, 1999). Beauvericin is very toxic to brine shrimp larvae (Hamill *et al.*, 1969); other biological activities include insecticidal properties, and toxicity to several human cell lines, causing apoptosis (Logrieco *et al.*, 1997). There are no reports of toxicity to experimental animals.

There is considerable evidence for co-occurrence of beauvericin with fusaproliferin in maize in Italy, South Africa and Slovakia (Logrieco *et al.*, 1996; Ritieni *et al.*, 1997b; Shephard *et al.*, 1999). Beauvericin has also been found in maize from Croatia, Poland, Austria, Switzerland, Peru and the USA (Shephard *et al.*, 1999; Noser *et al.*, 2001; Jurjevic *et al.*, 2002; Srobarova *et al.*, 2002) as well as in wheat affected by head blight in Finland (up to 3.5 mg/kg) (Logrieco *et al.*, 2002). As with fusaproliferin, co-occurrence with fumonisins has also been found. Up to 450  $\mu$ g/kg of beauvericin (together with moniliformin and fumonisins) was detected in human foods such as cornflakes and polenta in Switzerland (Noser *et al.*, 2001). It is important to consider any synergistic effects of these co-occurring mycotoxins when the possible human health hazard of beauvericin is eventually evaluated.

Maize can be analyzed for beauvericin after extraction with aqueous methanol or aqueous acetonitrile with omission of a hexane defatting step where considerable losses of beauvericin occur (Krska *et al.*, 1996). Clean-up by means of a silica SPE column (Krska *et al.*, 1996) or a Mycosep #224 column (Josephs *et al.*, 1999) and LC with UV detection at 192 nm or 209 nm resulted in methods with detection limits of about 50  $\mu$ g/kg. Jurjevic *et al.* (2002) were able to achieve a limit of detection of 10  $\mu$ g/kg with the SPE method. Using LC-positive ion electrospray ionization MS the detection limit was 0.5  $\mu$ g/kg for maize culture material extracted with methanol and not cleaned up (Sewram *et al.*, 1999b). Beauvericin has also been detected by LC-thermospray MS (Thakur and Smith, 1997).

### 17.3.4 Wortmannin

Wortmannin (H-1) is a mycotoxin produced by *Fusarium oxysporum*, *F. avenaceum*, *F. sambucinum* and other fungi (Abbas and Mirocha, 1988; Mirocha and Abbas, 1989; Xu and Mirocha, 1994). It has the sterol-like chemical structure 1,6b $\beta$ ,7,8,9a,10,11,11b-octahydro-11-hydroxy-1-methoxymethyl)-9ab,11b $\beta$ -dimethyl-3H-furo[4,3,2-*de*]indeno[4,5-*h*][2]benzopyran-3,6,9-trione acetate. Mirocha and Abbas (1989) suggested the possibility that it is the same as sporofusarin, a *Fusarium* steroid associated with human alimentary toxic aleukia in the USSR in the 1940s. The toxicological target organ of wortmannin in the rat is the heart, causing myocardial hemorrhage at an oral dose of 4 mg/kg bw, but it also causes hemorrhage in other organs. Wortmannin or *F. oxysporum* culture material had immunosuppressive effects in rats, mice and avian species (Gunther *et al.*, 1989, Xu and Mirocha, 1994). LC and GC methods for analysis of maize kernels have been developed with respective detection limits of 2.5 and 25  $\mu$ g/kg (Xu and Mirocha, 1994); LC detection was by UV at 250 nm and capillary GC detection was by flame ionization. There is no reported occurrence of wortmannin in agricultural commodities and no surveys have been carried out.

### 17.3.5 Apicidin

Apicidin is another hemorrhagic mycotoxin, produced by *Fusarium pallidoreseum* (*F. semitectum*) (Singh *et al.*, 1996) and by an unidentified *Fusarium* sp. isolated from soybean seeds (Park *et al.*, 1999). Its novel chemical structure is cyclo-{L-(2-amino-8-oxodecanoyl)-L-(*N*-methoxytryptophan)-L-isoleucyl-D-pipecolinyl}. When fed to rats it caused hemorrhages in the stomach, intestines and bladder. No analytical methods have been developed and its natural occurrence in agricultural commodities has not been reported.

### 17.3.6 Sambutoxin

Sambutoxin is 3-{5-methyl-6-[(*E*)-1,3,5-trimethylhept-1-enyl]tetrahydropyranyl}-5-(*p*-hydroxyphenyl)-1-methyl-4-hydroxy-2(1*H*)-pyridone, the only *Fusarium* toxin known with this type of structure. It was obtained from *Fusarium sambucinum* and *F. oxysporum* isolated from potato rot (Kim and Lee, 1994; Kim *et al.*, 1995). It too is a hemorrhagic mycotoxin, affecting the stomach and intestines of rats at an oral dose of 500 mg/kg diet. In the chicken embryo test it had an LD<sub>50</sub> of 30  $\mu$ g/egg. Detection in fungal cultures was by TLC and LC, with detection at 254 nm, but no foods or feeds have been analyzed.

### 17.3.7 Fusarochromanone

Fusarochromanone (TP-1) was first isolated from an Alaskan isolate of *Fusarium equiseti* (originally named *F. roseum* 'Graminearum') (Lee *et al.*, 1985) and structurally identified as 2,2-dimethyl-5-amino-6-(3'-amino-4'-hydroxybutyryl)-4-chromone (Pathre *et al.*, 1986). The mycotoxin causes tibial dyschondroplasia in

broiler chickens fed 37 and 75 mg/kg feed (Wu *et al.*, 1993). Tibial dyschondroplasia is a common skeletal abnormality in poultry. Both fusarochromanone and its 3'-*N*-acetate (TDP-2) (Xie *et al.*, 1989) are immunomodulatory and inhibited or increased bovine lymphocyte proliferation *in vitro* depending on the concentration (Minervini *et al.*, 1992). Other metabolites related to fusarochromanone, including four fatty acid esters of 3'-*N*-acetylfusarochromanone, have also been isolated from *F. equiseti* (Xie *et al.*, 1990, 1991a,b, 1995). Of human health interest, fusarochromanone may be involved in Kashin-Beck (Urov) disease, an endemic bone and joint deformation found in north China, Korea and Russia (Lee *et al.*, 1985; Xie *et al.*, 1989; Beardall and Miller, 1994); feeding *F. poae* cultures to young dogs reproduced bone necrosis observed in humans (Marasas *et al.*, 1984). Further work, such as carrying out this experiment with pure fusarochromanone, would appear to be warranted. Data on natural occurrence of fusarochromanone is limited to finding 4–59 µg/kg in 12 samples of poultry feed from two farms in Denmark where tibial dyschondroplasia was prevalent (Krogh *et al.*, 1989). Some maize and wheat samples from an area of China with Kashin-Beck disease were negative for fusarochromanone (< 1 µg/kg) (Luo *et al.*, 1994).

Fusarochromanone is a very fluorescent compound and readily analyzed by TLC and by reversed phase LC, with excitation at 384 nm and emission at 450 nm (Xie *et al.*, 1989; Xu *et al.*, 1994). The toxin can be extracted from maize and wheat with methanol-water-ammonia and cleaned up on a silica gel cartridge; using LC the method had a detection limit of 5 µg/kg (Xu *et al.*, 1994). Another LC-fluorescence method, with Florisil column clean-up, had a detection limit of 1 µg/kg (Luo *et al.*, 1994). GC-MS has been used for confirmation of identity of fusarochromanone (Krogh *et al.*, 1989). LC-continuous flow fast atom bombardment MS could detect 0.6 ng of fusarochromanone injected and was used to detect the toxin in maize at 50 µg/kg (Pawlosky and Mirocha, 1991). Immunochemical assay of fusarochromanone has also been reported (Yu and Chu, 1991); fractions eluted from TLC or LC were acetylated then determined by enzyme-linked immunosorbent assay (ELISA). Since the toxin was measured as diacetylfusarochromanone, total fusarochromanone and *N*-acetylfusarochromanone were determined. Detection limits were 5 µg/kg in barley and wheat by both TLC- and LC-ELISA and 20 µg/kg by ELISA alone.

### 17.3.8 Fusarin C

*Fusarium moniliforme* (*F. verticillioides*) produces fusarin C and other related fusarins (A, D, E, F and X) (Gelderblom *et al.*, 1988; Savard and Miller, 1992; Lu and Jeffrey, 1993). Earlier work on fusarin C was reviewed in 1989 (Farber and Scott, 1989). Fusarin C has received the most attention, as it is highly mutagenic after activation by S-9 mix. Metabolites FX and FZ (characterized as 1-hydroxyl derivatives) and PM<sub>1</sub>, obtained by treatment of fusarin C with the rat liver microsomal preparation, were also mutagenic (Gelderblom *et al.*, 1988; Zhu and Jeffrey, 1993). Structurally fusarin C consists of a polyene with all *trans* double bonds (2*E*, 4*E*, 6*E*, 8*E*, 10*E*) which is linked to a 2-pyrrolidone moiety (Gelderblom

*et al.*, 1984). UV irradiation produces 6Z, 8Z and 10Z isomers which have similar mutagenic activities to fusarin C; (8Z)-fusarin C is a natural product formed by *Gibberella fujikuroi* (perfect form of *F. moniliforme*) (Barrero *et al.*, 1991). Fusarin C is also produced by several other *Fusarium* species: *F. oxysporum*, *F. poae*, *F. sporotrichioides*, *F. acuminatum*, *F. crookwellense*, *F. dlamini* and *F. nygamai* (Golinski and Chelkowski, 1992; Cantalejo *et al.*, 1997). Fusarin C might be involved in human esophageal cancer in South Africa and China, but epidemiology is lacking. There is evidence for carcinogenicity of fusarin C. TLC-purified fusarin C (85 % pure) and non-purified fusarin C respectively caused esophageo-forestomach carcinomas in mice at total doses of 8–14 mg over 469–655 days and in rats at total doses of 294–537 mg over 531–814 days (Li *et al.*, 1990). Ninety five per cent pure fusarin C produced malignant transformation of rat esophageal cells (Lu *et al.*, 1991). It was also immunosuppressive in a mouse tumor cell line (Chen and Zhang, 1987).

Fusarin C is an unstable compound, even in the absence of heat (Farber and Scott, 1989; Jackson *et al.*, 1990). Almost complete loss occurred on heating in corn meal and wheat flour at 100 °C so it would not be expected to survive in baked foods. A study of a Chinese cooking process showed that after 30 minutes of steaming dumplings, only 11 % remained (Zhu and Jeffrey, 1992). So it is not surprising that little attention has been paid to the natural occurrence of fusarin C, although it has been found in *Fusarium* infected and 'healthy' maize samples in South Africa, maize screenings in the USA and maize in Linxian county, China (Farber and Scott, 1989).

