

Analysis of regulated and 'emerging' mycotoxins considered for regulation

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MYCOTOXINS

→ Toxic secondary metabolites of microscopic filamentous fungi



Fusarium spp.



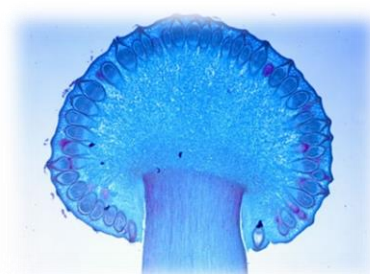
Aspergillus spp.



Alternarium spp.



Penicillium spp.



Claviceps spp.

→ Contaminants of plants and agricultural crops in the field

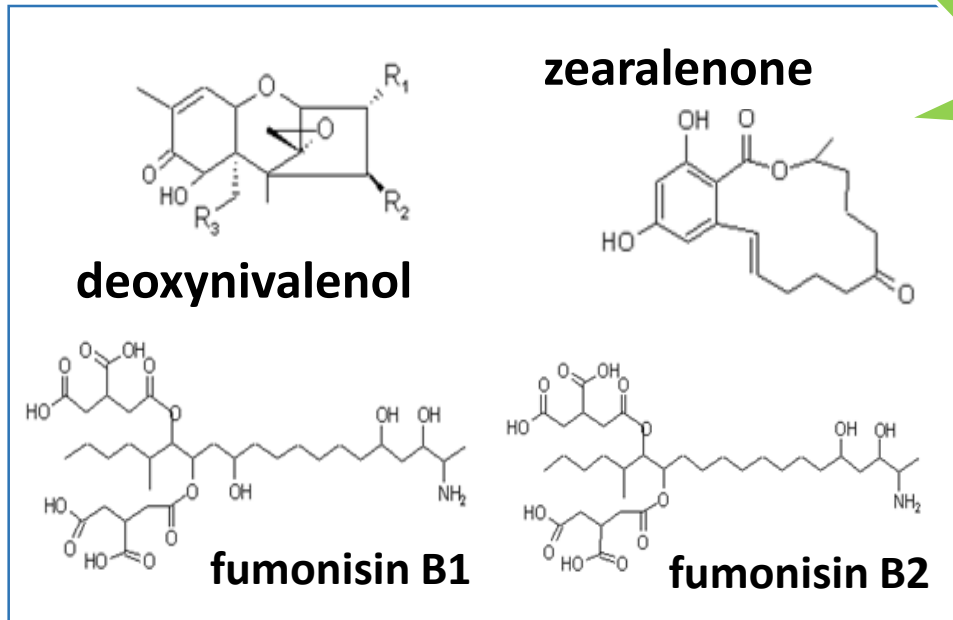
→ High thermal stability of mycotoxins → detected in many final products

Occurrence of mycotoxins in food and feed means a health risk for consumers and animals

Regulated mycotoxins

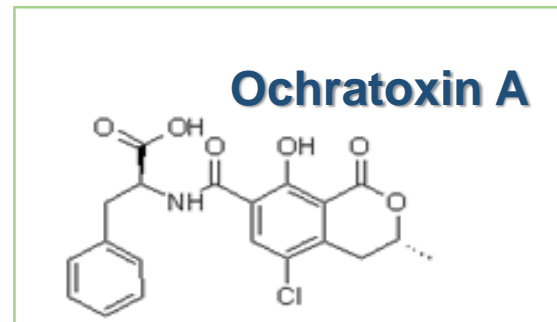
Commission Regulation (EC) No 1881/2006 amended by Regulation No 1126/2007, No 105/2010 and No 156/2010

11
mctx

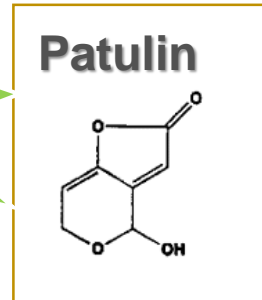


Fusarium ssp.

