



CONFIDENCE: Rapid methods: BSc education modules 4 and 11 October 2011



# CONFIDENCE: Rapid methods



**BSc education modules  
4 and 11 October 2011**

## **Mycotoxins and plant toxins in food**

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## Summary

Mycotoxins (toxic fungal metabolites) have challenged human and animal health since early times; dating back to the 900's. Some metabolites have pharmaceutical characteristics, but most are known for adverse effects on human and animal health.

Food and feed raw materials are seldom contaminated with one mycotoxin only, new mycotoxins are discovered and old ones re-emerge, while recent observations on masked mycotoxins may influence risk assessments.

This asks for wide range screening methods, new analytical approaches and low detection limits. Producers need fast methods for decisions on the use of raw materials. Effect based toxicity assays will improve the screening for unknown mycotoxins. Recent developments in analysis will be discussed.

Changing society and environment forces food safety officers and scientists to be aware of mycotoxin risks (occurrence and re-introduction). Trends in consumption may lead to (re-)introduction of risks from mycotoxins. Consumer demand for more healthy diets might increase the demand for oats (slow release of energy) and subsequently (re-)introduce or expand the area of cultivation in certain regions. The consumer perception of soy-GMO, and the demand for highly nutritious proteins for meat replacement will increase the demand for other legumes, such as lupine, with the risk for phomopsin contamination. Plant diseases and insects may spread due to alterations in climate and may force fungi to produce (more) mycotoxins. The use of food crops for biofuel production and the treatment of biofuel raw material (storage, use as animal feed) is a challenge for the agrichain and needs to be monitored closely.

The major challenge for food producers and food safety officers today is to incorporate the risk assessment for mycotoxins for every change in the agri-chain. This requires smart and reliable monitoring as well as close cooperation between governments, scientists, plant breeders and food and feed producers.



## Risks of mycotoxins and plant toxins in food and feed

Monique de Nijs, CONFIDENCE presentation October, 4, 2011



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## RIKILT Institute of Food Safety

[www.rikilt.wur.nl](http://www.rikilt.wur.nl)



- Detection, identification, functionality and effects of substances in food and feed;
- RIKILT:
  - Specific research, with CVI and RIVM, for the Netherlands new food safety authority (NVWA);
  - Is laboratory for VWA feed and AID;
- Contract work:
  - According to guidance document;
  - Report duty, as any laboratory, when legal limits are exceeded;

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## Program

1. RIKILT
2. Confidence 
3. Mycotoxins and plant toxins (Mw. de Nijs)
  1. Introduction
  2. Mycotoxins
  3. Plant toxins
  4. Conclusions
  5. Challenges
6. Risk assessment and regulations for mycotoxins (Mr. van Egmond)

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[www.rikilt.wur.nl](http://www.rikilt.wur.nl)



- Environment & process contaminants:
  - Dioxins in eggs in Germany;
  - Fire in Moerdijk (incl heavy metals);
- Radioactivity:
  - Imports from Japan;
- Pesticides;
- Natural toxins (mycotoxins, plant toxins, phycotoxins);
- Animal treatment medicines and residues;
- GMO s;
- Allergens;
- Nutrients / Quality:
  - Identify organically produced eggs;
  - Authenticity identification (is this the fruit juice I selected and ordered several months ago?);

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## RIKILT Institute of Food Safety

[www.rikilt.wur.nl](http://www.rikilt.wur.nl)

- Part of Wageningen UR since 1998
- About 200 employees



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### Confidence

- Contaminants in food and feed: inexpensive detection for control of exposure;
- LCP (large collaborative program) Food, Agriculture and Fisheries, and Biotechnology, 2008-2012;
- Simple, fast, multi-analyte, multi-class detection;
- Includes WP Biotoxins:
  - alkaloids (pyrrolizidine, tropane, ergot)
  - *Fusarium* mycotoxins (TCT, ZEA, FUM)
- Intra- and interlaboratory validation studies

   [www.confidence.eu](http://www.confidence.eu)

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### Introduction *Natural toxins in food and feed*

Compounds that are poisonous to humans and/or animals that naturally occur in food and feed can be produced by:

1. Microorganisms (fungi (mycotoxins) and bacteria);
2. Plants (plant toxins or phytotoxins);
3. Algae/shellfish
- (4. Zootoxins produced by snakes/ bees / frogs)

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### Introduction *Zootoxin*

#### Likken van kop hallucinerende pad kan levensgevaarlijk zijn



Poison, licked from the head of a certain toad, can give strong hallucinations. Can even kill a grown up person.

