DH13TP
Food Toxicology and Mycotoxins

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School of Food Science and Technology
Faculty of Science and Technology
Thammasat University

Who is Awanwee?

- B.Sc. Food Science and Technology, Thammasat University
- M.Sc. Environmental Sanitation, Mahidol University
- Ph.D Food Technology, Suranaree University of Technology
  and
  DOC (Ingenieries Microbienne et Enzimatique), ENSAT, INPT, France
- Postdoctoral Center for Analytical Chemistry, Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna (BOKU), Austria

Department of Food Science and Technology

Faculty of Science and Technology
Thammasat University

Dr. Awanwee Petchkongkaew

Research expertise

- Mycotoxins (Detection/Detoxification/and Biological decontamination methods)
- Application of Lactic Acid Bacteria (LAB) in food
- Food Fungi
- Food Fermentation
- Food Allergen

Thammasat University

- The *second* oldest university in Thailand
- Officially established on 27 June 1934 = **83** years
- The founder = Prof.Dr. Pridi Banomyong
- The University of Moral Science and Politics
- It had started out as an open university, with 7,094 people enrolled for its first academic year.
- The main goal of the University's foundation was *"To teach students to love and cherish democracy."*
- VDO presentation : https://youtu.be/5ksIQqLa-5s

Dr. Puey Ung-phakorn

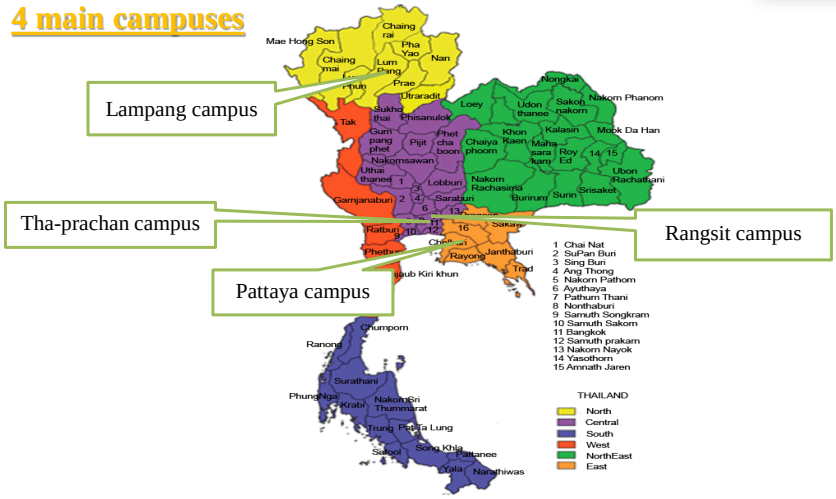
the tenth Rector of Thammasat University, *initiated the expansion into Science and Technology* with the establishment of Rangsit Center in 1986

Budgets

- In the fiscal year 2010, the total budget of the university is 6.425 billion baht
  - Government support : *2.16* billion baht
  - University income: *674* billion baht
  - Income from faculties/colleges/institutes/Thammasat Hospital and other units under Thammasat University's administration: *3.618* billion baht
- The main sources of our income are from the government and income generated by the units within the university.

Campuses

4 main campuses



Faculties/ Colleges/ Institutes

Social Science and Humanities

- Commerce and Accountancy
- Economics
- Fine and Applied Arts
- Journalism and Mass Communication
- Law
- Liberal Arts
- Political Science
- Social Administration
- Sociology and Anthropology
- College of Interdisciplinary Studies
- College of Innovative Education
- Graduate Volunteer Center
- Language Institute
- Pridi Banomyong International College

14

Science and Technology

- Architecture and Planning
- Engineering
- Science and Technology
- Sirindhorn International Institute of Technology (SIIT)

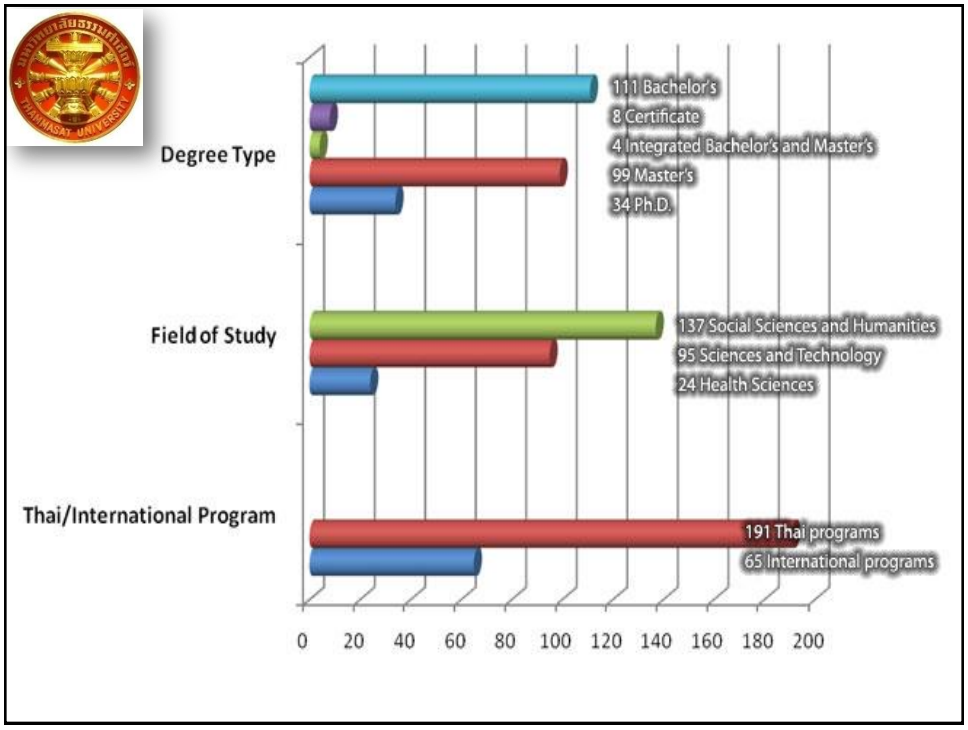
4

Total = 23

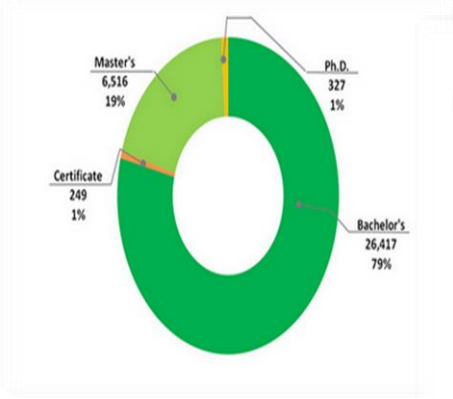
Health Science

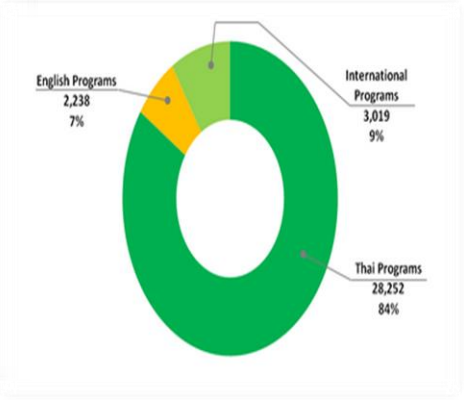
- Allied Health Sciences
- Dentistry
- Medicine
- Nursing
- Public Health

5



Students Enrolment





..The Rector

n

Prof. Dr. Somkit

(from 7 December 2010 – presently)

His international objectives:

- Develop the MOU between 2 universities
- Staffs and students exchange
- Collaborative research projects
- Joint seminar

Department of Food Science and Technology

Faculty of Science and Technology

Thammasat University

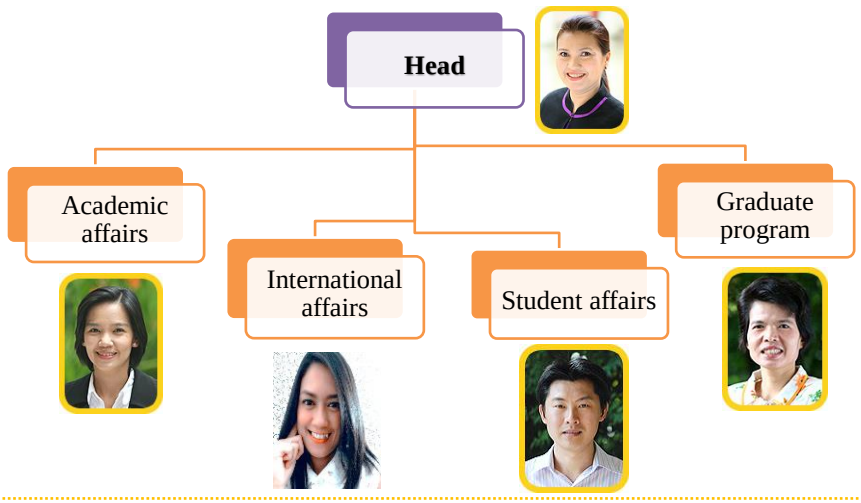
Department of Food Science and Technology

Faculty of Science and Technology
Thammasat University

- Head: Assoc.Prof.Dr. Prapasri Thepsugsa
- 15 full-time lecturers (all Ph.D)
  - 4 Associated Professors
  - 4 Assistant Professors
  - 8 lecturers
- 4 scientific staffs
- 4 administrative staffs
- Approx. 200 undergraduate students (Bachelor degree, 4 year)
- 20 Master students
- 6 Doctoral students

Department of Food Science and Technology

Faculty of Science and Technology
Thammasat University



THE MODULES

- Food Process and Engineering
- Food Chemistry
- Food Microbiology
- Food Product and Development

Our Research

- Mycotoxins reduction by biological method
- Bio-surfactant: application of LAB
- Fermentation technology (Wine production)
- Hydrocolloid
- Fat and oil
- Dairy product (Ice cream & yogurt production)
- Fishery product (Collagen from by-product of fish)
- Antioxidant
- Starch and modified starch
- Meat product (sausage)
- Food product development

ADMINISTRATIVE OFFICE

FOOD PROCESSING LABORATORY

FOOD MICROBIOLOGY LABORATORY

FOOD CHEMISTRY LABORATORY

FOOD PRODUCT DEVELOPMENT & SENSORY EVALUATION LABORATORY

OUR BAKERY HOUSE

- 4 branches
- More than 30 varieties of bakery and cake product

"We serve the fresh and tasty products from the oven to you everyday" ♥

OUR GRADUATE STUDENTS

COLLABORATION

- BIOTEC NSTDA , Thailand
  - Food Biotechnology Research Unit (FBU)
- BOKU Vienna, Austria
  - Center for Analytical Chemistry
- Koibuchi college of Agriculture and Nutrition, Japan
- AGROINNOVA, University of Torino, Turin, Italy
- International Association for Cereal Science and Technology (ICC) Vienna, Austria
- University of Porto
- MoniQA (Network of Excellence)

BIOTEC
a member of NSTDA

Welcome to BIOTEC, NSTDA

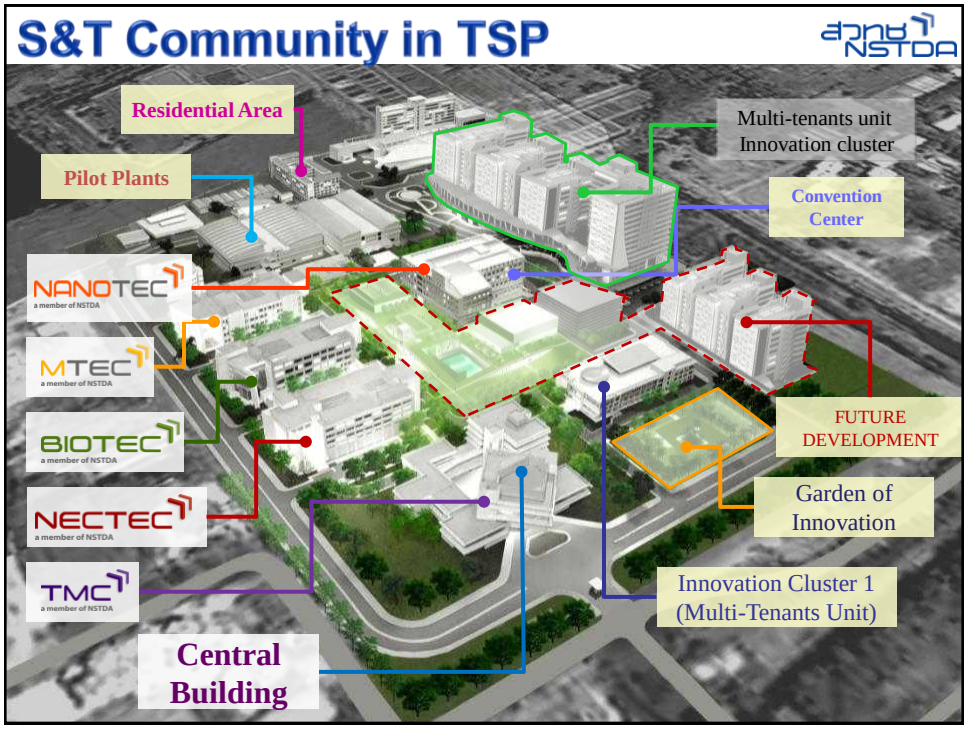
The first technology hub for Thailand is now ready to serve the country with its world-class facilities and pool of talented people.

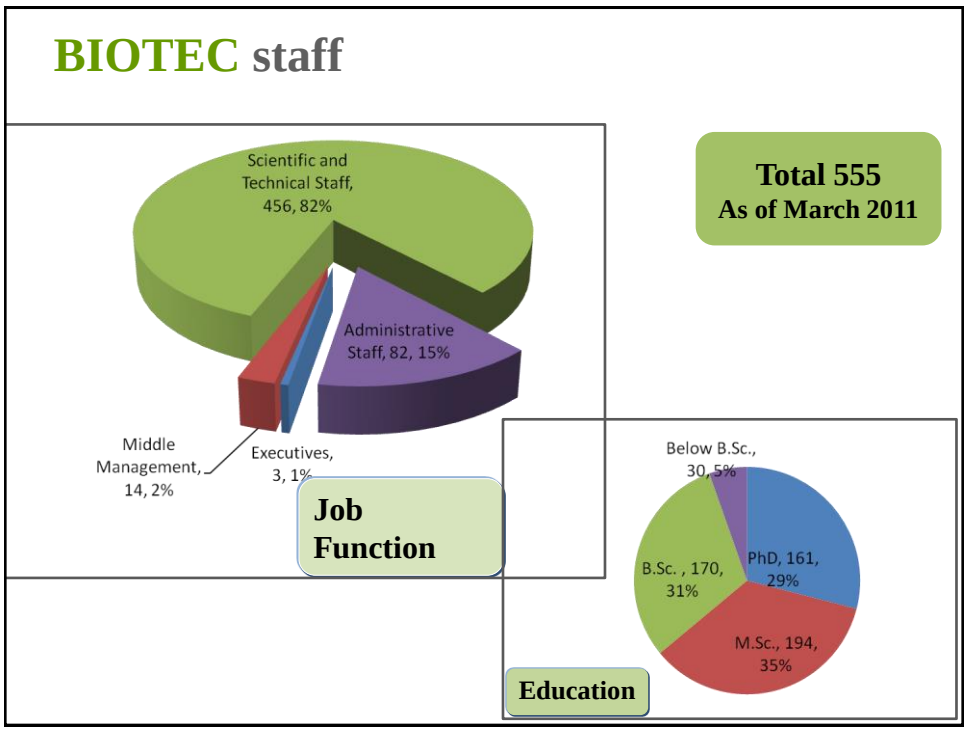
NSTDA at a glance

Established:	December 1991; member of Ministry of Science and Technology
Location:	In Thailand Science Park, 30 kilometers north of Bangkok
Vision:	A key partner for a knowledge-based society through science & technology
Employees:	2,700 (1,600 fulltime researchers and approx. 400 Ph.Ds)
Budget:	98 million USD (in 2010) allocated from the Government

4 National R&D Centers:

BIOTEC: National Center for Genetic Engineering and Biotechnology
MTEC: National Metal and Materials Technology Center
NECTEC: National Electronics and Computer Technology Center
NANOTEC: National Nanotechnology Center


S&T Community in TSP
NSTDA
Residential Area
Pilot Plants
Multi-tenants unit
Innovation cluster
Convention
Center
NANOTEC
a member of NSTDA
MTEC
a member of NSTDA
BIOTEC
a member of NSTDA
NECTEC
a member of NSTDA
TMC
a member of NSTDA
FUTURE
DEVELOPMENT
Garden of
Innovation
Innovation Cluster 1
(Multi-Tenants Unit)
Central
Building


BIOTEC staff
Scientific and
Technical Staff,
456, 82%
Administrative
Staff, 82, 15%
Middle
Management,
14, 2%
Executives,
3, 1%
Total 555
As of March 2011
Job
Function
Below B.Sc.,
30, 5%
PhD, 161,
29%
B.Sc. , 170,
31%
M.Sc., 194,
35%
Education

Food and Feed Innovation Center (FFIC)

National Center for Genetic Engineering and Biotechnology (BIOTEC)

BIOTEC
a member of NSTDA

Collaborative research projects (FD.TU vs. FFIC)

- Characterization of Probiotic properties
- Monitoring technology : using molecular techniques
- Enzyme characterization
- Mycotoxins degradation by biological methods : LAB & *Bacillus* spp.
- Bio-surfactant by LAB

Visit Us

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- www.sci.tu.ac.th
- www.sci.tu.ac.th/Food

CONTACT US

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  Faculty of Science and Technology

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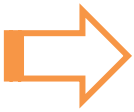
PRE-TEST

1. Definition of toxicology?
2. What is biotransformation?
3. What is toxin?

DH13TP
Food Toxicology and Mycotoxins

Toxicology

- Definition,
- Dose-response, Toxicity,
- Route of exposure,
- Biotransformation (CYP450)



Mycotoxins

- Type
- Top 8 mycotoxins
- Occurrence
- Toxicity
- Detection
- Degradation
- Effect of food processing

Toxicology

- Study of the adverse effects of ***chemicals*** ***(natural + synthetic)*** on living organisms.
- Draws heavily on knowledge in chemical and biological fields and seeks a detailed understanding of toxic effects.
- Much involved → studied of the effects of ***specific substances*** on specific biological and chemical mechanisms.

Paracelsus (1493-1541)

"All substances are poisons : there is none which is not a poison. **The right dose differentiates the poison from a remedy"**

-Founder of Toxicology-

Definition

Toxin (from Ancient Greek: *toxikon*)

- a poisonous substance produced within living cells or organisms
- synthetic toxicants created by artificial processes are thus excluded
- The term was first used by organic chemist Ludwig Brieger (1849–1919)

- Toxins can be small molecules, peptides, or proteins that are capable of causing disease on contact with or absorption by body tissues interacting with biological macromolecules such as enzymes or cellular receptors.
- Toxins vary greatly in their severity, ranging from usually minor (such as a bee sting) to almost immediately deadly (such as botulinum toxin).

Factors determining adverse effects

- **Intrinsic toxicity**
- Dose
- Exposure conditions
- Response of host

Intrinsic toxicity

1. Chemical properties

- molecular structure & functional groups
- solubility - insolubility
- volatility
- stability (light, water, acids, enzymes, …)
- Reactivity

2. Physical properties

- gas (density, …)
- liquid (vapor pressure, …)
- solid (crystal structure, size, shape, …)

Misunderstandings

Natural compounds ≠ safe compounds

Synthetic agents ≠ toxic agents

Factors determining adverse effects

- Intrinsic toxicity
- **Dose** = the amount of a substance administered at one time
- Exposure conditions
- Response of host

Types of doses

Exposure dose	the amount of a xenobiotic encountered in the environment
Absorbed dose	the actual amount of the exposed dose that enters the body
Administered dose	the quantity administered usually orally or by injection
Total dose	the sum of all individual doses

Toxicology terminology

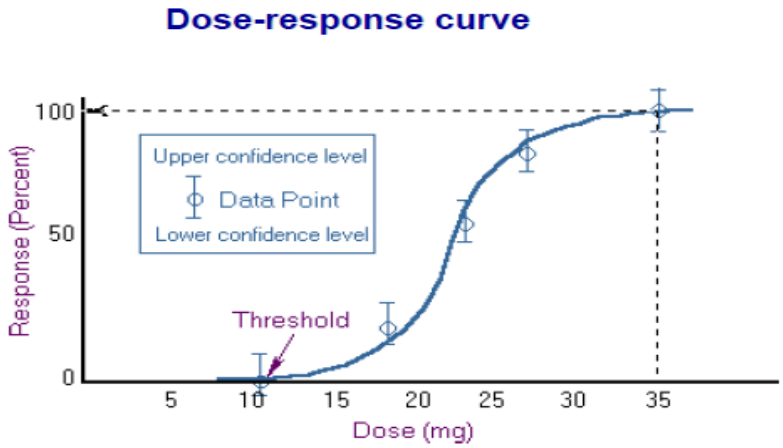
- **Toxicology**
- **Toxicologist** → scientist who conduct research on the harmful effects of the agent
- **Toxicant = xenobiotic = toxic substance = toxic agent** = substances that produce the biological adverse effect
- **Systemic toxin** → substance that affects the entire body or many organs rather than a specific site.
- **Organ toxin** → substances that affects only specific tissues or organs
- Target organ or tissue = specific sites of toxicants

Toxicology terminology (con't)

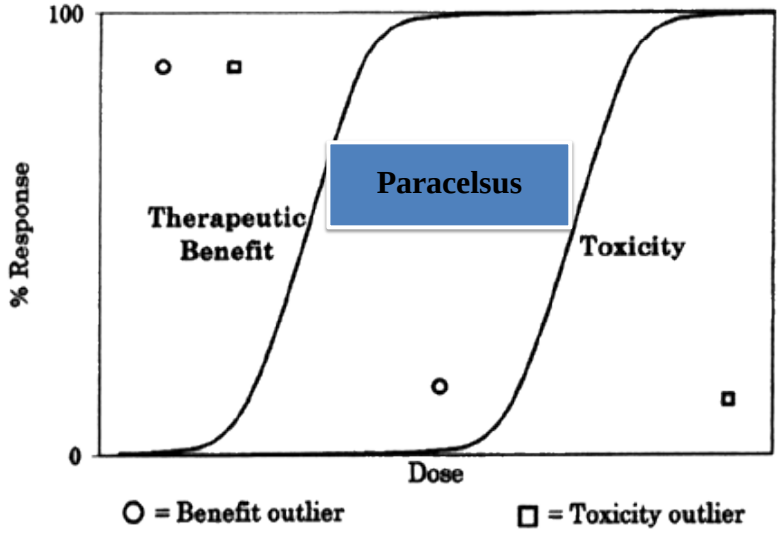
- Germ cells are those cells that are involved in the reproductive process and can give rise to a new organism. Toxicity to germ cells can cause effects on the developing fetus *(such as birth defects, abortions)*
- Somatic cells are all body cells except the reproductive germ cells. They have two sets *(or pairs)* of chromosomes.

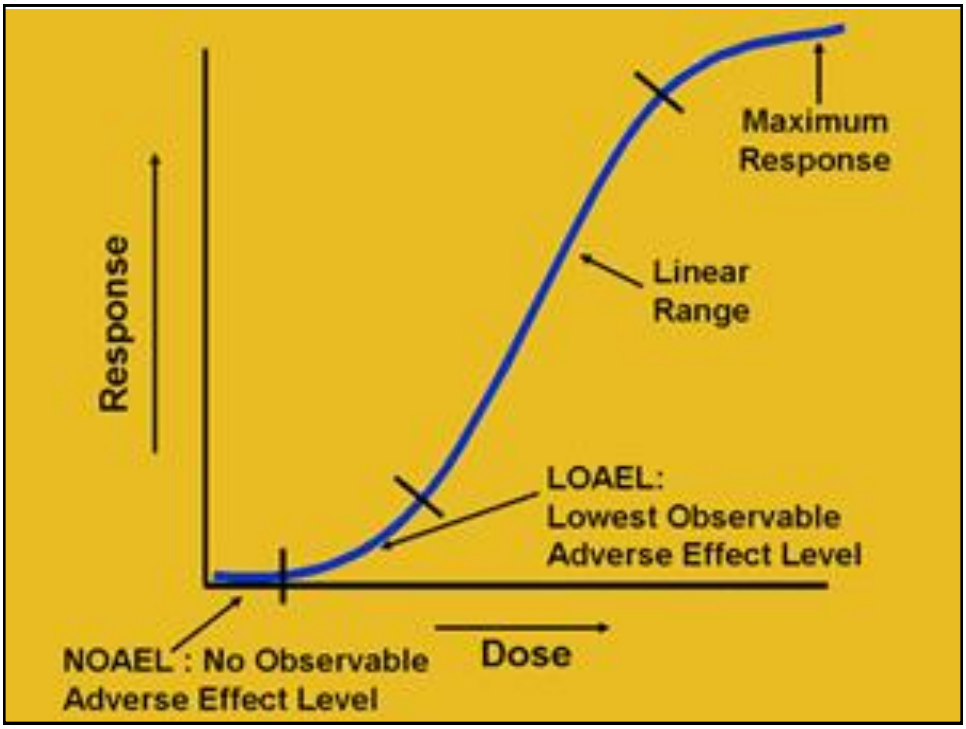
Toxicology terminology (con't)

Threshold = the point at which toxicity first appears

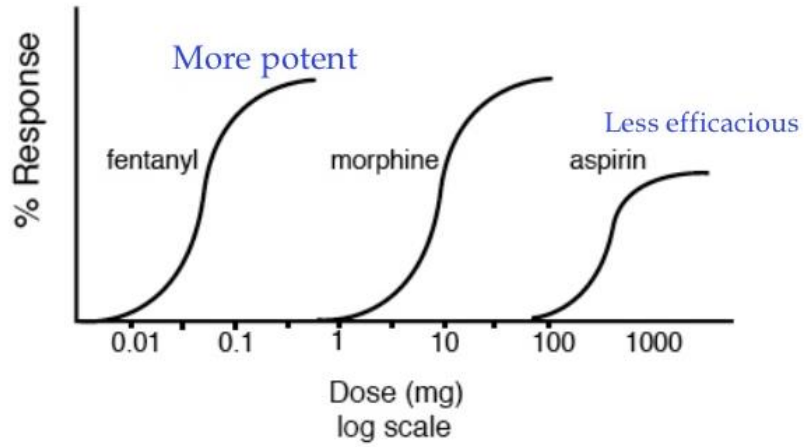


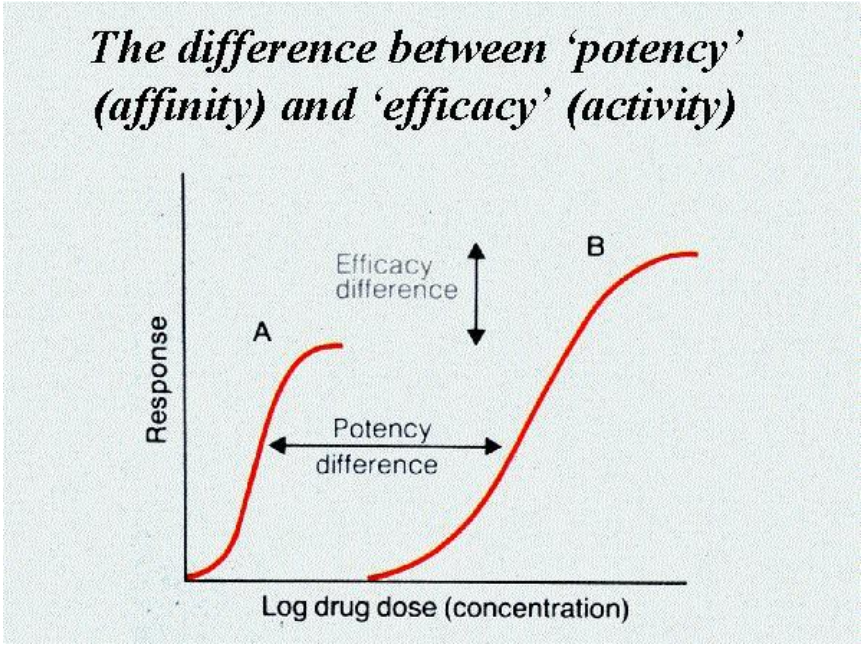
Dose-Response relationship



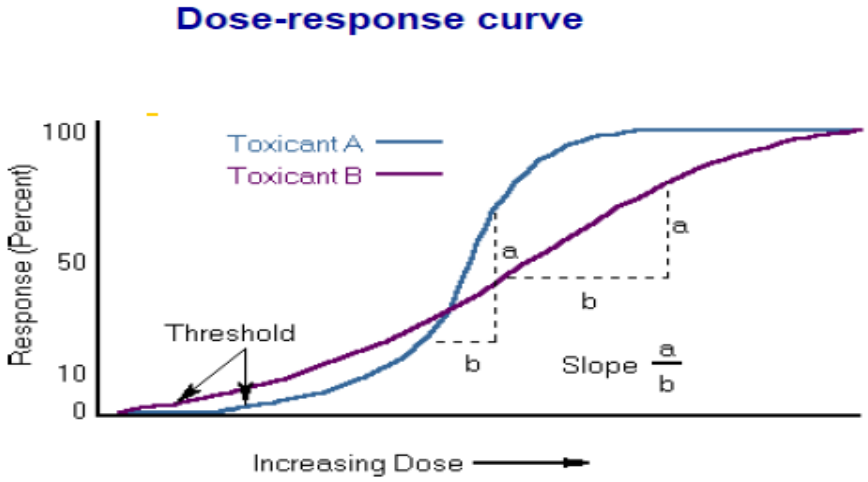


Potency vs. Efficacy

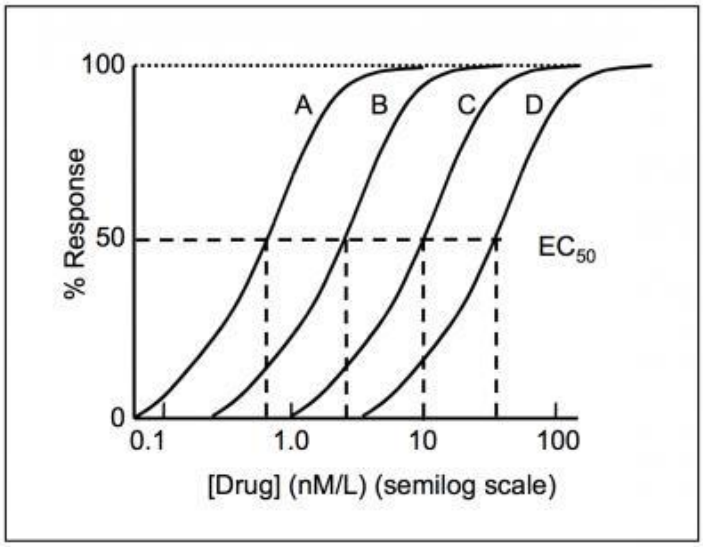




Quiz#1 : What do you think with this dose-response curve? Please explain (10 pts.)

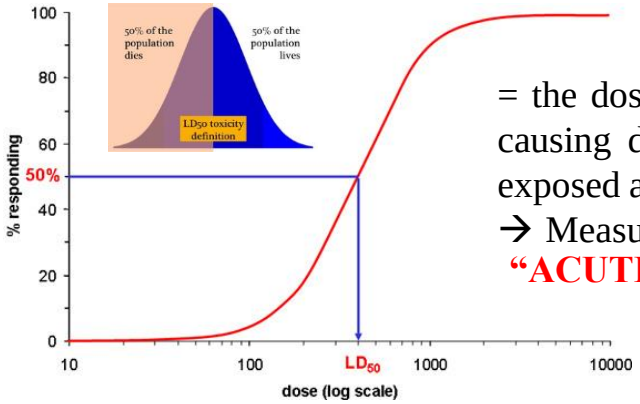


Quiz#2 : Please explain your idea about potency and efficacy of these 4 substances (5 pts.)