Extraction of maize can be achieved with a variety of solvent mixtures, including methylene chloride-isopropanol and acetonitrile; clean-up by SPE on amino and silica columns has been used (Farber and Scott, 1989). Procedures for determination of fusarin C are mainly based on TLC (as a yellow spot), normal phase LC and reversed phase LC (Farber and Scott, 1989; Jackson *et al.*, 1990; Cantalejo *et al.*, 1996), although GC after trimethylsilylation has also been reported (Tseng *et al.*, 1990). Detection for normal phase LC is by UV at 365 nm and for reversed phase LC (C<sub>18</sub> column) the wavelength is 350 nm; of interest is the use of a C<sub>18</sub> column with a mobile phase of chloroform-methanol (19:1,v/v) (detection at 365 nm) (Tseng *et al.*, 1990). Overall analyses should be carried out under 'gold' fluorescent lighting to avoid photodecomposition; however, formation of isomers is unavoidable and the sum of peak areas for the isomers has been used as the peak area response for fusarin C (Jackson *et al.*, 1990).

### 17.3.9 Chlamydosporol

Chlamydosporol has been isolated from several *Fusarium* species, namely *F. chlamydosporum*, *F. acuminatum*, *F. culmorum* and *F. tricinctum* (Savard *et al.*, 1990; Grove and Hitchcock, 1991; Solfrizzo and Visconti, 1991; Abbas *et al.*, 1992; Shier and Abbas, 1992). It exists in solution as two epimers of 7,8-dihydro-7-hydroxy-4-methoxy- *trans*-7,8-dimethyl-2*H*,5*H*-pyrano[4,3-*b*]pyran-2-one

(Grove and Hitchcock, 1991). Related metabolites have been isolated and characterized (Solfrizzo *et al.*, 1994b). It is a moderately toxic compound, causing feed refusal and weight loss (or reduced weight gain) in rats at 500–2000 mg/kg diet (Abbas *et al.*, 1992) and is toxic to brine shrimp (Savard *et al.*, 1990). No evidence links chlamydosporol to any mycotoxicoses. Chlamydosporol is not itself fluorescent but forms an intense blue fluorescent spot on TLC when sprayed with concentrated hydrochloric or sulfuric acids (Shier and Abbas, 1992). The two chlamydosporol epimers are detected on LC by UV at 287 nm (Solfrizzo *et al.*, 1994b) and could be separated by reversed phase LC after solvent optimization (Solfrizzo *et al.*, 1994a). LC-MS and LC-MS/MS of the trimethylsilyl ether has also been used (Abbas *et al.*, 1992). Although there is no method for analysis of grains, a method for determination of chlamydosporol in maize cultures involving extraction with aqueous methanol, hexane defatting and silica gel column clean-up had a rather high detection limit of 1 mg/kg using LC (Solfrizzo and Visconti, 1991).

#### 17.3.10 4-Acetamido-2-buten-4-olide

The *Fusarium* metabolite commonly known as butenolide has been more or less forgotten in recent years. An X-ray structure determination revealed the presence of two enantiomers and the compound lacks optical rotation (Ružić-Toroš and Kojić-Prodić, 1982). It was originally obtained from *F. equiseti* in New Zealand (White, 1967), then from *F. tricinctum* associated with 'fescue foot' in cattle (Tookey *et al.*, 1972) and later from other *Fusarium* species (Suzuki *et al.*, 1981; Marasas *et al.*, 1984). Oral administration of 22 mg/kg bw daily to cattle resulted in necrosis in the tail (Tookey *et al.*, 1972). It also has weak acute oral toxicity to mice (LD<sub>50</sub> 275 mg/kg bw) but there was no sub-acute toxicity even at a daily dose of 50 % of the LD<sub>50</sub> (275 mg/kg bw) (Burmeister *et al.*, 1980). It does cause subsurface hemorrhaging in a rabbit skin bioassay (Yates *et al.*, 1968). Determination of butenolide was by TLC (Burkhardt *et al.*, 1968) and packed column GC with electron capture (EC) detection (Suzuki *et al.*, 1981). LC is not useful (Frisvad and Thrane, 1987). With GC, a method of analysis for grain was developed which used acetonitrile-5 % lead acetate for extraction and silica gel for clean-up; it had a detection limit of 10 µg/kg. A limited survey of 34 samples of wheat and barley in Japan showed an incidence of 32 % at levels ranging from 10–430 µg/kg (Yoshizawa, 1984).

#### 17.3.11 Equisetin

The principal toxic metabolite of a strain of *Fusarium equiseti* was equisetin, characterized as an *N*-methyltetramic acid linked by a carbonyl group to a bicyclic hydrocarbon existing in two conformations (Phillips *et al.*, 1989). It is an antibiotic with an intraperitoneal LD<sub>50</sub> in mice of 63 mg/kg (Burmeister *et al.*, 1974), is toxic to mammalian cells and binds to DNA (Phillips *et al.*, 1989). Equisetin and a homolog, phomasetin, with the opposite stereochemistry were isolated from

*F. heterosporum* (Singh *et al.*, 1998). Packed column GC with flame detection and TLC have been used for its analysis (Burmeister *et al.*, 1974).

### 17.3.12 Fusaric acid

Fusaric acid (5-*n*-butyl-2-pyridinecarboxylic acid) has been primarily regarded as a phytotoxin but is also a mycotoxin. First isolated from *Gibberella fujikuroi*, it is produced by several species of *Fusarium* (Claydon *et al.*, 1977; Marasas *et al.*, 1984). Fusaric acid caused vomiting in orally dosed pigs (200 mg/kg bw), together with neurochemical changes (Smith and MacDonald, 1991). Fusaric acid caused depression in weight gain of immature pigs when fed at 12–16 mg/kg in the diet with a relatively constant concentration of deoxynivalenol (vomitoxin) (2.2–2.5 mg/kg) (Smith *et al.*, 1997). When rats were dosed intraperitoneally with 100 mg/kg of fusaric acid, it altered brain and pineal neurotransmitters and made the rats lethargic (Porter *et al.*, 1995). Significant concentrations of fusaric acid were found in 41 out of 48 samples of swine feed, dry maize and high-moisture maize, wheat and barley (commodity mean concentrations 12–36 mg/kg) and it was recommended that analyses for fusaric acid should accompany determinations of trichothecenes such as vomitoxin in swine feeds (Smith and Sousadias, 1993). For this survey reversed phase LC was used to detect fusaric acid at 271 nm. Another, smaller, survey also found mg/kg levels in feeds by capillary GC-MS of fusaric acid as its trimethylsilyl derivative (Porter *et al.*, 1995). 9,10-Dehydrofusaric acid and the methyl esters of both metabolites were included in reversed phase LC analysis of *Fusarium* cultures (Amalfitano *et al.*, 2002). Other related metabolites (from *Fusarium moniliforme*) are 10,11-dihydroxyfusaric acid and 5-(3'-carboxypropyl)-2-pyridine-carboxylic acid in which the methyl group of fusaric acid is oxidized to a carboxyl group (Burmeister *et al.*, 1985). TLC has also been used for detection of fusaric acid in cultures (Paterson and Rutherford, 1991).

### 17.3.13 Fusariocins

Fusariocins A and C are mycotoxins produced by *Fusarium moniliforme* (Itô, 1979; Marasas *et al.*, 1984). Both are toxic by intraperitoneal injection in mice and have LD<sub>50</sub>s of 2.9 and 4.0 mg/kg, respectively. The chemical structure of fusariocin C has been established by X-ray crystallography as containing two tropolone rings (Itô *et al.*, 1981).

## 17.4 *Aspergillus* toxins: chemical structure, toxicity, sources in foods and associated human health risks

In view of the numerous toxins produced by *Aspergillus* spp. (Moss, 1977; Cole and Cox, 1981; Davis 1981; Scott, 1994; Reijula and Tuomi, 2003), only a few are selected for inclusion here.

### 17.4.1 Sterigmatocystin

Sterigmatocystin is of interest because of its structural relationship to aflatoxin – it contains the same 7,8-dihydrofuro[2,3-*b*]furan moiety, which for sterigmatocystin is fused to a hydroxy- and methoxy-substituted xanthone (Rodricks, 1969). The mycotoxin is a metabolite not only of *Aspergillus* spp. – including *A. versicolor*, *A. flavus*, *A. sydowi* and *A. nidulans* – but also of *Bipolaris*, *Chaetomium* and *Emericella* spp. (Davis, 1981; Terao, 1983). Its acute toxicity (which is weak), mutagenicity, cytotoxicity and carcinogenicity have been reviewed by Terao (1983). The structure of the adduct of sterigmatocystin at the N<sup>7</sup>-guanine of oligodeoxynucleotides has been determined (Gopalakrishnan *et al.*, 1992). Sterigmatocystin is much less hepatocarcinogenic than aflatoxin B<sub>1</sub>, estimates varying from 10-fold to 150-fold lower (Terao, 1983; Gopalakrishnan *et al.*, 1992; Scudamore *et al.*, 1996). A ‘no significant risk’ level for humans of 8 µg/kg bw per day was estimated by the State of California (Scudamore *et al.*, 1996).

The incidence of sterigmatocystin in foods and feeds analyzed in the UK, Canada, Brazil, Italy and Denmark appears to be low (Pande *et al.*, 1990; Scudamore *et al.*, 1996). However, surveys in China using ELISA found a higher incidence of sterigmatocystin in foods and feeds from areas with a high rate of liver and stomach cancer compared to areas with a low rate (45 % versus 15 % with levels > 20 µg/kg) and average levels of 20 µg/kg versus 12 µg/kg (Lou *et al.*, 1995). Sterigmatocystin has been found at low incidence in cheese contaminated with *A. versicolor* (Scott, 1989). Detection limits by chromatographic methods were usually of the order of 20 µg/kg; unlike aflatoxins, sterigmatocystin is not a very fluorescent compound and UV or post-column fluorescence with aluminum chloride is the means of LC detection. Aluminum chloride was also used as a spray reagent in two-dimensional TLC; this procedure enabled densitometric detection of 1 µg/kg of sterigmatocystin in dairy feed (Domagala *et al.*, 1997). With LC-positive ion atmospheric chemical ionization (APCI) MS detection limits were less than 2 µg/kg for maize and bread and about 4 µg/kg for cheese (Scudamore *et al.*, 1996); samples were extracted with methanol-4 % KCl solution and partitioned into methylene chloride or chloroform while for cheese a Florisil column clean-up was added. GC-MS of underivatized sterigmatocystin was also sensitive and 1 µg/kg was reported as the detection limit for grains (Salhab *et al.*, 1976). Competitive ELISA methods have been applied to analysis of barley, wheat and other foodstuffs (Morgan *et al.*, 1986; Lou *et al.*, 1995; Li *et al.*, 1996).