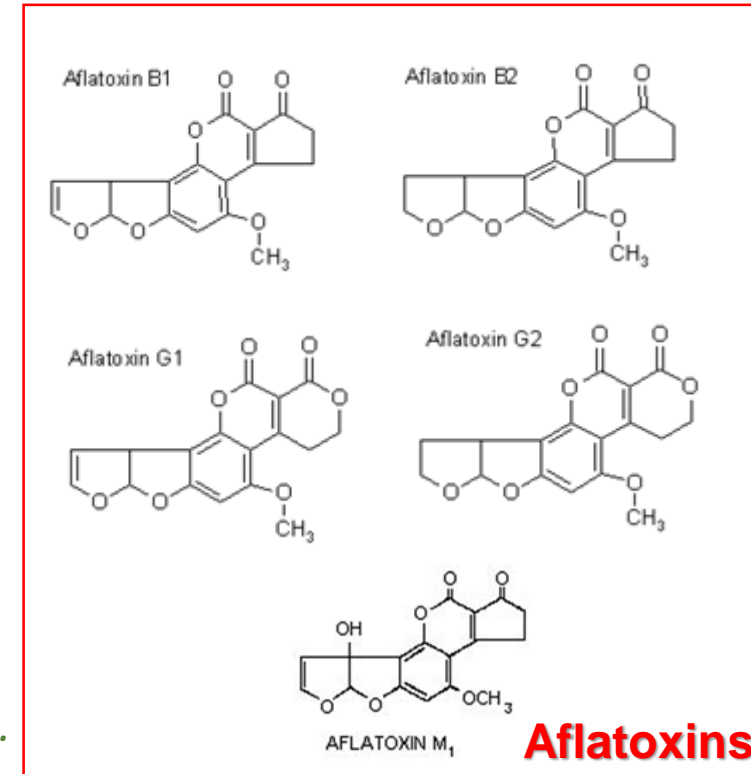
Fusarium ssp.



Aspergillus ssp.

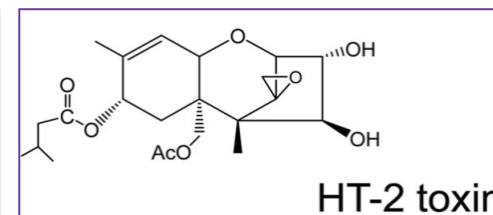
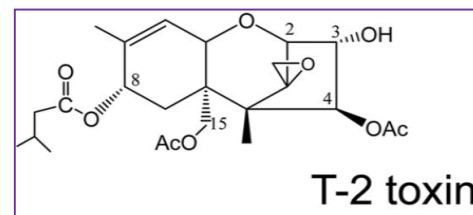


Penicillium ssp.



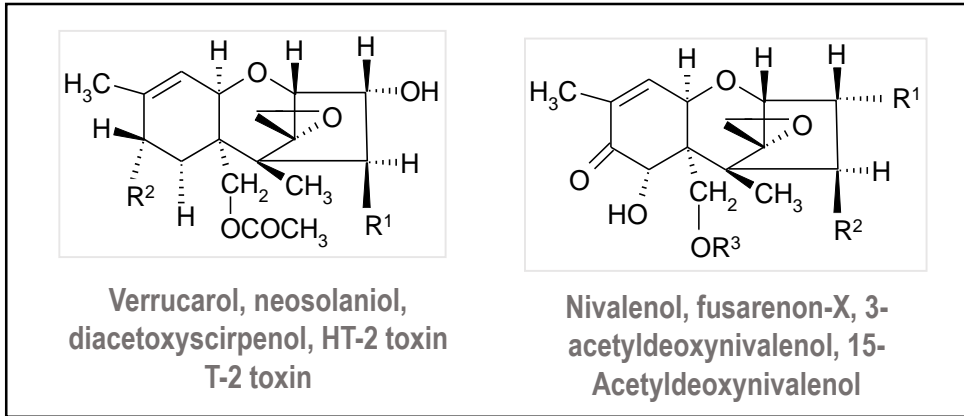
Aspergillus ssp.

Commission Recommendation (2013/165/EU)