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### Introduction *Secondary metabolites*

All natural toxins = secondary metabolites

Secondary metabolite ≠ toxin

**Secondary metabolites:** metabolites that are not directly related to growth of cells, or to development and reproduction of an organism;

**Primary metabolites:** amino acids and glucose and substances related to growth of cells, and to development or reproduction

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### Mycotoxins Incidents and issues

Year	Toxicosis	Toxin	Fungus
994	Holy fire	lysergic acid deriv.	<i>C. purpurea</i>
1890	Cardiac beriberi	citreoviridin	<i>P. citreo viride</i>
1913	Alimentary toxic aleukia	trichothecenes	<i>F. sporotrichioides</i>
1952	Balkan endemic nephropathy	ochratoxin	<i>P. verrucosum</i>
1960	Turkey X disease	aflatoxins	<i>A. flavus</i>
1988	Hole in the head syndr.	fumonisin	<i>Fusarium</i>
2004/8, 05/8, 10	Hum. aflatoxicosis	aflatoxins	Not determined 317 people ill, 127 fatalities (Kenya)

### Mycotoxins Challenges Products

- (Re)introduction of crops:
  - Introduction of lupin as GMO soy replacer: risk of phomopsis contamination (Australia & NZ limit of 5 µg/kg)
- Increased area of oats as healthy grain: no recent reports on occurrence of mycotoxins in oats in the Netherlands.

### Mycotoxins Toxic effects

	AFL	OTA	PAT	FUM	TCT	ZEA
carcinogenic	●	●	●	●	●	●
hepatotoxic	●	●	●	●	●	●
immunotoxic	●	●	●	●	●	●
nephrotoxic	●	●	●	●	●	●
neurotoxic	●	●	●	●	●	●
oestrogenic	●	●	●	●	●	●
teratogenic	●	●	●	●	●	●

### Mycotoxins Challenges Handling

- Up to now: patulin contamination of apple juice seems to be controlled:
  - bulk production of apples;
  - controlled storage and sorting;
  - quality control on juice;
- Currently: handicraft / local production:
  - lack of knowledge on contamination routes (uncontrolled storage, no sorting prior to processing);
  - use of new or old races (no experience);
 → patulin in artisanal produced apple juice (2009):  
(Gillard et al (2009) WMJ (1) 95 104)

### Mycotoxins Challenges Products

**994:** Holy fire (lysergic acid deriv. *C. purpurea*)

**1568:** Pieter Brueghel *The Cripples*  
Holy fire by ergot alkaloids in **rye**  
(gangren followed by *necrosis / hallucinations*)

**2011:** Ergots in **cereals**; EFSA opinion expected in 2012  
Regulatory limits on groups of toxins (as opposed to parts)

### Mycotoxins Challenges Masked mycotoxins

- Masked mycotoxin = mycotoxin that is metabolised by the plant into a, for the plant, harmless compound;
- Eg: deoxynivalenol 3 β D glucoside (D3G);
- First report 1985 by Miller JD & Young JC  
(Deoxynivalenol in an experimental Fusarium graminearum infection of wheat, Canadian Journal Plant Pathology, (1985), 7, 132-134);
- Relevance:
  - Occurrence: D3G analyzed by LC MS/MS;
  - Toxicity:
    - Increased exposure by release in intestinal tract?
    - Absorbed by intestinal cells?