### 17.4.2 α-Cyclopiazonic acid

Cyclopiazonic acid is an indole tetramic acid produced by several species of *Aspergillus* and *Penicillium* spp. It is sub-acutely toxic to rats, swine, guinea pigs, dogs and poultry, affecting several organs (Voss *et al.*, 1990; Smith *et al.*, 1992). Although it is not known to be an animal carcinogen, cyclopiazonic acid is weakly mutagenic to *Salmonella typhimurium* with metabolic activation (Sorenson *et al.*, 1984). There is a possibility it was involved together with aflatoxin in Turkey X disease in the UK. Originally isolated from *P. cyclopium*, it is also a metabolite of

*P. camemberti* and has been detected in Camembert cheese (Le Bars, 1979). Recently a sensitive method for such cheese with a detection limit of 7 µg/kg was developed which employed solid-phase microextraction and LC (Zambonin *et al.*, 2001). The main importance of cyclopiazonic acid is as a metabolite of *A. flavus*, often as a co-metabolite with aflatoxins, and as a result the two mycotoxins have been found co-occurring naturally in maize and peanuts (Urano *et al.*, 1992). It was detected in kodo millet seed in India, where an association with poisoning of humans was suggested (Rao and Husain, 1985), in tomato products (da Motta and Soares, 2001) and in corn flakes (Aresta *et al.*, 2003).

Several types of LC methods have been published for determination of cyclopiazonic acid in foodstuffs (Lawrence and Scott, 2000; Dorner, 2002). UV absorption at 279–284 nm or 225 nm was used for detection. As with tenuazonic acid (see Section 17.2.3), ligand exchange with zinc acetate or zinc sulfate in the mobile phase was useful to improve peak shape on reversed phase LC, and overall methods for maize and peanuts had quantitation limits in the 50–100 µg/kg range. LC-ion trap electrospray MS/MS was used for analysis of milk for cyclopiazonic acid with a limit of quantitation of 4 ng/mL (Losito *et al.*, 2002); three out of 20 samples analyzed in Italy were found to contain the toxin at 4.5–8.3 ng/mL. Capillary electrophoresis has also been used for analysis of milk for cyclopiazonic acid (Prasongsidh *et al.*, 1998). The recent interest in milk analysis arises from research showing carry-over of the toxin in lactating ewes from feed (Dorner *et al.*, 1994). Carry-over into meat and eggs has also been shown experimentally (Norred *et al.*, 1988; Dorner *et al.*, 1994). Immunochemical methods for cyclopiazonic acid have been developed, both as an immunoaffinity column for clean-up (Yu *et al.*, 1998; Dorner, 2002) and ELISA procedures for its determination in maize, peanuts and mixed feed (Yu and Chu, 1998) which, in combination, resulted in sensitive methods of analysis with low µg/kg detection limits.

### 17.4.3 Flavoglaucin

Flavoglaucin is a common metabolite of the *Aspergillus glaucus* group of fungi, notably *A. chevalieri*; this group is known to include mycotoxigenic species. Flavoglaucin has a 1,4-hydroquinone structure with a 3-aldehyde group and C<sub>7</sub> aliphatic and methylbutenyl side-chains in the 2 and 5 positions. Little is known about its toxicology; it caused mild liver damage when injected i.p. (10 mg/kg bw) into rabbits (Nazar *et al.*, 1984) and was highly toxic to isolated rat hepatocytes (Kawai *et al.*, 1983).

### 17.4.4 Echinulin

Also produced by *Aspergillus chevalieri*, as well as other *Aspergillus* species, the indole alkaloid echinulin may be regarded as a mycotoxin as it caused damage to the liver and lung of rabbits after i.p. injection (10 mg/kg bw) (Ali *et al.*, 1989) and caused mice to refuse to drink water containing 90 mg/L of it (Vesonder *et al.*, 1988). It was found at a concentration of 8 mg/kg in a sample of feed refused by

swine that had too low a level of vomitoxin in it to account for this refusal (Vesonder *et al.*, 1988).

#### 17.4.5 Xanthocillin X and xanthoascins

Another mycotoxin from *Aspergillus chevalieri* is xanthocillin X (Coveney *et al.*, 1966), which was reported in earlier literature as a *Penicillium* antibiotic (Rothe, 1954). It has the chemical structure 1,2-di-(*p*-hydroxybenzylidene)-1,2-di-isocyanoethane and caused severe hemorrhage of the lungs in mice. A related isocyanide metabolite xanthoascins, from *A. candidus*, was hepatotoxic, cardiotoxic and teratogenic to experimental animals (Ohtsubo *et al.*, 1976; Takahashi *et al.*, 1976).

#### 17.4.6 Gliotoxin

Gliotoxin has rather a novel relationship with food in that one of the fungal species which produce it, *Aspergillus fumigatus*, is pathogenic to animals as well as being saprophytic on plants. Thus gliotoxin has been isolated from an infected cow's udder (Bauer *et al.*, 1989) and from lung tissue of infected turkeys (Richard *et al.*, 1996). Gliotoxin is immunosuppressive, is positive in the 'Rec' assay for genotoxicity, causes liver lesions in hamsters and is toxic to turkeys (Richard *et al.*, 1989, 1996). Structurally it is an epidithiodioxopiperazine and can be determined by LC with UV detection at 254 nm (Richard *et al.*, 1996); for TLC, the spray reagent is ethanolic silver nitrate.

#### 17.4.7 Other *Aspergillus* toxins

Many other mycotoxins produced by *Aspergillus* species have been described in the literature (Cole and Cox, 1981; Davis, 1981). Some – for example, fumitremorgens A and B, TR-2 toxin, verruculogen, viriditoxin, tryptoquivalines, kojic acid, fumigatins and spinulosins – are additional *A. fumigatus* mycotoxins (Moreau, 1982; Tepšič *et al.*, 1997). Other mycotoxins produced by *Aspergillus* spp. and not specifically referred to above include cytochalasins E and K and ascladiol from *A. clavatus* (Flannigan, 1986), malformins from *A. niger* (Kim *et al.*, 1993), xanthomegnin, viomellein, vioxanthin from *A. ochraceus* (Scudamore *et al.*, 1986; Medentsev and Akimenko, 1998) and terretonin, terrein and terreic acid from *A. terreus* (Springer *et al.*, 1979; Cole and Cox, 1981).

### 17.5 *Penicillium* toxins: chemical structure, toxicity, sources in foods and associated human health risks

Mycotoxins produced by *Penicillium* spp. have been reviewed (Scott, 1977; Scott, 1994; Abramson, 1997). Toxicogenic *Penicillium* species have been reviewed by Frisvad and Filtenborg (1989).

### 17.5.1 Citrinin

Citrinin was one of the earliest antibiotics to be discovered (in 1931) but it was never used because of its mammalian toxicity. A review on citrinin was published by Frank (1992). It is produced by many *Penicillium* spp. and several *Aspergillus* spp.; recently it was found to be a metabolite (monascidin A) of some *Monascus* spp. (Blanc *et al.*, 1995; Xu *et al.*, 1999). The chemical structure of citrinin is (3*R*,4*S*)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid. Although citrinin is an important mycotoxin, particularly targeting the kidney, there are no international regulations specifically for it in foods.

Nephrotoxicity was observed in mice, rats, guinea pigs, rabbits, poultry, dogs and pigs (Frank, 1992). Citrinin is also embryotoxic, teratogenic, and genotoxic in short-term tests. Benign renal adenomas were observed in rats fed citrinin for 40 weeks. Citrinin can occur in grains and coconut products, often co-occurring with ochratoxin A, and in some cereal products – up to 98 µg/kg in maize flour (Nishijima, 1984), although usually levels in grain foods have been reported as less than 1 µg/kg (Frank, 1992). The low levels in processed foods may be because citrinin decomposes on heating in the presence of water, forming characterized products citrinin H1 and citrinin H2 with higher and lower cytotoxicity than citrinin, respectively (Trivedi *et al.*, 1993; Hirota *et al.*, 2002). Apples with rotten spots can contain citrinin (co-occurring with patulin) but as citrinin is unstable in apple juice it is not likely to occur in this food (Scott, 1977; Martins *et al.*, 2002).

A recent concern, although not related to citrinin as a *Penicillium* toxin, is the presence of citrinin in food colourings traditionally made in Asia from rice fermented with *Monascus* spp. Up to 360 000 µg/kg of citrinin has been found in these products (Dietrich *et al.*, 1999; Sabater-Vilar *et al.*, 1999; Xu *et al.*, 1999). The usual procedure for determination of citrinin in foods is reversed phase LC-fluorescence, which is most sensitive with an acidic mobile phase (Dick *et al.*, 1988; Sabater-Vilar *et al.*, 1999); if an ion pairing reagent (tetrabutylammonium hydroxide) is used in a pH 5.5 mobile phase then an acidic post column reagent (apparent pH 2.4) is required (Franco *et al.*, 1996). Similar attention to the pH applies to normal phase LC by treatment of the silica gel column with pH 2.5 buffer solution (Zimmerli *et al.*, 1989). Citrinin has been determined in barley and in *Monascus* colourings by ELISA (Abramson *et al.*, 1996; Dietrich *et al.*, 1999). An immunoaffinity column has been developed for clean-up (Dietrich *et al.*, 1999); liquid–liquid partition using bicarbonate is the usual clean-up procedure (Zimmerli *et al.*, 1989). Except for those using TLC, methods are very sensitive, with a limit of detection or quantitation as low as 0.1 µg/kg in cereals (Dick *et al.*, 1988; Zimmerli *et al.*, 1989).