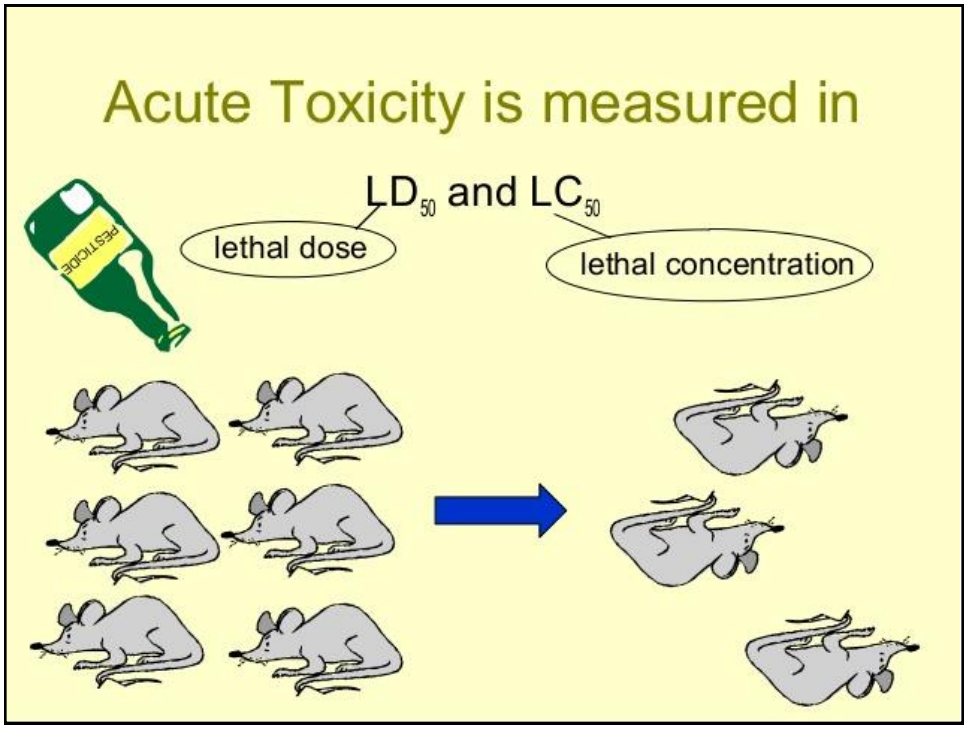
Toxicology terminology (cont)

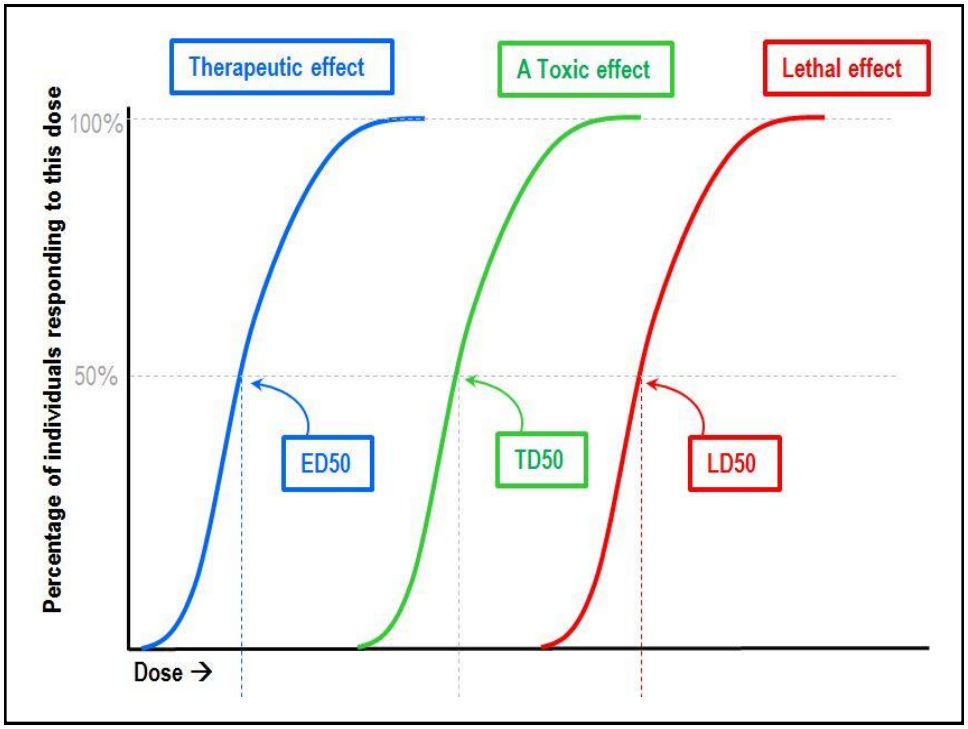
- Median lethal dose (LD_{50}) <u>or</u> Median lethal concentration (LC_{50}) <u>or</u> semi-lethal dose



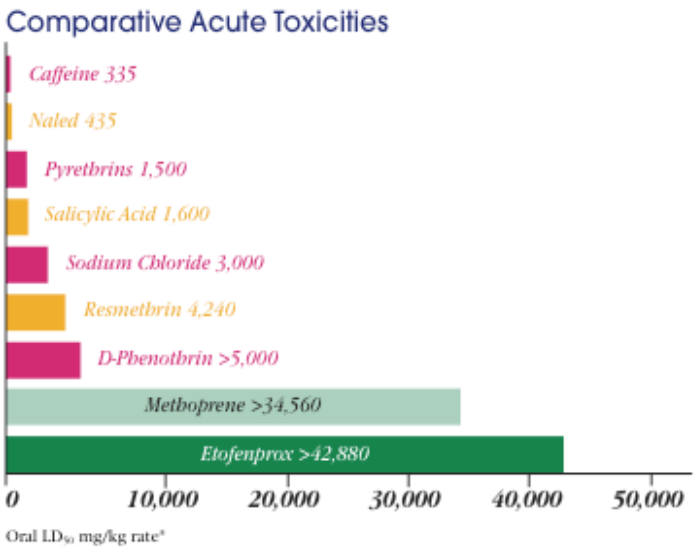
= the dosage (mg/kg bw) causing death in 50% of exposed animal

→ Measure **"ACUTE TOXICITY"**


Acute Toxicity is measured in
LD50 and LC50
lethal dose
lethal concentration
PESTICIDE


Therapeutic effect
A Toxic effect
Lethal effect
Percentage of individuals responding to this dose
100%
50%
ED50
TD50
LD50
Dose →

Quiz#3 : Which substance is more toxic? Which one is less toxic? Why? Please explain. (10 pts.)



Exposure and response

Patterns of exposure	Patterns of response
acute or high dose	clinically manifest
chronic low dose	subtle and/or long-term

- cancer
- reproductive effects
- neurodegenerative disease
- immunologic susceptibility
- …

epidemiology

Factors determining adverse effects

- Intrinsic toxicity
- Dose
- **Exposure conditions**
- Response of host

Exposure conditions

- Routes of exposure
- Frequency & duration of exposure
- Mixed exposures
- Environmental circumstances

Routes of exposure

- Oral
- Inhalation
- Dermal
- Parenteral = application outside the gastro-intestinal tract by e.g. intramuscular, intravenous or subcutaneous application of medicines

Oral exposure

- Drugs
  - therapeutic
  - non-therapeutic
- Water & soil pollutants
- Contamination of food & drinks
- Hand-mouth behavior children
  - occupational agents
  - hand hygiene

Inhalation

- Smoking
- Indoor air pollution
  - domestic
  - occupational
- Schools
- Hobby & sports
  - outdoor air pollution
- Urban, industry & vehicle traffic
- Natural sources

Dermal exposure

- Occupational agents
  - solvents
  - pesticides
- Cosmetics
- Some water pollutants

Factors determining adverse effects

- Intrinsic toxicity
- Dose
- Exposure conditions
- **Response of host**

Toxic effects

- Cellular, biochemical, or macromolecular changes
  - ☹ cell replacement, such as fibrosis
  - ☹ damage to an enzyme system
  - ☹ disruption of protein synthesis
  - ☹ production of reactive chemicals in cells
  - ☹ DNA damage

Factors Influencing Toxic Effects

- Form and innate chemical activity
- Dosage, especially dose-time relationship
- Exposure route
- Species
- Age
- Sex

Factors Influencing Toxic Effects (con't)

- Ability to be absorbed
- Metabolism
- Distribution within the body
- Excretion
- Presence of other chemicals

Systemic Toxic Effects

1. Acute Toxicity → occurs almost immediately *(hours/days)* after an exposure. An **acute exposure** is usually
   - a single dose or a series of doses received within a 24 hour period. Death is a major concern in cases of
   - acute exposures.
2. Sub-chronic Toxicity → results from repeated exposure for several weeks or months.
3. Chronic Toxicity → represents cumulative damage to specific organ systems and takes many months or years to become a recognizable clinical disease.

Developmental Toxicity

Embryolethality	failure to conceive, spontaneous abortion or stillbirth
Embryotoxicity	growth retardation or delayed growth of specific organ systems
Teratogenicity	irreversible conditions that leave permanent birth defects in live offspring *(e.g. cleft palate, missing limbs)*

Genetic Toxicity *(somatic cells)*

Gene mutation	change in DNA sequence within a gene
Chromosome aberration	changes in the chromosome structure
Aneuploidy / polyploidy	increase or decrease in number of chromosomes

Carcinogenicity

- *Multistage process*
  - Initiation → a normal cell undergoes irreversible changes
  - Promotion → initiated cells are stimulated to progress to cancer.
- Benign tumors
- Malignant tumors

Tumor *(neoplasm)*

= An uncontrolled growth of cells.

- **Benign tumors** grow at the site of origin; do not invade adjacent tissues or metastasize; and generally are treatable.
- **Malignant tumors *(cancer)*** invade adjacent tissues or migrate to distant sites ***(metastasis)***. They are more difficult to treat and often cause death

Other toxicity

- **Hepatotoxicity** is toxicity to the liver, bile duct, and gall bladder.
- **Immunotoxicity** relates to the immune system. It can take several forms: hypersensitivity *(allergy and autoimmunity)*, immunodeficiency, and uncontrolled proliferation *(leukemia and lymphoma)*.

- **Nephrotoxicity** is toxicity to the kidneys. It can result in systemic toxicity causing:
  - decreased ability to excrete body wastes
  - inability to maintain body fluid and electrolyte balance
  - decreased synthesis of essential hormones
- **Neurotoxicity** represents toxicant damage to cells of the central nervous system *(brain and spinal cord)* and the peripheral nervous system *(nerves outside the CNS)*

Frequency & duration

Acute toxicity (single dose or < 24h)

- may lead to immediate ("acute") effects
- may lead to delayed or persistent ("chronic") effects

Chronic toxicity (repeated doses)

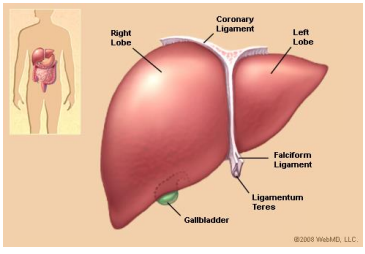
- may lead to sudden ("acute") effects
- results from accumulation of toxic agent or from cumulative effects
- may lead to delayed or persistent ("chronic") effects

Quiz#4 : How many toxic effects do you learn from this class? Please list and explain (10 pts.)

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

Response of host

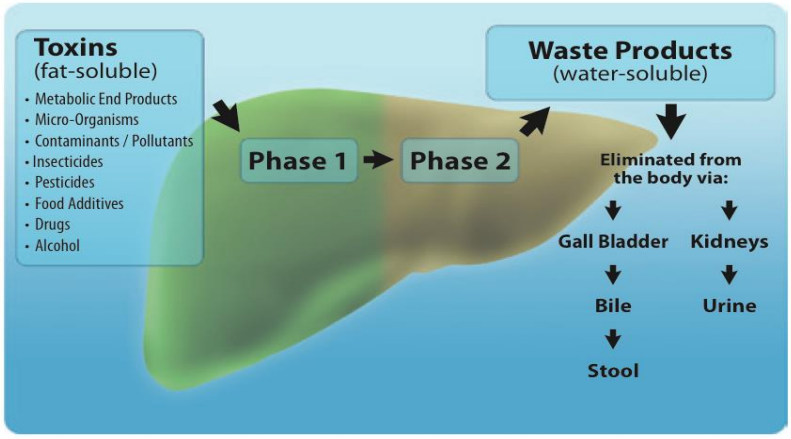
- Detoxification → *less toxic metabolites*
- Bioactivation → *more toxic metabolites*

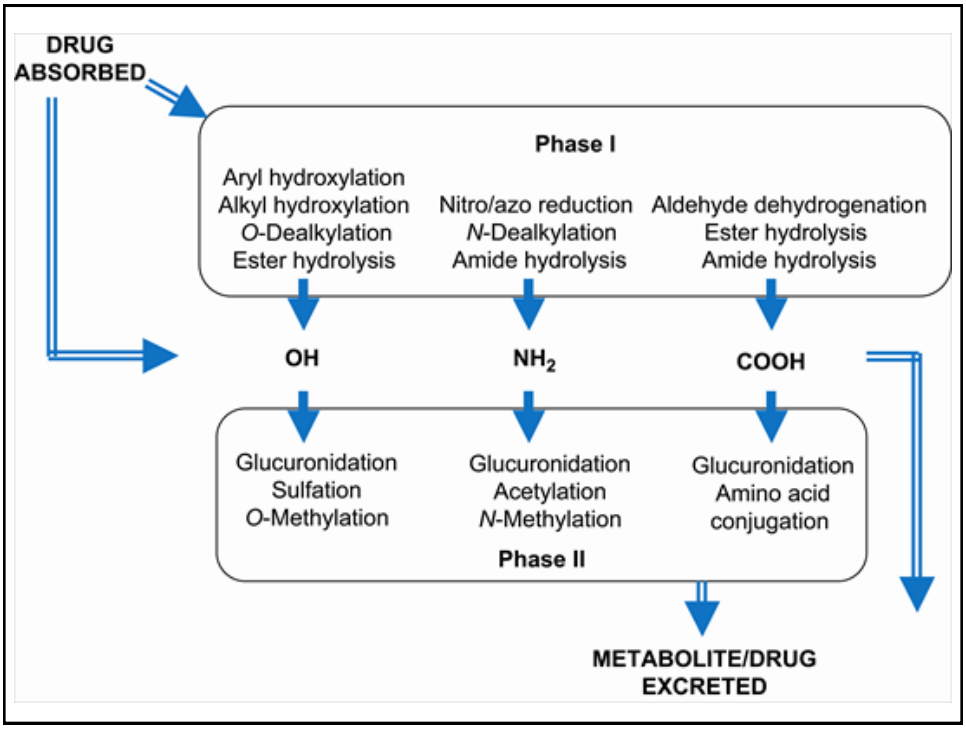


Liver

Biotransformation

BIOTRANSFORMATION

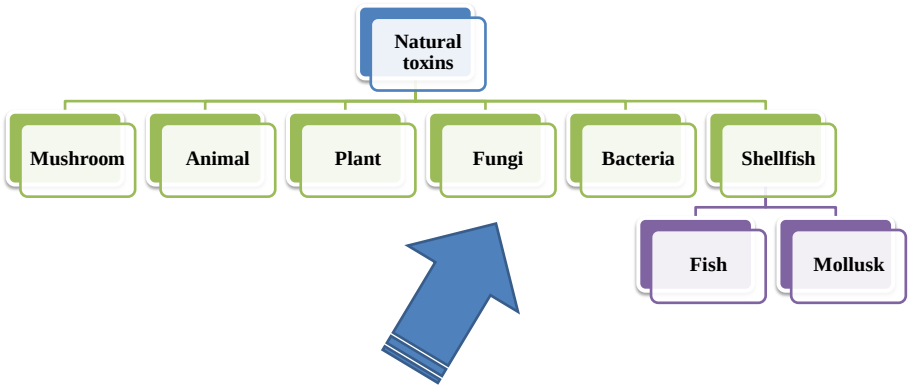




Quiz#5 : How the biotransformation importance to the organism? Please explain. (5 pts)

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

Classification of natural toxins



Food Fungi

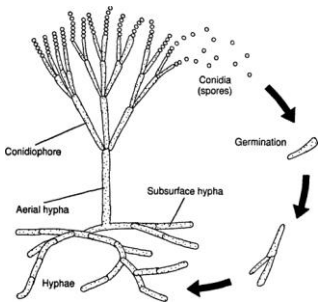
Fungi


Structure

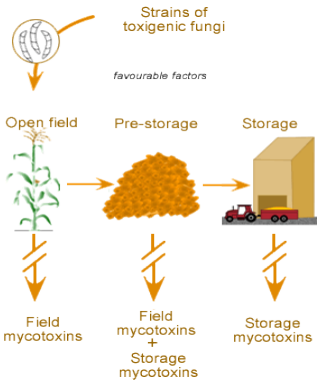

Growth


Enumeration


Identification



Mycotoxins



Fungi and Mycotoxins

- *Aspergillus* spp.
- *Penicillium* spp.
- *Fusarium* spp.
- *Clavicep* spp.

Mycotoxins-producing fungi
Or
Mycotoxigenic fungi

General Characteristics of True Fungi (Mycota or Eumycota)

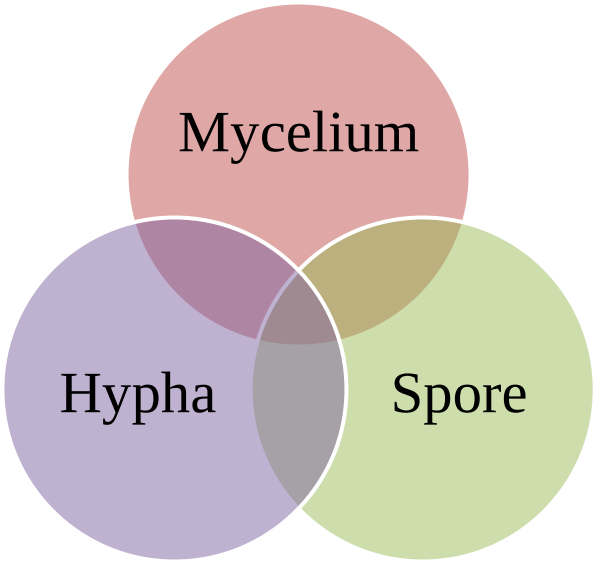
1. All are eukaryotic
2. Most are filamentous
3. Some are unicellular
4. Protoplasm of a hypha or cell is surrounded by a rigid wall
5. Many reproduce both sexually and asexually
6. Their nuclei are typically haploid and hyphal compartments are often multinucleate
7. All are achlorophyllous
8. All are chemoheterotrophic (chemo-organotrophic)
9. Possess characteristic range of storage compounds
10. May be free-living or may form intimate relationships with other organisms

Importance of Fungi

Fungi are important because they are:

- agents of biodegradation and biodeterioration
- responsible for the majority of plant diseases and several diseases of animals (including humans)
- used in industrial fermentation processes
- used in the commercial production of many biochemicals
- cultured commercially to provide us with a direct source of food
- used in bioremediation
- beneficial in agriculture, horticulture and forestry.

Fungal Basic Structure



Mycotoxigenic Fungi

Aspergillus spp.
Penicillium spp.
Fusarium spp.

Calvicep spp. ➔ Minor

Major



Pathogen and commensal
Contaminated during drying and storage

Plant pathogen

Quiz#6 : Explain the relationship between the fungi and the mycotoxins. (5 pts)

……
……
……
……
……

Genus *Aspergillus*

- The most significance mycotoxigenic species are *A. flavus*, A. parasiticus*, A. ochraceus (=A. westerdijkiae* or *A. steynii), A. niger*
- Color : Black, Yellow, Green, Brown and White

Enumeration using

1.) Dichloran rose bangal chloramphenicol agar (DRBC) or

2.) Dichloran 18% glycerol agar (DG18) or

3.) Aspergillus flavus and parasiticus agar (AFPA)

Factor influencing growth

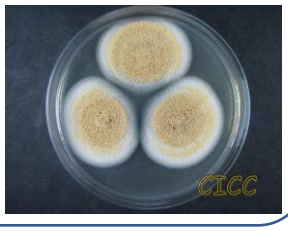
- Minimum temp 10-12 ◦ C
- Maximum temp 43-48 ◦ C
- Optimum temp 33 ◦ C
- Minimum Aw permitting growth
  - 0.82 at 25 ◦ C
  - 0.81 at 30 ◦ C
  - 0.80 at 37 ◦ C
- pH range 2.1-11.2
- Optimum broad pH 3.4-10
- D_{45} = 160 hour
- D_{50} = 16 hours
- D_{52} = 40-45 min
- D_{60} = 1 min

A. flavus & *A. parasiticus*

Factor influencing growth

- Optimum temp 24-31°C
- Optimum A_w 0.95-0.99
- Lower limit of A_w = 0.79
- Optimum pH 3-10

A. ochraceus



- *Aspergillus niger*
- *Aspergillus japonicus*
- *Aspergillus carbonarius*

Optimum temp 30°C
Optimum A_w 0.96-0.98

Black Aspergilli

Genus *Penicillium*

- More than 200 recognized species
- Grow slowly and have green conidia
- Enumeration using :

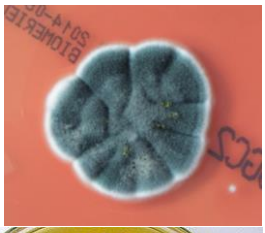
  PDA, DCPA, DRBC*, DG18*

  DRYS (selective media): Violet brown reverse color (for *P. verrucosum*)
- Toxigenic strain → *P. viridicatum* = *P. verrucosum*

  P. nordicum
- Ochratoxin producing strain

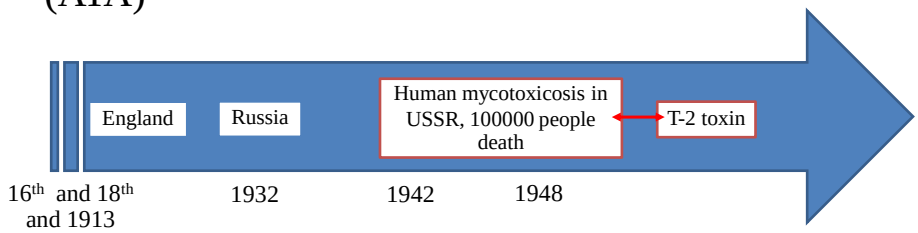
Factor influencing growth

- Range of temperature = 0-31°C
- Optimum temp 20 ° C
- Minimum A_w = 0.8
- pH range = 2.1-10.0
- Toxin production at 4 ° C A_w < 0.86



Genus *Fusarium*

- The most important genera of plant pathogenic fungi
- Infection may occur in developing seeds and also in maturing fruits and vegetables
- Strongly associated ***Alimentary Toxic Aleukia*** (ATA)



Alimentary Toxic Aleukia

- A form of toxicosis caused by ingestion of grain contaminated with any of several kinds of trichothecene mycotoxins; characterized by nausea, vomiting, diarrhea, leukopenia (aleukia), hemorrhaging, skin inflammation, and, in severe cases, death.
- **Leukopenia** = reduction of the number of leukocytes in the blood below about 5,000 per mm^3. Called also aleukemia, aleukocytosis, and leukocytopenia

Fusarium spp.

- Uncoloured / white, pink, purple, pale to red reverses
- Multi-septate
- Large curved conidia (25-50 μm or more)
- Some species produced microconidia
- Maximum temperature = 32-37°C
- Minimum temperature 2.5-5°C
- Optimum temperature 25°C
- Minimum A_w = 0.87

FUNGAL IDENTIFICATION

How to identify and differentiate the species of *Aspergiilus*, *Penicillium* and *Fusarium*?

A. flavus and *A. parasiticus*

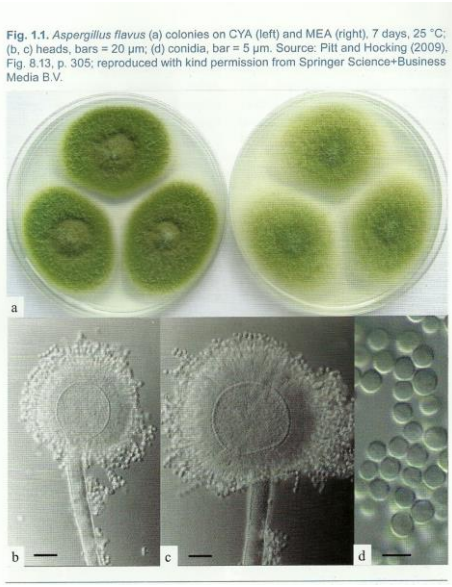


Fig. 1.1. *Aspergillus flavus* (a) colonies on CYA (left) and MEA (right), 7 days, 25 °C; (b, c) heads, bars = 20 µm; (d) conidia, bar = 5 µm. Source: Pitt and Hocking (2009), Fig. 8.13, p. 305; reproduced with kind permission from Springer Science+Business Media B.V.

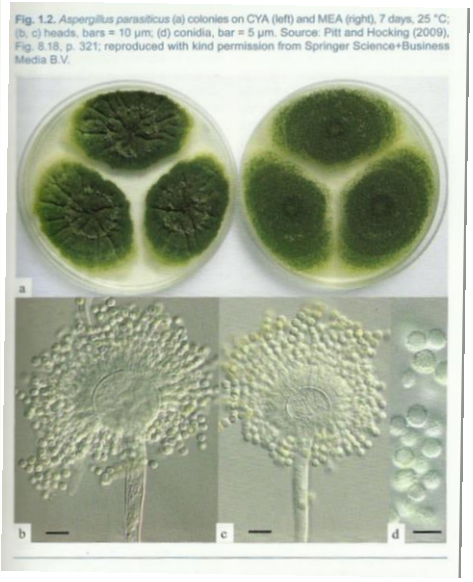


Fig. 1.2. *Aspergillus parasiticus* (a) colonies on CYA (left) and MEA (right), 7 days, 25 °C; (b, c) heads, bars = 10 µm; (d) conidia, bar = 5 µm. Source: Pitt and Hocking (2009), Fig. 8.18, p. 321; reproduced with kind permission from Springer Science+Business Media B.V.

A. ochraceus

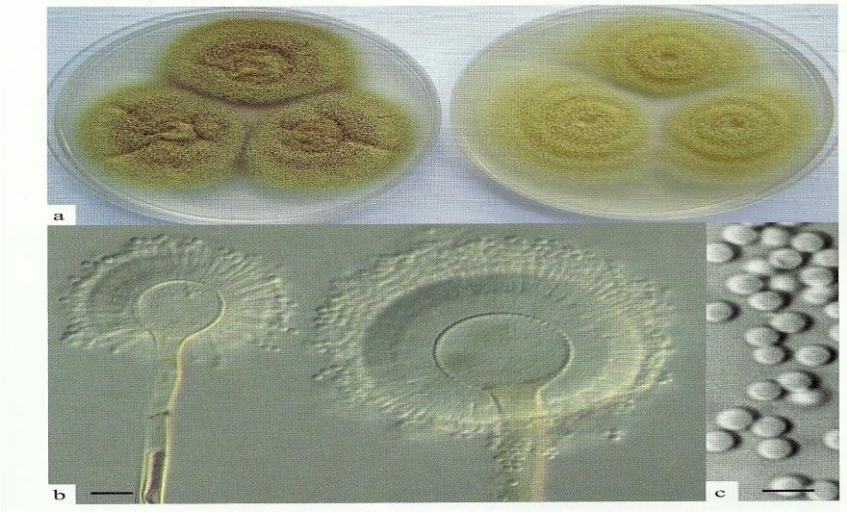


Fig. 1.3. *Aspergillus ochraceus* (a) colonies on CYA (left) and MEA (right), 7 days, 25 °C; (b) heads, bar = 20 µm; (c) conidia, bar = 5 µm. Source: Pitt and Hocking (2009), Fig. 8.17, p. 318; reproduced with kind permission from Springer Science+Business Media B.V.

Black aspergilli

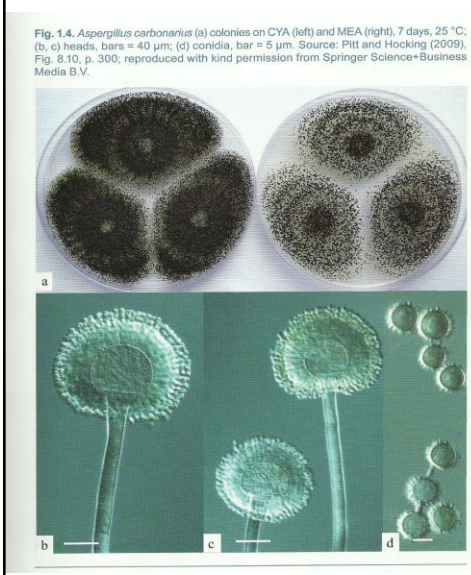


Fig. 1.4. *Aspergillus carbonarius* (a) colonies on CYA (left) and MEA (right), 7 days, 25 °C; (b, c) heads, bars = 40 µm; (d) conidia, bar = 5 µm. Source: Pitt and Hocking (2009), Fig. 8.10, p. 300; reproduced with kind permission from Springer Science+Business Media B.V.

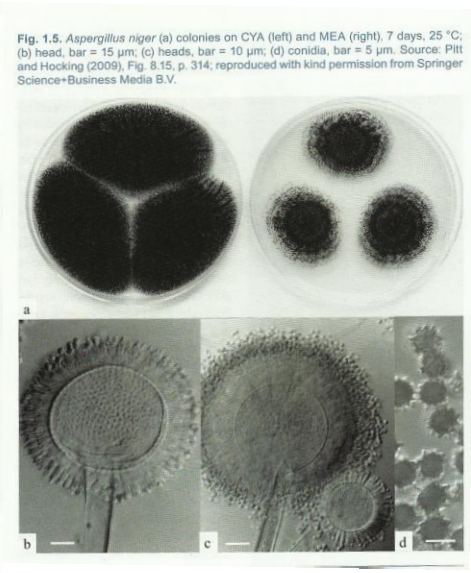


Fig. 1.5. *Aspergillus niger* (a) colonies on CYA (left) and MEA (right). 7 days, 25 °C; (b) head, bar = 15 µm; (c) heads, bar = 10 µm; (d) conidia, bar = 5 µm. Source: Pitt and Hocking (2009), Fig. 8.15, p. 314; reproduced with kind permission from Springer Science+Business Media B.V.

Penicillium and Fusarium

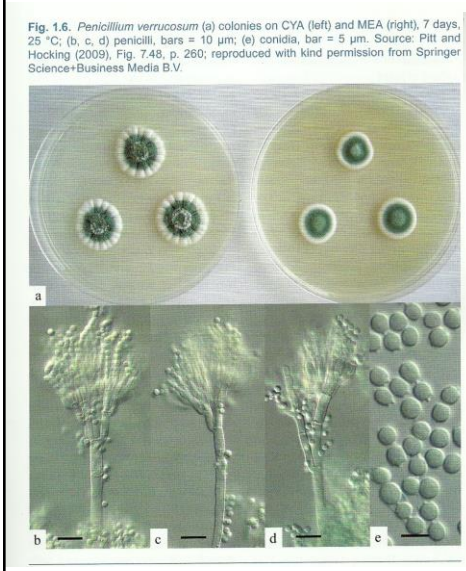


Fig. 1.6. *Penicillium verrucosum* (a) colonies on CYA (left) and MEA (right), 7 days, 25 °C; (b, c, d) penicilli, bars = 10 µm; (e) conidia, bar = 5 µm. Source: Pitt and Hocking (2009), Fig. 7.48, p. 260; reproduced with kind permission from Springer Science+Business Media B.V.

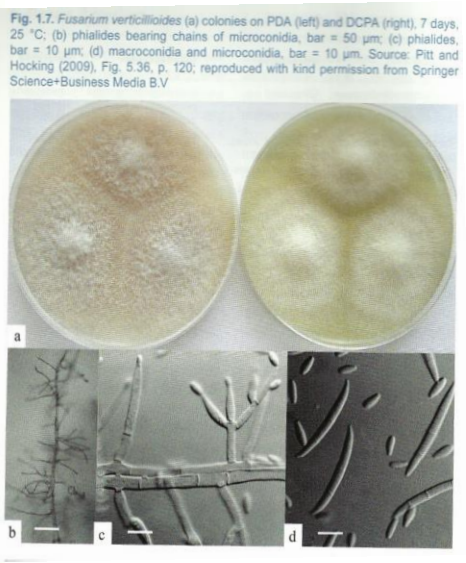


Fig. 1.7. *Fusarium verticillioides* (a) colonies on PDA (left) and DCPA (right), 7 days, 25 °C; (b) phialides bearing chains of microconidia, bar = 50 µm; (c) phialides, bar = 10 µm; (d) macroconidia and microconidia, bar = 10 µm. Source: Pitt and Hocking (2009), Fig. 5.36, p. 120; reproduced with kind permission from Springer Science+Business Media B.V

Fusarium spp.

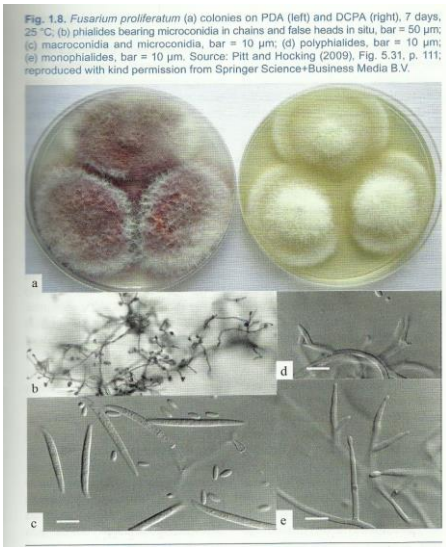


Fig. 1.8. *Fusarium proliferatum* (a) colonies on PDA (left) and DCPA (right), 7 days, 25 °C; (b) phialides bearing microconidia in chains and false heads in situ, bar = 50 µm; (c) macroconidia and microconidia, bar = 10 µm; (d) polyphialides, bar = 10 µm; (e) monophialides, bar = 10 µm. Source: Pitt and Hocking (2009), Fig. 5.31, p. 111; reproduced with kind permission from Springer Science+Business Media B.V.