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Mycotoxins Challenges Climate

- Expected changes in range of latitudes, where certain fungi are able to compete;
  - Example: *F. graminearum* growth: NIV increase;
- Drought, flooding and other consequences of climate change may result in more mycotoxins and changed toxin profiles;
  - Example: aflatoxins found in Italy since 2003, and in other parts of Central Europe;
- Response of insects and plant diseases to climate change poorly understood, but increases expected



Mycotoxins Regulations EU Feed (1/2)

- Commission Directive (EC) No 2002/32 (and its amendments): On undesirable substances in animal feed
- Commission Recommendation 2006/576/EC: On the presence of deoxynivalenol, zearalenon, ochratoxin A, T 2 and HT 2 and fumonisins in products intended for animal feeding



Mycotoxins Regulations EU Food (1/2)

- Commission Regulation (EC) No 1881/2006 (and its amendments): Setting maximum levels for certain contaminants in food
- Commission Regulation (EC) No 401/2006 (and its amendments): Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in food



Mycotoxins Regulations EU Feed (1/2)

- Commission Regulation (EC) No 152/2009: Laying down the methods of sampling and analysis for the official control of feed



Mycotoxins Regulations MS Food (2/2)

- The Netherlands: Warenwetbesluit bereiding en behandeling van levensmiddelen Artikel 12

*Fungal and bacterial toxins in quantities that can be harmful to the public health must be absent in food, drinks and raw materials*



Regulations EU import controls

- Commission Regulation (EC) No 669/2009 (and its amendments): Implementing Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards the increased level of official controls on imports of certain feed and food of non animal origin and amending Decision 2006/504/EC
- Commission Regulation (EC) No 1152/2009: imposing special conditions governing the import of certain foodstuffs from certain third countries due to contamination risk by aflatoxins and repealing Decision 2006/504/EC



**Mycotoxins** *Decontamination*

EU regulatory limit for mycotoxins in feed: aflatoxin  
 EU recommended limit for mycotoxins in feed: deoxynivalenol, zearalenon, ochratoxin A, T 2 and HT 2 and fumonisins

- Decontamination is not allowed in EU;
- Dilution to lower contamination is not allowed in EU;

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**Mycotoxins** *Decontamination*

- Feed: allowed are technological additives according to EU 1831/2003: substances for reduction of the contamination of feed by mycotoxins;
  - that can suppress or reduce the absorption;
  - promote the excretion of mycotoxins;
  - modify their mode of action.
 Need to be evaluated according to EU 429/2008
  - Toxicity of the substance to animals;
  - Efficacy;
- Only when EU regulations are met !

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**Plant toxins** *Contamination and exposure route*

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**Mycotoxins** *Decontamination*

Technological additives according to EU 1831/2003:

- **Biotransformation**
  - E.g.: enzymes; enzyme producing microorganisms;
- **Organic binders**
  - E.g.: yeast cell wall components; synthetic polymers; humic substances; dietary fibres;
- **Nutritional feed additives:**
  - E.g.: antioxidants; immunostimulatory agents;
- **Inorganic compounds (absorbents):**
  - E.g.: clays; activated carbons

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**Plant toxins** *factors affecting contamination*

- Role: protect plant against being eaten (insects and animals) and infections;
- Plant species (star anise);
- Environmental circumstances:
  - Temperature;
  - Growing season;
  - Insects;
  - Etc. more research.

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### Plant toxins *Challenges Carry over*

Carry over from feed to animal products:

- Jacoline from *Senecio jacobaea* (jacobs kruiskruid) to milk  
\* Hoogenboom et al. (2011) FAC Part A (28) 359-372;
- Incident in Afghanistan: possible PA s from other weeds carried over to goat milk/cheese;
- No reports on occurrence of plant toxins in meat.

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### Plant toxins *Challenges Climate*

- Growing area of weeds can change;
- Drought, flooding and other causes;
- (Re )introduction of crops;
- New plants /weeds due to import / export.

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### Challenges *Analytical methods*

Single compound chemical analysis

Multiple compound chemical analysis      Single / multiple compound effect analysis

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### Plant toxins *Legislation*

Food: 37/2010 *Aristolochia* spp. (table 2 forbidden substances, vet.drugs, AO)  
 396/2005 nicotine, pyrethrins, rotenone, azadirachtin (MRL pesticides)  
 Feed: 2002/32 hydrocyanic acid, gossypol, theobromine, allyl ITC (glucosinolates)  
 Botanical impurities: weed seeds/fruits with alkaloids, glucosides, other tox subst.