### 17.5.2 Penicillic acid

Penicillic acid was the first known mycotoxin to be isolated – from *Penicillium puberulum* in 1913 – and is now known to be produced by many *Penicillium* spp.

as well as by members of the *Aspergillus ochraceus* group (see review by Scott, 1977). Its structure is the lactone of 3-methoxy-5-methyl-4-oxo-2,5-hexadienoic acid. It has a variety of biological activities, including hepatotoxicity in experimental animals (Hayes *et al.*, 1977; Dierickx and De Beer, 1984). Interest in penicillic acid arose when it was found to produce malignant, transplantable tumours in rats and mice by subcutaneous injection (Dickens and Jones, 1961). No feeding studies to see if it is an oral carcinogen have been carried out and nor has it been shown to be mutagenic. However, a related metabolite from *A. ochraceus* – 5,6-dihydropenicillic acid – caused DNA damage in the *Drosophila* DNA-repair test (Obana *et al.*, 1995).

There is no additional evidence for natural occurrence of penicillic acid in foodstuffs since the earlier reports of its occurrence (up to 230 µg/kg) in commercial maize and beans (Scott, 1977); using a TLC method, an Indian survey of over 1000 samples of food grains was negative (Neelakantan *et al.*, 1978). This paucity of occurrence data may be due to its instability, e.g. it is reactive with glutathione (Dierickx and De Beer, 1984). GC methods, usually employing the trimethylsilyl derivative, have now been developed for analysis of a variety of foods for penicillic acid (Scott, 1993). LC methods for its determination in chicken tissues (Hanna *et al.*, 1981) and (together with other mycotoxins) in cocoa beans (Hurst *et al.*, 1987), a high performance TLC method for grains (Miguel and de Andres, 1987) and a two-dimensional TLC method for some foods of plant origin (Ehnert *et al.*, 1981) have also been published.

### 17.5.3 Roquefortine C

A metabolite of *Penicillium roqueforti* isolated in Japan as roquefortine C was found to be the same as roquefortine obtained in France from the same species of *Penicillium* and structurally characterized as 10b-(1,1-dimethyl-2-propenyl)-6,10b,11,11a-tetrahydro-3-(1*H*-imidazol-4-ylmethylene)-2*H*-pyrazin[1',2':1,5]-pyrrolo[2,3*b*]indole-1,4(3*H*,5*aH*)-dione with E configuration of the 3,12 double bond (Scott, 1984a). It is now known to be a metabolite of several other common *Penicillium* species (Reshetilova and Kozlovsky, 1990). There is some evidence for neurotoxic properties of roquefortine C (convulsive seizures, paralysis) but other experiments showed no toxicity in mice and sheep (Scott, 1984a; Bauer *et al.*, 1997, 2001). There are several reports (e.g. Boysen *et al.*, 2002; Naudé *et al.*, 2002; Young *et al.*, 2003) of its association with tremorgenic mycotoxicoses in dogs, although this may be just incidental as tremorgenicity of roquefortine C has not been demonstrated. Co-occurrence with penitrem A suggests that this is more likely to be the responsible mycotoxin (see Section 17.5.5). There is no information on the experimental toxicity of roquefortine to dogs. LC-MS/MS and MS/MS screening procedures were developed for roquefortine C and penitrem A in the stomach contents or vomit of dogs (Naudé *et al.*, 2002; Rundberget and Wilkins, 2002a). Roquefortine C also occurs in all samples of blue cheese consumed by humans, in a few cases in concentrations greater than 1 mg/kg (Finoli *et al.*, 2001). It has been determined by TLC and LC (with UV or electrochemical detection) in

blue cheese extracts; using on-line column-switching reversed phase LC, for which no clean-up was needed, the detection limit was 10 µg/kg (Noroozian *et al.*, 1998). Similarly reversed phase LC-MS and LC-MS/MS of a freeze-dried food mixture made clean-up unnecessary (Rundberget and Wilkins, 2002a). A structurally unrelated clavine alkaloid isofumigaclavine A (roquefortine A) is also produced by *P. roqueforti* and other *Penicillium* species; it was formed in homemade wine contaminated with *P. roqueforti* or *P. crustosum* before fermentation (Möller *et al.*, 1997).

#### 17.5.4 PR toxin

PR toxin is another mycotoxin produced by *Penicillium roqueforti*; it is acutely toxic to rodents, is mutagenic, inhibits protein and nucleic acid synthesis, and has DNA attacking ability in the *rec* assay (Scott, 1984b). There are indications that it is carcinogenic to rats by oral exposure (Polonelli *et al.*, 1982). Structurally PR toxin is an eremophilane derivative, namely 7-acetoxy-5,6-epoxy-3,5,6,7,8,8a-hexahydro-3',8,8a-trimethyl-3-oxospiro[naphthalene-2(1H),2'-oxirane]-3'-carboxaldehyde. Although PR toxin was not found in blue cheese (Engel and Prokopek, 1979; Siemens and Zawistowski, 1993) and in fact is unstable in blue cheese (Scott and Kanhere, 1979), the ammonia reaction product PR imine was found in 50/60 samples of blue cheese at low levels of 19–42 µg/kg (Siemens and Zawistowski, 1993). In mice, PR imine is reversibly converted to PR toxin so some potential hazard to humans is a possibility. PR toxin and PR imine were determined by reversed phase LC with UV (254 nm) detection (Siemens and Zawistowski, 1992, 1993). An antibody against PR toxin has been produced and used in developing a radioimmunoassay for detection of PR toxin in cheese (Wei and Chu, 1988). In addition to PR imine, PR amide is a degradation product of PR toxin in *P. roqueforti* culture (Chang *et al.*, 1993) and perhaps should also be looked for in blue cheese.

#### 17.5.5 Penitrem A

Originally isolated as tremortins A and B (Hou *et al.*, 1971), penitrems A and B are two of a group of six related tremorgenic mycotoxins, named penitrems A–F, produced by *Penicillium crustosum* (De Jesus *et al.*, 1983). Structurally they are complex indoloditerpenes with molecular formulae ranging from C<sub>37</sub>H<sub>45</sub>NO<sub>4</sub> (penitrem D) to C<sub>37</sub>H<sub>44</sub>NO<sub>6</sub>Cl (penitrem A). Several other related metabolites, including thomitrem A and E, have been obtained from *P. crustosum* (Yamaguchi *et al.*, 1993; Rundberget and Wilkins, 2002b) and from *Aspergillus sulphureus* (Laakso *et al.*, 1993). Penitrem A has received the most attention and is the most toxic of the penitrems. In low doses (0.024–0.025 mg/kg bw, i.v.) penitrem A caused sustained tremors in animals, including sheep, pigs and dogs (Peterson *et al.*, 1982). In both sheep and pigs, penitrem A was less potent than verruculogen (Peterson *et al.*, 1982). Dogs have been victims of poisonings by waste foods such as cream cheese and walnuts contaminated with *P. crustosum*. The probable cause

was penitrem A, as shown by finding the toxin in the dog's stomach contents, vomit or in the material that was eaten (see cases referred to in Section 17.5.3 in connection with roquefortine, as well as Richard and Arp, 1979, Richard *et al.*, 1981, and Hocking *et al.*, 1988).

Penitrem A was included in an MS/MS screening procedure for stomach contents or vomit (Braselton and Rumler, 1996). Reversed phase LC of penitrems has been carried out, with detection by UV, UV-DAD, MS, or MS/MS (Maes *et al.*, 1982; Musuki *et al.*, 1992; Rundberget and Wilkins, 2002a); substrates analyzed were fungal cultures and foods and the LC-MS/MS detection limit in analysis of a freeze-dried food mixture was 5 µg/kg (Rundberget and Wilkins, 2002a). Complete separation of penitrems A–F was obtained on a C<sub>8</sub> column with methanol–water (78:22, v/v) mobile phase (Maes *et al.*, 1982). Analysis for penitrem A has only been carried out on a case by case basis and no food surveys have been undertaken.

The penitrems are not the only tremorgenic mycotoxins and there exist many others isolated from *Penicillium* and *Aspergillus* species (aflatrem, paxilline, verruculogen, janthitrems, fumitremorgens, tryptoquivalines and related compounds, paspalinine, verrucosidin and territrems), from *Claviceps paspali*, including paspalitrems and paspalinine, and from *Acremonium lolii* (lolitrems) (Cole, 1981; Cole and Dorner, 1986; Moreau, 1990; Rowan, 1993).

### 17.5.6 Citreoviridin

Investigation of 'yellow rice' in Japan in the middle of the 20<sup>th</sup> century led to isolation of the mycotoxin citreoviridin (Ueno and Ueno, 1972). Yellow rice caused acute cardiac beri-beri in humans (Shoshin-Kakke), a neurotoxic disease which prevailed in eastern Asia for three centuries but disappeared after 1929. A crude extract from rice inoculated with *Penicillium toxicarium* (*P. citreoviride*) produced toxic symptoms in several animal species that closely resembled the human disease and citreoviridin had the same toxicity so is believed to be the responsible toxin. Administered to mice, cats and dogs, citreoviridin caused paralysis of the limbs, vomiting, convulsions, cardiovascular damage and respiratory arrest. The oral LD<sub>50</sub> in mice was 29 mg/kg bw. Chronic toxicity has not been studied, except for a teratogenicity study in rats which found maternal toxicity, skeletal retardation and developmental defects at daily gavage doses of 5–15 mg/kg bw (Morrissey and Vesonder, 1986).

Citreoviridin has been reviewed by Scott (1977) and by Abramson (1997). The structure consists of 4-methoxy-5-methyl-2-*H*-pyran-2-one substituted in the 6 position by a C<sub>8</sub> polyene side-chain terminating in a substituted tetrahydrofuran ring. Citreoviridin is produced by several other *Penicillium* species. It has been detected in moldy pecan fragments contaminated with *P. charlesii* (*P. fellutanum*) (Cole *et al.*, 1981) and in *Eupenicillium ochrosalmoneum* infected maize in the USA (Wicklow *et al.*, 1988). In the latter case, normal phase LC was used for determination of citreoviridin, with fluorescence excitation at 388 nm and detection limits of 10 ng for standard and 2 µg/kg maize for the overall method

(Stubblefield *et al.*, 1988); levels found in the maize ranged from 19–2790 µg/kg. Reversed phase LC with UV detection at 388 nm was used in an earlier investigation on infected maize (Wicklow and Cole, 1984) and the minimum detectable amount of standard was lower at 3–5 ng. UV-densitometric or fluorodensitometric TLC is a less sensitive procedure for determination of citreoviridin (Engel, 1977). Rather surprisingly, in view of the historical mycotoxicological importance of citreoviridin, there has been little further work on it in recent years.