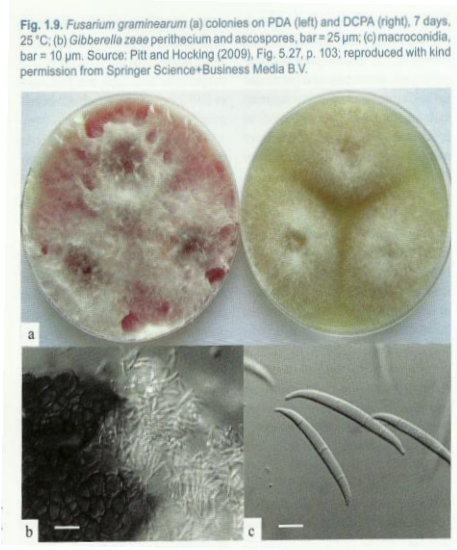


Fig. 1.9. *Fusarium graminearum* (a) colonies on PDA (left) and DCPA (right), 7 days, 25 °C; (b) *Gibberella zeae* perithecium and ascospores, bar = 25 µm; (c) macroconidia, bar = 10 µm. Source: Pitt and Hocking (2009), Fig. 5.27, p. 103; reproduced with kind permission from Springer Science+Business Media B.V.

Decision Tree

Fig. 1.10. Decision tree for directing risk management decisions or actions based on environmental considerations and probability of fungal contamination in warm climates. Expected toxic effects in susceptible animals are given for each group of mycotoxins.

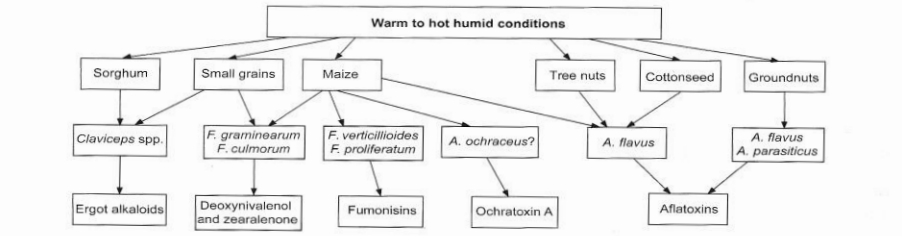
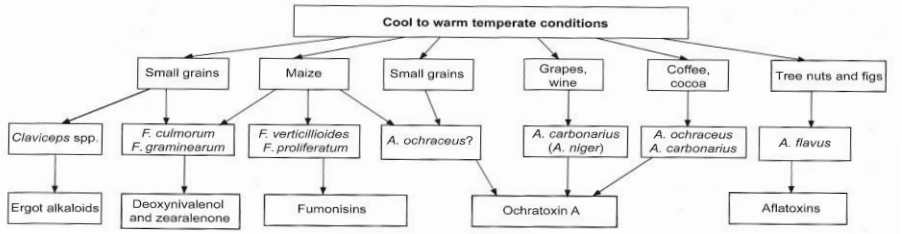
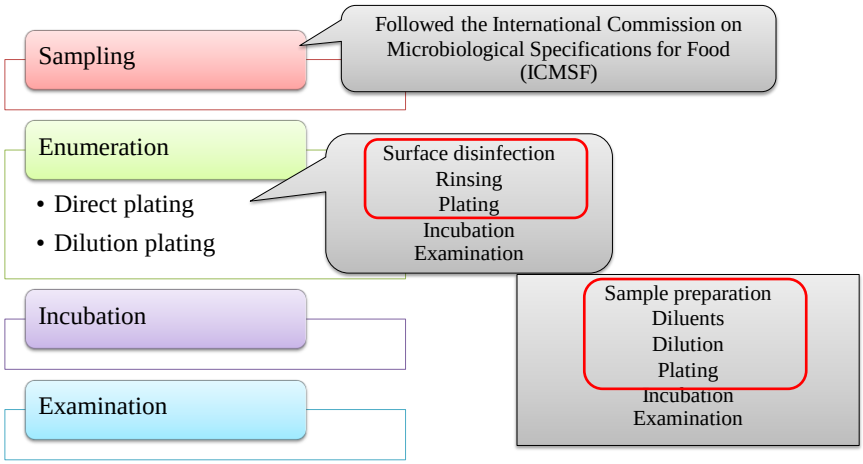


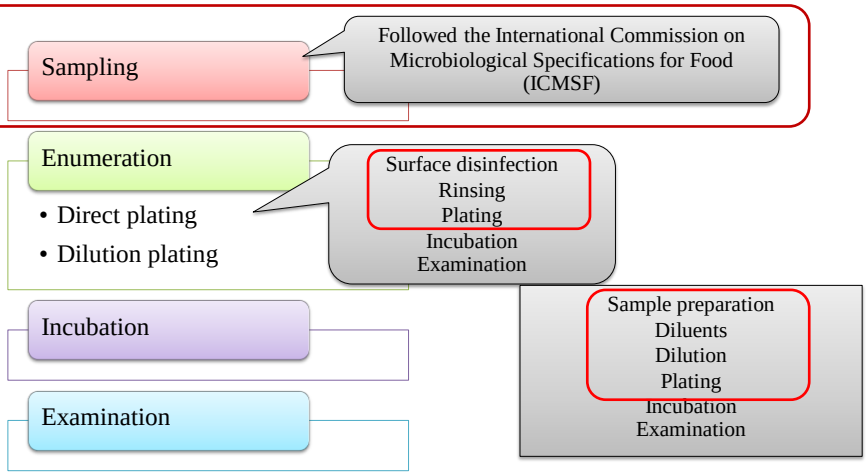
Fig. 1.11. Decision tree for directing risk management decisions or actions based on environmental considerations and probability of fungal contamination in cool climates. Expected toxic effects in susceptible animals are given for each group of mycotoxins.



FUNGAL ENUMERATION



FUNGAL ENUMERATION



How to sampling?

Using an effective sampling plan

- AOAC, 1985
- APHA, 1984
- Barrow, 1983
- FDA, 1978
- ICMSF, 1974

How to sampling? (cont.)

- Random sampling / good sampling plan
- Bulk sample → Sub-sample
- 1 inch in depth
- More than 25 gram
- Should have a good sample labeling (name of the people who do sampling, date, type of food, …)
- Keep at 4^{o}C

FUNGAL ENUMERATION

- **Sampling** — Followed the International Commission on Microbiological Specifications for Food (ICMSF)
- **Enumeration**
  - Direct plating
  - Dilution plating

  Surface disinfection
  Rinsing
  Plating
  Incubation
  Examination
- **Incubation**
- **Examination**

Sample preparation
Diluents
Dilution
Plating
Incubation
Examination

Enumeration : Direct plating

Removes the inevitable surface contamination arising from dust and other sources

50 or more particles in 250-500 beakers
↓
0.4% Chlorine 2 min + stir + cover the beaker
↓
Poured off
↓
Rinsed once with sterile water (1 min + stirring)*
↓
Plating (6-20 particles / plate)
↓
Incubated at 25°C 5 days / upright position
↓
Count the numbers of infected particles
↓
Express results as
percentage infection of particles

Enumeration : Dilution plating

Appropriate for liquid or powdered food

1. **Stomaching** – recommended by ICFM
   - ☺ dispersing and separating fungi from finely materials such as flour and spices
   - ☺ 2 min
2. **Blending** – should not exceed 60 sec
   - ☹ longer treatment may fragment mycelium into length too short to be viable

Sample size 10-40 g

Dilution plating : Procedure

Sample

↓

Soaking 30-60 min or up to 3 hr

↓

Stomaching or Blending + diluents

(0.1% peptone suitable for filamentous and yeast)

⌦ Saline solution, phosphate buffer or distilled water

↓

Dilution (1:10 = 1+9)

↓

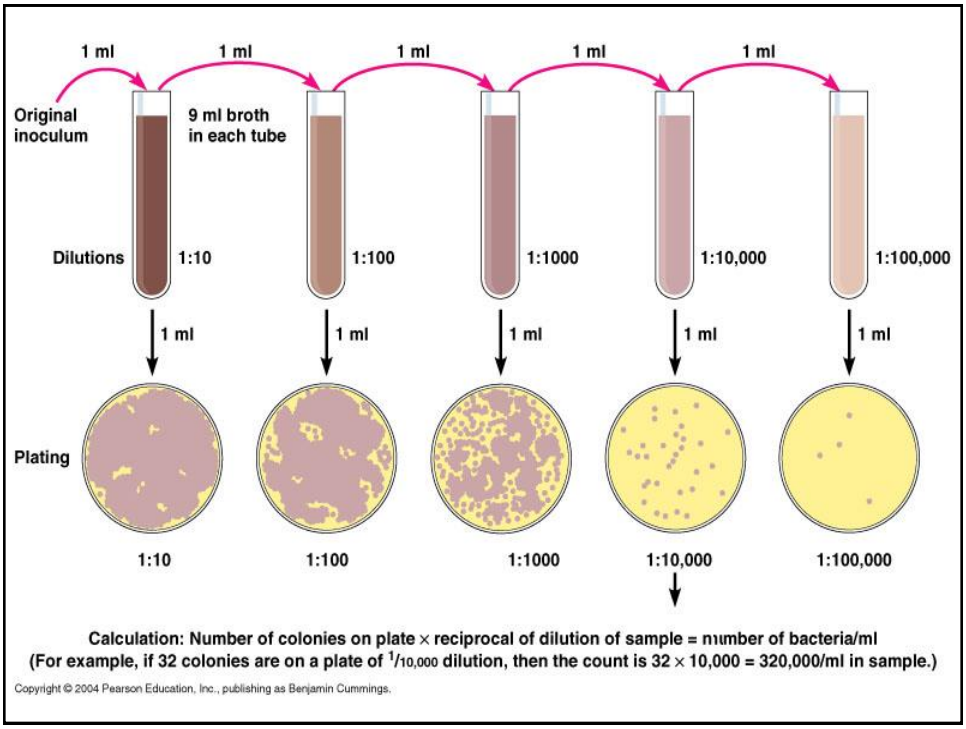
Spread plating

↓

Incubation at 25°C 5 days

↓

Report as viable count per gram of sample



Other plating techniques

1. Spiral plate count
   - PDA and DRBC
2. Hydrophobic grid membrane filters (HGMF)
   - Determined by a most probable number (MPN)
3. Petrifilm™ (3 M company)
   - Fungal enumeration on a layer of medium enclosed in a plastic film, which eliminates the use of Petridish
   - Not particularly effective in inhibiting the growth of *Rhizopus* and *Mucor*
   - Subculturing colonies for identification was more difficult
   - Do not recommend for the food A_w less than 0.95

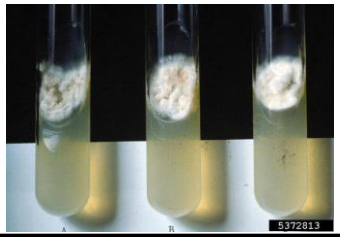
Isolation techniques

"Isolation" = The preparation of a pure culture, free from any contamination and ready for identification

1. Streaking techniques
   ☹ ineffective for filamentous fungi
   ☹ not recommended
2. *Simplest* with needle and inoculate a single point on a plate

Short term storage

- Stored on slant
- Cap must be kept loose
- Fungi require free access to oxygen for typical growth and sporulation
- Oxygen starvation during growth will at best lead to retarded sporulation or at worst death of the culture



Long term storage

1. Lyophilisation
2. Spore suspension + 60-80% glycerol (as a cryoprotectant)
3. Water storage → storage of agar block (7-10 mm2) in water → keep at 1-10°C (up to 7 years)

Choosing a suitable medium

1. ***General purpose enumeration media***
   - Completely inhibit bacterial growth, without affecting growth of food-borne fungi
   - Adequate nutrition and support growth of fastidious fungi
   - Suppress the growth of rapidly spreading fungi
   - Slow radial growth of all fungi, to permit counting of a reasonable number of colonies per plate, without inhibiting spore germination
   - Promote the growth of relevant fungi
   - Suppress the growth of soil fungi or others generally irrelevant in food spoilage
2. ***Selective isolation media***

General purpose enumeration media

1. Dichloran rose bengal chloramphenicol (DRBC)
   - Suit to fresh and high A_w foods
   - Contains Rose Bengal 25 mg/kg + dichloran 2 mg/kg
   - Restrict colony spreading (*Rhizopus* and *Trichoderma*) without affecting spore germination unduly
2. Dichloran 18% glycerol agar (DG18)
   - Suit for low Aw food (stored grain, nuts, flour and spices)
   - Support growth of the common *Aspergillus*, *Penicillium* and *Fusarium* spp. as well as most yeasts, and many other common foodborne fungi
   - Bacteria are totally suppressed
   - Useful medium for enumeration of airborne fungi
3. Rose bengal chloramphenicol agar (RBC)
4. Oxytetracycline glucose yeast extract agar (OGY)

Table 4.1 Recommended media for fungal detection, enumeration and isolation[a]

Type of food	Selecting for	Medium	Remarks
Fresh foods: milk and milk products, fruit, cheese, sea foods	Moulds	DRBC	Blend (where necessary) and dilution plate
	Yeasts	TGY, MEA, OGY	
	General	DRBC	
Freshly harvested grains, nuts	General	DRBC	Direct plate
	Dematiaceous Hyphomycetes	DRBC, CZID	Direct plate
	Fusarium	CZID	Direct plate
	Yeasts	TGY, MEA, OGY	Dilution plate
Fruit juices, fresh	Yeasts	TGY, MEA, OGY	Dilution plate
Fruit juices, preserved	Preservative resistant yeasts	TGYA, malt acetic agar	Dilution plate
Fruit juices, to be pasteurised, or pasteurised products	Heat resistant moulds	PDA, MEA	Special protocol
Fruit juice concentrates	Xerophilic yeasts	MY50G	Special diluents
Dried foods in general	General	DG18	Direct plate
Stored cereals, nuts	General	DG18	Direct plate
	Dematiaceous Hyphomycetes	DRBC, CZID	Direct plate
	Fusarium	CZID	Direct plate

Table 4.1 Recommended media for fungal detection, enumeration and isolation[a] (continued)

Type of food	Selecting for	Medium	Remarks
Grain for milling into flour	General	DG18	Stomach or blend and dilution plate
Dried fruit, confectionery, chocolate, etc.	Xerophilic moulds and yeasts	MY50G	Direct plate
	Fastidious xerophiles	MY50G	Direct plate
	– in presence of *Eurotium* spp.	MY70GF	Direct plate
Salt foods, e.g. salt fish	General	DG18	Direct plate or press plate
	Halophilic xerophiles	MY5-12, MY10-12	Direct plate or press plate
General	Fungi producing aflatoxins	AFPA	Direct or dilution plate
General	Fungi producing ochratoxins	DRYS	Direct or dilution plate

[a] For medium acronyms, see Section 4.6.

Selective isolation media

1. Media for *Asgergillus flavus* and related species
2. Media for fungi producing ochratoxin A
3. Media for *Fusarium* spp.
4. Detection of aflatoxin production using Coconut Cream Agar (CCA)

Media for *Asgergillus flavus* and related species

Aspergillus flavus and parasiticus agar (AFPA)

→Bright orange yellow reverse color

→Recommended for the detection and enumeration of potentially aflatoxigenic fungi in nuts, maize, spices and other commodities

☺Rapidly, 48 hour incubation is usually sufficient

☺Specificity, simplicity, little skill is required

Media for fungi producing ochratoxin A

- There are no selective or indicative media for these fungi
  - ➢DG18
  - ➢Coconut cream agar (CCA) } *Aspergillus* spp.
  - ➢Dichloran rose bengal yeast extract sucrose agar (DRYS) ⟶ *Penicillium verrucosum*

Media for *Fusarium* spp.

☺ Dichloran chloramphenicol peptone agar (DCPA)

- Found to be less effective in mixed population
- Induces the formation of macroconidia

Fungicide

☺ Czapek iprodione dichloran agar (CZID)

- Suitable for isolation of *Fusarium* spp. either direct plating or dilution plating
- Too selective and not support growth of all foodborne *Fusarium* spp.

Detection of aflatoxin production using Coconut Cream Agar (CCA)

Canned coconut cream
↓
Dilute 50:50 with water
↓
Added agar 1.5%
↓
Autoclave
↓
Inoculate up to 4 colonies
↓
Incubated at 30C 5-7 days
↓
Examine under long wave length UV light
(Fluoresce bluish white or white)
*Control = uninoculated coconut cream agar plate

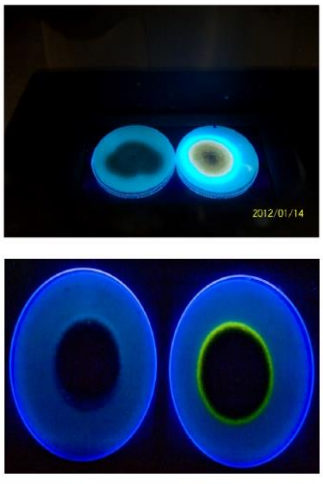


Figure 1. Characteristic beige ring shown by aflatoxigenic *Aspergillus flavus* (right side) on coconut agar medium

Culture media

ANNEX. MEDIA

The formulations given below are from Pitt and Hocking (2009).

Aspergillus flavus and parasiticus agar (AFPA)
Peptone, bacteriological: 10 g
Yeast extract: 20 g
Ferric ammonium citrate: 0.5 g
Chloramphenicol: 100 mg
Agar: 15 g
Dichloran (0.2% in ethanol, 1.0 mL): 2 mg
Water, distilled: 1 L

Sterilize by autoclaving at 121 °C for 15 minutes. Final pH is 6.0–6.5.

Czapek concentrate
$NaNO_3$: 30 g
KCl: 5 g
$MgSO_4.7H_2O$: 5 g
$FeSO_4.7H_2O$: 0.1 g
Water, distilled: 100 mL

Czapek concentrate will keep indefinitely without sterilization. The precipitate of $Fe(OH)_3$ that forms in time can be resuspended by shaking before use.

Czapek–Dox iprodione dichloran agar (CZID)
Sucrose: 30 g
Yeast extract: 5 g
Chloramphenicol: 100 mg
Dichloran (0.2% in ethanol, 1.0 mL): 2 mg
Czapek concentrate: 10 mL
Trace metal solution: 1 mL
Agar: 15 g
Water, distilled: 1 L
Iprodione suspension: 1 mL

Sterilize by autoclaving at 121°C for 15 minutes. Add iprodione suspension (0.3 g Roval 50WP [Rhône-Poulenc Agrochimie, Lyon, France] in 50 mL sterile water, shaken before addition to medium) after autoclaving. This formulation is an adaptation of the original published formulation (Abildgren *et al.*, 1987), made from basic ingredients rather than using commercial Czapek–Dox broth. Chloramphenicol (100 mg/L) replaces the original combination of chlortetracycline (50 mg) and chloramphenicol (50 mg).

Czapek yeast extract agar (CYA)
K_2HPO_4: 1 g
Czapek concentrate: 10 mL
Trace metal solution: 1 mL
Yeast extract, powdered: 5 g
Sucrose: 30 g
Agar: 15 g
Water, distilled: 1 L

Refined table grade sucrose is satisfactory for use in CYA provided it is free of sulfur dioxide. Sterilize by autoclaving at 121 °C for 15 minutes. Final pH is 6.7.

Dichloran chloramphenicol peptone agar (DCPA)
Peptone: 15 g
KH_2PO_4: 1 g
$MgSO_4.7H_2O$: 0.5 g
Chloramphenicol: 0.1 g
Dichloran (0.2% in ethanol, 1.0 mL): 2 mg
Agar: 15 g
Water, distilled: 1 L

Sterilize by autoclaving at 121 °C for 15 minutes. Final pH is 5.5–6.0.

Dichloran 18% glycerol agar (DG18)
Glucose: 10 g
Peptone: 5 g
KH_2PO_4: 1 g
$MgSO_4.7H_2O$: 0.5 g
Glycerol, A.R.: 220 g
Agar: 15 g
Dichloran (0.2% w/v in ethanol, 1.0 mL): 2 mg
Chloramphenicol: 100 mg
Water, distilled: 1 L

Add minor ingredients and agar to about 800 mL of distilled water. Steam to dissolve agar, then make up to 1 L with distilled water. Add glycerol; note that the final concentration is 18% w/w, not w/v. Sterilize by autoclaving at 121 °C for 15 minutes. Final a_w is 0.955; final pH is 5.5–5.8.

Dichloran rose bengal chloramphenicol agar (DRBC)
Glucose: 10 g
Peptone, bacteriological: 5 g
KH_2PO_4: 1 g
$MgSO_4.7H_2O$: 0.5 g
Agar: 15 g
Rose bengal (5% w/v in water, 0.5 mL): 25 mg
Dichloran (0.2% w/v in ethanol, 1.0 mL): 2 mg
Chloramphenicol: 100 mg
Water, distilled: 1 L

Sterilize by autoclaving at 121 °C for 15 minutes. Final pH is 5.5–5.8. Store prepared medium away from light; photoproducts of rose bengal are highly inhibitory to some fungi, especially yeasts. In the dark, the medium is stable for at least 1 month at 1–4 °C. The stock solutions of rose bengal and dichloran need no sterilization, and are also stable for very long periods.

Dichloran rose bengal yeast extract sucrose agar (DRYS)
Yeast extract: 20 g
Sucrose: 150 g
Dichloran (0.2% in ethanol, 1.0 mL): 2 mg
Rose bengal (5% w/v in water, 0.5 mL): 25 mg
Chloramphenicol: 50 mg
Agar: 20 g
Water, distilled: to 1 L
Chlortetracycline (l% in water, filter-sterilized, 5.0 mL): 50 mg

Sterilize all ingredients except chlortetracycline by autoclaving at 121 °C for 15 minutes. Add chlortetracycline after tempering to 50 °C. Chloramphenicol at twice the concentration specified (i.e. 100 mg/L) adequately controls bacteria in most situations, and this avoids the need for a second antibiotic that must be filter-sterilized.

25% Glycerol nitrate agar (G25N)
K_2HPO_4: 0.75 g
Czapek concentrate: 7.5 mL
Yeast extract: 3.7 g
Glycerol, analytical grade: 250 g
Agar: 12 g
Water, distilled: 750 mL

Glycerol for G25N should be of high quality, with a low (1%) water content. If a lower grade is used, allowance should be made for the additional water. Sterilize by autoclaving at 121 °C for 15 minutes. Final pH is 7.0.

Malt extract agar (MEA)
Malt extract, powdered: 20 g
Peptone: 1 g
Glucose: 20 g
Agar: 20 g
Water, distilled: 1 L

Commercial malt extract used for home brewing is satisfactory for use in MEA, as is bacteriological peptone. Sterilize by autoclaving at 121 °C for 15 minutes. Do not sterilize for longer as this medium will become soft on prolonged or repeated heating. Final pH is 5.6.

Potato dextrose agar (PDA)
Potatoes: 250 g
Glucose: 20 g
Agar: 15 g
Water, distilled: to 1 L

PDA prepared from raw ingredients is more satisfactory than commercially prepared media. Wash the potatoes, which should not be of a red skinned variety, and dice or slice, unpeeled, into 500 mL of water. Steam or boil for 30–45 minutes. At the same time, melt the agar in 500 mL of water. Strain the potato through several layers of cheesecloth into the flask containing the melted agar. Squeeze some potato pulp through also. Add the glucose, mix thoroughly, and make up to 1 L with water if necessary. Sterilize by autoclaving at 121 °C for 15 minutes.

Quiz#7 : The Vietnamese company which manufacture the dried lotus seed would like to investigate the contamination of mycotoxigenic fungi in their product. Unfortunately, they don't know how to do? You are the food technologist who are specialize in this area. What will you tell/ explain and design the experiment for them. Please explain. (20 pts.)

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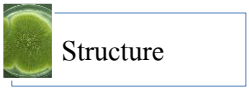
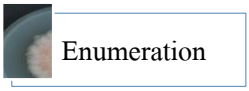
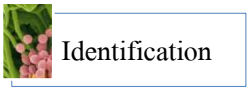
Food Fungi

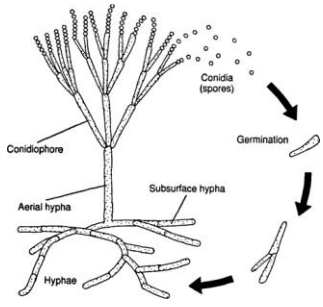
Fungi


Structure

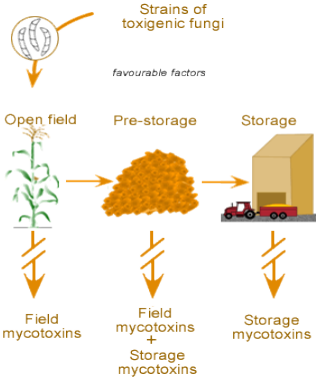

Growth


Enumeration

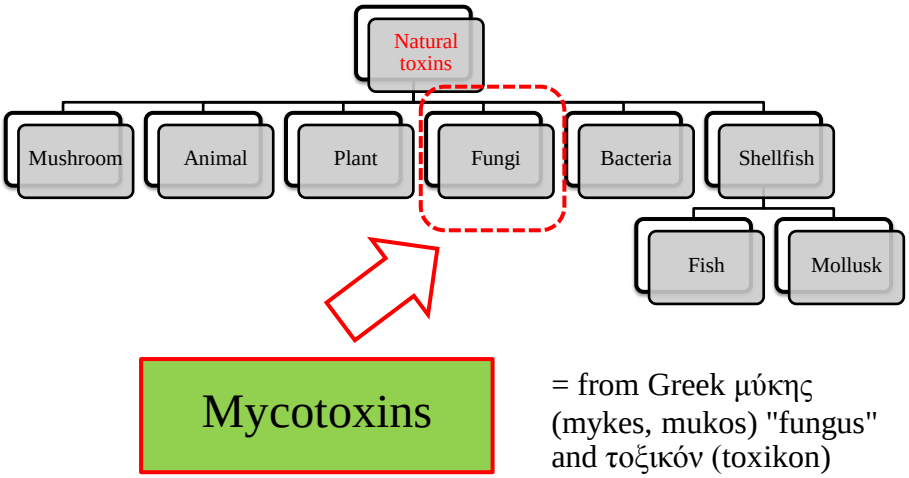

Identification



Mycotoxins



Classification of natural toxins



= from Greek μύκης (mykes, mukos) "fungus" and τοξικόν (toxikon)

Definition

Toxin (from Ancient Greek: *toxikon*)

- a poisonous substance produced within living cells or organisms
- synthetic toxicants created by artificial processes are thus excluded
- The term was first used by organic chemist Ludwig Brieger (1849–1919)

- Toxins can be small molecules, peptides, or proteins that are capable of causing disease on contact with or absorption by body tissues interacting with biological macromolecules such as enzymes or cellular receptors.

- Toxins vary greatly in their severity, ranging from usually minor (such as a bee sting) to almost immediately deadly (such as botulinum toxin).

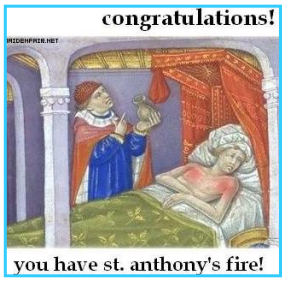
MYCOTOXINS

"Fungal metabolites which when ingested, inhaled or absorbed through the skin cause lowered performance, sickness or death in man or animals including birds"

Pitt, 1996

Outbreak!!!

Ergotism or St. Anthony's Fire



Turkey X Disease 1960, England

The Kenyan episode, 2004

Mycotoxins

- Over 300 fungi produced toxic compounds that contaminate a wide variety of agricultural commodities
- Secondary metabolites produced by mould (mainly *Aspergillus*, *Penicillium*, *Fusarium*) that are known to cause toxic effects in human or animals
- Low molecular weight
- Resistance to common thermal treatments
- Contamination occurs in the field and can increase during harvesting and storage

Mycotoxins

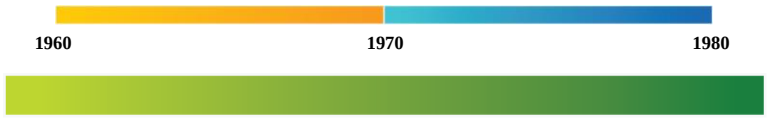
- Ingestion cause a range of toxic response, from acute to chronic health disorders
- Affect trade
- Cereal and cereal products, dried fruit, spices, coffee, wine, beer, milk, cheese, meat and egg may be contaminated by mycotoxins
- More than 25% of foods are considered significantly contaminated by mycotoxins
- Contamination and severity of the problem vary from year to year also from one geographic region to another
- More than 400 different mycotoxins are known about 10% of which occur in feed/foods, being the main source for animal/human exposure

1960 **1970**

- Classes of toxins and associated fungi
- Physical, chemical properties of the secondary metabolites
- Detection and analysis (TLC)
- Preliminary toxicity studies
- Storage issues/ sorting, etc

1970 **1980**

- Surveys conducted
- Fungal diversity
- Toxic effects
- Novel methods of detection (HPLC)
- PRE-HARVEST to POST-HARVEST contamination
- Control measures (Plant breeding)
- factors affecting storage.. moisture, temp etc
- First consideration of stored grain as an ecosystem
- consideration of physical measures; multivariate approach

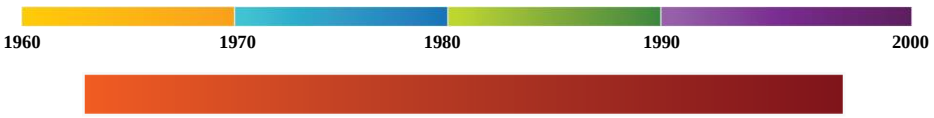


1980 **1990**

- Chemistry/biochemistry of toxin synthesis
- Storage and transportation structures ; Detoxification (ammoniation etc)
- Still novel methods of detection (ELISA/ GC-MS)
- Genetics of fungi
- Advent of biocontrol

1990 **2000**

- Gene clusters for toxin synthesis
- Mechanism of crop resistance
- Breeding for resistance
- Epidemiology
- Fumigation for insect and mould control – especially using SO2 and modified atmospheres; heated drying as opposed to ambient drying
- Clays etc for detoxification
- Large scale biocontrol project



2000 **2010**

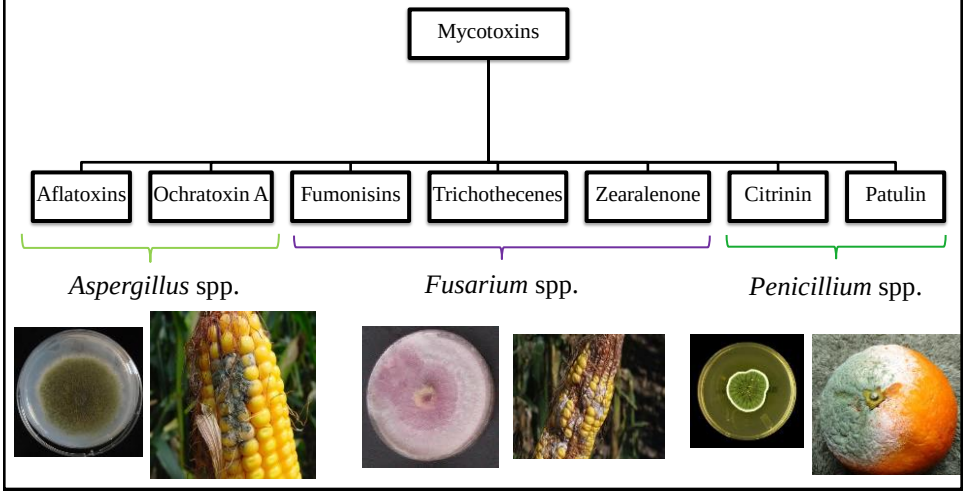
- "omics era (genomics/ metabolomics of fungi, proteomics of crops)
- Fungal diversity based on genetics/metabolite profiles
- Biosensors, hyperspectral imaging,
- integrating ecological data and physical data for predicting risk in store
- remote sensors for measuring real time changes in silos and integrating knowledge centrally (remotely) for mycotoxin risk measurement using the available ecological data/models for mycotoxigenic fungi
- biocontrol globally

Table 1
2012 Notifications by hazard category in the EU.

Hazard category	Alert	Border rejection	Information for attention	Information for follow-up
Allergens	64	3	17	1
Biocontaminants	6	9	26	2
Food additives and flavourings	10	59	23	47
Foreign bodies	24	61	26	47
GMO/novel food	2	52	14	22
Heavy metals	57	108	79	24
Industrial contaminants	16	9	18	14
Mycotoxins	38	425	53	9
Parasitic infestation	4	13	13	25
Pathogenic microorganisms	162	159	168	103
Pesticides residues	19	320	90	18
Residues of veterinary medicinal products	12	18	16	14

Rapid Alert System for Food and Feed (RASFF) Annual Report, 2012.