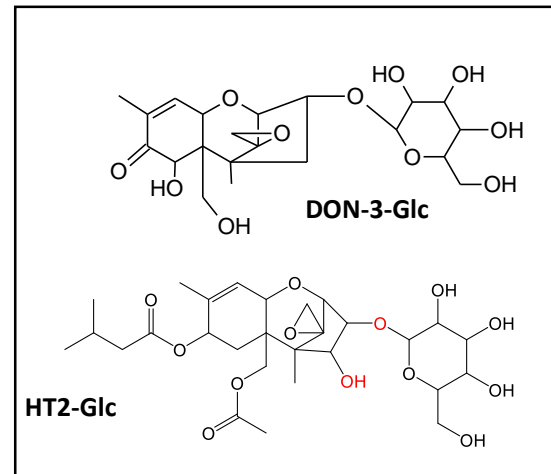


Mycotoxins considered for regulation

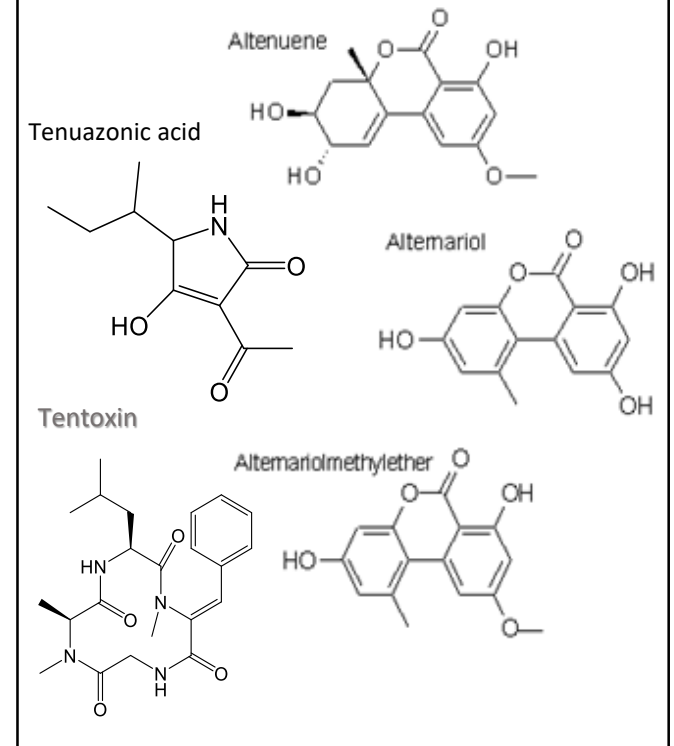
Other trichothecenes *Fusarium ssp.*



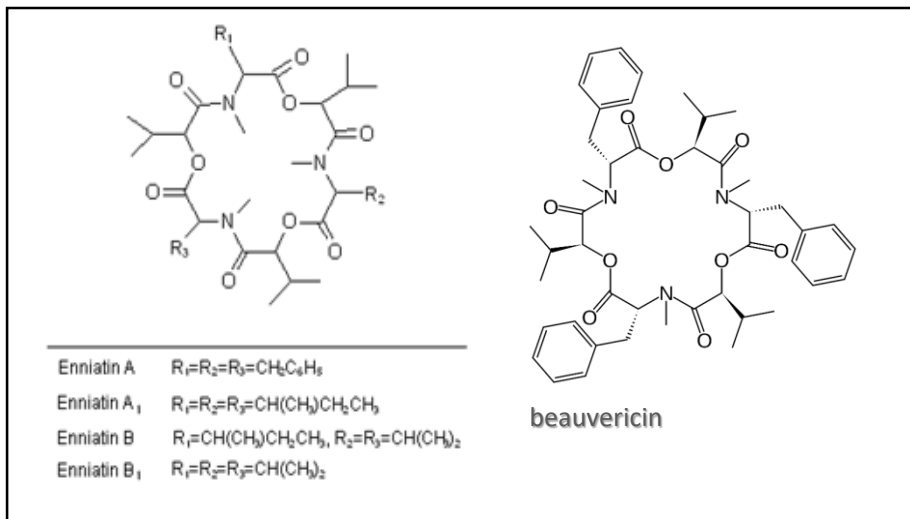
Conjugated mycotoxins



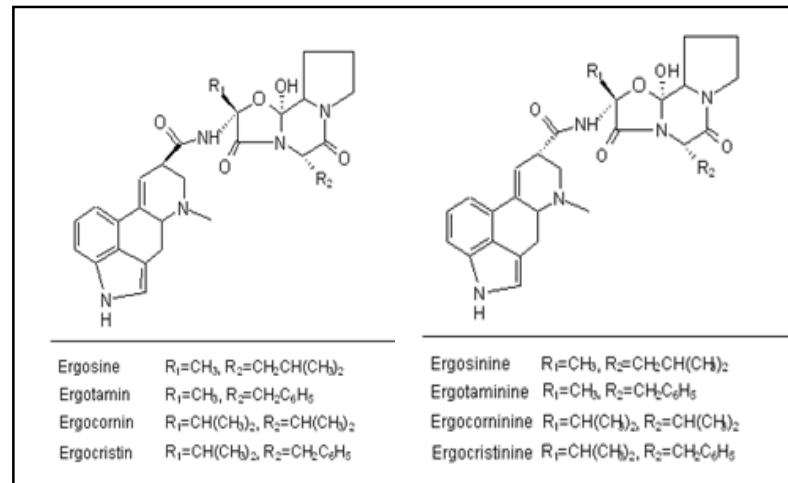
Alternaria mycotoxins



Alternaria alternata



Enniatins and beauvericin *Fusarium ssp.*



Ergot alkaloids *Claviceps purpurea*

ANALYSIS OF MYCOTOXINS

Sampling



- Mycotoxins: **unevenly distributed** in agricultural commodities
- Detection of „**HOT SPOTS**“
- **Legislation: EC 401/2006**, sampling for cereals, dried fruit, dried figs, peanuts, spices, milk, coffee, fruits, etc.

- ✓ Important to take large number of small samples at various places distributed throughout the dose and so obtained a representative sample