### 17.5.7 Luteoskyrin and other mycotoxins from *Penicillium islandicum*

Many pigments – monomeric anthraquinones (e.g. islandicin), dimeric anthraquinones (e.g. (+)-skyrin), modified bianthraquinones (notably (–)-luteoskyrin, (–)-rubroskyrin, (–)-rugulosin and (+)-rugulosin), and a non-quinonoid red pigment erythrokyrin – have been isolated from *Penicillium islandicum* (Takeda *et al.*, 1973; Enomoto and Ueno, 1974; Scott, 1977). In addition, two toxic chlorine containing cyclic peptides, cyclochlorotine and islanditoxin, and the diastereoisomeric indole alkaloids rugulovasines A and B and their 8-chloro derivatives are products of *P. islandicum* (Enomoto and Ueno, 1974; Cole *et al.*, 1976; Ghosh and Ramgopal, 1980). Luteoskyrin, cyclochlorotine and (+)-rugulosin are potent hepatotoxins and have been shown to be carcinogenic in mice; in the case of luteoskyrin, 50 mg/kg in the diet caused liver tumours. Also several of the pigments, including luteoskyrin, (+)-rugulosin and rubroskyrin, are mutagens (Mori *et al.*, 1998). Luteoskyrin and (+)-rugulosin inhibit replication, transcription and DNA repair in bacteria, yeast and animal cells and form chelate complexes with nucleic acids (Bouhet and Pham Van Chuong, 1977). Thus it is important to have methods for detecting *P. islandicum* and/or its major toxins in foodstuffs. Monoclonal antibodies have been raised against *P. islandicum* and incorporated into ELISA and dipstick immunoassays to detect the fungus in naturally contaminated rice grains (Dewey *et al.*, 1990). TLC on oxalic acid impregnated silica gel plates had a detection limit (by fluorescence) of 500 µg luteoskyrin/kg rice (Doncheva and Tsanev, 1993). UV-visible diode array LC of luteoskyrin was included in a study of 182 standard mycotoxins (Frisvad and Thrane, 1987) but there has been no application of LC to analysis of agricultural commodities for luteoskyrin.

### 17.5.8 Secalonic acid D

Secalonic acids A–G are a group of *bis*-tetrahydroxanthone pigments isolated from *Claviceps purpurea*, *Aspergillus ochraceus*, *A. aculeatus*, *Phoma terrestris*, *Penicillium oxalicum* and/or *P. atramentosum* (Reddy *et al.*, 1979; Fujimoto *et al.*, 1983). Most toxicological studies have been with secalonic acid D. The toxin caused pulmonary injury and was teratogenic and fetotoxic in rats and mice (Reddy *et al.*, 1979, 1981b; Mayura *et al.*, 1982; Sorenson *et al.*, 1982) and caused neurobehavioural changes in suckling mice whose mothers were exposed both prenatally and postnatally (Bolon and St Omer, 1989). It was also weakly muta-

genic in mice (Reddy *et al.*, 1980). Secalonic acid D has been detected in maize dust obtained from grain storage elevators in New Orleans, Louisiana and Manhattan, Kansas at levels up to 4.5 mg/kg (Ehrlich *et al.*, 1982) but not in freshly harvested maize, even in samples heavily contaminated with *P. oxalicum* (Palmgren and Fleischhacker, 1987). These analyses were made by reversed phase LC using a procedure developed by Reddy *et al.* (1981a) for biological fluids with UV detection at 340 nm. The detection limit of the method used for analysis of maize dust was 10–40 µg/kg (Ehrlich and Lee, 1984; Ehrlich *et al.*, 1982).

### 17.5.9 Rubratoxin B

Rubratoxin B and rubratoxin A (dihydrorubratoxin B) are mycotoxins obtained from *Penicillium rubrum* and *P. purpurogenum* and they have been reviewed by Richmond *et al.* (1980) and by Davis and Richard (1984). Most research has been carried out with rubratoxin B as rubratoxin A is only a minor metabolite. In 1957 (before the discovery of aflatoxins) it had been found that maize infected with *P. rubrum* caused death of swine in the southeastern USA and rubratoxins were isolated from cultures of the fungus. Rubratoxin B is acutely toxic in various animal species, causing kidney and liver damage (Hayes *et al.*, 1977). Most studies used intraperitoneal injection as the route of administration, including a sub-acute study in Syrian golden hamsters over a one week period (Engelhardt *et al.*, 1987). The mycotoxin is teratogenic and fetotoxic in mice (Davis and Richard, 1984).

Rubratoxins A and B can be separated by reversed phase LC (Davis and Richard, 1984) with detection at 254 nm. Normal phase LC of rubratoxin B was incorporated into a method for analysis of mixed feed, which was first frozen to minimize decomposition of the toxin and then extracted with ethyl acetate–acetic acid (95:5); various feed ingredients were also analyzed by this method (Engstrom and Richard, 1981). Details of TLC procedures for rubratoxin B were described by Hayes and McCain (1975) but TLC did not give a sensitive method for analysis of maize. Rubratoxin B has been included in multimycotoxin TLC procedures for grain analysis (Whidden *et al.*, 1980; Grabarkiewicz-Szcześna *et al.*, 1985) and a multimycotoxin reversed phase LC procedure for standards (Hill *et al.*, 1984). Anti-rubratoxin antibody has been produced and a radioimmunoassay for rubratoxin B developed (Davis and Stone, 1979). There appears to be only one report of natural occurrence of rubratoxin B – in an Indian sample of wheat at a concentration of 245 µg/kg (Pande *et al.*, 1990).

### 17.5.10 Other *Penicillium* toxins

There is a long list of other *Penicillium* mycotoxins (Scott, 1977, 1994; Abramson, 1997); some of these, such as kojic acid, xanthomegnin, viomellein, vioxanthin and several of the tremorgenic toxins, are also *Aspergillus* toxins. β-Nitropropionic acid is described below with reference to *Arthrimum*.

## 17.6 $\beta$ -Nitropropionic acid from *Arthrinium* species: chemical structure, toxicity, sources in foods and associated human health risks

A safety assessment of  $\beta$ -nitropropionic acid (3-nitropropionic acid) was recently published (Burdock *et al.*, 2001). An acceptable daily intake of 25  $\mu\text{g}/\text{kg}$  bw per day for humans was calculated based on chronic rodent studies and a safety factor of 100.  $\beta$ -Nitropropionic acid was not carcinogenic or mutagenic. The primary toxic feature is pathological change in the brain. In addition to being present in some plants, it is a metabolite of several *Aspergillus* and *Penicillium* species, including starter cultures for such foods as katsuobushi, miso and shoyu.  $\beta$ -Nitropropionic acid consumption in Japan from miso and soy sauce was estimated to be 5.5 mg/day. It should be considered as an *Arthrinium* mycotoxin as there is considerable evidence that it is responsible for numerous acute poisonings in China due to eating improperly stored sugarcane infected with *Arthrinium*. During 1972–1989 there were 217 outbreaks of this poisoning, with 884 patients and 88 deaths (Ludolph *et al.*, 1991; Liu *et al.*, 1992). Victims were mostly children and young people and the main clinical symptoms included vomiting, dystonia and coma. The highest concentration of  $\beta$ -nitropropionic acid in a moldy sugarcane sample which caused a human poisoning was 6600 mg/kg (Liu *et al.*, 1992). Methods of analysis were reviewed by Burdock *et al.* (2001); they use TLC, GC and reversed phase LC for the determination. GC of the pentafluorobenzyl derivative of  $\beta$ -nitropropionic acid has been applied to food and has a limit of detection for cheese of 3 mg/kg but no  $\beta$ -nitropropionic acid was detected in 12 cheese samples analyzed, most of which were mold ripened (Gilbert *et al.*, 1977).

## 17.7 Future trends

It is apparent from this review that research on mycotoxins has developed either in response to mycotoxicosis in animals (or in some cases in humans) or because of important toxicology found experimentally for a fungal metabolite. Most of the present day effort is being devoted to the mycotoxins described in Chapters 13–16, but it is evident that there are gaps in our knowledge of the other known mycotoxins (and new mycotoxins will continue to be discovered). Thus for *Alternaria* toxins, research gaps or needs include development of immunoaffinity columns for clean-up of food extracts and beverages as well as ELISAs in order to carry out surveys of foods and obtain an estimate of human exposure to *Alternaria* toxins; additional toxicological work with purified *Alternaria* toxins, such as sub-acute toxicity and cancer studies, are also required. For the *Fusarium* toxins, attention should be paid to co-occurrence of moniliformin, fusaproliferin and beauvericin with fumonisins and possible synergistic toxicological effects; surveys to check for the hemorrhagic toxins wortmannin, apicidin and sambutoxin in foods and feeds should be conducted; further surveys for occurrence of fusarochromanone would be desirable; and improved analytical methods for

chlamydosporel in grains are needed. Transmission studies of cyclopiazonic acid in cows have yet to be done as have further surveys for this mycotoxin in milk and other dairy products. The continuing reports of neuromycotoxicoses in dogs attributed to penitrem A in waste food point to the need for experimental toxicological studies on this tremorgen (as well as on roquefortine C) in dogs. Exposure of humans and animals to 'forgotten' mycotoxins such as citreoviridin and luteoskyrin needs to be assessed by surveys; there is no LC method for determination of luteoskyrin in grains. The foregoing are examples of topics concerning 'other mycotoxins' that should be addressed in the future.

## 17.8 References

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**Part IV**

**Appendix**

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## Control of mycotoxins: raw material production

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### 18.1 Introduction

Mycotoxins found in the field are mainly associated with infections caused by *Fusarium* species, particularly on cereal crops. The mycotoxins linked with fusarium infections of cereals include fumonisins, trichothecenes, zearalenone, enniatins and moniliformin. Of these the trichothecenes are probably regarded as the most important group, believed to be responsible for a variety of diseases in both man and domestic animals. The trichothecene group is made up of over 150 toxins; however, the toxins most commonly associated with the group are deoxynivalenol (DON), nivalenol (NIV), diacetoxyscirpenol, T-2 toxin and HT-2 toxin. The biochemical basis of trichothecene toxicity is non-competitive inhibition of protein synthesis. The point of attack for individual toxins varies and this accounts for the differences in toxicity.