2001 act on herbal preparations (= food)  
 No aristolochic acids  
 Pyrrolizidine alkaloids < 1 µg/kg  
 List of plants not to be used in herbal preparations (monk's hood, fox glove,...)

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### Challenges *Chemical analysis*

Single compound methods:

- Optimized extraction;
- SPE and/or IAC clean up (derivatisation);
- LC UV, LC Flu, GC ECD

Multi compound methods:

- Generic extraction;
- (limited) no clean up;
- LC MS/MS  
(RIKILT 37 mycotoxins + 10 plant toxins)

Spencer et al. Food Additives and Contaminants (2008), 25, 472.  
 Mal et al. Anal. Chem. (2008), 80, 9450.  
 Mal et al. Food Additives and Contaminants (2011) accepted.

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### Challenges *Effect analysis (1/2)*

*In vitro* testing: effects of mycotoxins on gene expression

1. Culture human Caco 2 cells
2. Seed cells; expose to compounds or sample extracts of interest
3. Isolate RNA from Caco 2 cells;
4. Conversion in cDNA, amplify cDNA, labelling;
5. Hybridisation, detection and data acquisition;
6. Data analysis.

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### Challenges

Challenge: perform risk assessment on mycotoxins and plant toxins with any change in agrichain (new supplier, new recipe, new harvest);

This demands exchange of information through the whole production chain and close cooperation between authorities, science, plant breeders and producers of feed and feed.

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### Challenges *Effect analysis (2/2)*

Affected genes whole array  
(44000 spots from 25000 genes)

- Red: up-regulated
- Green: down-regulated

Further method development, e.g.:

- Caco 2 cell line clones with luciferase expression when exposed to mycotoxins;
- multiplex qRT-PCR on several specific up- and down regulated genes;

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### Natural toxins

Past performance is no guarantee of future results!

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### Conclusions

- Similarities in occurrence and toxic effects of mycotoxins and plant toxins:
  - Route of exposure: mainly through plant materials;
  - Carry over through animals (meat, milk) is known;
  - Toxin profiles occur and toxicity differs between toxins;
  - Animal species vary in sensitivity;
  - Climate will affect occurring profiles and concentration;
  - Gaps in knowledge about and toxicity of many myco- and plant toxins;
- Plant toxin intoxication often resulting from mistake. The results can be devastating.

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### Thank you for your attention

### Questions ?

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MoniQA Fact Sheet No 8 on:

## Mycotoxins



Network of Excellence supported by the European Commission under FP6

Coordinator: Roland Poms (ICC)

Mycotoxins are small (low-molecular weight) secondary metabolites (products of metabolism not essential for survival), produced by some filamentous fungi and often found in food. Chemically-diverse, they can cause disease and death to man and livestock. Adverse effects include tissue damage, immuno-suppression and neurological disorders following short- (acute) or long-term (chronic) exposure. In many countries, mycotoxin contamination is addressed by legislation. Successful management of mycotoxins is, however, effected thorough knowledge of their cause and implementation of appropriate control mechanisms, which are verified through use of reliable sampling and robust analytical methods. European Union Rapid Alert System for Food and Feed (RASFF) data suggest that harmonisation of mycotoxin detection methods would improve compliance at regulatory and commercial limits.



**Aspergillus carbonarius: a) black rot of berries caused by A. carbonarius; b) A. carbonarius artificially inoculated on grape berry; c) A. carbonarius on PDA plate –**

Dr Giancarlo Perrone, Institute of Sciences of Food Production, National Research Council, Italy

**Aflatoxins** are produced by many *Aspergillus* species, most notably *A. flavus* and *A. parasiticus*. There are four different types of aflatoxins: B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>. Aflatoxin B<sub>1</sub>, the most toxic, is a potent carcinogen linked to adverse health effects in humans including liver cancer. Aflatoxins are associated with commodities produced in the tropics and subtropics such as cotton, maize, spices, peanuts, pistachios and dried fruits. Aflatoxin M<sub>1</sub> is found in the milk of animals that are fed aflatoxin B<sub>1</sub>-contaminated feed. Susceptibility to aflatoxin-related diseases is affected by species, age, nutrition, sex and exposure to other toxins.