- ✓ Important to avoid the cross-contamination

ANALYSIS OF MYCOTOXINS

Sampling

Single mycotoxin analysis

- Extraction
- Extract purification
- Analysis (LC-MS, LC-UV/FLD, GC-ECD, ...)

Multiple mycotoxin analysis

- Extraction
- Minimal or no extract purification
- Analysis (LC-MS)

Single mycotoxin screening

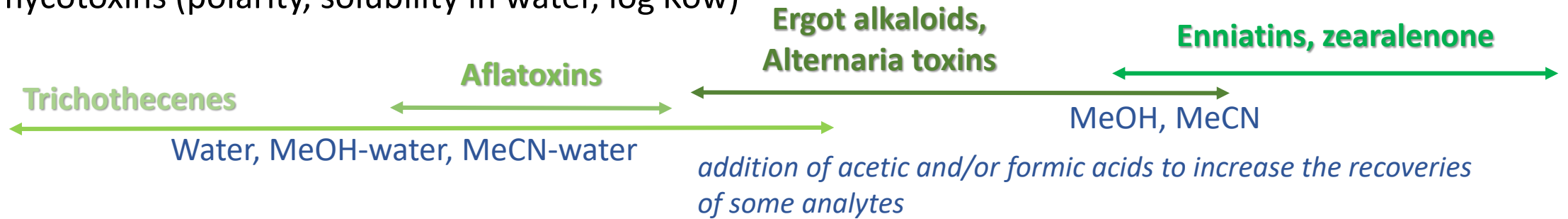
- Extraction
- No extract purification
- Analysis (ELISA, biosensors)

ANALYSIS OF MYCOTOXINS

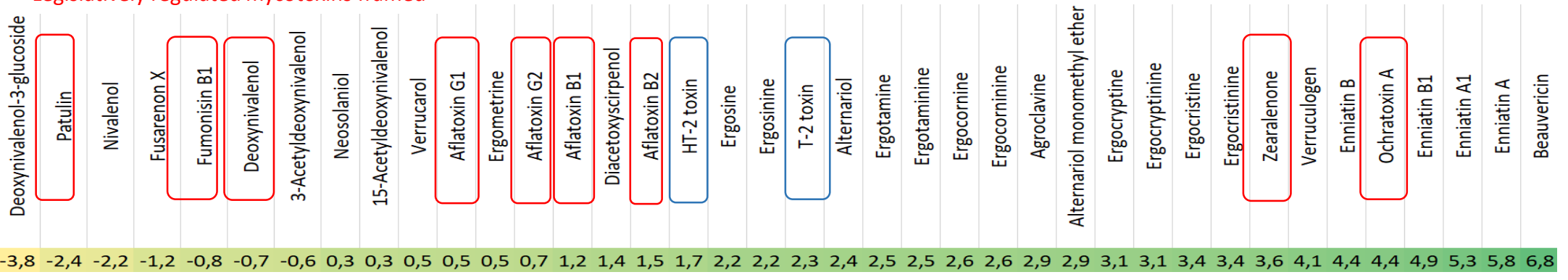
Single mycotoxin analysis (analysis of structurally similar mycotoxins)

EXTRACTION

- Selection of **extraction solvent** is based on physico-chemical properties of particular mycotoxin / group of mycotoxins (polarity, solubility in water, log Kow)