Worldwide plant diseases caused by *Fusarium* species are varied, with *Fusarium* head blight (FHB) probably being the most economically important. As a result it is the control of this disease and associated mycotoxins that will be covered in this chapter. FHB is a potentially devastating fungal disease of small grain cereals such as wheat and barley, whose significance has increased in recent years as more and more countries introduce mycotoxin legislation for food and feedstuffs. The major pathogens associated with FHB are *Fusarium culmorum*, *F. graminearum* (teleomorph: *Gibberella zeae*), *F. avenaceum*, *F. poae* and *Microdochium nivale*

(formerly *F. nivale*), although a number of other species, including, *F. cerealis*, *F. tricinctum* and *F. sporotrichioides* have also been associated with the disease. The predominant species associated with the disease varies between regions of the world with *F. graminearum* predominant in hotter regions and *F. culmorum*, *F. poae* and *M. nivale* in cooler maritime regions (Parry *et al.*, 1995). However, results from surveys looking at the pathogens responsible for FHB in The Netherlands and the UK have shown that *F. graminearum* is replacing *F. culmorum* as the predominant toxigenic *Fusarium* spp. involved in the FHB complex (Daamen *et al.*, 1991; De Nijs *et al.*, 1996; Waalwijk *et al.*, 2003; Jennings *et al.*, 2004). Of the major pathogens involved in FHB, all but *M. nivale* are capable of producing mycotoxins.

The most susceptible stage for infection of a crop by FHB pathogens is during crop flowering. Spores alight on the ear as a result of splash or wind dispersal from crop debris in the soil or weed hosts found in the field or at the field margins. Optimal conditions for disease development are warm, wet and humid conditions for a period of 24–48 hours after spores land on the ear. Trichothecene toxins, in particular DON and NIV, are phytotoxic to plant tissue and, as such, are primarily thought to be produced during the initial stages of infection to aid the entry of the fungus into the plant.

There are several routes by which FHB infection and the subsequent contamination of grain by mycotoxins may be controlled. These include the use of resistant cultivars, fungicides, biological control and cultural practices. The use of resistant cultivars is potentially the best approach for control as this would prevent disease development in the plant no matter how large the inoculum potential for the disease. Cultural practices focus on reducing the level of inoculum in the field through measures such as crop rotation and the removal or ploughing-in of crop debris. Fungicides and biological control agents are both protective/curative methods which work through eradication of pathogens as they arrive on the crop or by reducing inoculum in the soil and on crop debris. The following sections of this chapter discuss further the role of each method in the control of FHB and mycotoxin contamination of grain. However, as will become apparent, no one control method is one hundred per cent effective against FHB and so an integrated approach to crop protection against FHB and mycotoxin contamination should always be considered.

## 18.2 Resistance breeding

### 18.2.1 Origins of resistance

Large-scale FHB disease trials consisting of hundreds of wheat varieties of diverse geographic origin have confirmed that, while there is significant genotypic variation for resistance (Bai and Shaner, 1994; Buerstmayr *et al.*, 1996; Yong-Fang *et al.*, 1997), complete immunity has yet to be found. In general, tetraploid durum wheats appear to be more susceptible than bread wheats and, as yet, no source of durum resistance has been found (Miedaner, 1997; Gilbert and Tekauz, 2000).

In terms of their prevalence in bread wheat breeding programmes worldwide,

Sumai-3 (China), Frontana (Brazil) and Nobeokabozu Komugi (Japan) are the most widely used sources of resistance (Miedaner, 1997; Gilbert and Tekauz, 2000; Chen *et al.*, 2001a). However, the dependence of plant breeders on such a narrow genetic base may place an undue selection pressure on the genes responsible, so novel sources need to be identified to protect against, or prevent, a potential breakdown in resistance.

### 18.2.2 Introgression of resistance from wide crosses

Fédak *et al.* (1997) and Chen *et al.* (2001b) attempted to identify additional resistance genes in wild relatives for introgression into adapted germplasm. Alternative sources included Brazilian rye land-races, and a number of wheat and durum addition and hybrid lines involving rye, *Dasyphyrum villosum*, *Hordeum californicum*, *Thinopyrum intermedium* and *T. distichum*. Attempts have also been made to introduce FHB resistance into highly susceptible tetraploid durum wheats using a Sumai-3 pentaploid F<sub>2</sub> population (Gilbert *et al.*, 1997; Stack *et al.*, 1999). However, for reasons that are unclear, these attempts have so far been unsuccessful.

### 18.2.3 Species and race specificity of resistance

In common with other members of the *Fusarium* genus, *F. graminearum* and *F. culmorum* have a wide host range and no host specialization has been detected among strains of these two pathogens (Cook, 1981a; Miedaner *et al.*, 1996). However, some studies have detected limited significant host genotype/fungal isolate interactions for *F. culmorum* and *F. graminearum* infections in wheat (Mesterhazy, 1988; Snijders and van Eeuwijk, 1991), but these were not found to be stable over years.

The lack of evidence for host/pathogen structures in *F. culmorum* and *F. graminearum* epidemics does, therefore, suggest that a single, aggressive fungal isolate should be adequate for resistance phenotyping (Miedaner, 1997). However, Mesterhazy (1988) proposed that the use of several fungal strains of varying aggressiveness in parallel may stabilize the effect of environment.

### 18.2.4 Stability of resistance

Many studies of FHB resistance over the years have identified a highly significant environmental component to total variance (Mesterhazy, 1995; Mesterhazy *et al.*, 1999; Buerstmayr *et al.*, 2000) which is generally attributed to differences in weather conditions. In view of this, it is desirable that evaluation of cultivars should be carried out over several years (Mesterhazy, 1987, 1995). However, the value of FHB testing in different environments, thought to be important by Scott (1927) and Cook (1981a), has been questioned. Liu and Wang (1991) suggested that FHB trials should be carried out under controlled environment conditions in order to reduce the influence of environmental factors.

### 18.2.5 Components of genetic resistance

A model of host resistance to FHB consisting of two components (type I and type II) was first proposed by Schroeder and Christensen (1963). Type I resistance is that which is active against initial infection and type II operates against fungal colonization of the wheat spike. However, subsequent work has hypothesized four further components: type III is the ability of hosts to degrade DON (Miller and Arnison, 1986); type IV is tolerance of high mycotoxin concentrations (Wang and Miller, 1988); type V is resistance to kernel infection; and type VI is tolerance of yield, whereby yields are maintained despite the presence of visual disease (Mesterhazy, 1995; Mesterhazy *et al.*, 1999). The postulated relationships between the characteristics of FHB infection and the morphological, developmental and physiological factors, which may underlie the above components, are summarized in Ban and Svenaga (2000).

### 18.2.6 Passive resistance

A number of aspects of host life-cycle and morphology have been identified which may enhance resistance to FHB in cereals (Parry *et al.*, 1995; Miedaner, 1997). Several authors have observed that early flowering varieties tend to be more resistant (Hanson *et al.*, 1950; Cook, 1981b; Boosalis *et al.*, 1983). The presence of awns has also been reported to enhance infection by FHB in natural epidemics, and a negative correlation between plant height and FHB infection has been reported in both wheat (Mesterhazy, 1995; Miedaner, 1997; Buerstmayr, 2000) and barley (Steffensen, 1998; Zhu *et al.*, 1999). Although variation for FHB resistance associated with aspects of plant morphology is generally thought to be the result of environmental interaction (Anderson, 1986; Mesterhazy, 1995), quantitative trait loci (QTL) analysis suggests that certain components of resistance are associated with morphological traits in some instances. A field-based study of FHB resistance in barley by Zhu *et al.* (1999) indicated that QTL positions for most of the resistance traits were coincident with QTL for morphological characters. In wheat, Somers *et al.* (2003) detected coincident QTL for plant height and DON content of grain on chromosome 2D of Maringa, and Ban and Svenaga (2000) reported linkage in repulsion between the *B1* awning suppressor gene and a resistance QTL on the long arm of chromosome 5A in Sumai-3. However, none of the data from these studies were able to resolve if there was a true pleiotropic effect of morphology on FHB resistance or if there were linked genes controlling the traits independently.

### 18.2.7 QTL for resistance breeding

As discussed above, inheritance of host resistance to FHB is quantitative (van Ginkel *et al.*, 1996; Miedaner, 1997; Buerstmayr *et al.*, 1999) and influenced significantly by prevailing weather conditions (Mesterhazy, 1995; Mesterhazy *et al.*, 1999). Consequently, genotype differences tend to be inconsistent between years (van Eeuwijk *et al.*, 1995). Therefore, it is generally accepted that evaluation of cultivars should be carried out at several trial sites over a period of several years

(Cook, 1981a; Mesterhazy, 1987). However, the costs associated with such trials can be considerable and may hinder efforts to breed varieties with improved FHB resistance (Campbell and Lipps, 1998). In view of the above difficulties, the development of molecular marker assisted selection (MAS) for FHB resistance within breeding programmes is highly desirable.

There have, in recent years, been a large number of studies to identify QTL associated with FHB resistance in the spring wheat cultivar Sumai-3 and its derivatives. Depending on the cross, major QTL explaining up to 60 % of the phenotypic variation for type II resistance have been detected on 3BS in several studies (Waldron *et al.*, 1999; Anderson *et al.*, 2001; del Blanco *et al.*, 2003). Smaller effect QTL explaining up to 20 % of phenotypic variation for type I and type II resistance have also been detected on 5A (Buerstmayr *et al.*, 2003) and 6B (Anderson *et al.*, 2001). QTL analyses have also been carried out on alternatives to Sumai-3-based resistance. Four QTL on chromosomes 1B, 3A, 3D and 5A together accounted for 32.7 % of the phenotypic variation for resistance in the Romanian spring wheat cultivar Fundulea (Shen *et al.*, 2003). Gervais *et al.* (2003) detected two stable QTL on 2B and 5A which, together with other minor QTL, explained between 30–45 % of the total variance in the European winter wheat variety Renan. Unpublished studies of resistance in other winter wheat materials have detected a stable QTL for resistance on 4A in the hexaploid wild wheat *Triticum macha* (Steed *et al.*, 2002) and on 1B and 2D in the North American breeding line WEK0609 (Gosman *et al.*, 2004).

The analyses reviewed above confirm that resistance to FHB is polygenic, conditioned by several QTL of relatively minor effect dispersed across the genome. However, plant breeders should, with the help of MAS, be able to pyramid QTL, preferably from several different germplasm sources, to achieve significant levels of FHB resistance in their breeding material.

## 18.3 Fungicides

Fungicides have been used successfully to control many diseases since their introduction in the late 1800s. However, their effectiveness against FHB has been inconsistent. In many instances this inconsistency may be attributed to incorrect application of the fungicide by the user. However, even when applied optimally the best products currently available are still likely to be only 60–70 % effective. FHB is a complex disease in terms of the pathogens involved, inoculum sources and toxins produced and, as such, it is a complex disease to control with fungicides. To achieve optimal control of FHB pathogens and their associated mycotoxins there are several areas where important decisions have to be made; these include product choice, application rate and application timing.