Principal mycotoxins



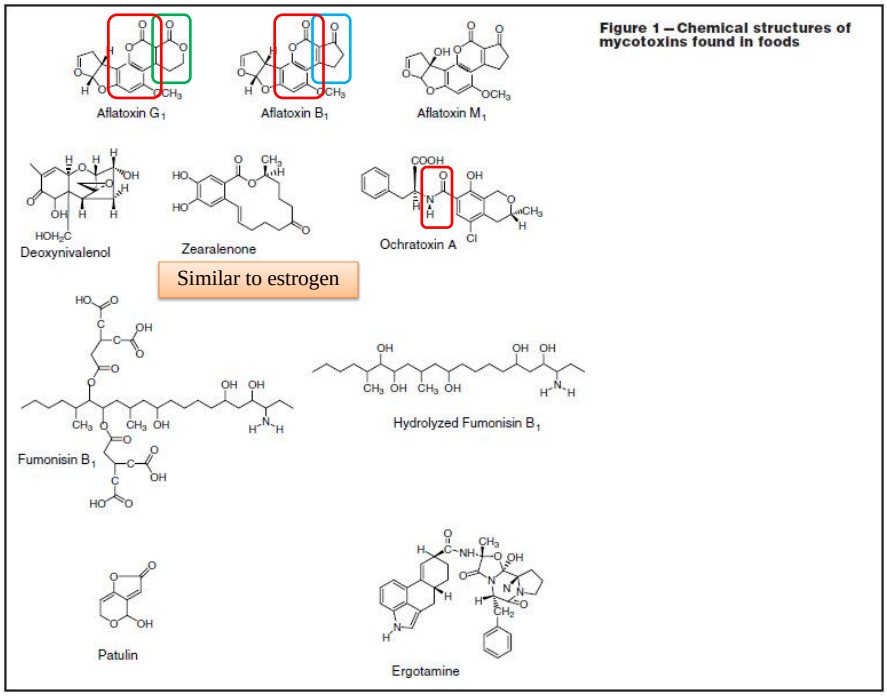


Figure 1 – Chemical structures of mycotoxins found in foods

Table 4
Non-exhaustive list of mycotoxins and producing species.

Mycotoxin	Acronym	Species producing
Aflatoxins B1, B2, G1, G2	AFB1 AFB2 AFG1 AFG2	*Aspergillus* section *Flavi*
Alternariol	AOH	*Alternaria alternata*
Alternariol monomethyl ether	AME	*Alternaria alternata, A. solani*
Tenuazonic acid	TeA	*Alternaria alternata,*
Altertoxins	ALTs	*A. tenuissima*
Altenuene	ALT	*Alternaria alternata* *Alternaria alternata*
Beauvericin	BEA	*F. sporotrichioides, F. poae,* *F. langsethiae, Fusarium* section *Liseola, Fusarium avenaceum*
Enniatins	ENNs	*Fusarium avenaceum, F. tricinctum*
Fusaproliferin	FUS	*F. poae, F. langsethiae, F. sporotrichioides* *F. proliferatum, F. subglutinans*
Moniliformin	MON	*Fusarium avenaceum, F. tricinctum, Fusarium* section *Liseola*
Ergot alkaloids	EAs	*Claviceps purpurea, C. fusiformis, C. africana, Neotyphodium* spp.
Fumonisins B1, B2	FB1, FB2	*Fusarium* section *Liseola*
Ochratoxin A	OTA	*Aspergillus* section *Circumdati* *Aspergillus* section *Nigri* *Penicillium verrucosum* *Penicillim nordicum*
Patulin	PAT	*Penicillim expansum, Bysochlamis nivea, Aspergillus clavatus*
HT-2 and T-2 toxin (type A trichothecenes)	HT-2 T-2	*Fusarium acuminatum, F. poae,* *F. sporotrichioides, F. langsethiae*
Deoxynivalenol (type B trichothecenes)	DON	*Fusarium graminearum, F. culmorum, F. cerealis*
Zearalenone	ZEN	*Fusarium graminearum (F. roseum), F. culmorum, F. equiseti, F. cerealis, F. verticillioides, F. incarnatum*

Mycotoxins of major concern occuring in food/feed and relevant fungal producer

Mycotoxins	Matrix	Fungus
Aflatoxins (B_1, B_2, G_1, G_2)	Maize, peanuts, nuts, spices, dried fruit	*Aspergillus flavus, A. parasiticus*
Aflatoxin M_1	Milk, cheese	
Ochratoxin A	Wheat, barley, maize, coffee, wine, beer	*Aspergillus ochraceus, A. carbonarius, A, niger, Penicillium verrucosum*
Deoxynivalenol	Wheat, maize, barley	*Fusarium graminearum, F. culmorum*
T-2/HT-2 toxins	Wheat, maize, barley, rye, oats	*F. sporotrichioides, F. langsethiae, F. poae*
Zearalenone	Maize, wheat	*F. graminearum, F. culmorum, F. crookwellense*
Fumonisins (B_1, B_2, B_3)	Maize	*F. verticillioides (F. moniliforme), F. proleferatum*
Patulin	Apple products, fruit juice	*P. expansum*

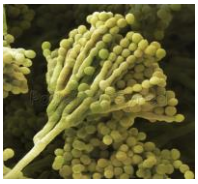
Major genera of mycotoxigenic fungi

Aspergillus

- Large family of fungi generally regarded as **saprophytes**
- Worldwide in distribution but primarily occupy **subtropical** and **warm temperate climates**
- Growth at **high temperatures** and **low water activity**

Penicillium

- Grow and produce mycotoxins over a **wide range of temperatures**
- More abundant in **temperate climates**
- Commonly associated with **storage**

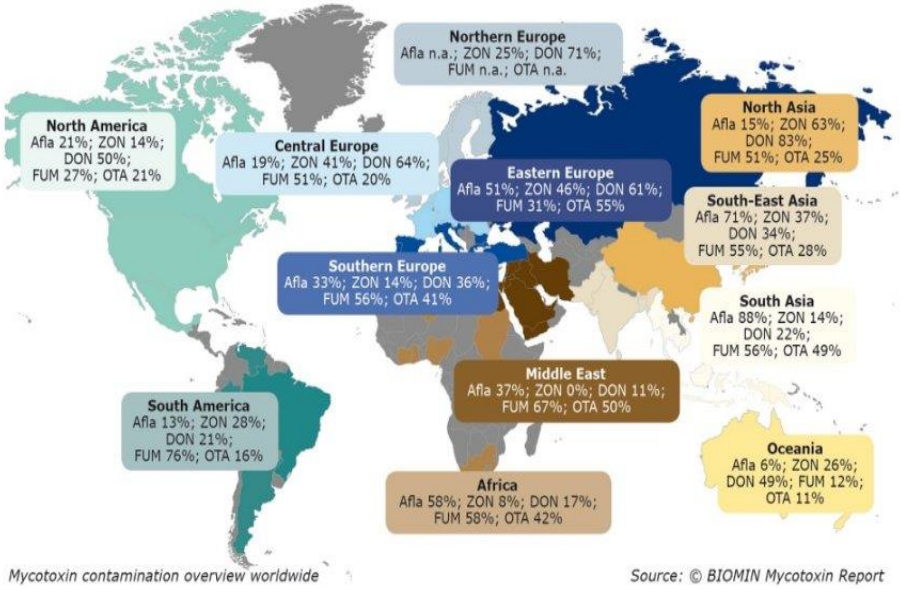


Fusarium

- Adapted to a wide range of habitats and **worldwide in distribution**
- Important **plant pathogens**
- Few species are significant **mycotoxin producers**

Global Occurrence

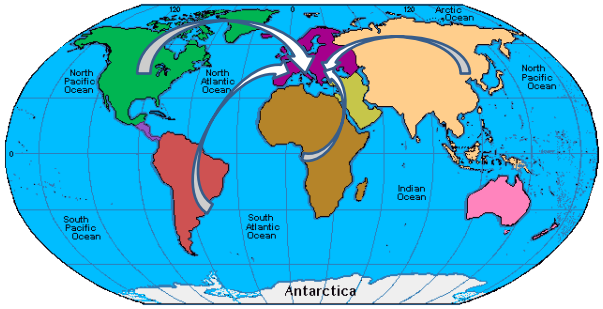
More info : BIOMIN Mycotoxins Survey



Mycotoxin contamination overview worldwide

Source: © BIOMIN Mycotoxin Report

Mycotoxin problems due to trade exchanges



Imported products with high risk of mycotoxin contamination :

- **Maize** (fumonisin and aflatoxins) **from all continents**
- **Cereals** (deoxynivalenol, OTA) **mostly from North and South America**
- **Coffee** (OTA) **mostly from South America & Africa**
- **Pistachio nuts** (aflatoxins) **mostly from North Africa & Asia**
- **Peanuts and other nuts** (aflatoxins) **mostly from North, South America & Africa**
- **Spices** (aflatoxins) **mostly from Asia & Africa**

Quiz#8 : Why mycotoxins are more receive increasing interest from both scientists and industries? (5 pts.)

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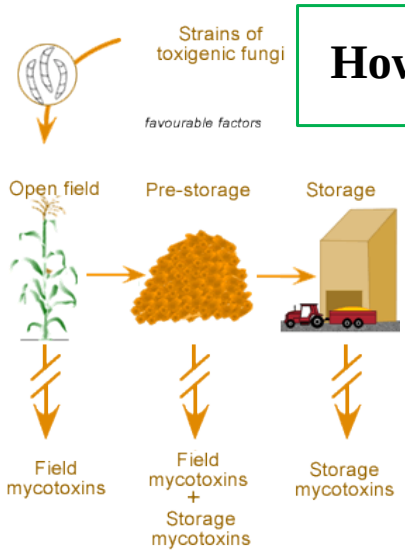
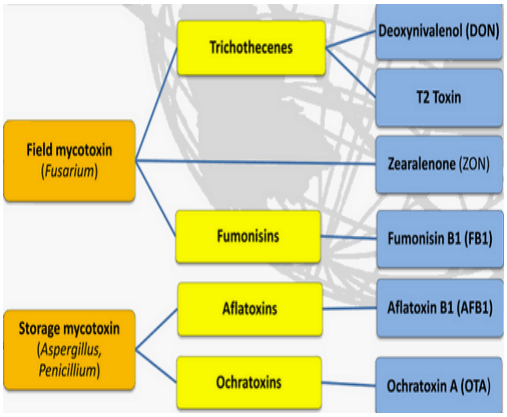
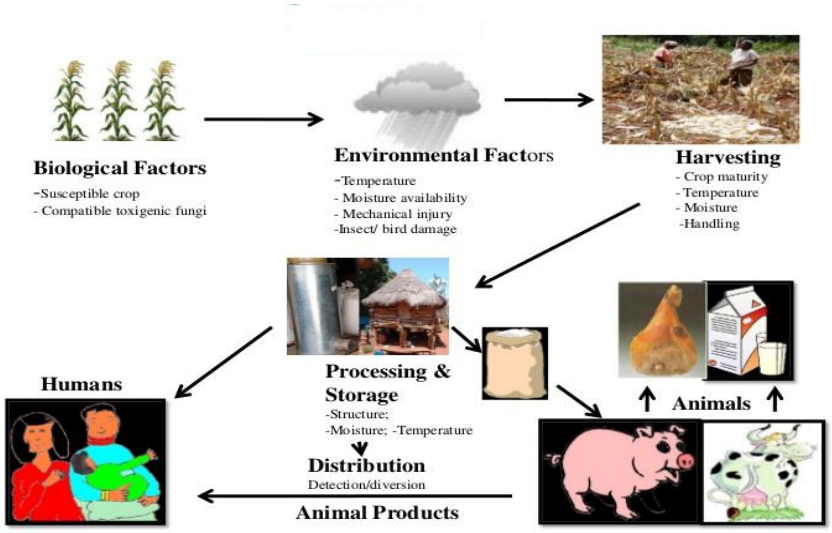
How does mycotoxins occur?





Factor affecting mycotoxin occurrence in the food chain



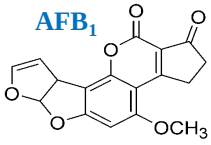
Mycotoxins can be grouped into *2 categories*

1. FIELD MYCOTOXINS
   - Fumonisins, deoxynivalenol, zearalenone, T2/HT-2 toxins, aflatoxins, patulin

2. STORAGE MYCOTOXINS
   - Aflatoxins, ochratoxin A

Some mycotoxins can develop both in the field and in storage

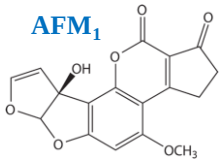
Mycotoxins of MAJOR Concern



Aflatoxins (B_1, B_2, G_1, G_2)

- *Aspergillus flavus, A. parasiticus, A.nomium*
- **PRE- and POST-HARVEST** production under certain conditions of temperature, water activity and availability of nutrients
- **FOODSTUFFS AFFECTED :** groundnuts, nuts, maize, rice, figs and other dried foods, spices, cocoa beans, copra, palm kernels, cottonseeds
- **Aflatoxin B_1** is the most common amongst aflatoxins in food
- **GEOGRAPHICAL AREAS AFFECTED :** South America, Africa, Middle East (very frequent); South-East Asia (frequent); North America (occasional); Europe-Russia, North Asia (rare)

Mycotoxins of MAJOR Concern

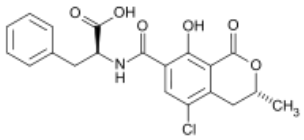


Aflatoxin M_1

- **Aflatoxin M_1** is a major metabolite of aflatoxin B_1 in human and animals, which may be **present in milk (and derived products)** from animals fed with aflatoxin B_1 contaminated feed
- **Average transfer of 0.3 to 6% of AFB_1 to AFM_1 in milk** (generally 1-3% of the feed aflatoxin consumed is excreted in the milk)

Mycotoxins of MAJOR Concern

OTA

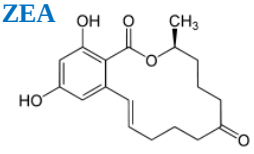


Ochratoxin A (OTA)

- ***Penicillium verrucosum*** (temperate climates), ***Aspergillus ochraceus*** and ***A. carbonarius*** (warm regions)
- **FOODSTUFF AFFECTED :** common in cereals (maize, wheat, rye, barley, oats), grape (wine), coffee, cocoa, bean, soybean
- **TOXIN PRODUCTION :** usually during **STORAGE**
- **OTA occurrence :** poor or inadequate drying of cereals prior to storage, or poor storage conditions leading to **"hot-spots"** of contamination (heterogeneous distribution in stored grains).
- **GEOGRAPHICAL AREAS AFFECTED :** North Europe-Russia (frequent); North America, Africa, Middle East, North Asia, South-East Asia (occasional); South America (rare)

Mycotoxins of MAJOR Concern

ZEA

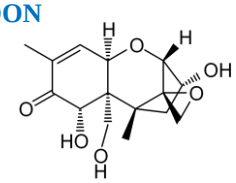


Zearalenone (ZEA)

- Produced by several **FIELD FUNGI**, mainly ***Fusarium graminearum*** and ***F. culmorum***
- **TOXIN PRODUCTION :** mainly at the **PRE-HARVEST STAGE** and can not be avoided under the conditions of current agricultural practice
- **FOODSTUFF AFFECTED :** common in **maize** and maize products, but also in **soybeans** and various **cereals** (barley, oats, rye, sorghum, wheat, rice)
- **CO-OCCURRENCE** with other **Fusarium toxins (DON, NIV,** and **FBs)**
- **GEOGRAPHICAL AREAS AFFECTED :** South America, North Asia, South-East Asia (frequent); North America, Europe-Russia, Africa, Middle East (occasional)

Mycotoxins of MAJOR Concern

DON

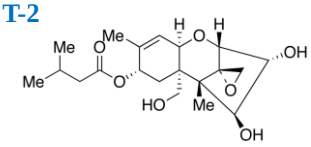


Deoxynivalenol (DON)

- Produced by several **FIELD FUNGI**, including ***Fusarium graminearum*** and ***F. culmorum***, which commonly contaminate cereal crops in **Europe**. It is one of the World's best-known and most common mycotoxins.
- **TOXIN PRODUCTION :** mainly at the **PRE-HARVEST STAGE** and can not be avoided under the conditions of current agricultural practice
- **FOODSTUFF AFFECTED :** various cereals including wheat, barley, maize, rye, oats and derived products, beer)
- **CO-OCCURRENCE** with other trichothecenes **(NIV, 3-** and **15-acetylDON)**
- **GEOGRAPHICAL AREAS AFFECTED :** South America, North Asia, South-East Asia, North America, Europe-Russia, Africa, Middle East (frequent)

Mycotoxins of MAJOR Concern

T-2

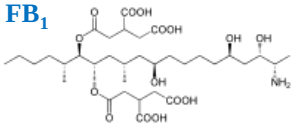


T-2 and HT-2 Toxins

- Produced by several **FIELD FUNGI** (including ***Fusarium sporotrichoides, F. poae, F. langsethiae, F. equiseti*** and ***F. acuminatum***)
- **TOXIN PRODUCTION :** mainly at the **PRE-HARVEST STAGE** under moist cool conditions (overwintered cereals)
- **FOODSTUFF AFFECTED :** various **cereals** including wheat, barley, maize, oats, rye and derived products)
- Notable levels have been found in **oats** and **oat by-products**
- **HT-2 concentration** represents two-thirds of the sum of T-2 and HT-2 concentration
- **GEOGRAPHICAL AREAS AFFECTED :** North America, South America, Europe-Russia, North Asia, (frequent); South-East Asia, Africa, Middle East (occasional).

Mycotoxins of MAJOR Concern

FB_1

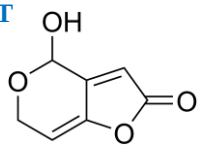


Fumonisins

- Produced by several **FIELD FUNGI**, including ***Fusarium verticillioides, F. proliferatum and Aspergillus niger***
- **TOXIN PRODUCTION : PRIOR TO HARVEST** or during the **EARLY STAGE of STORAGE**
- **Main fumonisins : FB_1, FB_2 and FB_3**
- **FOODSTUFF AFFECTED : MAIZE and derived products, grape** (mainly FB_2)
- Extensive **world-wide survey data** indicate that the **vast majority of MAIZE** is contaminated by fumonisins (it is very difficult to found uncontaminated maize)
- **GEOGRAPHICAL AREAS AFFECTED :** North America, Africa, Middle East, North Asia, South-East Asia (very frequent); South America, Europe-Russia (frequent).

Mycotoxins of MAJOR Concern

PAT



Patulin

- Produced by ***Penicillium expansum***, *P. claviforme*, *P. roquefortii*, *Aspergillus clavatus* and *A. terreus*
- **TOXIN PRODUCTION :** mainly during fruit spoilage
- **FOODSTUFF AFFECTED :** rotted **apples, apple juice**, moldy feed

Mycotoxins of MINOR concern

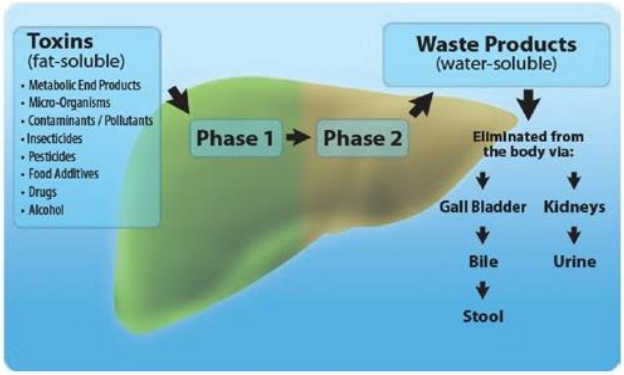
Mycotoxins that pose a minor risk to human and animal health as food and feed contaminants are these following;

Mycotoxins	Fungal species
Tenuazonic acid alternariol	*Alternaria* spp.
Cyclopiazonic acid	*Aspergillus flavus, A. tamarii, A. versicolor, Penicillium camembertii, P. cyclopium*
Citrinin	*P. citrinum, P. verrucosum, P. expansum*
Sterigmatocystin	*A. versicolor, A. flavus, A. parasiticus*
Moniliformin	*Fusarium proliferatum, F. oxysporum*
Beauvericin	*Beaveria bassiana, Fusarium* spp.
Enniatins	*Fusarium* spp.

MODE OF MYCOTOXIN ACTIONS

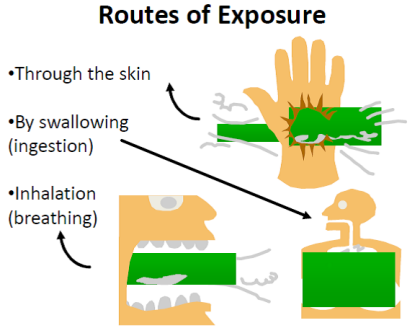
Toxicity

- Mycotoxins are generally lipophilic (except for FB) and, therefore, they tend to accumulate in the fat fraction of plants and animals.



Routes of exposure to mycotoxins

1. In humans and animals, by ingestion of contaminated food/feed
2. Other routes such as inhalation and skin-contact must be considered



Mycotoxicoses

= Disease resulting from exposure to mycotoxins

1. **ACUTE mycotoxicoses**
   - ❖ Due to the ingestion of **high amounts** of mycotoxins
   - ❖ Usually restricted to livestock or to **human population of developing countries**
2. **CHRONIC mycotoxicoses**
   - ❖ Produced by mycotoxins occurring in foods and feeds at **lower levels**
   - ❖ Affect **animal** and **human health** in the long-term

Biological effects

- For their diversity of chemical structures and physical properties, mycotoxins exhibit a wide range of biological effects

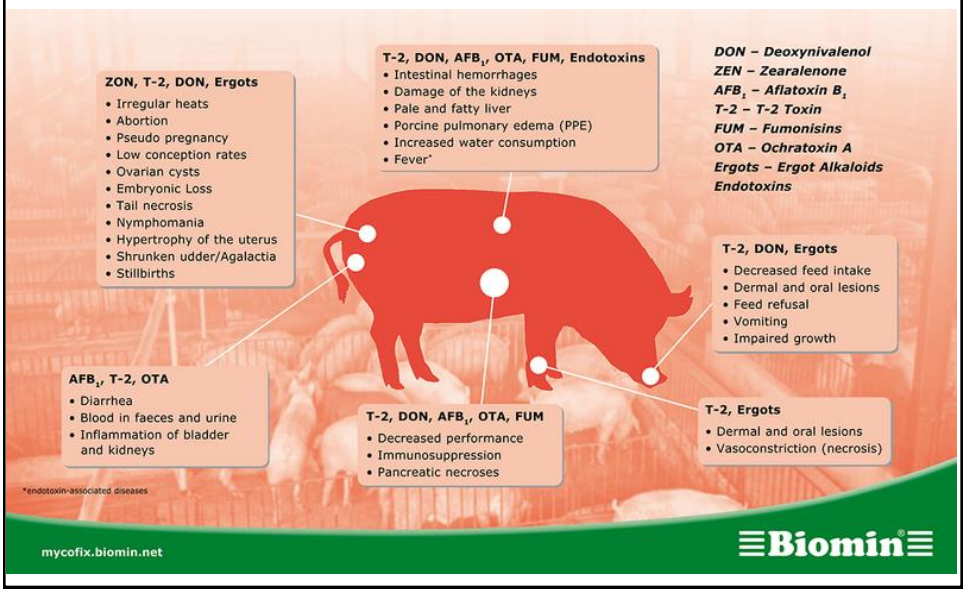
- ❖ GENOTOXIC
- ❖ MUTAGENIC
- ❖ CARCINOGENIC
- ❖ EMBRYOTOXIC
- ❖ TERATOGENIC
- ❖ OESTROGENIC

Toxic effects of mycotoxins

	AFL	OTA	FUM	TRICH*	ZEA
Carcinogenic	•	•	•		
Hepatotoxic	•	•	•		
Nephrotoxic		•	•		
Neurotoxic			•	•	
Teratogenic	•	•			•
Oestrogenic					•
Immunotoxic	•	•	•	•	•

*TRICH = trichothecenes including DON, NIV, T-2 and HT-2

Effects of mycotoxins on the health of pigs



Some human diseases in which analytical and/or epidemiologic data suggest or implicate mycotoxin involvment

Disease	Substrate	Etiologic agent
Alimentary Toxic Aleukia (ATA)	Cereal grains	*Fusarium spp.*
Balkan nephropathy	Cereal grains	*Penicillium spp., Aspergillus ochraceus*
Ergotism	Rye	*Claviceps purpurea*
Kashin-Beck disease	Cereal grains	*Fusarium spp.*
Esophageal tumors	Maize	*Fusarium verticillioides*
Hepatocarcinoma (acute aflatoxicosis)	Cereal grains, peanuts	*Aspergillus flavus, A. parasiticus*
Kwashiorkor	Cereal grains	*Aspergillus flavus, A. parasiticus*
Reye's syndrome	Cereal grains	*Aspergillus flavus, A. parasiticus*
Onyalai	Millet	*Phoma sorghina*

IARC EVALUATION OF CARCINOGENIC HAZARD OF MYCOTOXINS TO HUMNS

International Agency for Research on Cancer (IARC)

OVERALL EVALUATION		MICOTOXIN
Group 1	Carcinogenic to humans	Aflatoxins (naturally occurring mixture of B_1, B_2, G_1 e G_2)
Group 2B	Possibly carcinogenic to humans	Aflatoxin M1 Ochratoxin A Fumonisin B_1
Group 3	Not classifiable as its carcinogenicity to humans	Toxins derived from *Fusarium graminearum, F. culmorum, F. crookwellense and F. sporotrichioides* (zearalenone, deoxynivalenol, T-2 toxin, nivalenol)

Adapted from: *IARC Monographs on the Evaluation of Carcinogenic risk to humans*. VOL. 56, 1993 and Vol. 82, 2002, International Agency for Research on Cancer (IARC), Lyon, France.

IARC classification of the main mycotoxins

RF EXPOSURE		HUMAN EVIDENCE			
		Sufficient	Limited	Inadequate	Lack of Evidence
ANIMAL EVIDENCE	Sufficient	Group 1	Group 2-A	Group 2-B	Group 3
	Limited		Group 2-B	Group 3	
	Inadequate				
	Lack of Evidence				Group 4

Toxicity of Major Mycotoxins

Mycotoxin	Commodities	Toxicity
Ochratoxins	Cereals, coffee beans, grapes	Nephrotoxin, immunosuppressant, teratogen, suspected carcinogen
Trichothecenes	Maize (corn) , cereals, wheat, barley, oats, rye, rice, others	Cytotoxicity, protein synthesis inhibition, emetic toxicity
Fumonisins	Maize (corn), wheat and other cereals	Hepatotoxin
Aflatoxins	Maize (corn), nuts, copra, cottonseed, milk	Hepatotoxin, synergistic toxicant, potent carcinogen, growth inhibitor

Aflatoxins

- It is well established that AFB_1 is both carcinogenic and cytotoxic
- AFB_1 can inhibit cyclic nucleotide phosphodiesterase activity in the brain, liver, heart, and kidney tissues.

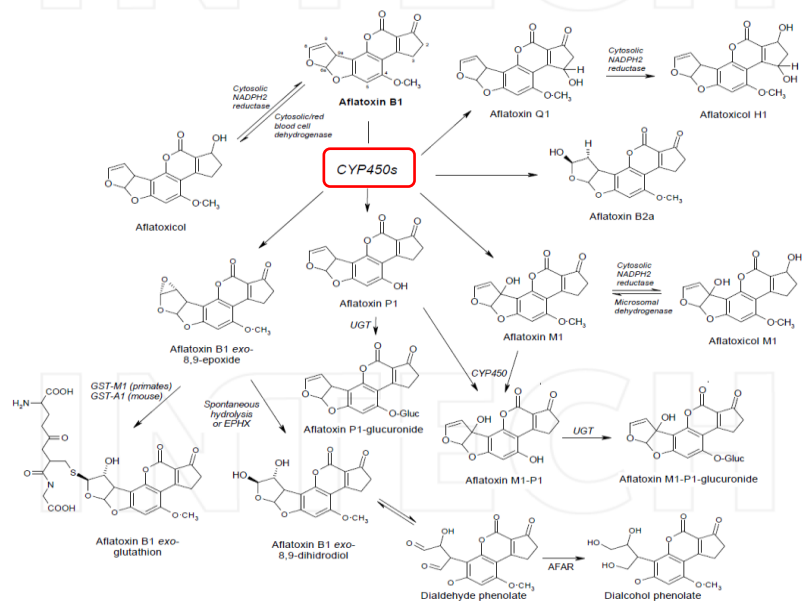
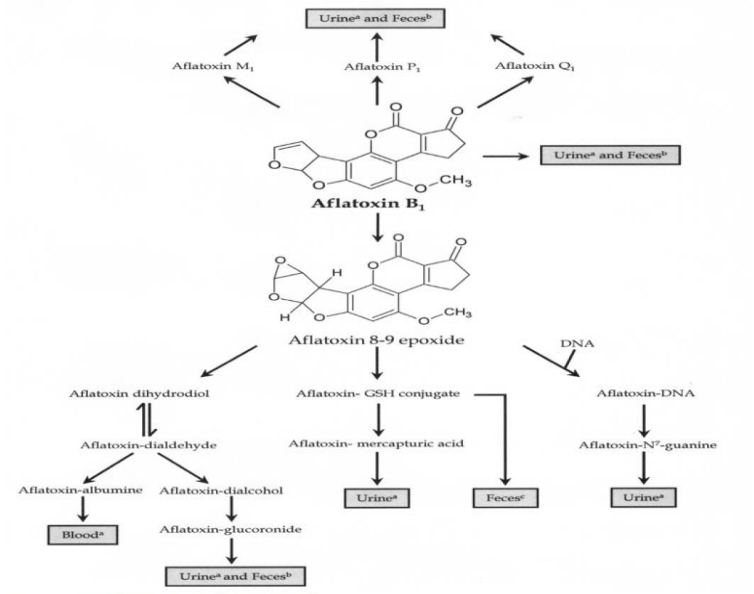


Fig. 1. Biotransformation reactions of aflatoxin B1 in poultry and mammals, including humans. The main CYP450s involved in these reactions are CYP1A1, CYP1A2, CYP2A6 and CYP3A4. Not all reactions occur in a single species.



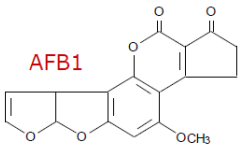
Source: Adapted from Mykkanen et al., 2005.

Fig. 1. Pathways of aflatoxin B_1 biotransformation and excretion in humans. (a) Experimental and human evidendence of excretion of this metabolite; (b) Scarce or no evidence available; (c) only experimental evidence available (no data for humans).

HUMAN AFLATOXICOSIS

Aflatoxin B1 is the most potent natural **hepato-carcinogen** and it is **genotoxic**

- **Acute toxicity in humans rare but**
  - 40 deaths in 1974 (India)
  - 13 deaths in 1990 in Malaysia
  - 14 deaths in 2001 (Kenya)
  - 125 deaths in 2004 (Kenya)
  - 16 deaths in 2005 (Kenya)
  - .....



- **Sub-acute and chronic effects in humans**
  - Possible causative role in human liver cancer, chronic hepatitis, jaundice, enlarged liver, cirrhosis in Asia and Africa
  - Possibly associated with Kwashiorkor
  - Child growth impairment , e.g. in West-Africa
  - Suppressed immune system

It is of interest that aflatoxicoses have been reported only in communities where maize is the dietary staple

Hepato-carcinogenicity AFLATOXIN B_1

- **Liver cancer:** positive correlation between estimated aflatoxin intake and epatocellular carcinoma (HCC) rates
- **Hepatitis B virus (HBV):** Strong evidence of an additive interaction between aflatoxin intake and HBV, in relation to increased HCC risk.
- The overall incidence from epidemiological studies shows a **particular high risk of HCC from aflatoxin exposure in individuals chronically infected with HBV** and **reasonable evidence that an increased risk also exists in individuals exposed to aflatoxins without chronic HBV infection**

Given that >350 million chronic HBV carriers exists in the world, many living in aflatoxin-endemic area, the need to reduce aflatoxin exposure remain highly relevant for cancer prevention

Hepato-carcinogenicity AFLATOXIN M_1

- Since **aflatoxin M_1 is a metabolite of aflatoxin B_1** and is presumed to **induce liver cancer in rodents by a similar mechanism**, estimates of the potency of aflatoxin B_1 can be used for determining the risk due to intake of aflatoxin M_1.
- No adequate epidemiological studies exist on the dose–response relationships between the intake of aflatoxin M_1, exposure to hepatitis B or C virus, and liver cancer. **JECFA** (Joint FAO/WHO Expert Committee on Food Additives) therefore **assumed that aflatoxin M_1 acts similarly to aflatoxin B_1 with hepatitis B (and possibly) C virus.**
- **The Committee assumed that the potency of aflatoxin M_1 was one-tenth that of aflatoxin B_1 in rat.**

Ochratoxins

- International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B).
- Currently, the mode of carcinogenic action by OTA is unknown.
- OTA is genotoxic following oxidative metabolism.
- This activity is thought to play a central role in OTA-mediated carcinogenesis and may be divided into
  - direct (covalent DNA adduction) and
  - indirect (oxidative DNA damage) mechanisms of action

Mycotoxicoses in humans associated to ochratoxin A

- **OTA** is suspected to be involved in the **Balkan Endemic Nephropathy (BEN)**, a fatal kidney disease occurring in some areas of eastern Europe (Bosnia, Serbia, Croatia, Bulgaria and Romania) and to be associated with **urinary tract tumors (UTT)** and with cases of **chronic nephropathy** of unknown aetiology in Tunisia and Egypt.
- Evidence of relationships between hepatocellular carcinoma (HCC) and ochratoxicosis (by the analysis of the presence of OTA in the serum of HCC patients)

Fumonisins

- Both cytotoxic and carcinogenic to animals.
- The modes of such actions, however, are not completely understood
- FB1 exerts its cytotoxicity by inhibiting sphingolipid metabolism, protein metabolism, and the urea cycle.
- The carcinogenic role of FB1 has been linked to the accumulation of sphingoid bases that cause unscheduled DNA synthesis (Schroeder et al., 1994), alteration of signaling by cAMP (Huang et al., 1995) and protein kinase C (Yeung et al., 1996), and disruption of normal cell cycling

Mycotoxicoses in humans associated to *Fusarium* toxins - Fumonisins

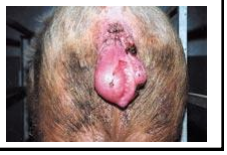
- Ecological studies in the former Transkei region of **South Africa** showed that *F. verticillioides* and **fumonisins contamination of maize was positively correlated with oesophageal cancer incidence** rates.
- **Similar correlations** have been reported in **China** and in **North-East Italy.**

Tricothecenes

- Cytotoxicity of trichothecenes has been attributed to their potent inhibition of protein, RNA, and DNA synthesis (Liao et al., 1976)
- Other toxic effects of trichothecenes include disruption of membrane transport and function, suppression of the immune response, and abnormal blood function

Zearalenone

- Has been known for its estrogenic effects on animals.
- It binds to estrogen receptors influencing estrogen dependent transcription in the nucleus (Kolb, 1984).
- Receptor binding by ZEN has been shown to inhibit the binding estrogenic hormones in rat mammary tissues (Boyd and Wittliff, 1978).
- Recent studies (Ahamed et al.,2001; Withanage et al., 2001) have demonstrated the potential for ZEN to stimulate growth of human breast cancer cells containing estrogen response receptors.