Legislatively regulated mycotoxins framed



Log K_{ow}

ANALYSIS OF MYCOTOXINS

Single mycotoxin analysis (analysis of structurally similar mycotoxins)

EXTRACTION

- Selection of **extraction solvent** is based on physico-chemical properties of particular mycotoxin / group of mycotoxins (polarity, solubility in water, log Kow)
- Selection of appropriate **extraction method**

- **Shaking:** most often used extraction process, **30 – 120 min**
- **Blanding:** with solvent, approx. **15 min**
- **Ultra-turrax:** high performance disperser, approx. **3 min**
- **Pressurized Liquid Extraction (PLE):** intensive extraction under high temperature and pressure, reduced time – **30 sec**



ANALYSIS OF MYCOTOXINS

Single mycotoxin analysis (analysis of structurally similar mycotoxins)

EXTRACT CLEAN-UP

- Removal of impurities and co-extracts which influence further determinative and quantitative steps
- Pre-concentration of analytes prior to analysis

Clean-up procedures ...

- *„Classical“ SPE columns*
- *SPE „pass-through“ cartridges*
- *Molecularly imprinted polymers (MIP)*
- *Immunoaffinity columns (IAC)*

ANALYSIS OF MYCOTOXINS

Single mycotoxin analysis (analysis of structurally similar mycotoxins)

EXTRACT CLEAN-UP

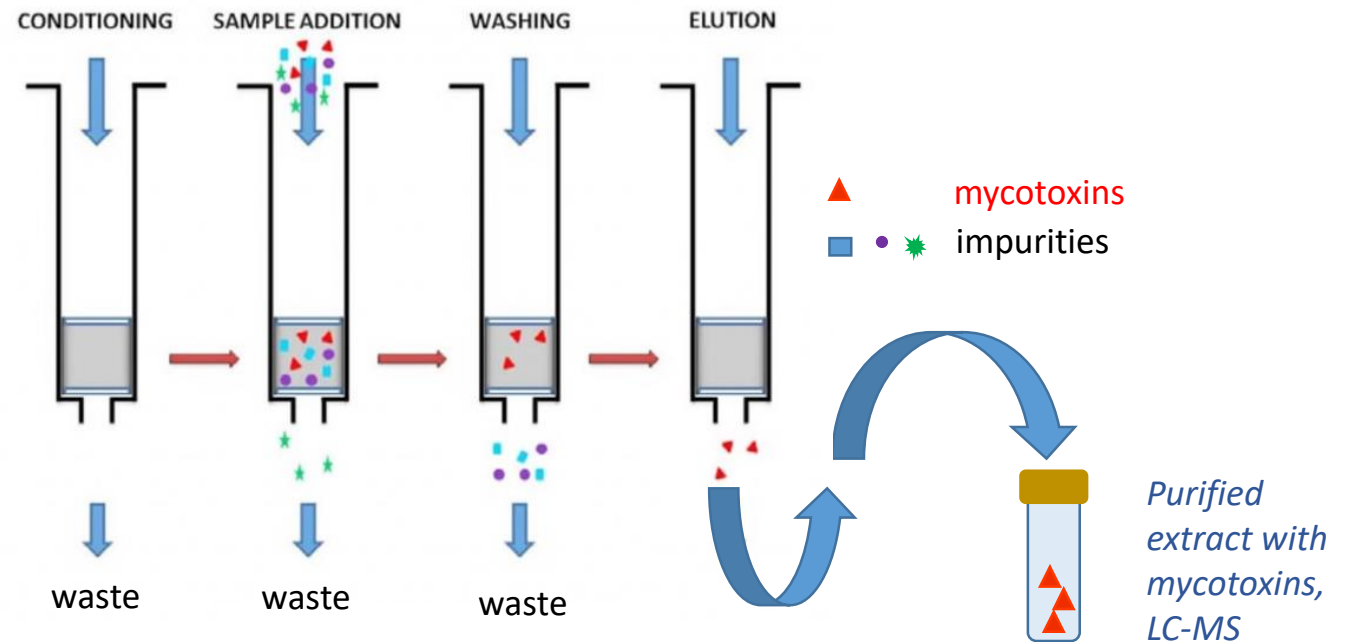
- Removal of impurities and co-extracts which influence further determinative and quantitative steps
- Pre-concentration of analytes prior to analysis

Clean-up procedures

o „Classical“ SPE columns



e.g. Oasis HLB SPE
(Waters)



ANALYSIS OF MYCOTOXINS

Single mycotoxin analysis (analysis of structurally similar mycotoxins)