### 18.3.1 Product choice

As previously indicated, FHB symptoms can result from infection caused by either one species or a complex of different species on the ear. As a consequence, for a

single product to be completely effective it must control all pathogens involved in the disease complex. Most currently registered products with activity against FHB are active against one or more FHB pathogens involved in the complex but are less effective or completely ineffective against the remaining pathogen or pathogens. In most cases this difference in activity of an active ingredient to FHB pathogens is between the true fusaria and *M. nivale*, i.e. between toxin producing and non-producing species. So product choice is of particular importance in the control of grain contamination by mycotoxins.

Trials which demonstrate differential control of FHB pathogens have been carried out by Jennings *et al.* (2000). In a series of trials inoculated with a mixed conidial suspension of FHB pathogens at mid-anthesis, the demethylation inhibiting (DMI) fungicide tebuconazole (as Folicur®) effectively controlled the toxigenic *Fusarium* species present on the ear, but showed little control of the non-toxicogenic *M. nivale*. Conversely, application of the strobilurin fungicide, azoxystrobin (as Amistar®), controlled *M. nivale* but not the *Fusarium* species present. The differences in activity between tebuconazole and azoxystrobin has also been reported in other trials naturally infected by FHB pathogens (Simpson *et al.*, 2001). Differential control of fusaria and *M. nivale* also exists within the methyl benzimidazol carbamate (MBC) fungicide group; however, this has arisen through the widespread development of resistance in populations of *M. nivale* (Locke *et al.*, 1987; Pettitt *et al.*, 1993).

Other active ingredients which have shown consistently good control of the toxin-producing species involved in the FHB complex come primarily from the same fungicide group as tebuconazole and include metconazole [as Caramba® (Dardis and Walsh, 2000; Jennings *et al.*, 2000; Siranidou and Buchenauer, 2001)], epoxiconazole (as Opus®) prochloraz [as Sportak® 45 (Matthies and Buchenauer, 2000)], and propiconazole (Martin and Johnson, 1982). Active ingredients with differing chemistry from the DMIs have also significantly reduced toxin production, e.g. carbendazim [as Bavistin DF (Cromey *et al.*, 2001)] and benomyl [as Benelate 50WP (Jones, 2000)].

Several *in vitro* experiments have indicated that the use of fungicides may lead to increased mycotoxin production by *Fusarium* species (Moss and Frank 1985; Matthies and Buchenbauer, 1996; Matthies *et al.*, 1999; D'Mello *et al.*, 1998). Following field application of fungicide, a reduction in disease symptoms generally results in reduced DON contamination of grain compared to control plots. However, instances of increased DON contamination of grain have been reported following the application of azoxystrobin (Hart and Ward, 1997; Jennings *et al.*, 2000; Simpson *et al.*, 2001; Siranidou and Buchenauer, 2001). However, not all applications of azoxystrobin resulted in increased DON levels (Jennings *et al.*, 2000; Jones, 2000; Matthies *et al.*, 2000; Cromey *et al.*, 2001). In a series of trials carried out in 1998 and 1999, Jennings *et al.* (2000) showed that, following the application of azoxystrobin, DON levels in grain increased in 1998 but not in 1999. Polymerase chain reaction (PCR) analysis of the grain indicated that the major difference between the two years was make up of FHB pathogens on the ear. In 1999 only *Fusarium* species were detected on control plots, with *F. culmorum*

being the predominant pathogen. However, in 1998 a combination of *M. nivale* and *Fusarium* species were detected, again with *F. culmorum* the predominant pathogen. This suggested that the combination of FHB pathogens present on the ear was the determining factor for increased DON levels in grain following applications of azoxystrobin. Where a mixed population of *M. nivale* and *F. culmorum* existed, the removal of *M. nivale* by azoxystrobin opened a niche for *F. culmorum* to colonize, resulting in increased mycotoxin levels in grain. Application of azoxystrobin where *M. nivale* was absent did not affect the status quo of the FHB pathogens and hence no change in toxin levels occurred.

Similar increases in DON have also been seen from 'on-farm' data. In 1998, a survey carried out on UK wheat grain indicated that the predominant pathogen was *M. nivale*, with low levels of *Fusaria* also present (Turner *et al.*, 1999). Analysis of data on fungicide application, mycotoxin and FHB pathogen levels from the surveyed fields indicated that, on average fields where azoxystrobin had been applied as an ear application had levels of DON and NIV than those where no fungicide had been applied to the ear.

Increase in NIV production following fungicide application in the field has also been reported by Gareis and Ceynowa (1994). Application of tebuconazole/triadimenol (as Matador) to wheat ears either 3 hours before or 24 hours after inoculation with *F. culmorum* resulted in a considerable decrease in the incidence of FHB but a 16- and 6-fold increase in NIV, respectively, compared to the controls.

### 18.3.2 Timing of fungicide application

Arguably the timing of fungicide application is more important than product choice when trying to control FHB and mycotoxin contamination of grain; no matter how effective the fungicide, if it is applied at the wrong time it will not control FHB. Mid-anthesis is the most susceptible time for infection of wheat by FHB pathogens (Sutton, 1982) and, as such, is the most appropriate time to apply a fungicide spray aimed at controlling FHB. Work carried out by both Homdork *et al.* (2000) and Matthies and Buchenauer (2000) has highlighted just how narrow the window for fungicide application is for optimum control of FHB pathogens.

Matthies and Buchenauer (2000) investigated the effect of timing of fungicide application on disease development using trials artificially inoculated with *F. culmorum* at mid-anthesis. Fungicide treatments applied were tebuconazole or prochloraz at eight days pre and two or nine days post inoculation. The most effective treatment timing for both fungicides was two days post inoculation. The efficacy of the fungicide treatments decreased with increasing time interval between fungicide application and inoculation. A similar set of experiments carried out by Homdork *et al.* (2000) using tebuconazole sprayed at either three days pre and/or five days post inoculation again showed that the closer together the timing of the spray and inoculation, the better the efficacy of the fungicide.

Some reports of fungicide failure in the field can be directly attributed to

incorrect timing of application. Milus and Parsons (1994) concluded after testing the efficacy of seven fungicides against *F. graminearum* that the prospects for chemical control of head blight were poor. Fungicides were applied to plots at the end of heading growth stage. To each fungicide treated plot *F. graminearum* was inoculated three times, at the beginning, mid and end of anthesis (this equated to two, five and seven days post fungicide application). The results showed no reduction in levels of either head blight or DON following fungicide treatment. However, as already highlighted, inoculum landing on the ear seven days post fungicide treatment would not be effectively controlled. A more appropriate growth stage for the fungicide application would have been mid-anthesis. At this growth stage the time interval between fungicide application and inoculum arrival at the ear would be at its optimum at between two and three days.

### 18.3.3 Application rate

To achieve the optimum efficacy against FHB pathogens, a fungicide must be applied at the manufacturer's recommended rate. Work carried out by Nicholson *et al.* (2003) showed that halving the rate of several fungicides led to significant reductions in control of FHB disease levels and mycotoxin production.

With any fungicide application there is always the potential for the development of resistance within a population, as has been the case with *M. nivale* to the MBC fungicides. To date there have been no reports of fusarium populations developing resistance to fungicide. However, the inappropriate use of fungicide and previous experience suggest this is likely to occur sooner rather than later. Results from *in vitro* investigations (D'Mello *et al.*, 1998) indicated that more persistent patterns of toxin production may also develop in fusarium populations showing resistance to fungicides.

Agrochemical companies are addressing the problem of fungicide activity against FHB, and improved active ingredients are continually being tested. One way to improve the efficacy of a fungicide would be to prevent the FHB pathogen from gaining a quick entry inside the glume. Mycotoxins, such as DON, are thought to be virulence factors, which aid penetration by the fungus (Proctor *et al.*, 1995). The development of inhibitors of trichothecene biosynthesis to restrict penetration, combined with a fungicide, may help future development of fungicide use. At present, the challenge of controlling FHB and mycotoxin contamination of wheat grain can only be met through an integrated approach to crop protection. The use of fungicides forms an essential part of this approach. However, inappropriate use of fungicides through incorrect product choice, timing of application or rate of product can significantly reduce the efficacy of a fungicide application.

## 18.4 Biological control

Toxigenic *Fusarium* spp. compete for nutrients and space throughout their life-cycle with other microbial populations. Antagonistic micro-organisms have developed various methods in order to gain an advantage while competing for a

common nutrient source. These include production of antibiotics, cell-wall degrading enzymes which attack competitors or more efficient enzyme. Antagonists may also differ in their ecological competence resulting in faster growth under both optimal and sub-optimal conditions, better survival during unfavourable conditions, or faster re-growth after periods unfavourable for growth (Köhl and Molhoek, 2001).

Two different strategies can be considered for biological control of necrotrophic pathogens such as FHB pathogens (Köhl and Fokkema, 1998). These are either direct application of the antagonistic micro-organisms to ears during flowering or targeting a crop residue with an antagonist which either enhances the decomposition of crop debris or reduces survival and multiplication of the pathogen. The advantages of the first strategy are that ears can easily be targeted by spray applications and the time where protection is required is relatively short. The main disadvantages of this strategy are the short period of interaction between antagonist and pathogen, and that the microclimate around the ear may not favour the antagonist. The advantage of the second strategy is that the interaction period between antagonist and pathogen lasts for several months and the microclimate in the crop residue is more likely to be favourable to the antagonists.

During the infection process, competing micro-organisms may be present at the infection site. Interaction between pathogens and beneficial yeasts has been demonstrated in the presence of pollen on cereal leaves (Fokkema, 1971), suggesting that antagonistic yeasts may also compete with *Fusarium* spp. for nutrient on the ear. Several research groups have selected antagonistic micro-organisms for application to the ear in order to prevent infection by *Fusarium* spp. In one study carried out under glasshouse conditions (Khan *et al.*, 2001) 26 out of 54 isolates of yeast and bacteria isolated from wheat anthers prevented FHB caused by *F. graminearum*. The most promising of these isolates were further evaluated in field experiments carried out on durum wheat varieties at two different locations (Schisler *et al.*, 2002). Several *Cryptococcus* spp. significantly reduced FBH severity by about 60 %, however, results for individual yeast isolates proved inconsistent. Using a similar approach, Luz (2000) screened thousands of bacteria and yeasts and selected antagonistic strains which controlled FHB under field conditions. The most promising antagonists were *Bacillus* spp. and *Paenibacillus macerans*, which not only decreased disease severity, but also increased yield by between 7 and 31 % compared to the controls. Fungal isolates have also been investigated. Dawson *et al.* (2002) tested over 100 fungal isolates for antagonism towards *F. culmorum* on wheat under glasshouse conditions, isolates showing the greatest level of antagonism were subsequently tested under field conditions. Four isolates of non-pathogenic *Fusarium* spp. gave results similar to those of the fungicide tebuconazole which reduced FHB incidence from 35 to 9 %. Two of the four isolates also significantly reduced the DON content of grain as effectively as the fungicide. Diamond and Cooke (2003) in glasshouse experiments applied the non-host pathogens, *Pythium ultimum* and *Phoma betae*, or the germination fluids of these fungi to wheat ears during anthesis. This resulted in reduced symptom development by *M. nivale* and *F. culmorum* or a longer latent period after

infection. Pathogen-induced systemic resistance was suggested as a possible control mechanism in this system.