**Patulin** is produced by *Aspergillus*, *Penicillium*, *Byssoschlamys* and *Paecilomyces* species, but mostly *P. expansum*, which is found in a range of moldy fruits and vegetables, especially rotting apples, but destroyed by fermentation. Patulin is not carcinogenic, but it can damage the immune system and intestine. In 2004, the EU set limits on patulin in food at 50 µg/ kg (fruit juice), 25 µg/ kg (solid apple products for direct consumption) and 10 µg/ kg (children's apple products including juice), respectively.



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**Ochratoxins** are produced mainly by *A. carbonarius*, *A. ochraceus* and *P. verrucosum*. Ochratoxin B is a non-chlorinated form of Ochratoxin A whilst Ochratoxin C is an ethyl ester form Ochratoxin A. They are one of the most abundant food-contaminating mycotoxins, largely arising from improper storage. Ochratoxin A is a carcinogen and nephrotoxin, affecting kidney function, and it has been linked with urinary tract tumours but research in humans is limited.

**Citrinin** was originally isolated from *P. citrinum* as an antibiotic but it is also produced by *Penicillium* and *Aspergillus* spp. including those used in food manufacturing (e.g. cheese – *P. camemberti*, sake, miso and soy sauce – *A. oryzae*). However, although associated with many human foods (wheat, rice, corn, barley, oats, rye, and food coloured with *Monascus* pigment), its full significance in human health is unknown.

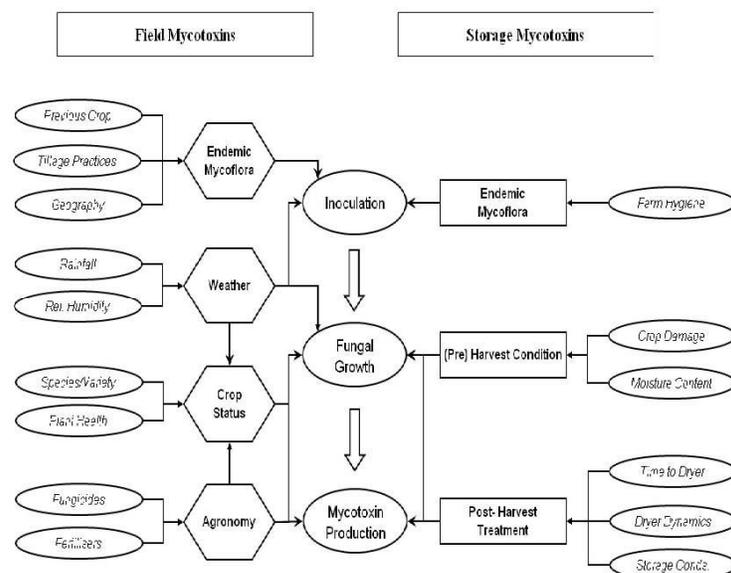
**Ergot Alkaloids:** Ergot or ergot fungi refers to a group of fungi of the genus *Claviceps*. The most prominent member of this group is *Claviceps purpurea*. This fungus grows on rye and related plants, and produces alkaloids that can cause ergotism in humans and other mammals consuming grains contaminated with its fruiting structure (ergot sclerotium). Ergot alkaloids cause vasoconstriction leading to gangrene and loss of limbs and/ or affect the central nervous system causing hallucinations and irrational behaviour and convulsions. However, ergot alkaloids have and continue to be used in medicine including control of post-partum haemorrhage.

**Fusarium** is a large genus of filamentous fungi widely distributed in soil and in association with plants, which are generally harmless. However, in cereal crops such as wheat and maize, some produce a range of mycotoxins including fumonisins – affecting the nervous systems of horses and cause cancer in rodents – trichothecenes, which are associated with chronic and fatal toxic effects in animals and humans and zearalenone, which causes hyperestrogenism, particularly in swine. Other major *Fusarium* toxins include beauvercin and enniatins, butenolide, equisetin and fusarins.