EXTRACT CLEAN-UP

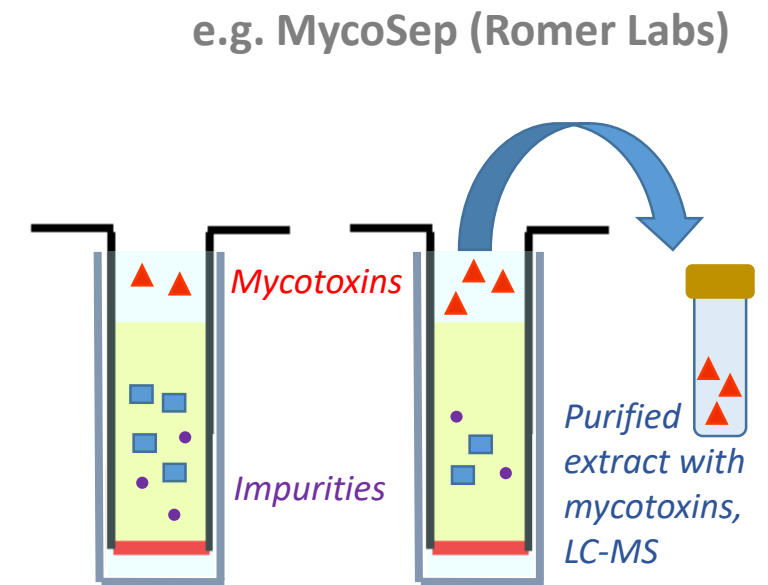
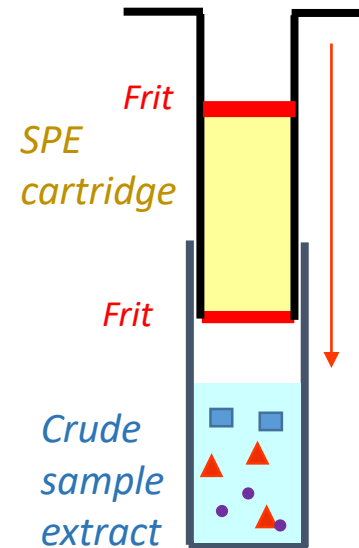
- Removal of impurities and co-extracts which influence further determinative and quantitative steps
- Pre-concentration of analytes prior to analysis

Clean-up procedures

o SPE „pass-through“ cartridges

Currently available for:

- ✓ Trichothecenes
- ✓ Aflatoxins + zearalenon
- ✓ Aflatoxins + patulin
- ✓ Ochratoxin A
- ✓ Fumonisin
- ✓ Ergot alkaloids
- ✓ Moniliformin



ANALYSIS OF MYCOTOXINS

Single mycotoxin analysis (analysis of structurally similar mycotoxins)