Antagonists have also been screened for suppression of *Fusarium* spp. sporulation on crop residues. Application of a *Microsphaeropsis* sp. to autoclaved wheat straw two weeks pre, at the same time as or four weeks post inoculation with *G. zeae* all significantly reduced ascospore production after incubation under controlled conditions (Bujold *et al.*, 2001). A similar effect by the antagonist was found on maize stalks. Control effects of applications of the same antagonist to residues of an infected wheat crop were also significant but less pronounced. A similar strategy was followed by Köhl *et al.* (2003) and Corazza (unpublished data) using gamma irradiated wheat straw and maize stalks as the target substrates respectively. On straw segments approximately 100 fungal isolates were tested in a bio-assay against *F. culmorum* and *F. graminearum*, Corazza extended the range of *Fusarium* pathogens to include *F. proliferatum* and *F. verticillioides* on the maize substrate. Amongst the various fungal species tested, only isolates of *Clonostachy rosea* were consistently effective against all the *Fusarium* spp. tested on both substrates. Species such as *Trichoderma harzianum*, *T. viride* and non-pathogenic *F. equiseti* were less effective or showed a high degree of variability in antagonism against the different *Fusarium* spp. The antagonistic effect of *C. rosea* against *Fusarium* spp. was confirmed in preliminary field experiments carried out on artificially inoculated maize stalks (Corazza *et al.*, 2003).

## 18.5 Cultural practices: crop rotation, soil preparation, weed control

Preventive measures are generally aimed at the reduction of inoculum present in the susceptible crop. In order to achieve this it is important to understand what are the primary sources of inoculum for the FHB pathogens and how they survive and develop, particularly under differing environmental conditions. The saprophytic part of the pathogen life-cycle has been investigated for several *Fusarium* spp.; however, studies have mainly focused on single species, in particular *F. graminearum*. Studies which consider the complex interaction between the various pathogens involved in the head blight complex are rare and generally incomplete. Thus it is difficult to determine the overall effect of any control measures; for instance, does the reduction of one FHB pathogen enable another to proliferate in the vacant niche?

As already indicated, the most susceptible stage for infection of wheat ears by the FHB pathogens is during anthesis. Following infection, polycyclic development and spread of the disease do not occur within the crop. This has been particularly demonstrated in *G. zeae* where steep disease gradients are produced around a source of inoculum indicating that secondary spread within the crop does not occur (Fernando *et al.*, 1997). Consequently, any inoculum source is only a threat during a short period of the growing season, with disease incidence and

severity being closely related to the amount of primary inoculum present (Sutton, 1982). Inoculum sources can be found both within and outside the wheat crop. Most of the toxigenic FHB pathogens produce inoculum in the form of splash-dispersed conidia. Using this method of dispersal, inoculum in a wheat crop has been shown to move only small distances, ranging from a few centimetres up to over a metre (Jenkinson and Parry 1994b; Parry *et al.*, 1995; Fernando *et al.*, 1997, 2000). *G. zeae* differs from many of the other toxigenic FHB pathogens as it can also produce ascospores. Unlike conidia, ascospores are predominantly wind-dispersed and can travel distances from several metres up to several kilometres (Fernando *et al.*, 1997; Maldonado-Ramirez and Bergstrom, 2000). Thus FHB infections are generally caused by an inoculum source originating from the same field or, in the case of infections caused by *G. zeae*, from a neighbouring field.

Infected seeds, wheat plants or weeds, crop residues of preceding host crops and saprophytically colonized non-host necrotic tissues can all potentially act as a source of inoculum and, as a result, are areas where control measures can be targeted. Birzele *et al.* (2002) demonstrated a close correlation between the level of seed infected by *M. nivale* and the level of grain infection at harvest. However, similar correlations have not been demonstrated for other *Fusarium* spp. (Gilbert, 2001; Birzele *et al.*, 2002).

*Fusarium* spp. may be present as non-host specific pathogens on weeds or under sown crops found within a cereal crop, thus acting as a potential source of inoculum. Jenkinson and Parry (1994a) isolated *Fusarium* spp., such as *F. avenaceum*, *F. culmorum*, *F. poae*, *F. sambucinum* and *F. graminearum* from 14 of 15 weed species evaluated. The majority of the isolates obtained proved to be pathogenic to wheat seedlings. Meier *et al.* (2001) identified seeds of the weed *Gallium aparine* as a possible inoculum source of *Fusarium* spp. In organically grown wheat 46 % of *G. aparine* seeds were infested with the same spectrum of *Fusarium* spp. as the wheat crop.

Crops grown in rotation with wheat could potentially affect levels of FHB infection in the following wheat crop. Wheat rotations with maize are the major source for increased inoculum (Dill-Mackey and Jones, 2000); however, other crops may also harbour FHB pathogens. When seedlings of various non-gramineous crops, such as field pea, bean, chickpea and lentil, were inoculated with *F. graminearum* their roots became infected by the pathogen; however, the roots of *Brassica* spp. were not infected (Chongo *et al.*, 2001). Thus choice of crop to be grown in rotation with wheat can be vital for the reduction of FHB, particularly in relation to crop residue. In many cases, crop residues from a preceding infected crop are the main source of inoculum for the subsequent wheat crop, e.g. the incidence of *Fusarium* spp. on organic particles of field soil correlated significantly with the incidence of *Fusarium* spp. in grain (Birzele *et al.*, 2002).

Over the last four decades FHB has become one of the main limiting factors in north American wheat production (Sutton, 1982; Gilbert and Tekauz, 2000). In this region monoculture, reduced tillage or no-tillage systems have been shown to favour the development of *F. graminearum* within the crop (Dill-Mackey and Jones, 2000). Infection by *F. graminearum* is initiated by the release of ascospores

from perithecia formed in crop residues at the soil surface. Perithecia have been shown to survive for 18 months on crop residues buried in soil and for up to three years for crop residues on the soil surface (Sutton, 1982; Khonga and Sutton, 1988). However, only perithecia found on the soil surface contained ascospores, thus putting subsequent crops at risk.

In addition to crop residues, it has been shown that *Fusarium* damaged wheat kernels remaining in the field can serve as a source of inoculum for the FHB pathogens. Inch and Gilbert (2003) demonstrated that *F. graminearum* could still be isolated from more than 85 % of *F. graminearum* damaged kernels 24 months after burial in soil at various depths. This study also re-enforced the finding that ascospores were only formed in perithecia on kernels found at the soil surface. For western Europe, the role of wheat as a preceding crop and reduced tillage intensity as risk factors for the occurrence of FHB is not as clear as in north America, possibly because climatic conditions in western Europe favour decomposition of crop residues more than in north America. In surveys carried out between 1989 and 1999 (Beck and Lepschy, 2000), it was shown that the occurrence of *Fusarium* spp. was no higher in samples from fields in which wheat had been grown the previous year or where the soil had not been ploughed.

In the same studies, the significant role of maize in crop rotation with wheat, especially in combination with reduced tillage practices, was confirmed. In western Europe, *F. graminearum* is a major pathogen of maize; thus the increase in maize acreage grown during the last decades may explain the increased importance of *F. graminearum* in the head blight complex of wheat. Crop residues of maize such as stalks are decomposed slowly and are present in subsequent crops. Cotton and Munkvold (1998) demonstrated that *F. moniliforme*, *F. proliferatum* and *F. subglutinans* could survive in maize residue left at the soil surface for at least 630 days under conditions found in north America; however, when buried under soil, survival of the pathogens was reduced to at least 340 days. Thus, long survival periods, at least from maize harvesting until flowering of wheat in the following year, can also be expected for *F. graminearum* in maize stalks under conditions prevalent in western Europe. The importance of maize stalks as an inoculum source of *F. graminearum* is underlined in a survey carried out by Windels *et al.* (1988) who isolated the pathogen from the majority of symptomless maize stalks tested. Saprophytic colonization of maize stalks by soil- and air-borne inoculum of *Fusarium* spp. has been observed by Cotton and Munkvold (1998). Competition between toxigenic *Fusarium* spp. in maize grains has been studied by Marín *et al.* (1998). *F. moniliforme* and *F. proliferatum* were superior to *F. graminearum* because they needed shorter wetness periods for successful infection and colonization of the substrate. Similar studies on competitive colonization of *Fusarium* spp. on crop residues relevant for European cropping conditions are needed for a better understanding of population dynamics and inoculum build-up of specific *Fusarium* spp. under various environmental conditions.

Crop rotation, tillage and weed control are thus all important components for the development of wheat cropping systems which reduce the risk of FHB occurrence. In general, to avoid FHB, it is important to ensure that crop residues

from a previously infected crop do not remain in the following wheat crop. *Fusarium* spp. have a high saprophytic competitive ability in crop residues and can survive in this niche. Rapid decomposition of crop residues colonized by *Fusarium* spp. will decrease inoculum pressure since *Fusarium* spp. are not well adapted to survival in soil (Gilbert and Tekauz, 2000). Maize should not be grown in rotation with wheat. If this cannot be avoided, maize stubble should be ploughed carefully to avoid its remaining on the soil surface thus preventing maturation of perithecia; wheat crop residues should also be avoided. In many cases, the need to protect soil from erosion and save costs through conservation of energy may force the farmer to apply reduced or no-tillage systems. In such cases, new methods are needed to enhance decomposition of maize stalks on the soil surface. The role of crop residues from other crops is not as clear as for crop residues of maize and wheat. There may be differential effect of such crops and their residues on the different populations of *Fusarium* spp. causing FHB. Research is needed to come to a better understanding of the saprophytic development of such *Fusarium* spp. populations and their competitive interactions in crop rotation systems.

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