**MoniQA and mycotoxin analysis methods:** In Europe, sampling and analysis for the control mycotoxins in foods must be in accordance with those set out in Commission Regulation 401/2006. For each mycotoxin, analytical method performance (recovery, repeatability and reproducibility) must fall within the range of acceptability. MoniQA undertook a survey on current laboratory practices for commercially-significant mycotoxins [aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub> and M<sub>1</sub>, fumonisins FB<sub>1</sub> and FB<sub>2</sub>, ochratoxin A, deoxynivalenol, patulin, zearalenone, and T-2 and HT-2 toxins]. Nineteen control, commercial and research laboratories from 12 countries (UK, IT, BE, ES, DE, NL, BG, HU, GR, TR, NZ and CN) participated, 14 of which were accredited to ISO 17025:2005. Participants stated they receive laboratory samples weighing between 4g-1kg. The number and types of food matrices varies for the different mycotoxins with the highest number recorded for ochratoxin A. Most participants use HPLC coupled with either a fluorometer, ultraviolet or mass spectrometric (MS) detectors; one uses GC-MS for analysis of T-2 and HT-2 toxins and two use TLC-based methods for all mycotoxins except fumonisins. The use of LC-MS methods by eight (42%) laboratories is remarkable because these are not CEN- or AOAC-recognised method for mycotoxin analysis. ELISA kits are used by three (16%) laboratories for a range of mycotoxins whilst other test kits are used for the determination of ochratoxin A and deoxynivalenol, specifically. Six definitions of limit of detection (LOD) and nine definitions of limit of quantification (LOQ) were reported with signal-noise ratio being the most popular. In some cases, the values of LOD, LOQ and the measurement uncertainty varied from laboratory to laboratory for the same mycotoxin. This survey suggests laboratory accreditation, sample size, most appropriate and validated analytical methods, proficiency testing, reference/ certified materials/ standard solutions, and use of the same definition/ calculation for LOD, LOQ, recovery and measurement uncertainty need to be addressed as a priority. Solfrizzo et al. (2009) The use of mycotoxin methodology in practice: a need for harmonization *QAS Crops & Foods* 1(2): 121–132 DOI: 10.1111/j.1757-837X.2009.00021.x



**Mycotoxins and rapid test methods:** Rapid test methods are promoted as tools for food companies to validate and/ or verify efficacy of food-safety management systems. Little is known about their uptake by the industry or current and future needs. In order to obtain more information, MoniQA conducted a survey in 17 countries (AL, BE, BG, CN, FI, FR, DE, GR, HU, IT, NZ, NO, PL, ES, TR, UK, VN) designed to explore routine analytical regimens operated by the food industries and the use of rapid test methods. More than 2600 questionnaires were sent to companies across the food chain (661 replies). At a strategic level, the survey revealed raw materials and final products are most commonly analysed, and the major analytes are microbiological contaminants, heavy metals, pesticides, foreign bodies, mycotoxins and allergens. Two-thirds of respondents use rapid test methods; almost all would be interested in extending the range of tests performed particularly for microbiological, food allergens and mycotoxins analyses. Lebesi et al. (2010) Rapid test methods: a versatile tool to assist food-safety management QAS Crops & Foods 2(4): 173–181, DOI: 10.1111/j.1757-837X.2010.00080.x



Formation of both field or storage mycotoxins in commodities and the interaction of a selection of external modifying factors on their formation – Dr Anton J. Aldrick, Campden BRI, United Kingdom

### More about mycotoxins

- European Mycotoxins Awareness Network – [www.mycotoxins.org](http://www.mycotoxins.org)
- HGCA – Agriculture and Horticulture Development Board (AHDB) – [www.hgca.com](http://www.hgca.com) (crop research)
- Food Standards Agency – <http://www.food.gov.uk/safereating/chemsafe/mycotoxins>
- European Food Safety Authority – <http://www.efsa.europa.eu/de/topics/topic/mycotoxins.htm>
- Europa – [http://ec.europa.eu/food/food/chemicalsafety/contaminants/index\\_en.htm](http://ec.europa.eu/food/food/chemicalsafety/contaminants/index_en.htm)
- PubChem – <http://pubchem.ncbi.nlm.nih.gov/>

For further information please visit: [www.moniqa.org/xxx](http://www.moniqa.org/xxx) or contact [moniqa@moniqa.org](mailto:moniqa@moniqa.org).

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