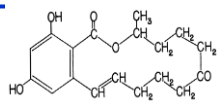


THE MILESTONE :

- The first report on the condition which called **vulvovaginitis**.(Iowa, USA) (McNutt *et al*.)

- The syndrome was occurred from pigs fed corn infected *Gibberella zeae*

- The active fungal metabolite of *F. graminerum* is **ZEARALENONE** (Urry *et al*.)



1928 1952 1957-8 1962 1966 1977-2002 **Current research**

- The hypothesis was that the syndrome was caused by an estrogen produced by molds in feeds (McErlean)

- Purified the metabolite from *Gibberella zeae* (*F. graminearum*) exhibiting anabolic and uterotrophic activity (Stob *et al*.)

The Milestone :

- The biotranformation pathways of ZEA was discovered (Olsen *et al*.)

- There were many researches study on the toxicity of ZEA.

- Found the human symptom from received ZEA (Bilgrami & Choudhary)

1977 1981 1982 1987 1993 1994 1998 2002 **Current research**

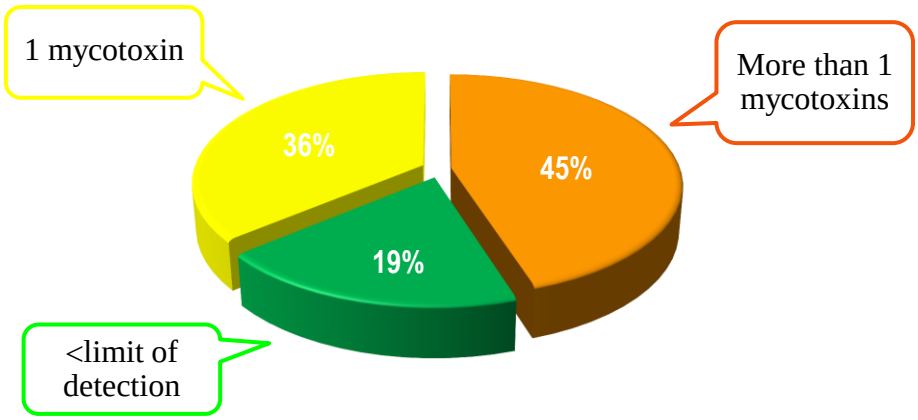
- First data about the capacity of ZEA to induce adverse liver lesions (NTP)

- Research started on the using of LAB to inhibit the growth of *Fusarium* sp. (Roy *et al*.)

- ZEA was detected in maize in Yugoslavia & Zambia (lovelace & nyathi; Balzer *et al*.)

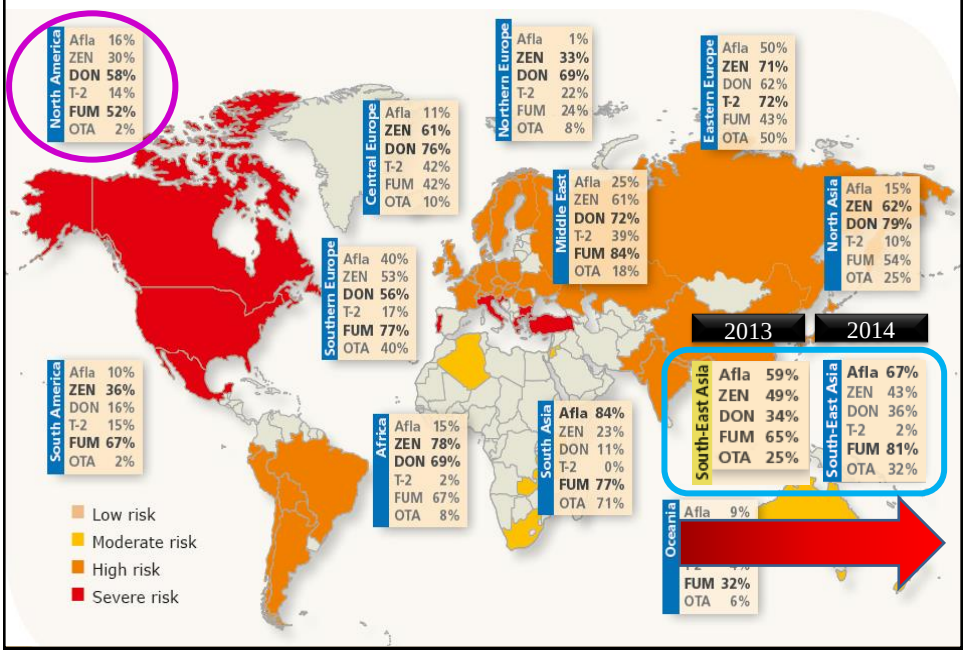
- The economic loss >200 M euros from contamination of mould in bread (Legan)

BIOMIN MYCOTOXIN SURVEY (2013)

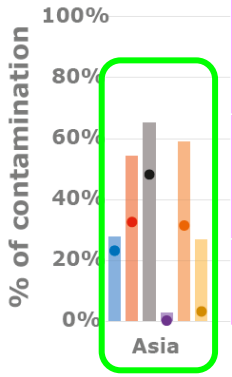


Global co-ocurrence of mycotoxin in raw material of feedstuff (4,218 samples)

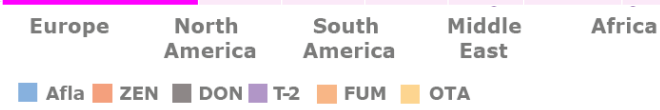
BIOMIN MYCOTOXIN SURVEY (2013-2014)



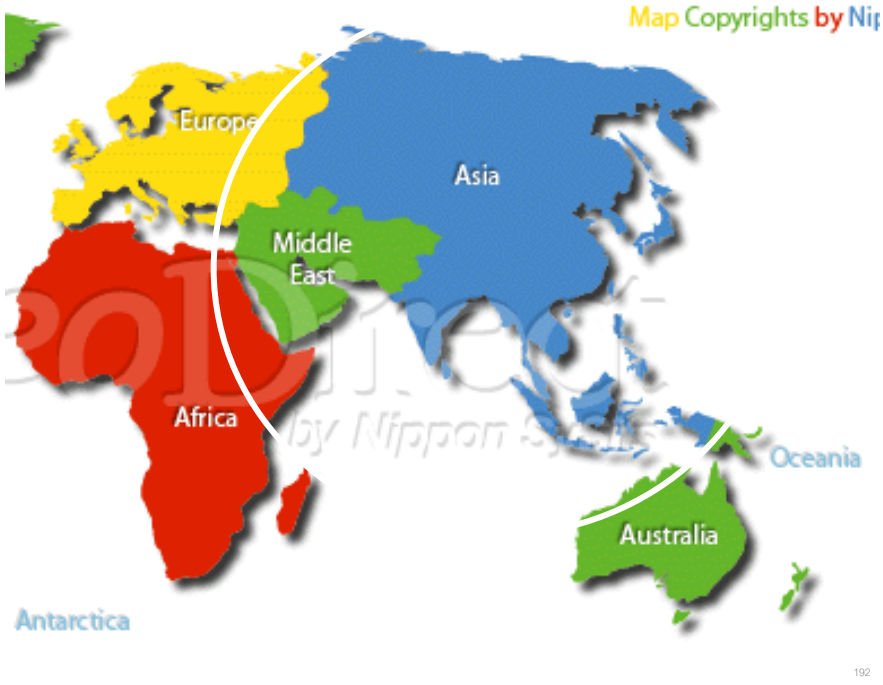
BIOMIN MYCOTOXIN SURVEY (2014)



	Afla	ZEA	DON	T-2	FUM	OTA
Number of samples tested (n)	1,751	1,767	1,762	436	1,651	1,686
Average of positive (ppb)	104	167	512	18	1,399	7
Maximum (ppb)	5,155	6,215	8,901	61	130,246	854



Prevalence of major mycotoxins by region.



Borutova *et al* (2012)

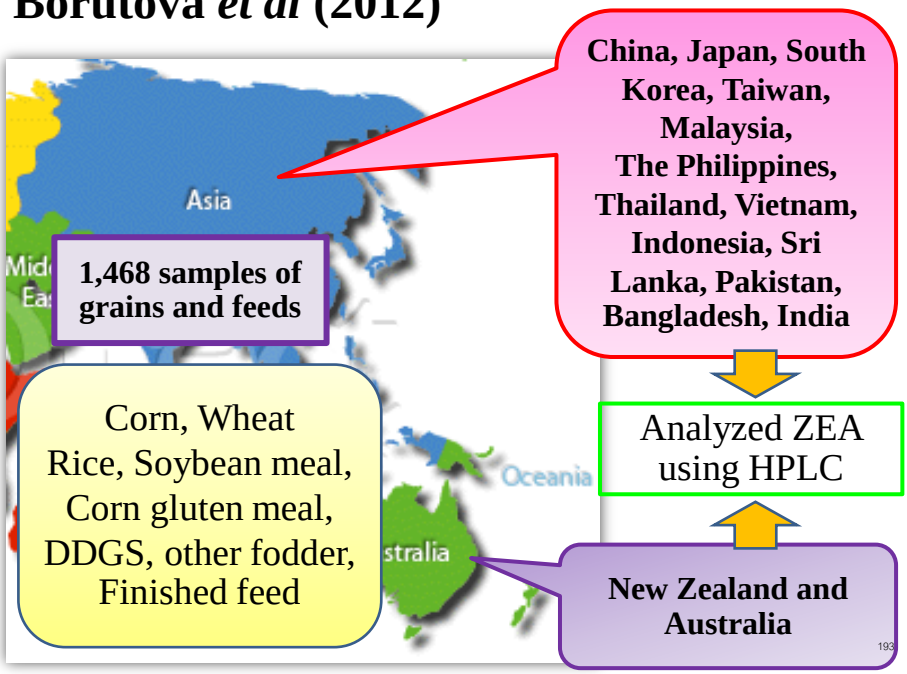


Table 1 Zearalenone contamination levels (μg/kg) detected in samples from different regions in Asia and Oceania

Statistical parameter	Zearalenone
Total samples analyzed	**1,464**
Percentage of positive samples (%)	**47.5**
Mean of positive[a] (μg/kg)	**311.6**
Maximum concentration[b] (μg/kg)	**16,712**

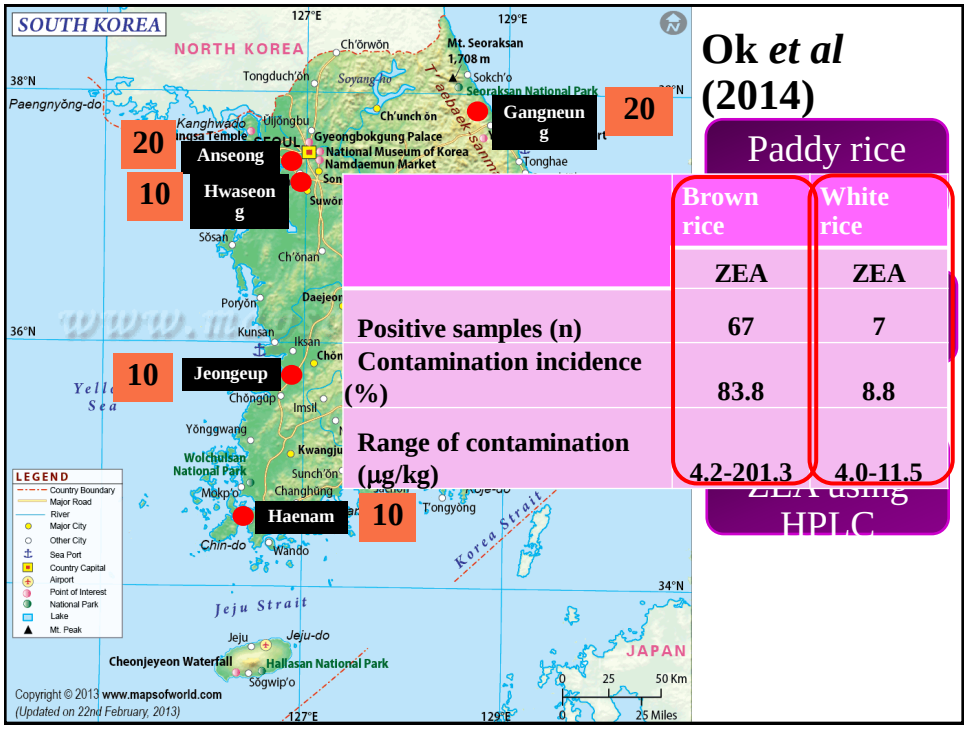
[a] Percentage of positive samples refers to results above the limit of quantification of the HPLC method.
[b] Mean of positive excludes results below the limit of quantification.

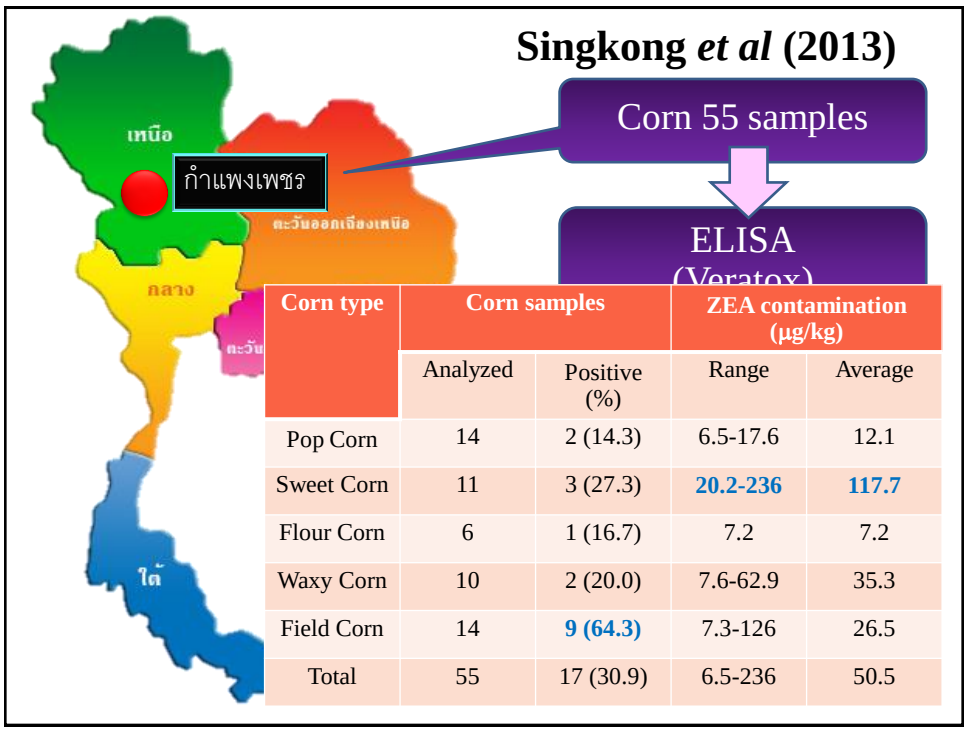
Li *et al* (2014)

Mycotoxins	Zearalenone
Positive samples (n)	21
Contamination incidence (%)	27.6
Mean value of positive samples ($\mu g.kg^{-1}$)	76.5
Range of contamination ($\mu g.kg^{-1}$)	10.0~440.0

Table 2 Frequency of co-occurrence of mycotoxins in analyzed samples.

Sample types	Co-occurrence of mycotoxins (%)				
	AFs-ZEA	AFs-OTA-ZEA	OTA-ZEA	ZEA-DON	OTA-ZEA-DON
Rice	-	-	14.3	-	-
Wheat	-	-	-	7.6	-
Oil	22.2	-	-	-	-
Maize	7.1	-	7.1	7.1	7.1
Peanut, Soybean	27.3	12.5	-	-	-
Oats	-	-	9.1	-	-





Corn type	Corn samples		ZEA contamination (µg/kg)	
	Analyzed	Positive (%)	Range	Average
Pop Corn	14	2 (14.3)	6.5-17.6	12.1
Sweet Corn	11	3 (27.3)	**20.2-236**	**117.7**
Flour Corn	6	1 (16.7)	7.2	7.2
Waxy Corn	10	2 (20.0)	7.6-62.9	35.3
Field Corn	14	**9 (64.3)**	7.3-126	26.5
Total	55	17 (30.9)	6.5-236	50.5

Unpublished Data

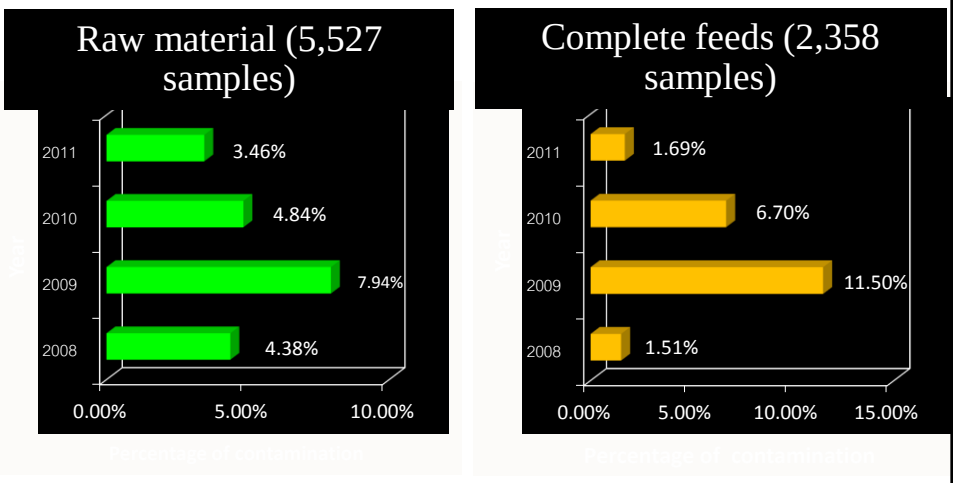
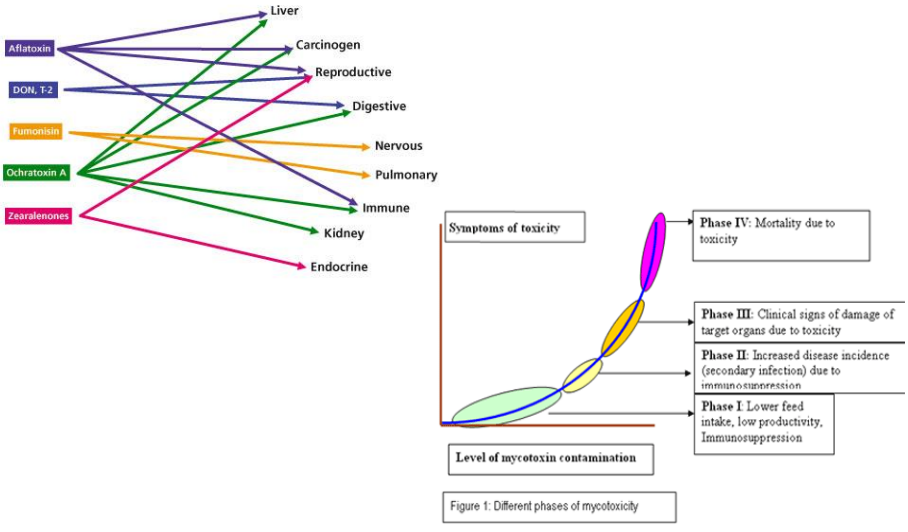


Fig. 3 The contamination of zearalenone (>1,000 ppb) in raw material and feedstruffs (Tangmunkhong,P., 2010)

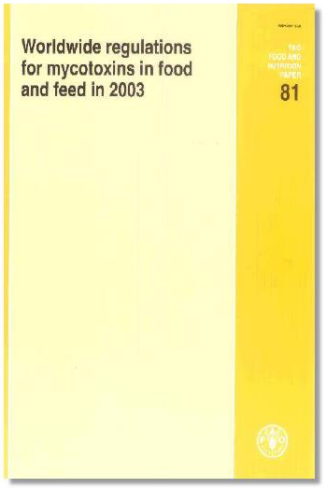
Toxicity-Summary



Enquiries on mycotoxin regulations

Hans van Egmond - RIKILT

- Worldwide enquiries: 1981, 1987, 1995, 2003, 2012, resulting in various publications
- in 2012: enquiry on economically important regions (not published)
- Enquiry 2003 published as FAO FNP 81 (2004)
- French, Spanish and Chinese translations available
- Mycotoxin regulations exist in at least 100 countries and for 13 different toxins



FAO FNP 81, 2004

Worldwide mycotoxin regulations exist for:

- **Aflatoxins B_1, B_2, G_1 and G_2; aflatoxin M_1**
- **Trichothecenes** (**DON**, DAS, **T-2/HT-2 toxins**)
- **Fumonisins B_1, B_2 and B_3**
- Ergot alkaloids
- **Ochratoxin A**
- **Patulin**
- Sterigmatocystin
- **Zearalenone**
- Agaric acid
- Phomopsins

Table 3
European Union regulations for aflatoxins (µg/kg)

Feed	AfB1	Feed	AfB1
Feed (exceptions below)	50	Complete feedstuff for pigs and poultry	20
Groundnuts, copra, palm kernel, cottonseed, babasu, maize and products derived from processing thereof	20	Other complete feedstuffs	10
		Complementary feedstuffs for cattle, sheep, goats (except dairy, calves and lambs)	50
Complete dairy feed	5	Complementary feedstuffs for pigs and poultry (except for young animals)	30
Complete feed for lambs and calves	10		

Table 2
European Union regulations for aflatoxins (µg/kg)

Human food	AfB1	AfB1, B2, B3, B4	M1
Groundnuts, dried fruit and processed products thereof	2	4	–
Groundnuts subjected to sorting or phys. treating	8	15	–
As above but for nuts and dried fruits	5	10	–
Cereals (including maize) and processed products thereof	2	4	–
Milk	–	–	0.05

Table 9
European Union regulations for ochratoxin (μg/kg)

Product	Concentration
Raw cereal grains	5
All products derived from cereals intended for direct human consumption	3
Dried vine fruit (currants, raisins and sultanas)	10

Table 8
European Union regulations for zearalenone (μg/kg)

Product	Concentration
Unprocessed cereals other than maize	100
Unprocessed maize	200
Cereal flour except maize flour	75
Maize flour, meal, grits and refined maize oil	200
Bread, pastries, biscuits, other cereal snacks and breakfast cereals	50
Maize snacks and maize-based breakfast cereals	50
Processed maize-based foods for infants and young children	20
Other processed cereal-based foods for infants and young children and baby food	20

Table 6
U.S. Food and Drug Administration guidelines for fumonisins in human foods and animal feeds (μg/g)

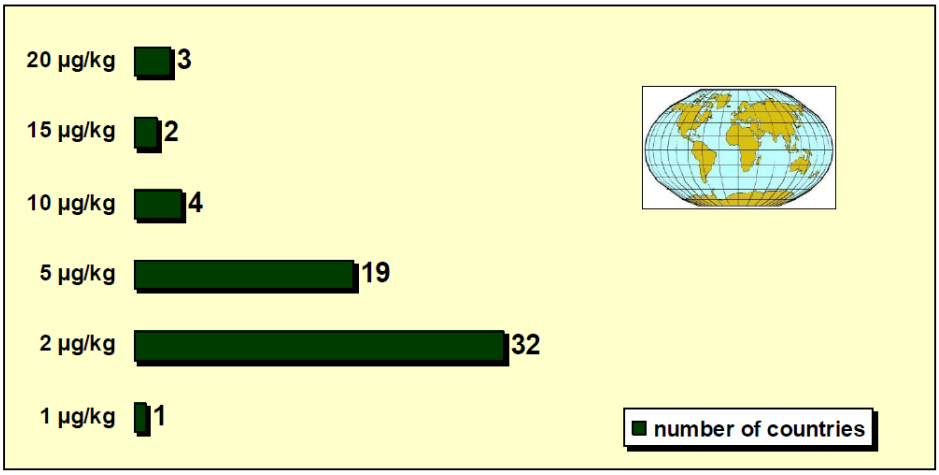
	Concentration total fumonisins (FB1, FB2, FB3)
Human foods	
Degermed dry milled corn products	2
Whole/partially degermed dry milled corn product	4
Dry milled corn bran	4
Cleaned corn intended for mass production	4
Cleaned corn intended for popcorn	3
Corn and corn byproducts for animals	
Equids and rabbits	5 <20% diet
Swine and catfish	20 <50% diet
Breeding ruminants, poultry, mink, dairy cattle, laying hens	30 <50% diet
Ruminants >3 mos. before slaughter and mink for pelts	60 <50% diet
Poultry for slaughter	100 <50% diet
All other livestock and pet animals species	10 <50% diet

Table 7
European Union regulations for fumonisins (μg/kg)

Product	Concentration
Unprocessed maize	2000
Maize grits, meal and flour	1000
Maize-based food for direct consumption except maize grits, meal, flour and processed maize-based foods for infants and young children and baby food	400
Processed maize-based foods for infants and young children and baby food	200

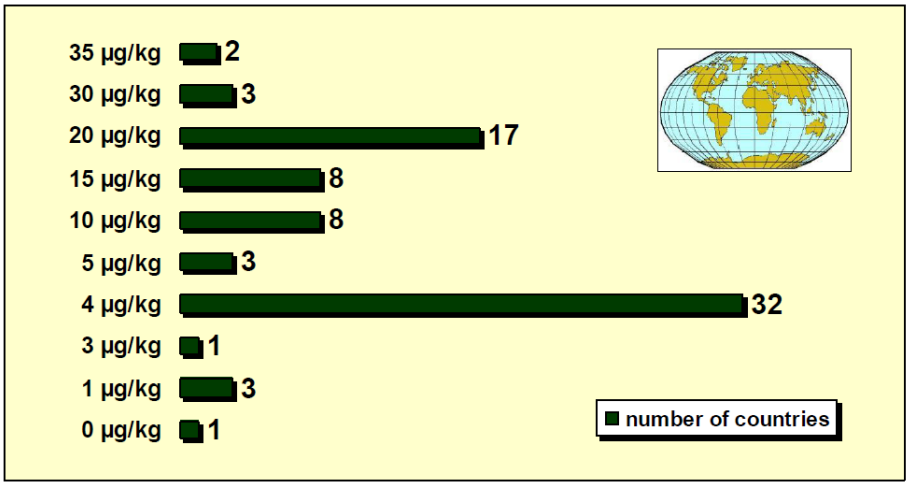
Aflatoxin B_1 in food

(Enquiry 2012)



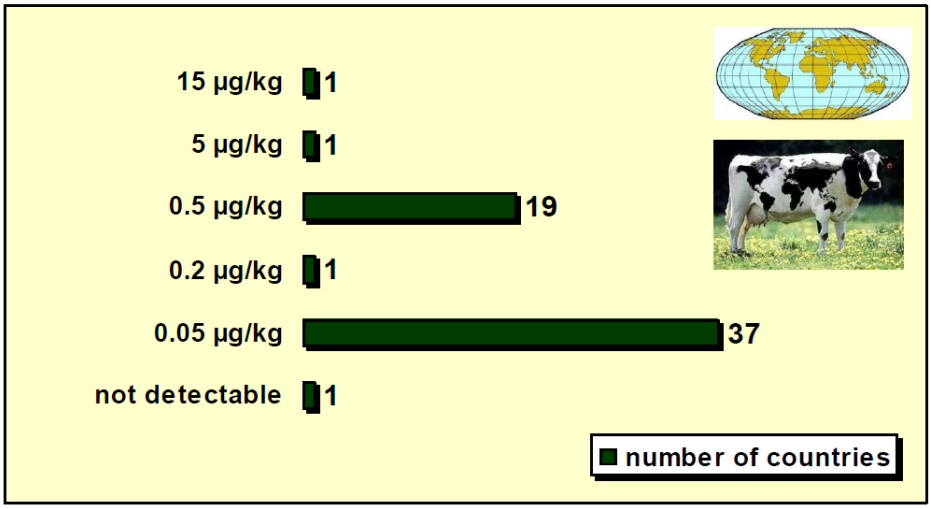
Total aflatoxins in food

(Enquiry 2012)



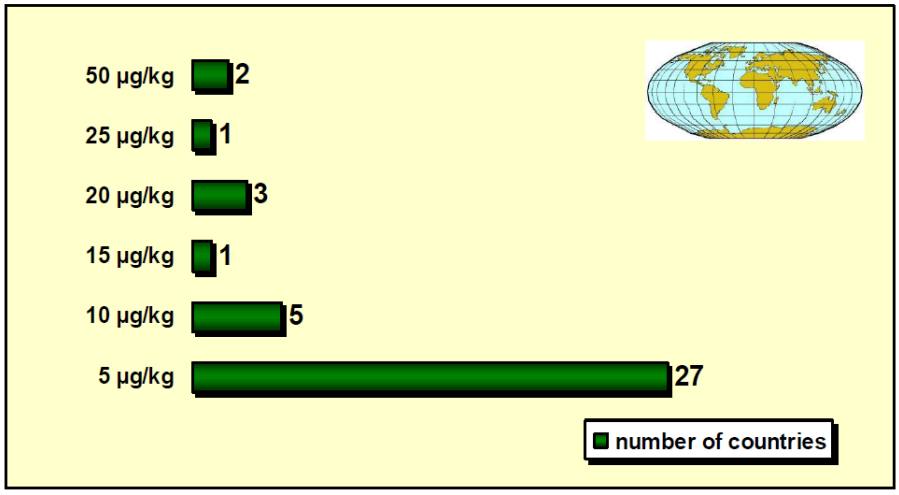
Aflatoxin M_1 in milk

(Enquiry 2012)



Aflatoxin B_1 in feed for dairy cattle

(FAO FNP 81, 2004)



Mycotoxin regulating countries in the European Union

28 member countries (accession of Croatia on 1 July 2013)

- EU-harmonized legal limits exist for aflatoxins, ochratoxin A, patulin, DON, zearalenone, fumonisins
- EU indicative levels for T-2/HT-2
- EU feed limits exist for aflatoxin B_1
- EU feed guidance values exist for ochratoxin A and some *F.* toxins

EU maximum permitted levels

EC Regulations **No. 1881/2006, 1126/2007, 105/2010, 165/2010, 594/2012, 1058/2012, 212/2014**

Mycotoxins under regulation	Matrices considered by EC regulations
Aflatoxins B_1, B_2, G_1, G_2, M_1	**Milk** (AFM1)
Deoxynivalenol	**Dried fruits, nuts**
Fumonisins (B_1, B_2)	**Cereals, cereal based foods**
Zearalenone	**Baby food**
Ochratoxin A	**Roasted coffee**
Patulin	**Dried vine fruits, wine**
T-2 and HT-2 toxins *(recommandation)*	**Spices, liquorice**
	Fruit juice, apple products
	Animal feed (AFB1)

Maximum permitted levels range: from ng-µg/kg (ppt-ppb) **to mg/kg** (ppm)

COMMISSION REGULATION (EC) No. 1881/2006

setting maximum levels for certain contaminants in foodstuffs (including some mycotoxins)

MYCOTOXINS	*FOODSTUFFS*
AFLATOXINS	Groundnuts, nuts, and processed products; dried fruit and processed products; all cereals and all products derived from cereals; milk; spices; baby foods; dietary foods for special medical purposes
OCHRATOXIN A	Unprocessed cereals and derived products; dried vine fruit; roasted and soluble coffee; wine, fruit wine, aromatised wine, grape juice, grape must; baby foods; dietary foods for special medical purposes
PATULIN	Fruit juices; spirit drinks; solid apple products; baby foods
DEOXYNIVALENOL **ZEARALENONE** **FUMONISINS**	*Amended by Commission Regulation (EC) No 1126/2007*

COMMISSION REGULATION (EC) No 1126/2007

amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants **in foodstuffs** as regards ***Fusarium* toxins** in maize and maize products

	Maxim. Levels (µg/kg)		
Foodstuffs	**DON**	**ZEA**	**FUM**
Unprocessed cereals other than maize	**1250**	**100**	**-**
Unprocessed durum wheat and oats	**1750**	**100**	**-**
Unprocessed maize	**1750**	**350**	**4000**
Cereals intended for direct human consumption, cereal flour, bran, germ	**750**	**75**	**-**
Pasta (dry)	**750**	**-**	**-**
Bread, pastries, biscuits, cereal snacks and breakfast cereals	**500**	**50**	**-**
Maize and maize-based foods intended for direct human consumption	**-**	**100**	**1000**
Maize based snacks and maize-based breakfast cereals	**-**	**100**	**800**
Processed cereal-based foods and baby foods for infants	**200**	**20**	**200**
Milling fractions of maize with particle size >500 micron not used for direct human consumption	**750**	**200**	**1400**
Milling fractions of maize with particle size ≤500 micron not used for direct human consumption	**1250**	**300**	**2000**
Refined maize oil	**-**	**400**	**-**

Prevention strategies

General CODEX (FAO/WHO) **codes for the prevention and reduction of mycotoxins available free at** ***www.codexalimentarius.org***

- CAC/RCP 45-1997: AFB_1 in feeding stuffs for cows
- CAC/RCP 50-2003: PAT in apple juice
- CAC/RCP 51-2003: Mycotoxins in cereals (OTA, ZEA, FB, TRIC)
- CAC/RCP 55-2004: AF in peanuts
- CAC/RCP 59-2005: AF in tree nuts
- CAC/RCP 63-2007: OTA in wine
- CAC/RCP 65-2008: AF in dried figs
- CAC/RCP 69-2009: OTA in coffee

Off. J. EU (2006), L 234/35-40
Commission recommendation on prevention and reduction of *Fusarium* toxins in cereals

PRE-HARVEST PREVENTION

RECOMMENDED PRACTICES BASED ON GOOD AGRICULTURAL PRACTICES (GAP)

Pre-harvest control of fungal infection and toxin formation is the best way to manage post-harvest contamination

PREVENTION IN THE FIELD

- **Reduction of plant stress** (drought stress, plant-spacing, irrigation, mineral nutrition, protection from insect or mechanical damage)
- **Crop rotation** (wheat and maize have been found to be particularly susceptible to *Fusarium* species and they should not be used in rotation with each other - potatoes, vegetables, sugar beet, etc. in rotation with cereals)
- **Avoidance of critical environmental conditions** (timing of planting)
- **Minimization of crop residues** and other **sources of inoculum** by soil preparation (weed control)
- **Selection of cultivars resistant** to fungal infection
- **Plant protection** (use of fungicides, biocompetitive agents)
- Development of **transgenic plants**

PREVENTION AT HARVEST

Commercial peanut drier

- **Clean containers** (e.g., wagons, trucks) to be used for collecting and transporting the harvested grain from the field to drying facilities.
- **Avoid mechanical damage to the grain** and avoid contact with soil during the harvesting operation.
- **Dry the crop** to the moisture content recommended for storage of that crop (this is necessary to prevent further growth of a number of fungal species).
- **Remove damaged kernels** and other foreign matter.