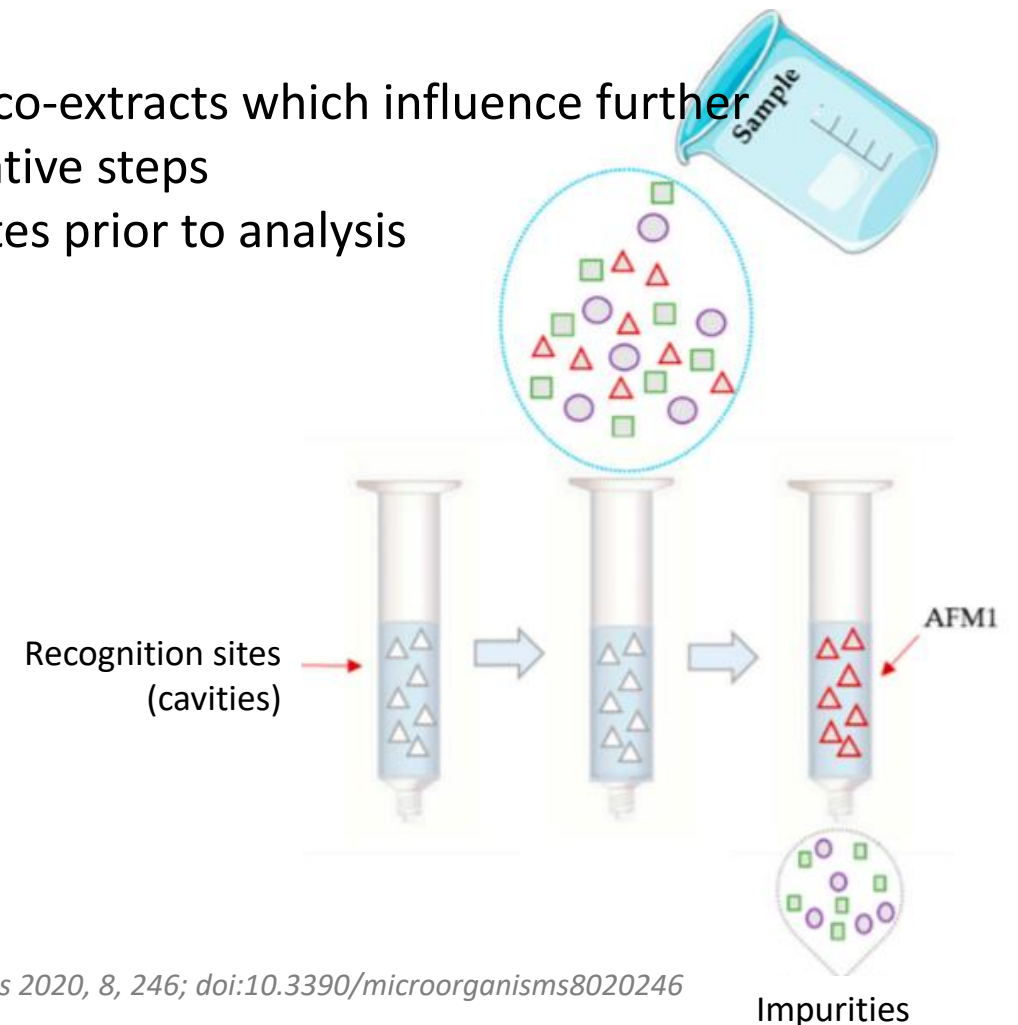
EXTRACT CLEAN-UP

- Removal of impurities and co-extracts which influence further determinative and quantitative steps
- Pre-concentration of analytes prior to analysis

Clean-up procedures

o *Molecularly imprinted polymers (MIPs)*

- A polymer with an imprinted cavity, originated by imprinting of a „template molecule“ (mycotoxin)
- The cavities have an affinity for specific mycotoxins („lock and key“ principle)



ANALYSIS OF MYCOTOXINS

Single mycotoxin analysis (analysis of structurally similar mycotoxins)

EXTRACT CLEAN-UP

- Removal of impurities and co-extracts which influence further determinative and quantitative steps
- Pre-concentration of analytes prior to analysis

Clean-up procedures

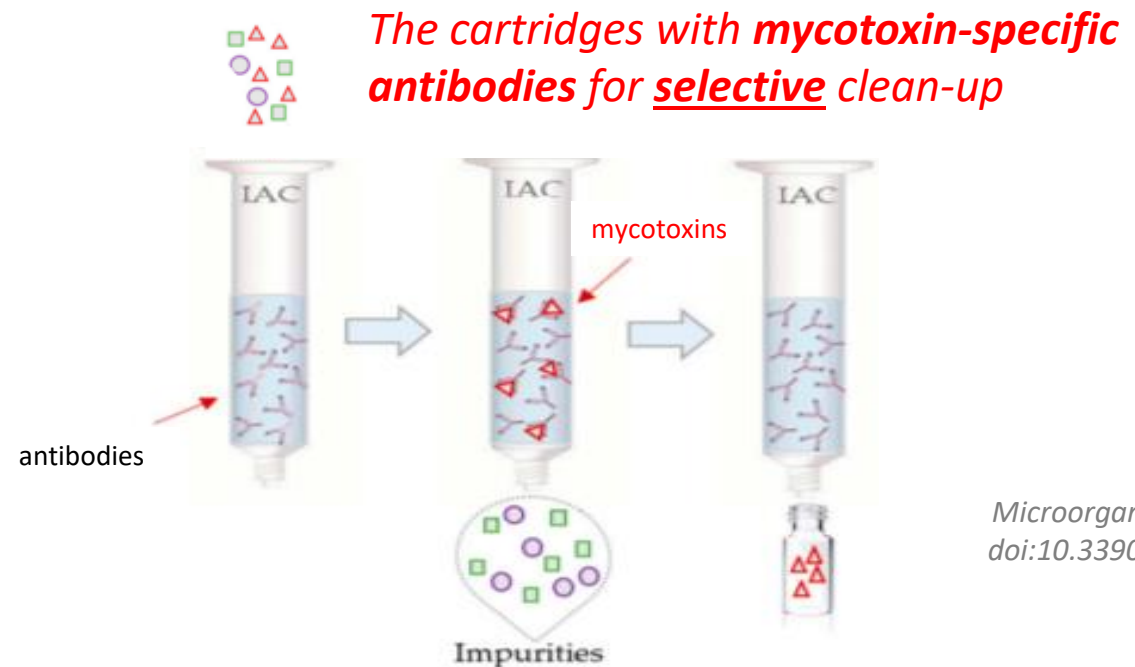
o Immunoaffinity columns (IACs)