PREVENTION IN STORAGE

- **FUNGAL CONTROL**
  Use approved preservatives, to limit fungal development, modified atmospheres, sulphur dioxide gas, ozone, irradiation

- **MOISTURE CONTROL**
  - Store crops at a suitable moisture content and temperature *(at 20°C is 15% for wheat, 14% for maize, 7% for groundnuts)*
  - Avoid piling or heaping wet, freshly harvested commodities for more than a few hours prior to drying
  - Use storage facilities that provide protection from rain, drainage of ground water, and minimum temperature fluctuations
- **INSECT CONTROL**
  - Use good procedures to minimize the levels of insects, rodents and birds in storage facilities

Management of mycotoxin contamination

- **Pre-harvest** and **post-harvest preventive strategies** are the best way to manage mycotoxin contamination
- However under certain environmental conditions the **contamination of crops/commodities with fungi and mycotoxins could be unavoidable**

- **Destruction** of contaminated products or **diversion** to non-animal/human uses (e.g. bioethanol production)
- **Redirection into feed** for less-susceptible animals
- **Blending** of non-contaminated material with material above the limits to lower the levels ***(prohibited in Europe!!)***
- **DECONTAMINATION / DETOXIFICATION** strategies to recuperate mycotoxin contaminated commodities

Strategies for the Decontamination / Detoxification of Mycotoxins

Physical	Removal of fines or screenings; γ-irradiation; thermal treatments; adsorbent materials
Chemical	Sodium bisulfite, calcium hydroxide, ozone, chlorine, hydrogen peroxide, ascorbic acid, sulfur dioxide, formaldehyde, ammonia, etc.
Biological	Microbial degradation; transgenic plants
Feed/Food Processing	**Chemical**, **Biological** or **Thermal** and **Mechanical Treatments** applied to raw materials to produce the final products

Physical Treatments

Known to Remove Mycotoxins

- **Cold processing methods:**
  cleaning (screening), decortication or pearling (removal of the outer bran layers), dehulling (removal of hull or chaff), density segregation and fractionation, washing/soaking, dry/wet milling
- **Hot processing methods:**
  roasting/baking, extrusion, steam rolling/flaking
- **Irradiation** (including microwaves), ultrasound
- **Solvent extraction**

Physical decontamination

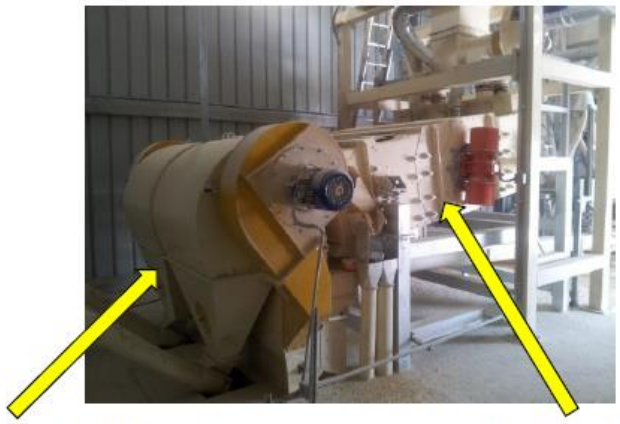
SCREENING/CLEANING

- **High levels of mycotoxins** in dust, debris and damaged kernels
- **Screening out fine materials** reduces mycotoxin content
- The process is **noninvasive**, and does not alter the product
- ***It is simple but incomplete !!!***

EFFECT OF THE PROCESS ON MYCOTOXIN LEVELS

Aflatoxins Fumonisins	Significant reduction (30-80%)
Deoxynivalenol Zearalenone	Small reduction

Separator and Aspiration channel



Aspiration Channel
Removes dust and light impurities by air resistance

Separator
Removes coarse and fine impurities by size

Physical decontamination

Optical Sorter *(Pearson et al. 2004)*

AFs and FBs co-contaminated maize (pre-cleaned)

- **AFLATOXINS** at 51 ppb reduced by **83%** by removing 5%
- **FUMONISINS** at 18 ppm reduced by **82%** by removing 5%

Manual Sorting *(van der Westhuizen et al. 2010)*

- **FUMONISINS** at 1.67 ppm reduced by **84%** by removing 3.9%

Reduction of aflatoxins in maize by combining mechanical and optical sorting technologies

Separator – Aspirator – Sortex

Lot	Fraction	Aflatoxin B_1 (µg/kg) mean ± SD*	Total AFs (µg/kg) mean ± SD*	AFB_1 reduction (%)	Total AFs reduction (%)
A	Incoming product	24.2 ± 0.3	25.4 ± 0.3	65	65
	End product	8.4 ± 0.3	8.8 ± 0.3		
B	Incoming product	62.0 ± 1.5	64.3 ± 1.6	78	78
	End product	13.5 ± 0.2	14.3 ± 0.2		

* (n= 3 replicates)

Pascale et al., International Mycotoxin Conference 2014, Beijing, P.R. China

Physical decontamination

DEHULLING / DECORTICATION

- **DEHULLING: removing of seed coat or outer shell of kernels** (hulls or chaff)
- **DECORTICATION (or PEARLING): removal of the outer bran layers by machine brushing or abrasion**

- Mycotoxins are found on the **outer surface of grains**
- They can be reduced by removing the outer parts of kernels
- The process is **incomplete** and the efficacy depends on the **degree of fungal penetration** into the kernel

Physical decontamination

DENSITY SEGREGATION AND FRACTIONATION

Mould-damaged, contaminated kernels exhibit different physical properties from non-damaged kernels

DENSITY SEGREGATION

removal of kernels floating in water and/or saturated NaCl solution

Mycotoxin reduction in cereals:

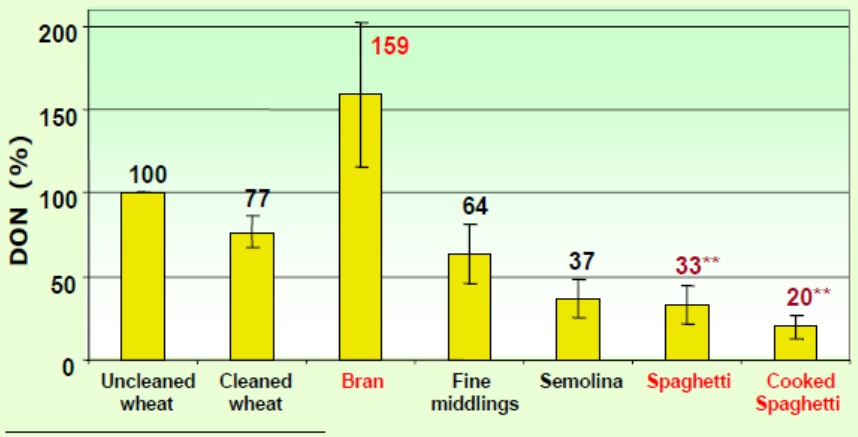
AFLA (70%) - DON (69%) - ZEA (61%)

FRACTIONATION

Gravity table separators are used for sorting of materials of the same size of particles in different specific weight.

Reduction of DON (68-85%) in wheat

Distribution of DON (%) in Milling Fractions and Spaghetti after Processing*



* mean ± SD (n = 9) from processing of durum wheat contaminated with DON in the range 0.3 - 13.1 µg/g; ** dry weight basis

Visconti et al., 2004

Effect of some Thermal Treatments on Mycotoxin Levels

	Extrusion	Baking
Afla	10-95% reduction	Some degradation depending on T
Ota	8-42% reduction	Small loss depending on conditions
Don	21-99% reduction	Stable at T < 120°C
Zea	66-83% reduction	Little effect
Fum	31-95% reduction	Unstable during roasting, but fairly stable during baking and canning

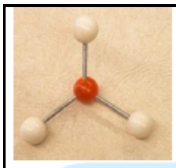
CHEMICAL TREATMENTS

KNOWN TO DESTROY/INACTIVATE MYCOTOXINS

- **Acids** (formic and propionic acids)
- **Alkali** (ammonium, sodium hydroxide)
- **Oxidizing reagents** (hydrogen peroxide, ozone)
- **Reducing agents** (bisulphite, sugars)
- **Chlorinating agents** (chlorine)

Detoxification ability depends on:

- **parameters related to the contaminated products**
- **parameters associated with the process** (such as T and P)
- **incubation time**
- **level of the mycotoxin in the product**



Chemical decontamination

AMMONIATION

- **Ammonium hydroxide** or **gaseous ammonia** (7%)
- **AFLATOXIN reduction up to 99%**
  corn, peanut meal-cakes, cottonseed
- **Irreversible process** (if the reaction proceeds to completion)
- Identification of several **decomposition compounds**
- **No toxic effects** related to the process in animals
- **Reduction/Elimination of aflatoxicosis** signs in animals

- **Some changes in the nutritional quality** of the feed (decrease in lysine and sulphur containing amino acids)
- **Adequate aeration** after ammoniation for acceptance of the feed by animals

REDUCTION OF MYCOTOXINS IN FOOD/FEED

- …Complete elimination of mycotoxin contaminated commodities is not achievable at this time
- Large-scale, practical, costs-effective methods for a complete decontamination are currently not available
- No single decontamination method effective against the variety of occurring mycotoxins has been developed

"Prevention is better than cure …"

What is the mycotoxin ???

- They are the secondary metabolite(s) which produced from fungi.

- They can occur under natural conditions in foods and feeds.

- They are six major classes of mycotoxin that frequently occurs: aflatoxins, trichothecenes, fumonisins, zearalenone, ochratoxin, and T-2 toxin

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Why we have to reduce the mycotoxin ???

1. They are cause of abnormality in animal and human health.
2. They bring enormous economic loss in food industry, crop products, and animal husbandry annually.



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Harmful effects of mycotoxin-contaminated food can be avoid by:

1. Preventing contamination
2. Removing contaminated material from the food communities
3. Mitigating mycotoxin content in food
4. Treating exposed individuals

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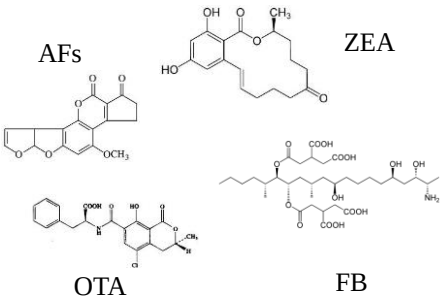
How to degrade the mycotoxin ???

1. Chemical
2. Physical
3. Biological



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Chemical strategies

- Acid treatment
- Base treatment
- Ammoniation
- Peroxidation
- Ozonation

Chemical treatment for detoxification and decontamination is not authorized within the EU for human food.

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➢ Acid treatment

AFB_1 —Strong acid→ AFB_{2a}

AFG_1 → AFG_{2a}

AFB_1 —Lactic acid→ AFB_2 (Traces)

AFB_{2a} (Main product)

➢ Base treatment

- Increase the efficiency of some physical methods to reduce mycotoxin
- Degrade AFs by opening the lactone ring

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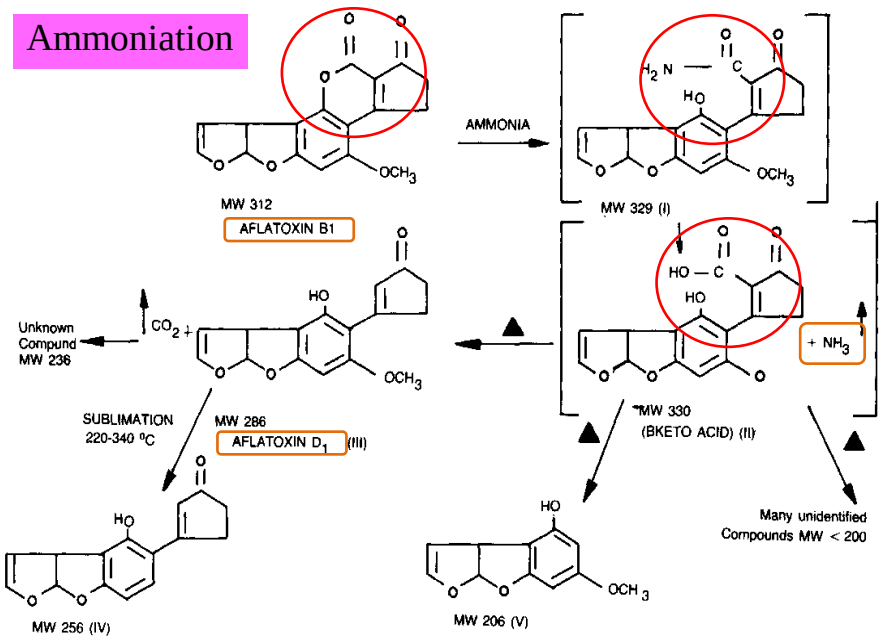
Ammoniation



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➢ Peroxidation

- H_2O_2 was used on a commercial scale to detoxify aflatoxins.
- OTA was resistant to the H_2O_2

Foods	Toxins	Reduction
Corn, peanut meal, milk	AFs	More than 50%
Corn	ZEA*	Decreased the ZEA toxicity

*Depended on the concentration of H_2O_2 , temperature, and period of exposure

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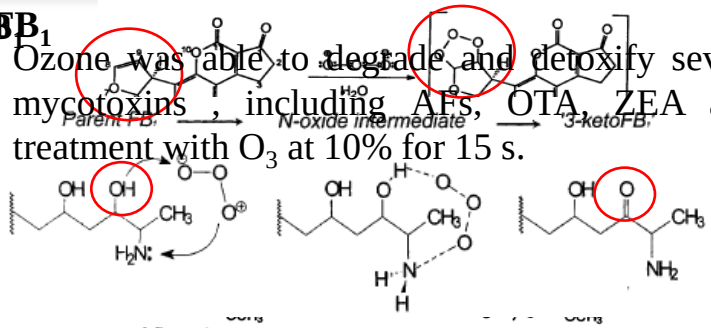
• Ozonation

AFB$_1$

- Ozone was able to degrade and detoxify several mycotoxins , including AFs, OTA, ZEA after treatment with O_3 at 10% for 15 s.



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Physical strategies

- Sorting
- Sieving cleaning
- Flotation and density segregation
- Washing
- Dehulling (or Decortication)
- Milling
- Heating
- Irradiation
- Mycotoxin binder

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Sorting

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Sieving cleaning

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Material	Solution	Mycotoxin	Mycotoxin removal	Mass loss
Corn	Water	AFs	60%	22%
material	30% Sucrose	AFs	87%	50%
Maize	Saturated sodium chloride	AFs	74%	3%
Maize	Water and 30% sucrose	DON	More than 53%	-
Wheat	Water and 30% sucrose	DON	More than 68%	-
Maize	Saturalted brine	FBs	86%	20%

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Washing

Sodium carbonate :
- Reduced DON by 72-72%
- Reduced ZEA by 80-87%

Name	Solubility in water (mg/L)
OTA	25.6
ZEA	117
T-2 toxin	347
AFB1	233
DON	36,000
Patulin	163,000
FB1	>20,000

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Dehulling

- This condition is fulfilled for aflatoxins in maize.
- This method can remove up to 93% of aflatoxins.

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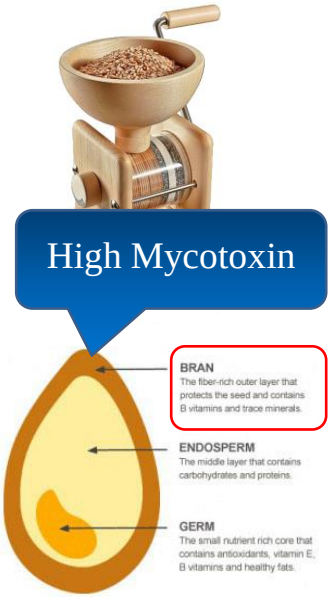
Milling



- **Dry milling** of maize results in germ and bran fractions.
- **Aflatoxins** are concentrated in germ, 20-38% remained in endosperm fraction, 11-17% in the gluten fraction, 6-11% in the germ, and 1-4% in the bran fractions after dry milling.
- **Wet milling** of maize results in concentration of mycotoxins in germ and bran fractions. **Aflatoxins** move from steep water in the gluten, **Fumonisins** remain in the fiber fraction, **Zearalenone** is mainly found in germ and bran.
- For **fumonisins**, they are partly dissolved in steep water, the amount of FBs remained in gluten and fiber.

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Heating

- **Extrusion + alkaline treatment:**
  - Reduced **AFs** by 50-80%
  - Reduced **ZEA** by 65-83% in maize
  - Reduced **FBs** by 34-95% in maize
- **Roasting:**
  - Reduced **AFs** by 50-70% in peanuts and pecans
  - Reduced **AFs** by 40% in maize
  - Reduced **OTA** in coffee bean by up to 97%




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Irradiation

- **Sunlight**
  - 40% of AFs was reduced after 3 h, up to 75% after 30 h
- **Gamma radiation**

Foods	Toxin	Reduction	Gamma radiation
Prenuts, pistachios, rice, & corn	AFs	59-88%	10 kGy
Cereals	AFs	43%	20 kGy
Almonds	OTA	24%	15 kGy
Maize	FBs	20%	15 kGy

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Mycotoxin binder

- **Chemical Binder:**
  - Activated charocal
  - Montmorillonite
  - Cholestyramine
  - Bentonite
  - Zeolite
- **Biological Binder:**
  - Yeast cell wall
  - Bacteria
  - Fungi

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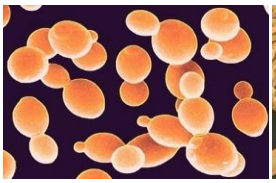
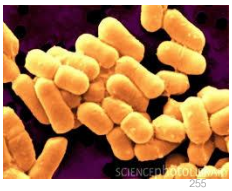
Biological strategies

- Yeast
- Mold
- Bacteria

- Decomposition
- Transformation
- Biodegradation
- Adsorption





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Mycotoxin Degradation

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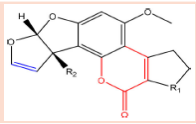
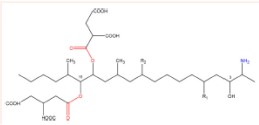
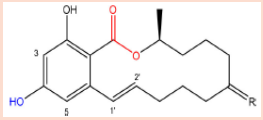
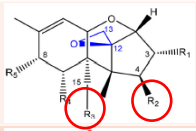
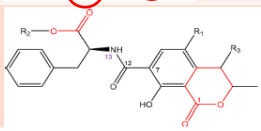
Mycotoxin		Main toxic structural group
(1)	 **AFs**	- Lactone ring - Double bond in difuran ring moiety
(2)	 **FB**	- Two carballylic acid side chains - Free amino group
(3)	 **ZEA**	- Lactone ring - C-4 hydroxyl group
(4)	 **HT-2**	- Epoxide group - Acylated side groups
(5)	 **OTA**	- Isocoumarin moiety - Carboxyl group of the phenylalanine moiety Cl group

Aflatoxins

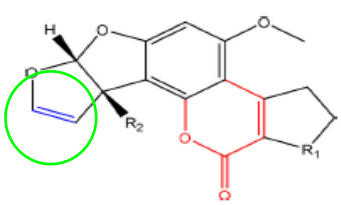
There are **two** main mechanism to detoxify of AFS.

- Modification of the **difuran ring**
- Modification of the **coumarin structure**

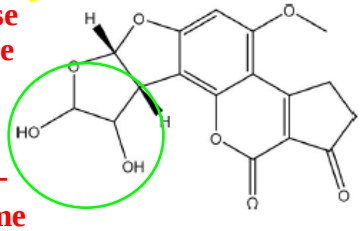
- Modification of the difuran ring

1). AFB$_1$-8.9-dihydrodiol

Phanerochaete sordida



Manganese peroxidase



Aflatoxin-detoxifizyme (ADTZ)

Aflatoxin B1

A[illegible]-8.9-dihydrodiol

Armillarriela tabescens

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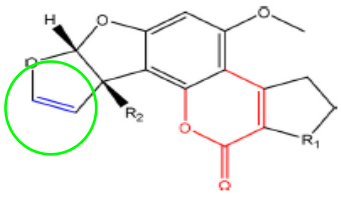
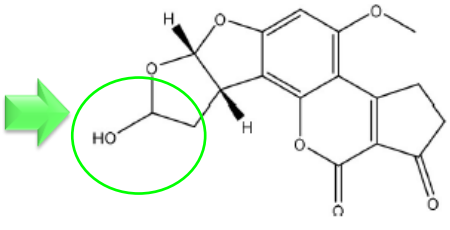
- Modification of the difuran ring

2). Dihydrohydroxyaflatoxin B$_1$ (AFB2a)

Pleurotus ostreatus





Aflatoxin B$_1$

AFB$_{2a}$

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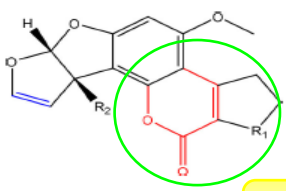
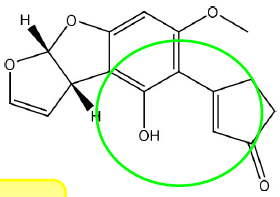
- Modification of the lactone ring

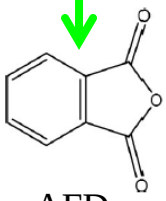


Aflatoxin B_1

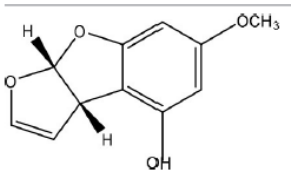
Pseudomonas putida



AFD_1



AFD_3



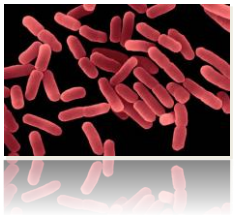
AFD_2

261

- Other degradation of aflatoxins

- **Laccase enzyme**
  - *Trametes versicolor*

- **Extracellular enzymes**
  - *Rhodococcus eryhtropolis*
  - *Bacillus subtilis*



262

Fumonisins

The main mechanism which used for detoxification of fumonisins is:

- The removal of tricarballylate side chains & amino group from fumonisin structure

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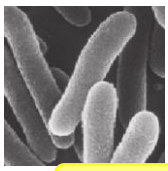
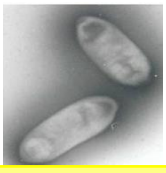
- Removal of tricarballylate side chains & amino group

- **Carboxylesterase & Aminotransferase**

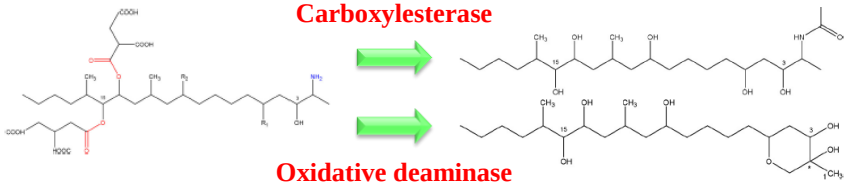
  - *Sphingomonas* sp.
  - *Sphingopyxis* sp.





- **Carboxylesterase & Oxidati**

Exophiala sp.



264

Zearalenone

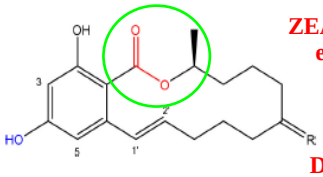
There are **two** main mechanism to detoxify of ZEA.

- Cleavage of the **lactone ring**
- Cleavage of the **aromatic ring**

265

- Cleavage of the lactone ring

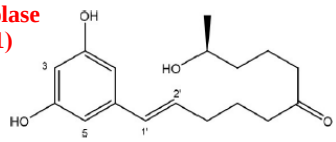
1.) Fungi & bacteria



ZEA

ZEA lactonohydrolase enzyme (zhd101)

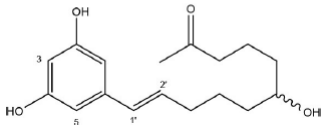
Decarboxylation



1-(3,5-dihydroxyphenyl)-10'-hydroxy-1'E-undecene-6'-one

- *Clonostachys rosea*
- *Trichoderma aggressivum*
- *Bacillus natto*
- *Bacillus subtilis*

Gliocladium roseum

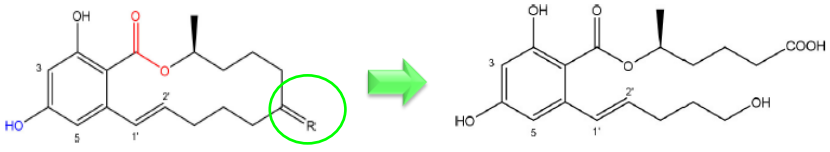


266

- Cleavage of the lactone ring

2.) Yeast

- *Trichosporum mycotoxinivorans*



ZEA ZOM-1

C_6-ketone group

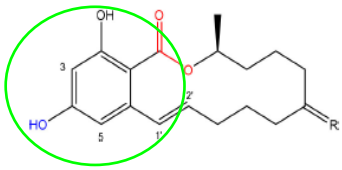
- Nor show any estrogenic activity
- Nor interact with the human estrogen receptor

267

- Cleavage of the aromatic ring

- **Fungi**
  - *Aspergillus niger*

ZEA-A & ZEA-B



ZEA

Less severe liver & kidney damage in rat

- **Bacteria**
  - *Acinetobactor* sp.

ZEA-1 & ZEA-2

Reduced estrogenic effect

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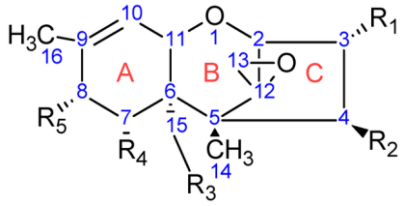
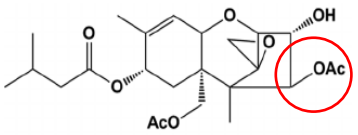
Teichothecenes

The mechanisms for detoxification including:

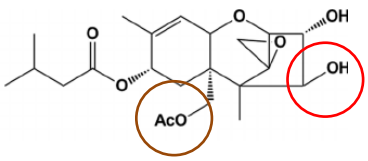
- Deacetylation
- De-Epoxidation
- 3-acetylation



- Deacetylation (T-2 toxin & HT-2 toxin)



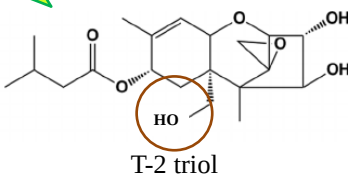
T-2 toxin



HT-2 toxin

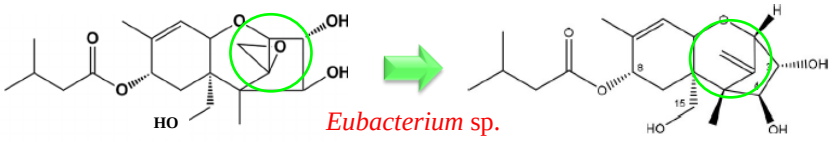
Less toxic than T-2 toxin & HT-2 toxin

***Curtobacterium* sp.**



T-2 triol

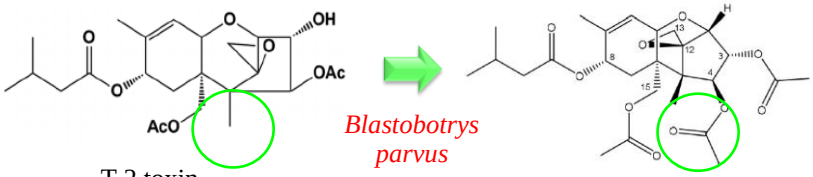
- De-epoxidation (DON & NIV)



T-2 triol

De-epoxy

- 3-acetylation (T-2 toxin)



T-2 toxin

3 acetyl T-2 toxin

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Ochratoxins

The main detoxification pathway of OTA is the **hydrolysation of the amide** bond between the isocoumarin residue and phenylalanine by a carboxypeptidase.

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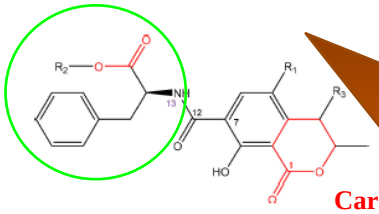
- Removal of phenylalanine group

➢ **Bacteria, Fungi, & Yeast**



OTA

Carboxy

Bacteria:
- *Bacillus linens*
- *Acinetobactor calcoaceticus*
- *Pediococcus pavulus*
- *Lactobacillus acidophilus*

Fungi:
- *s clavatus*
- *s niger*
- *s ochaceus*
- *on mycotoxinivorans*
- *odozyma*

Other enzymes:
- Deoxygenases
- Lipases
- Amidases
- Protease

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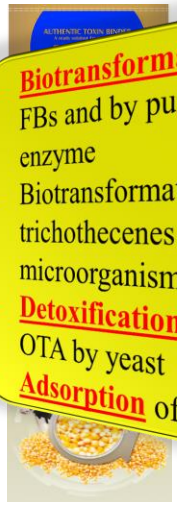
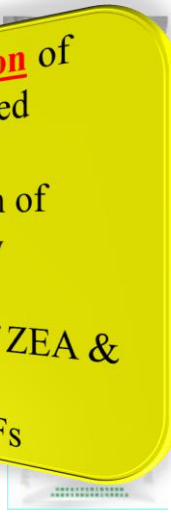
Commercial Products for Mycotoxin removal

Binder & inactivation of mycotoxins

- **Biotransformation** of FBs and by purified enzyme
- Biotransformation of trichothecenes by microorganism
- **Detoxification** of ZEA & OTA by yeast
- **Adsorption** of AFs



Silicates & yeast cell wall component



Mix enzymes

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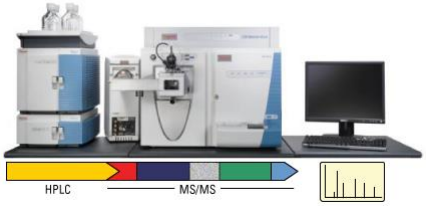
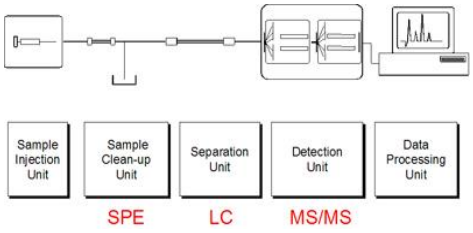
MYCOTOXINS SAMPLING & ANALYSIS



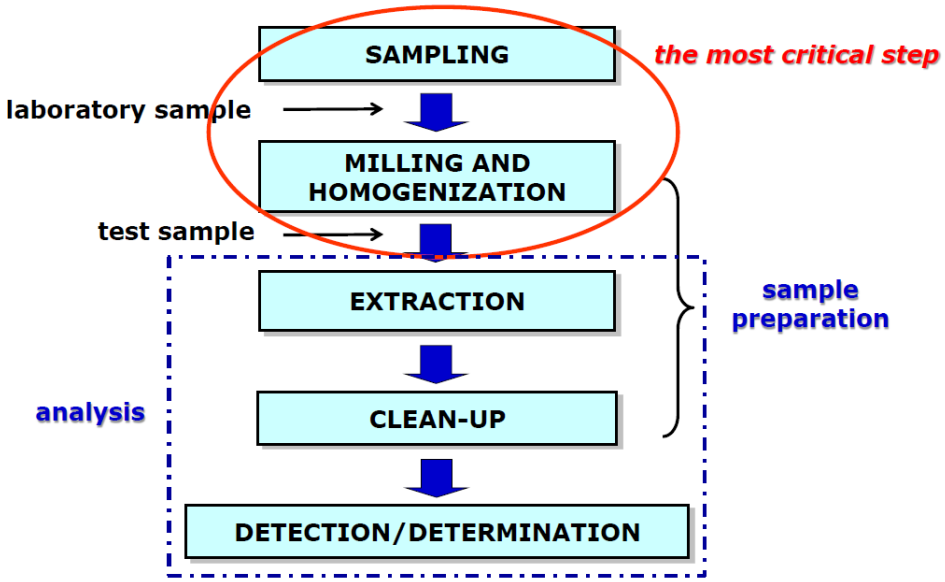


Analytical methods for mycotoxins

An analytical method for mycotoxins should:

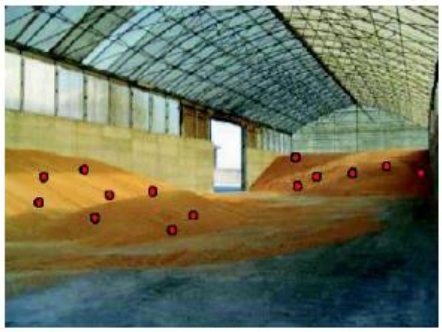
- **fulfill international regulatory limits** (must allow determination of mycotoxin at levels lower than legal limit, with set performance criteria)
- **be suitable for monitoring programs** (screening for quality control of materials in food/feed production)
- **gather information on human and animal exposure to mycotoxins** (risk assessment studies)

Typical procedure for the determination of mycotoxins in solid samples (e.g. cereals)



What is the problem? Non-homogeneous distribution of mycotoxins

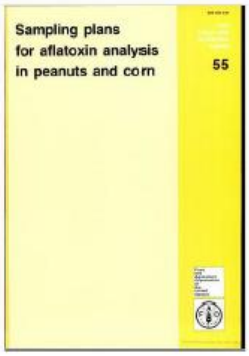
Most mycotoxins, including **aflatoxins, fumonisins and ochratoxin A**, are **unevenly distributed** in grains, so that high concentrations of toxins could be found in "**hot spots**" or "**pockets**" in bulk storage of commodities or sometimes in a single seed of dried fruit or single ear or few kernels of maize.

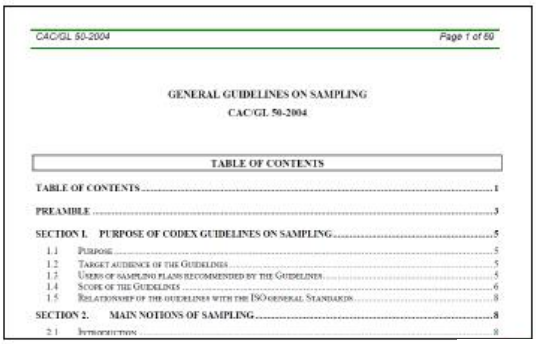


Representative samples

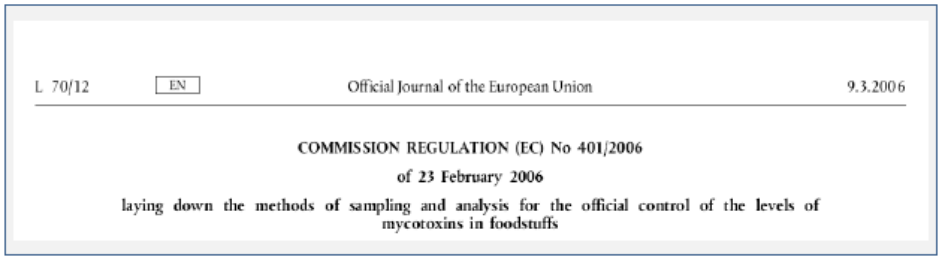
- ✓ If the **mycotoxin concentration** in the test portion **does not accurately reflect** the concentration in the **entire lot**, then the lot **maybe misclassified.**
- ✓ The **collection of truly representative samples requires** carefully **designed sampling protocols.**

Guidelines on Sampling





EU Commission Regulation for Sampling



L 70/12 EN Official Journal of the European Union 9.3.2006

COMMISSION REGULATION (EC) No 401/2006

of 23 February 2006

laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs

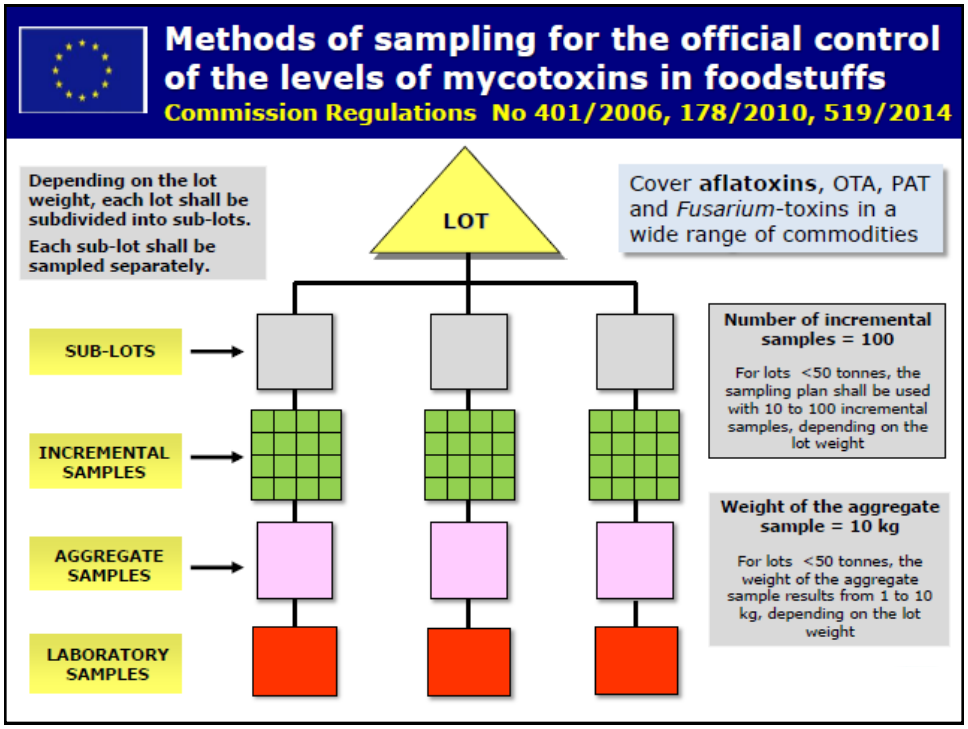
COMMISSION REGULATIONS (EC) N. 178/2010 and 519/2014

amending Regulation EC No. 401/2006

EU mycotoxin sampling plan

COMMISSION REGULATIONS (EC) N. 401/2006, 178/2010, 519/2014

AFB_1, AFBs, OTA, *Fusarium* toxins	Cereals and cereal products
AFB_1, AFBs	Dried fruits, dried figs, groundnuts, nuts, spices
AFM_1	Milk and milk products, infant formulae
OTA	Coffee, coffee products, wine, grape juice, grape must, dried vine fruits, spices, liquorice
PAT	Fruit juice, apple product for infant
AFB_1, AFBs, OTA, *Fusarium* toxins, *PAT*	Baby food, processed infant foods



Mycotoxins sampling video

- http://www.mycored.eu/d/34/Training_video_for_mycotoxin_sampling/

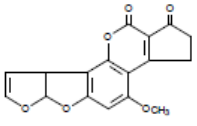
Analytical methods for mycotoxins

Conventional/routine methods
(GC, HPLC, ELISA, LC-MS/MS)

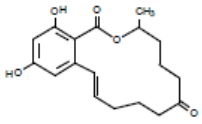
Rapid/Emerging methods
(dipsticks, LFD, FPIA, immunosensors, NIR, ...)

Standard/Official methods
(AOAC International, CEN ...)

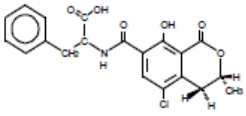
Some major mycotoxins occurring in food, feeds and derived products



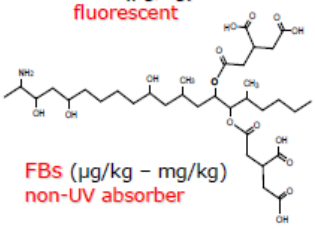
AFB_1 (µg/kg)
Low fluorescent



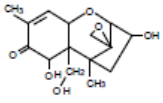
ZEA (µg/kg)
fluorescent



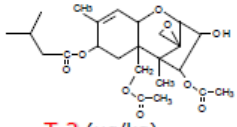
OTA (ng/kg - µg/kg)
fluorescent



FBs (µg/kg – mg/kg)
non-UV absorber



DON (µg/kg - mg/kg)
UV absorber



T-2 (µg/kg)
non-UV absorber

- Different polarities
- Different UV absorption and fluorescence spectra
- Different ionic nature, dependent on pH
- Different levels of contamination
- Occurrence in different commodities

different extraction, clean-up and detection strategies

Typical procedure :

1. Sampling
2. Milling
3. Extraction – organic solvent (methanol, acetonitrile, chloroform,…)
4. Clean-up
   - Solid phase extraction (SPE)
   - Immuno –affinity column (IAC)
5. Analysis
   - ELISA
   - HPLC
   - LC/MS-MS

Sampling

Ideal sample

- identical in all of its intrinsic properties
- homogenous

In practice

- similar properties or close to original

Consideration in sampling

- Identification of the population from which the sample is to be obtained
- Gross sample
- Reduction by 4-quarter technique = lab size sample

Types of samples

1. Random samples – bias ↑
2. Systematic samples
3. Representative samples
4. Composite samples

Extraction

Extraction

Choice of a suitable solvent

shaking
blending

- water
- methanol/water
- acetonitrile/water
- acetonitrile/methanol/water

✓ Filter or centrifuge to obtain clear supernatant

CLEAN-UP

DETECTION/ DETERMINATION

Clean-up

EXTRACTION

✓ **to remove impurities from sample extract** and to concentrate mycotoxin before detection/determination

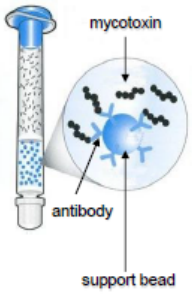
CLEAN-UP

- ✓ **Solid Phase Extraction** (SPE)
- ✓ **Multifunctional columns** (e.g. MycoSep®; Trichothecene P&EP)
- ✓ **Immunoaffinity columns** (IAC)

DETECTION/ DETERMINATION

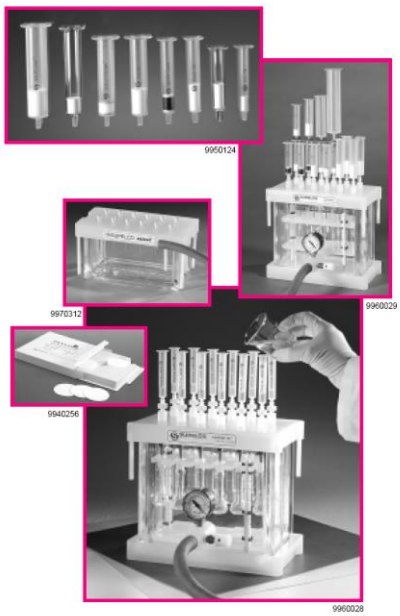
Immunoaffinity columns (IAC)

Based on monoclonal or polyclonal antibodies immobilized on agarose, sepharose or dextran beads



- Total aflatoxins (B_1, B_2, G_1, G_2)
- Aflatoxin B_1
- Aflatoxin B_1, B_2
- Aflatoxin M_1
- Ochratoxin A (OTA)
- Zearalenone (ZEA)
- Fumonisin B_1, B_2, B_3
- Deoxynivalenol (DON)
- Deoxynivalenol/Nivalenol
- T-2 + HT-2 toxins
- Total aflatoxins/OTA
- Total aflatoxins/OTA/ZEA
- Total aflatoxins/OTA/ZEA/DON/T-2/HT-2/FB_1/FB_2

Solid Phase Extraction (SPE)



- More efficient than liquid/liquid extraction
- Easy to perform
- Rapid
- Automated
- Reduced lab time and solvent

SPE Phase Types

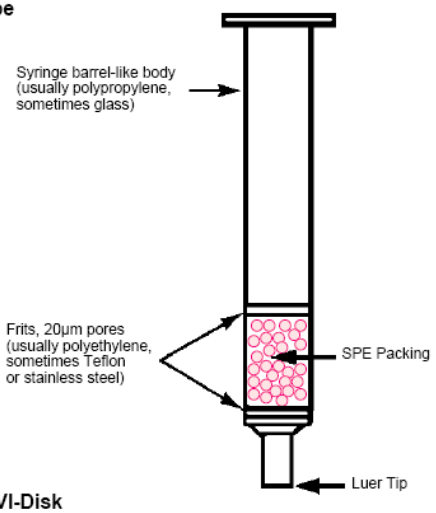
Reversed Phase	**Silica-Based Packing – 40µm particles, 60Å pores (unless otherwise noted).**		
	LC-18	octadecyl bonded, endcapped silica	For reversed phase extraction of nonpolar to moderately polar compounds, such as antibiotics, barbiturates, benzodiazepines, caffeine, drugs, dyes, essential oils, fat soluble vitamins, fungicides, herbicides, pesticides, hydrocarbons, parabens, phenols, phthalate esters, steroids, surfactants, theophylline, and water soluble vitamins.
	ENVI™-18	octadecyl bonded, endcapped silica	Higher phase coverage and carbon content than LC-18, greater resistance to extreme pH conditions, and slightly higher capacity for nonpolar compounds. For reversed phase extraction of nonpolar to moderately polar compounds, such as antibiotics, caffeine, drugs, dyes, essential oils, fat soluble vitamins, fungicides, herbicides, pesticides, PNAs, hydrocarbons, parabens, phenols, phthalate esters, steroids, surfactants, water soluble vitamins. Also available in disk format.
	LC-8	octyl bonded, endcapped silica	For reversed phase extraction of nonpolar to moderately polar compounds, such as antibiotics, barbiturates, benzodiazepines, caffeine, drugs, dyes, essential oils, fat soluble vitamins, fungicides, herbicides, pesticides, hydrocarbons, parabens, phenols, phthalate esters, steroids, surfactants, theophylline, and water soluble vitamins. Also available in disk format.
	ENVI-8	octyl bonded, endcapped silica	Higher phase coverage and carbon content than LC-8, greater resistance to extreme pH conditions, and slightly higher capacity for nonpolar compounds. For reversed phase extraction of barbiturates, benzodiazepines, caffeine, drugs, dyes, essential oils, fat soluble vitamins, fungicides, herbicides, pesticides, PNAs, hydrocarbons, parabens, phenols, phthalate esters, steroids, surfactants, theophylline, water soluble vitamins.
	LC-4	butyldimethyl bonded, end-capped silica (500Å pores)	Less hydrophobic than LC-8 or LC-18. For extraction of peptides and proteins.
	LC-Ph	phenyl bonded silica	Slightly less retention than LC-18 or LC-8 material. For reversed phase extraction of nonpolar to moderately polar compounds, especially aromatic compounds.
	Hisep™	hydrophobic surface enclaved by a hydrophilic network	For exclusion of proteins in biological samples; retains small molecules such as drugs under reversed phase conditions.
Normal Phase	**LC-CN**	cyanopropyl bonded, endcapped silica	For reversed phase extraction of moderately polar compounds, normal phase extraction of polar compounds, such as aflatoxins, antibiotics, dyes, herbicides, pesticides, phenols, steroids. Weak cation exchange for carbohydrates and cationic compounds.
	LC-Diol	diol bonded silica	For normal phase extraction of polar compounds.
	LC-NH_2	aminopropyl bonded silica	For normal phase extraction of polar compounds, weak anion exchange for carbohydrates, weak anions, and organic acids.
Ion Exchange	**LC-SAX**	quaternary amine bonded silica with Cl⁻ counterion	For strong anion exchange for anions, organic acids, nucleic acids, nucleotides, and surfactants. Capacity: 0.2meq/g.
	LC-SCX	sulfonic acid bonded silica with Na^+ counterion	For strong cation exchange for cations, antibiotics, drugs, organic bases, amino acids, catecholamines, herbicides, nucleic acid bases, nucleosides, and surfactants. Capacity: 0.2meq/g.
	LC-WCX	carboxylic acid bonded silica with Na^+ counterion	For weak cation exchange of cations, amines, antibiotics, drugs, amino acids, catecholamines, nucleic acid bases, nucleosides, and surfactants.
Adsorption	**LC-Si**	silica gel with no bonded phase	For extraction of polar compounds, such as alcohols, aldehydes, amines, drugs, dyes, herbicides, pesticides, ketones, nitro compounds, organic acids, phenols, and steroids.
	Alumina-Based Packing – Crystalline, chromatographic grade alumina, irregular particles, 60/325 mesh.		
	LC-Alumina-A	acidic pH ~5	For anion exchange and adsorption extraction of polar compounds, such as vitamins.
	LC-Alumina-B	basic pH ~8.5	For adsorption extraction of polar compounds, and cation exchange.
	LC-Alumina-N	neutral pH ~6.5	For adsorption extraction of polar compounds. With pH adjustment, cation or anion exchange. For extraction of vitamins, antibiotics, essential oils, enzymes, glycosides, and hormones.
	Florisil®-Based Packing – Magnesium silicate, 100/120 mesh particles.		
	LC-Florisil		For adsorption extraction of polar compounds, such as alcohols, aldehydes, amines, drugs, dyes, herbicides, pesticides, PCBs, ketones, nitro compounds, organic acids, phenols, and steroids.
	ENVI-Florisil▲		For adsorption extraction of polar compounds, such as alcohols, aldehydes, amines, drugs, dyes, herbicides, pesticides, PCBs, ketones, nitro compounds, organic acids, phenols, and steroids.
	Graphitized Carbon-Based Packing – Nonbonded carbon phase.		
	ENVI-Carb	nonporous, surface area 100m^2/g, 120/400 mesh	For adsorption extraction of polar and nonpolar compounds.
	ENVI-Carb C	nonporous, surface area 10m^2/g, 80/100 mesh	For adsorption extraction of polar and nonpolar compounds.
	Resin-Based Packing – 80-160µm spherical particles.		
	ENVI-Chrom P▲▲		For extraction of polar aromatic compounds such as phenols from aqueous samples. Also for adsorption extraction of nonpolar to midpolar aromatic compounds.

▲ SPE tubes that are packed with this material contain stainless steel or Teflon® frits, required by US Environmental Protection Agency Contract Laboratory Program (CLP) pesticide methods.

▲▲ Highly crosslinked, neutral, specially cleaned styrene-divinylbenzene resin. Very high surface area, mean pore size 110-175Å.

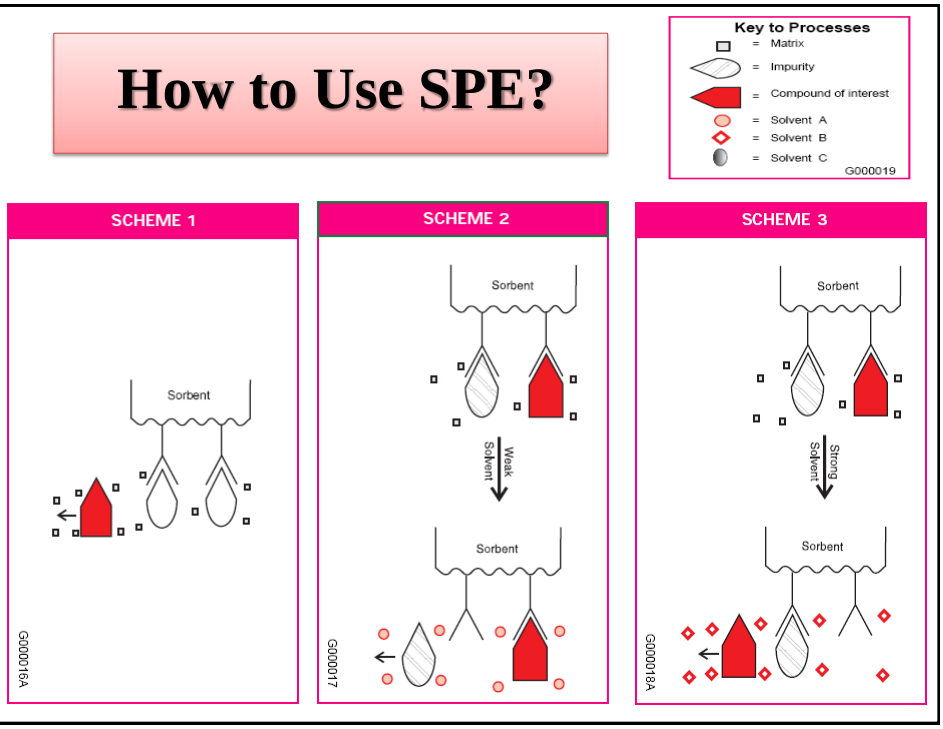
Typical SPE Tube and Disk

SPE Tube

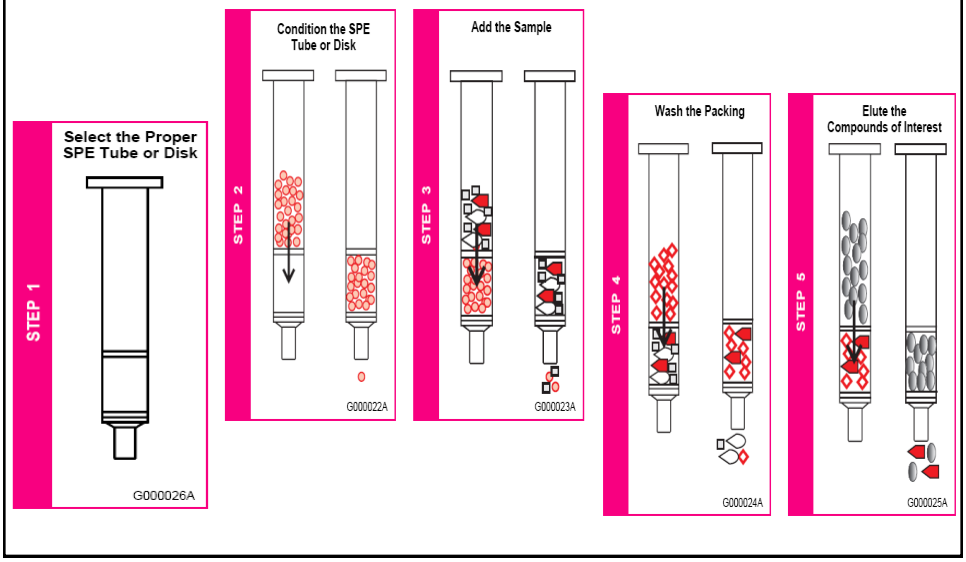


SPE ENVI-Disk

How to Use SPE?



SPE is a five-step process



Selecting an SPE Tube or Disk: Size

Selecting SPE Tube Size	
If Your Sample Is . . .	**Use Tube Size . . .**
< 1mL	1mL
1mL to 250mL and the extraction speed is not critical	3mL
1mL to 250mL and a fast extraction procedure is required	6mL
10mL to 250mL and higher sample capacity is needed	12, 20, or 60mL
< 1 liter and extraction speed is not critical	12, 20, or 60mL
Selecting SPE Disk Size	
If Your Sample Is . . .	**Use Disk Size . . .**
100mL to 1 liter	47mm
>1 liter and higher sample capacity is needed	90mm

Table A. Characteristics of Solvents Commonly Used in SPE

Polarity			Solvent	Miscible in Water?
Nonpolar	Strong Reversed Phase	Weak Normal Phase	Hexane	No
			Isooctane	No
			Carbon tetrachloride	No
			Chloroform	No
			Methylene chloride (dichloromethane)	No
			Tetrahydrofuran	Yes
			Diethyl ether	No
			Ethyl acetate	Poorly
			Acetone	Yes
			Acetonitrile	Yes
			Isopropanol	Yes
	Weak Reversed Phase	Strong Normal Phase	Methanol	Yes
Polar			Water	Yes
			Acetic acid	Yes

Hardware and accessories for processing samples

Figure A. Single Tube Processor

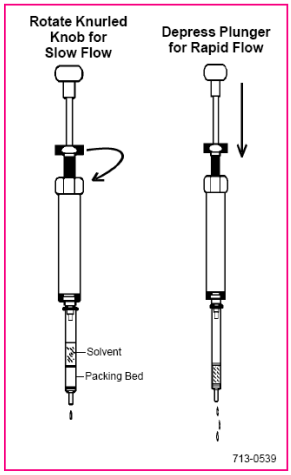


Figure B. Process Using Applied Pressure

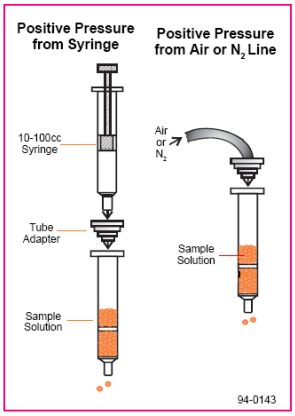


Figure C. Configuration Using a Vacuum Flask

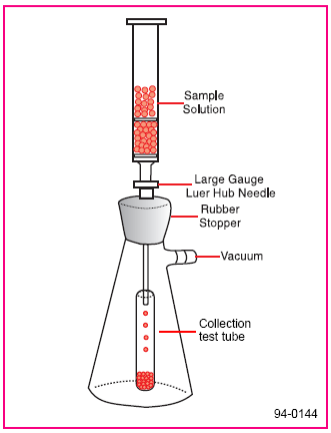


Figure D. Processing Several Tubes Using a Centrifuge

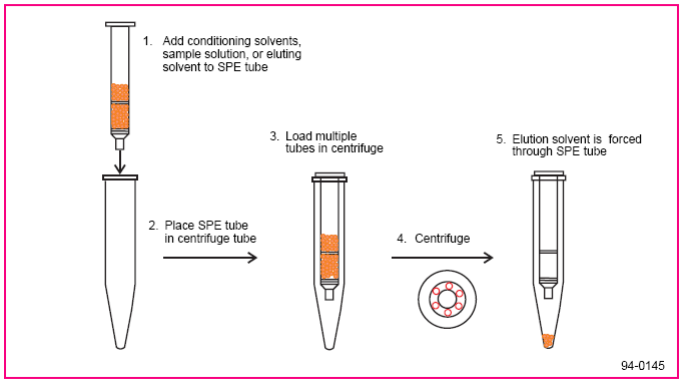


Figure E. Visiprep Vacuum Manifold with Standard Lid

Figure F. Visiprep Vacuum Manifold with Disposable Liner

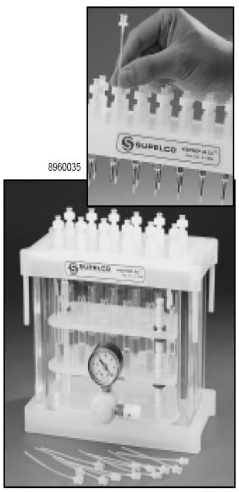


Figure G. SPE Vacuum Pump Trap

Figure H. Preppy Vacuum Manifold

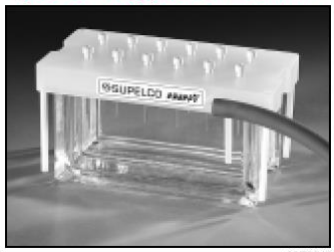


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Figure I. Visiprep
Large Volume Sampler

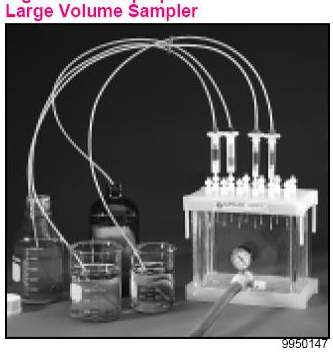

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Figure J. Visidry Drying Attachment

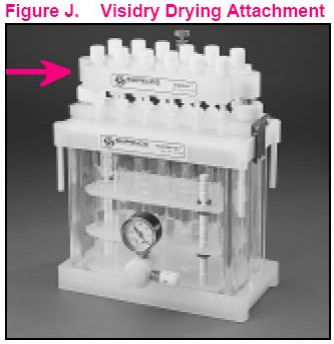

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Figure K.

7130554

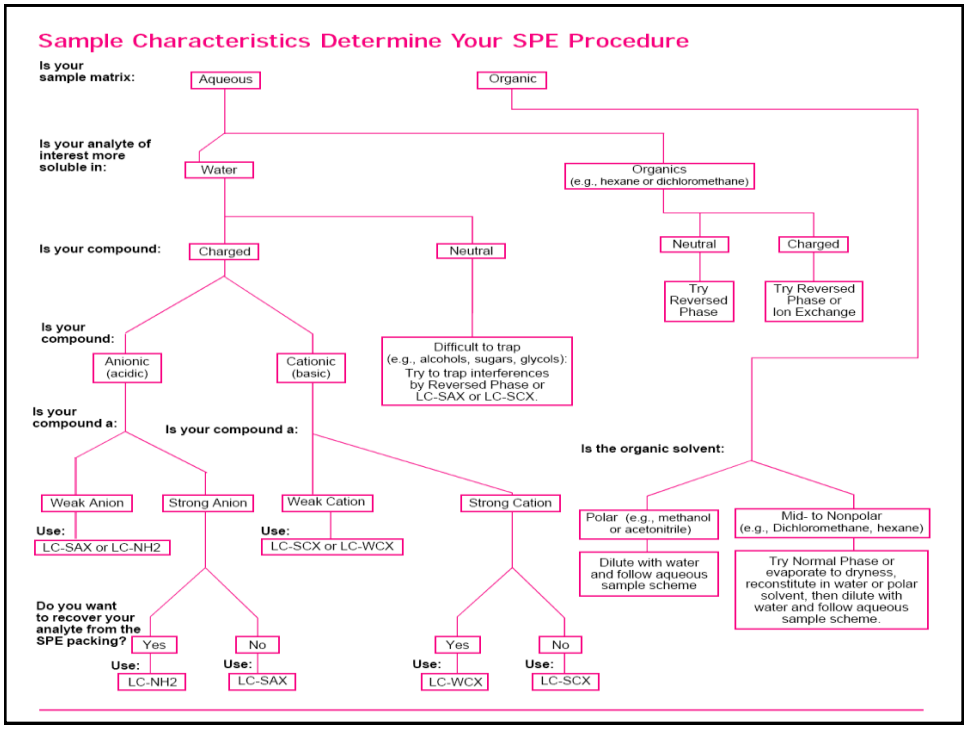
Figure M.

9960280

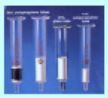
Figure N.

9950117

Figure L.



Major advantages and drawbacks of clean-up methods for mycotoxins

Method	ADVANTAGES	DRAWBACKS
SPE 	• Simultaneous batch processing • Automation • Analyte enrichment • Multi-toxin clean-up	• Conditioning of the column • Limited selectivity • Time consuming
MycoSep® 	• Easy and rapid clean-up • Multi-toxin clean-up	• Limited selectivity • No automation • No analyte enrichment
IAC 	• High selectivity • Applicability to complex matrices • Rapid sample preparation and clean-up procedure • Simultaneous batch processing • Automation • Analyte enrichment (sensitive) • Multi-toxin clean-up (few)	• High costs • Single use • Analysis of individual mycotoxins (most of IAC)

ANALYSIS

Detection/determination

SAMPLING

EXTRACTION

CLEAN-UP

DETECTION/
DETERMINATION

GC, HPLC, UHPLC,
LC-MS(MS)

GAS CHROMATOGRAPHY (GC)

Mycotoxins: **Type A-trichothecenes** (T-2, HT-2, DAS, T-2 tetraol)
Type B-trichothecenes (DON, 3- and 15-AcDON, NIV, FUS X)

Clean-up: **charcoal-alumina, florisil, silica, MycoSep® columns**

Derivatization: **Trimethylchlorosilane** (TMCS),
N-trimethylsilyl-imidazole (TMSI),
N,O-bis-(trimethylsilyl) acetamide (BSA),
Heptafluorbutyryl-imidazole (HFBI),
Heptafluorobutyric anhydride (HFBA)
Tri-Sil® TBT (3:3:2 mixture of TMSI:BSA:TMCS)
(trimethylsilyil, trifluoroacetyl, pentafluoropropionyl heptafluorobutyryl derivatives)

Detector: **FID, ECD, MS**

GAS CHROMATOGRAPHY (GC)

ADVANTAGES

- **simultaneous analysis of several trichothecenes**
- **good sensitivity**
- **can be automated** (autosampler)
- **confirmation** (MS detector)

DISADVANTAGES

- **relatively expensive** (30-40 k€ GC-FID/ECD; 70-80 k€ bench-top GC-MS)
- **specialist expertise requested**
- **derivatization** (to increase volatility)
- **matrix effect** (higher trichothecenes response for calibrants in presence of matrix than pure calibrants)
- **non-linear calibration curve**
- **drifting response**
- **carry-over or memory effects from previous samples**
- **matrix interferences**
- **high variation in reproducibility and repeatability**

High Performance Liquid Chromatography (HPLC)

Mycotoxins: **Aflatoxins** (AFB_1, AFB_2, AFG_1, AFG_2), **Aflatoxin M1** (AFM1), **Ochratoxin A** (OTA), **Fumonisins** (FB_1, FB_2, FB_3), **Deoxynivalenol** (DON), **Zearalenone** (ZEA), **type A-trichothecenes** (T-2, HT-2); **Patulin** (PAT)

Clean-up: **Solid phase extraction, MycoSep®, immunoaffinity columns**

Detector: **UV (or DAD), FLD, MS**

Derivatization: **TFA, iodine, bromine, UV irradiation** (AFB_1, AFG_1)
OPA reagent (FB_1, FB_2, FB_3)
1-anthroylnitrile (T-2, HT-2)

Mycotoxins marked in blue need to be derivatized

High Performance Liquid Chromatography (HPLC)

ADVANTAGES

- **good sensitivity**
- **good selectivity**
- **good repeatability**
- **short times of analysis**
- **can be automated** (autosampler)

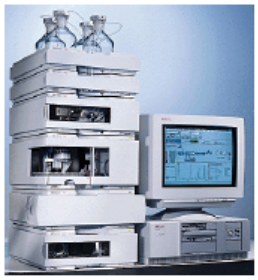
DISADVANTAGES

- **expensive** (instrumentation 50-70 k€)
- **specialist expertise requested**
- **derivatization** (in some cases)

Aflatoxins in maize by HPLC/FLD

Extraction
(methanol:water, 70:30, v/v)

- Filtration (Whatman Nº 4)
- Dilution 1:3 (v/v) with water
- Filtration (Whatman GF/A)



Immunoaffinity column clean-up

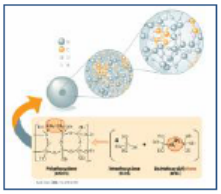
Post-column derivatization

HPLC/FLD determination

(λ_{ex} = 365 nm, λ_{em} = 435 nm)

AOAC Official Method 991.31

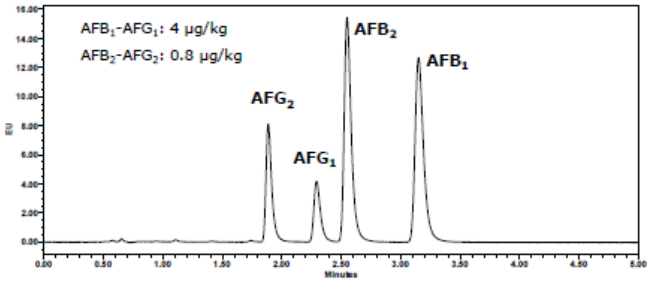
Ultra High Performance Liquid Chromatography (UHPLC)



New stationary-phases (particle sizes <2.0 µm)

Advantages (improved performances):
- **improved resolution;**
- **improved sensitivity;**
- **improved speed;**
- **reduced solvent use;**
- **no derivatization** (AFs,T2/HT2 toxins)

UHPLC runs up to 9 times lesser than HPLC runs



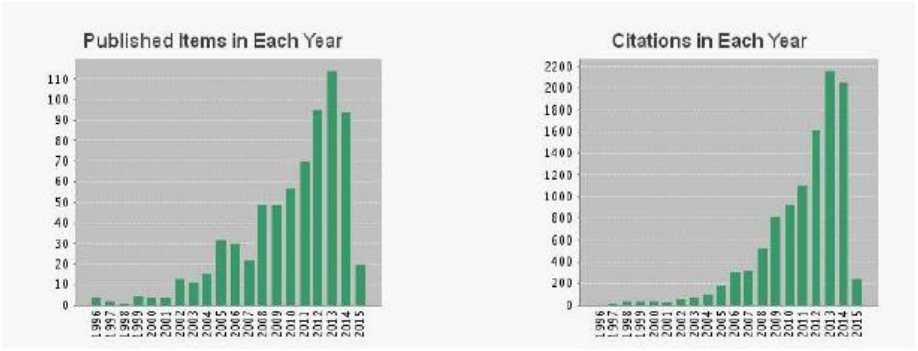
Column: Acquity UPLC® BEH C18 (100 x 2.1 mm, 1.7 µm)

Mobile phase: water:acetonitrile:methanol (64:18:18, v/v/v);

Flow rate: 0.4 mL/min

Detection: fluorescence ($\lambda_{ex.}$ = 365 nm, $\lambda_{em.}$ = 435 nm)

LC-MS methods for mycotoxin determination in the literature



Multi-mycotoxin methods by LC-MS/MS

Mycotoxins: **aflatoxins, ochratoxin A, zearalenone, trichothecenes, fumonisins .... modified mycotoxins**

Applicability: **wide range of matrices** (including biological tissues and fluids)

Clean-up: **not required** (analysis of diluted crude extract), **required** (SPE, MycoSep®, IAC)

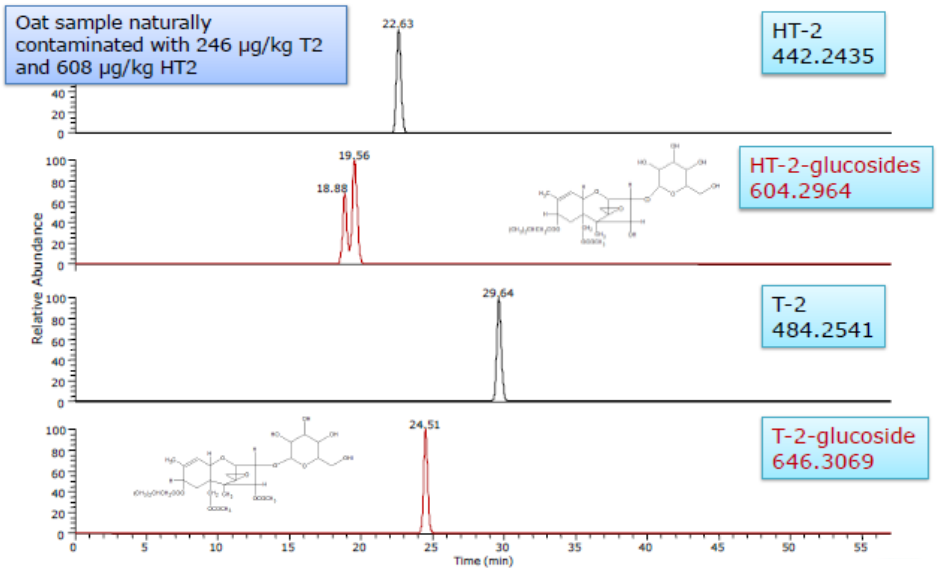
ADVANTAGES

- **simultaneous analysis of several mycotoxins** (decreased cost per analysis)
- **high sensitivity**
- **high selectivity** (tandem MS)
- **confirmation** (MS spectra)
- **no derivatization procedure**
- **allows detection of mycotoxin conjugates** ("modified" or "masked" mycotoxins)

DISADVANTAGES

- **equipment very expensive** (200-600 k€)
- **specialist expertise requested**
- **low accuracy** (for some mycotoxins)
- **matrix effect** (ion suppression, ion enhancement)
- **matrix assisted calibration curve** (for quantitative analysis)
- **use of isotope labeled internal standard**
- **internal standards not available for several mycotoxins**

Identification and characterization of T-2 and HT-2 glycosides in wheat and oats



Lattanzio, A. Visconti, M. Haidukowski, M. Pascale. *Journal of Mass Spectrometry, 2012, 47:466-475*

Why rapid methods?

Conventional method

EXTRACTION

↓

CLEAN-UP

↓

DETECTION
(GC, HPLC)

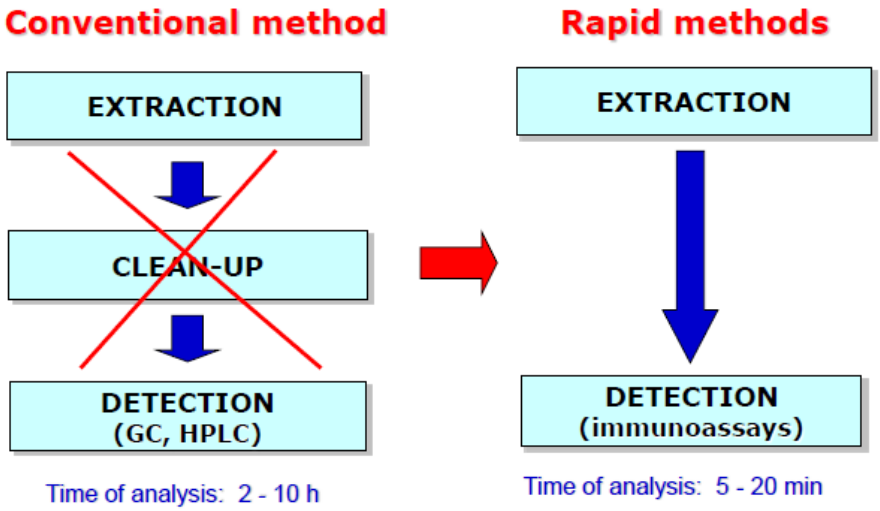
Time of analysis: 2 - 10 h

- **Tedious sample preparation**
  - Grinding of sample
  - Extraction
  - Clean-up
- **Time consuming separation and detection**
  - GC-ECD (MS)
  - HPLC-DAD (FD, MS)
- **Expensive equipments and operation costs**

↓

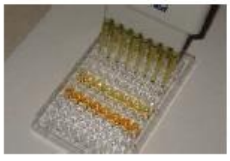
Growing demand for rapid and easy-to-perform methods

Rapid methods



Rapid/emerging methods

- **Immunoassays/immunosensors:**
  - (Fast)Enzyme Linked Immunosorbent Assay (ELISA)
  - Flow Through Immunoassay (FIA)
  - Lateral flow devices (LFD) or dipsticks
  - Fluorescence polarization immunoassays (FPIA)
  - Surface plasmon resonance (SPR) biosensors
  - Electrochemical immunosensors (ES)
  - Biosensor arrays
- **Indirect screening methods:** Infrared spectroscopy (FT-IR), Electronic noses
- **Methods using alternative receptor:** aptamers, antibody fragments, molecularly imprinted polymers, peptides




ELISA : Introduction

Early 1900	First use of immunoassay for clinical diagnosis of Typhoid
1939-1985	3 methods for food-borne pathogen diagnosis had been validated by AOAC
1986-1993	More than 30 commercial kits available
1993-Presently	> 200 commercial kits available

Immunoassay has become very popular because

1. Easy to use
2. Cheap
3. Specific and sensitive
4. Can be used quantitatively

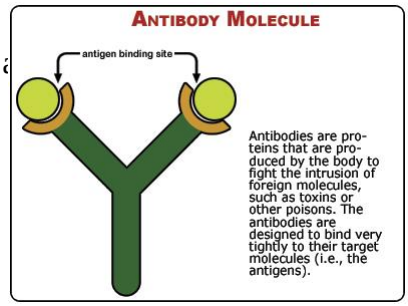
Basic principle in Serology

1. Body Defense Mechanism
   - *Innate immunity* – Macrophage (WBC) : continually search for foreign (nonself) antigenic molecules, viruses, or microbes. When found, the macrophages engulfs and destroys them.
   - *Adaptive (Induced) immunity*-Antibody (Ab)
2. Antigen (Immunogen)
   = foreign substance (s) with specific surface topography which can elicit the immune response in an animal resulting in a specific Antibody production

   Ex. Microbes, macromolecules (protein, CBH, nucleic acid, MW>5,000)
3. Antibody (Immunoglobulin : Ig)
4. Serological reaction

Antibody (Immunoglobulin : Ig)

- Antibodies are Y-shaped molecules composed of two identical long polypeptide (Heavy or H chains) and two identical short polypeptides (Light or L chains). Function of antibodies includes:
  - Recognition and binding to a
  - Inactivation of the antigen



Structure of Antibody

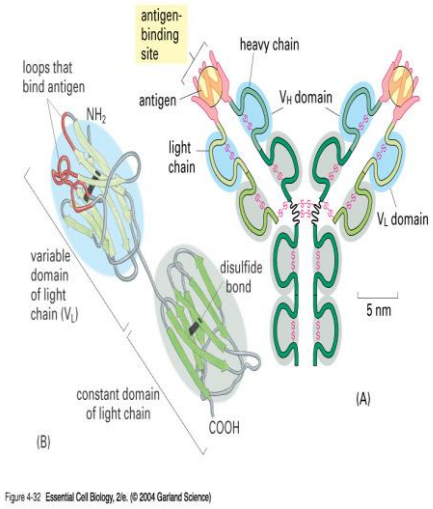
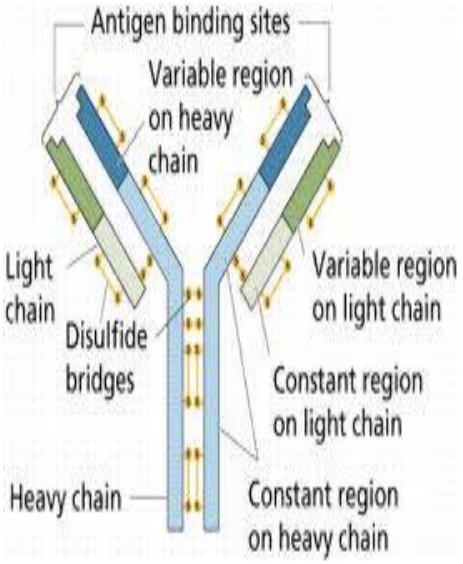


Figure 4-32 Essential Cell Biology, 2/e. (© 2004 Garland Science)

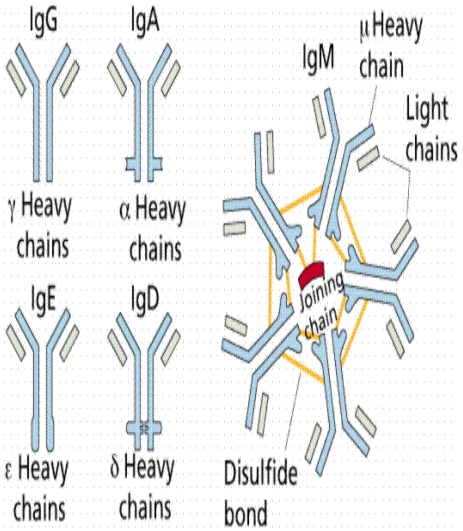


Types of Antibody

Antibodies bind to specific antigens in a lock-and-key fashion, forming an antigen-antibody complex.

Antibodies are a type of protein molecule known as immunoglobulins.

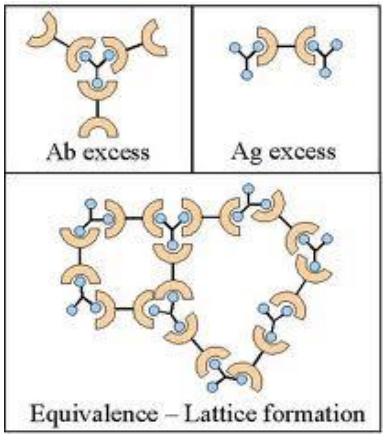
There are five classes of immunoglobulins: IgG, IgA, IgD, IgE, and IgM.



Serological reaction

Ag + Ab ⟶ Ag-Ab complex

result in : Precipitation or Agglutination



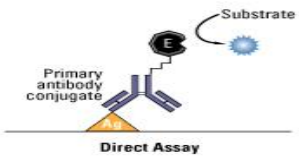
Enzyme-Linked Immunosorbent Assay (ELISA)

- Antigen or antibody is passively adsorbed to solid surface (polystylene surface)
- Separation of bound and the free (unbound) reactants are made by washing
- Result of an ELISA is a (soluble) color reaction

ELISA format

1. Direct
2. Indirect
3. Sandwich direct
4. Sandwich indirect
5. Competitive

Direct ELISA



Antigen

- Should be in the buffer
  - Carbonate coating buffer (pH 9.6) เพื่อให้ประจุของ Ag ตรงกันข้ามกับประจุของ Plastic → จะได้เกาะกันดีขึ้น
  - Phosphate buffer saline – Tween (pH 7.4) เป็นการปรับสภาพให้เหมือนน้ำเลือด ใช้เพื่อล้าง
  - Conjugate buffer pH 7.4 ใช้ทำ dilution ของ Antibody
  - Substrate buffer

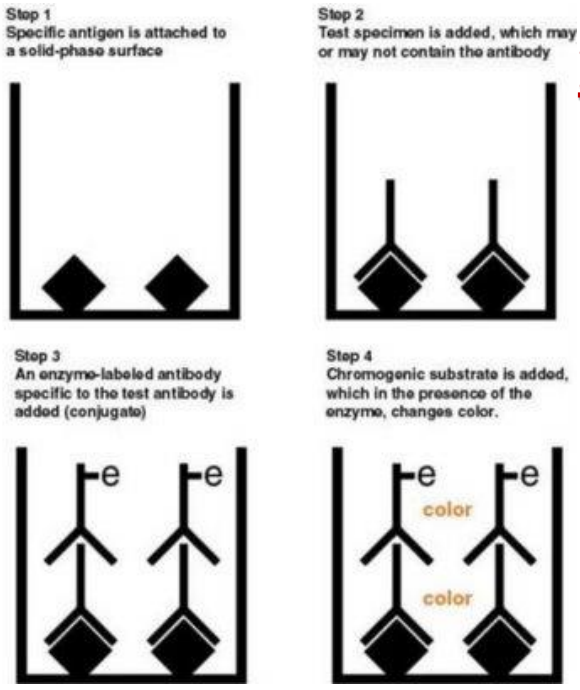
Enzyme

- Alkaline phosphatase (ALP)
- Horse radish peroxidase (HRP)
- Penicillase

Substrate

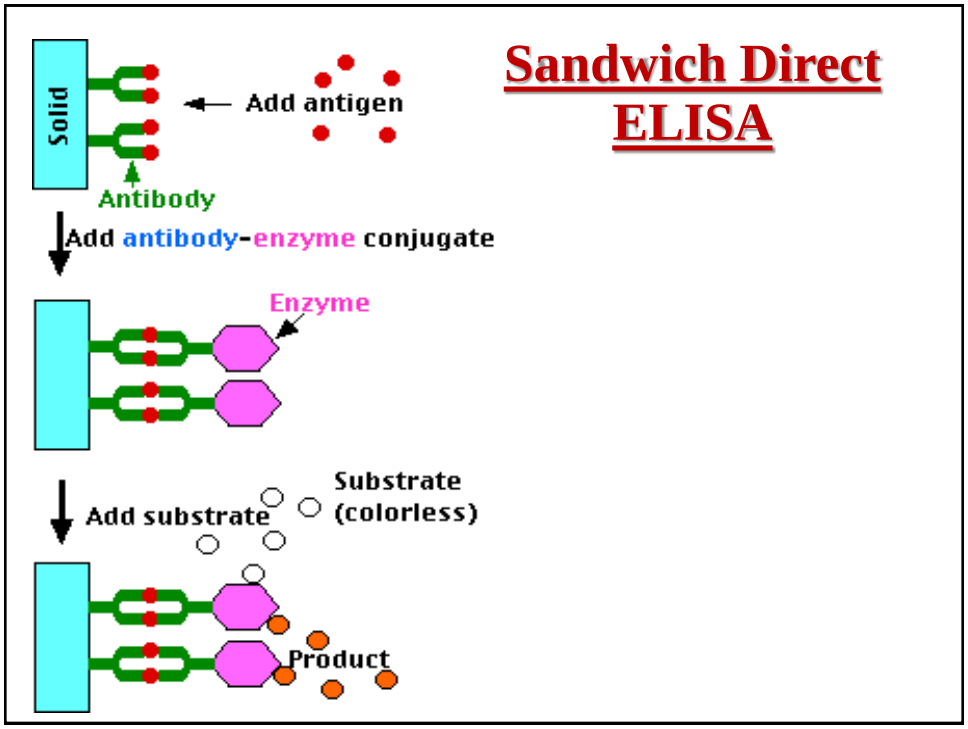
- ALP (p-dinitrophosphate = ให้สีเหลือง, Nitro-blue tetrazolium chloride (NBT) ให้สีน้ำเงิน)
- HRP (Tetramethyl benzidine dihydrochloride (TMB))

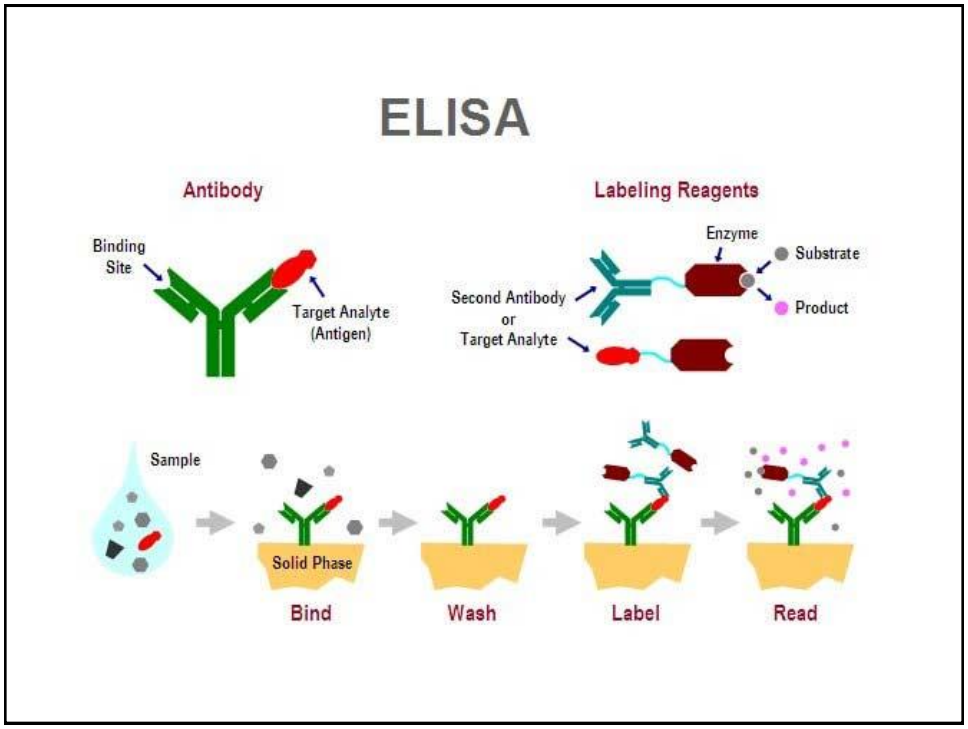
Indirect ELISA



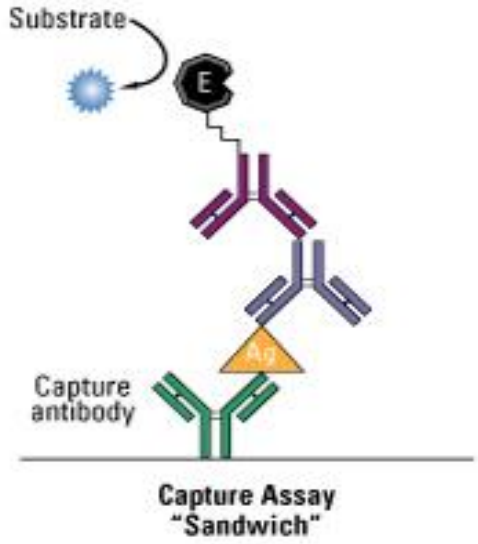
Comparison of Direct and Indirect ELISA Detection Methods

Direct Detection	**Advantages**	•Quick because only one antibody and fewer steps are used. •Cross-reactivity of secondary antibody is eliminated.
	Disadvantages	•Immunoreactivity of the primary antibody might be adversely affected by labeling with enzymes or tags. •Labeling primary antibodies for each specific ELISA system is time-consuming and expensive. •No flexibility in choice of primary antibody label from one experiment to another. •Minimal signal amplification.
Indirect Detection	**Advantages**	•A wide variety of labeled secondary antibodies are available commercially. •Versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection. •Maximum immunoreactivity of the primary antibody is retained because it is not labeled. •Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification. •Different visualization markers can be used with the same primary antibody.
	Disadvantages	•Cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal. •An extra incubation step is required in the procedure.

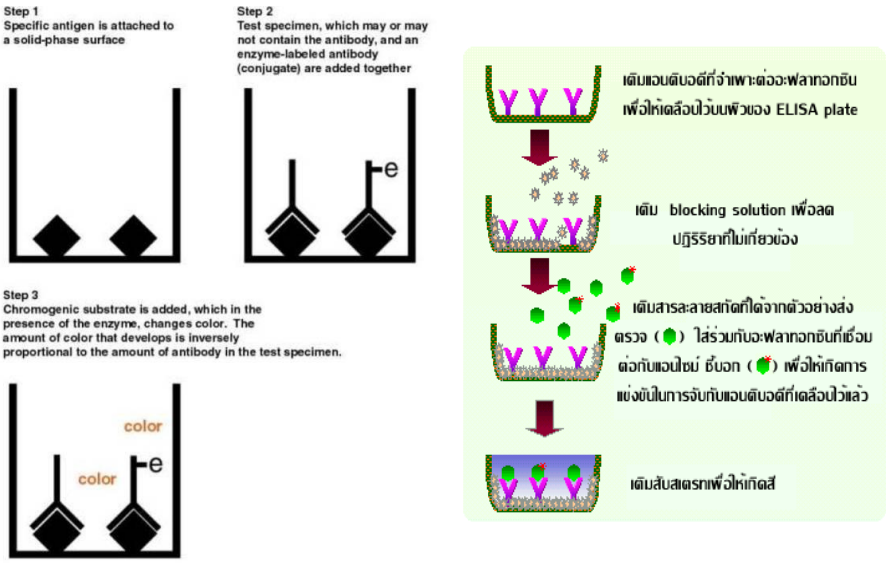

Sandwich Direct ELISA
Solid
Add antigen
Antibody
Add antibody-enzyme conjugate
Enzyme
Add substrate
Substrate (colorless)
Product


ELISA
Antibody
Binding Site
Target Analyte (Antigen)
Labeling Reagents
Enzyme
Substrate
Product
Second Antibody or Target Analyte
Sample
Solid Phase
Bind
Wash
Label
Read

Sandwich Indirect ELISA



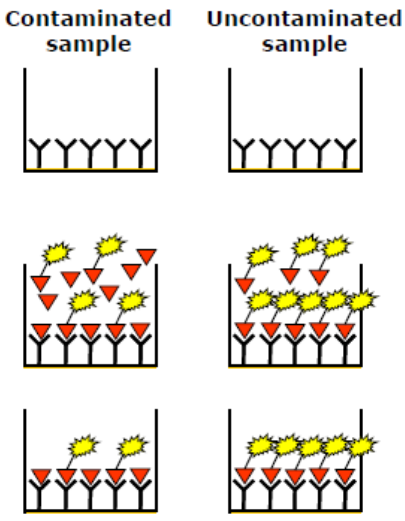
Competitive ELISA



ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

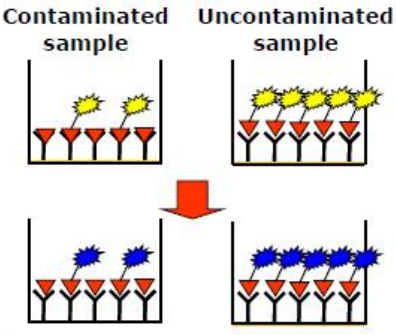
Competitive ELISA

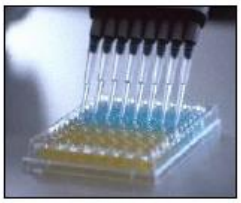
1. Antibodies (mono- o polyclonal) immobilized on microwell-plates
2. Addition of mycotoxin-enzyme conjugate to the sample extract. Addition of extract to the surface–immobilized antibody (competition with mycotoxin). Incubation time.
3. Washing

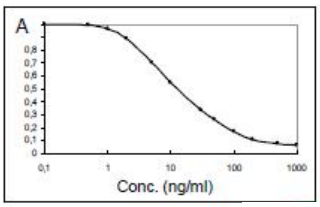


ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

4. Addition of a specific substrate; reaction with the mycotoxin-enzyme conjugate to give a chromogenic product
5. Colorimetric measurement (signal is inversely proportional to toxin concentration in the sample)



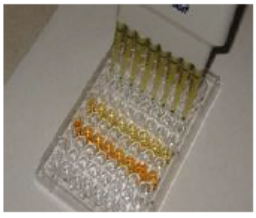




ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Advantages:

- antibodies available for all major mycotoxins;
- good sensitivity (µg-ng/kg level);
- simultaneous analysis of a large number of samples;
- simple sample preparation and inexpensive equipments;
- limited use of organic solvents;
- suitable for screening purposes.

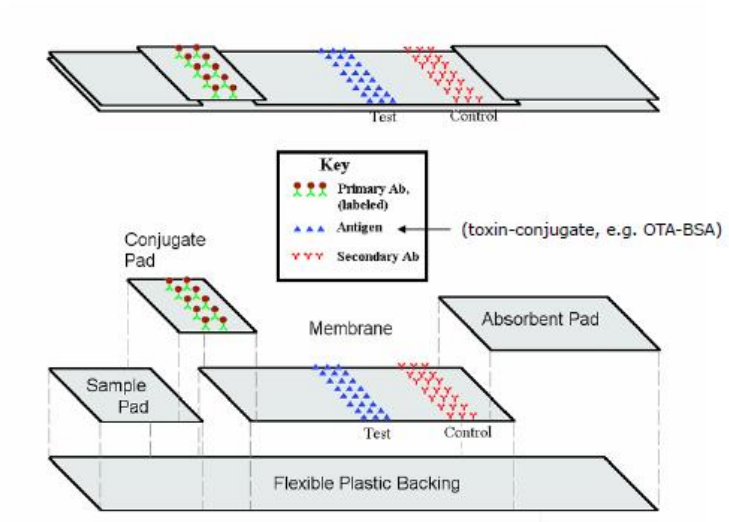


Disadvantages:

- total testing times (including sample preparation) from 5 (fast-ELISA) to 100 min (ELISA)
- cross-reactivity of antibody with other mycotoxins;
- matrix interferences may affect result;
- possible false positive/negative results;
- confirmatory analysis LC required.

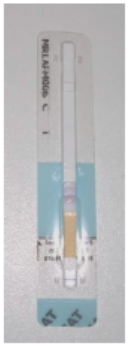
- ELISA kits are of particular interest for screening of raw materials.
- ELISA kits should be used only for analysis of matrices that have been extensively tested.

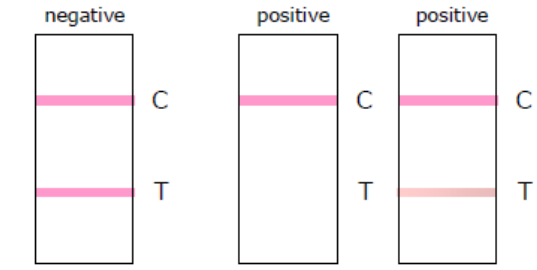
Lateral flow devices (LFD) or "dipsticks"



Rapid tests (based on antigen-antibody reaction)

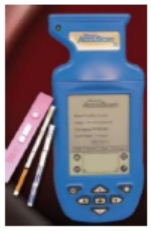
Lateral Flow Device (LFD)





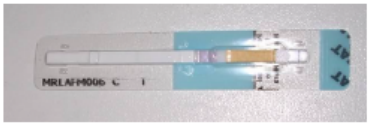
Negative sample with Control line (C) and Test line (T) present

Positive (contaminated) sample with Control line (C) present and Test line (T) absent or sligthly visible



- LFDs commercially available for AFs and FBs in maize, DON in wheat, OTA, ZEA, T-2 and HT-2 in cereal grains.
- Photometric strip readers allow quantitative analysis

Rapid tests (based on antigen-antibody reaction)



- **dipsticks or lateral flow device**
- **membrane-based flow-through immunoassay**

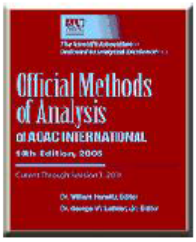
ADVANTAGES: rapid (5-10 min), simple, no expensive equipments required, portable, limited use of organic solvents, suitable for screening purposes, can be used *in situ*

DISADVANTAGES: qualitative or semi-quantitative (cut off level), matrix interferences may affect result, possible false positive/negative results, cross-reactivity of antibody with other mycotoxins, sensitivity not acceptable at levels close to regulatory limits

Standard/Official methods

- validated by inter-laboratory studies
- performance characteristics are established (recovery, repeatability, reproducibility, limit of detection, range of application)

- 21 CEN and 45 AOAC methods are official reference methods (aflatoxins, fumonisins, deoxynivalenol, ochratoxin A, zearalenone, patulin in various matrices)
- can be used in case of official control and for resolving any disputes between parties



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AOAC = Association of Official Analytical Chemists
CEN = European Committee For Standardization

Physical methods Montmorillonite Activated charocal
Aflatoxins Mycotoxin degradation
binders Milling
Ochratoxins **Thank You** Zearalenone Bacteria
Chemical methods Fumonisins Fungi
Trichothecenes Yeast Biological methods

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