Commercially available for:

- ✓ Deoxynivalenol
- ✓ HT2+T2
- ✓ Aflatoxins
- ✓ Zearalenon
- ✓ Ochratoxin A
- ✓ Fumonisin
- ✓ Ergot alkaloids
- ✓ Moniliformin



VICAM



Microorganisms 2020, 8, 246;
doi:10.3390/microorganisms8
020246

ANALYSIS OF MYCOTOXINS

Multiple mycotoxin analysis

EXTRACTION

- Extraction of **multiple analytes with wide range of physico-chemical properties** by one solvent / solvent mixture
- **No or minimal extract clean-up**
- Increased risk of „matrix effects“ caused by matrix co-extracts and influence on the analytical result → increased demands on analytical instrumentation (**mass spectrometry is needed**)

o „*Dilute-and-shoot*“

o *QuEChERS*

ANALYSIS OF MYCOTOXINS

Multiple mycotoxin analysis

EXTRACTION

- Extraction of **multiple analytes with wide range of physico-chemical properties** by one solvent / solvent mixture
- **No or minimal extract clean-up**
- Increased risk of interferences of matrix co-extracts and influencing the analytical result → increased demands on analytical instrumentation (**mass spectrometry is needed**)

o *Dilute-and-shoot*

- Extraction of the matrix with solvent mixture followed by dilution of the extract by the same solvent mixture (e.g. MeOH/water)

↑ Decreased matrix effects

↓ Increased LOQs (after recalculation on the equivalent of the original matrix)

Extraction
MeOH/water, shaking



Dilution
By MeOH/water, e.g. 1:1, v/v



LC-MS



ANALYSIS OF MYCOTOXINS

Multiple mycotoxin analysis

EXTRACTION

o *QuEChERS*



Soaking of dry matrix sample + water

Extraction

Addition of MeCN, shaking

Salts addition
NaCl + MgSO₄

Centrifugation

Partitioning of phases
removing of acetonitrile layer

LC-MS



Extraction

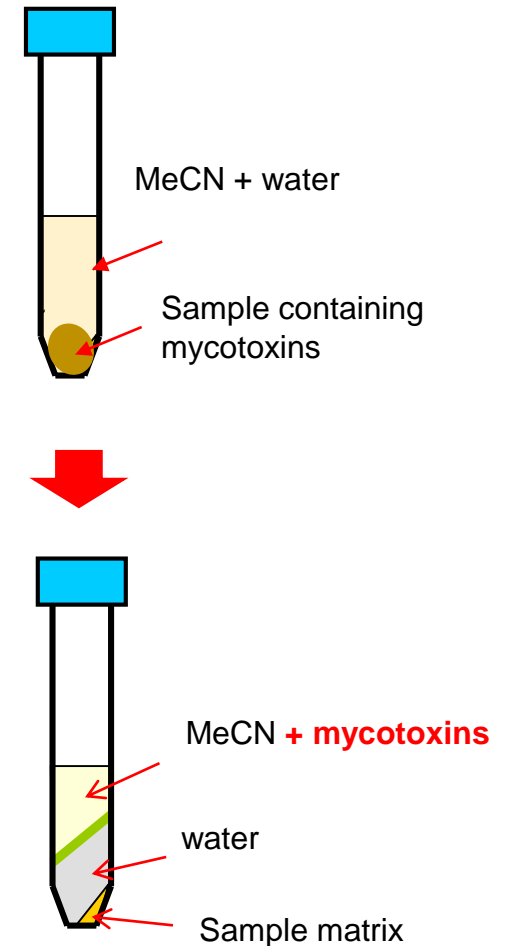
Addition of MeCN, shaking

Salts addition
NaCl + MgSO₄

Centrifugation

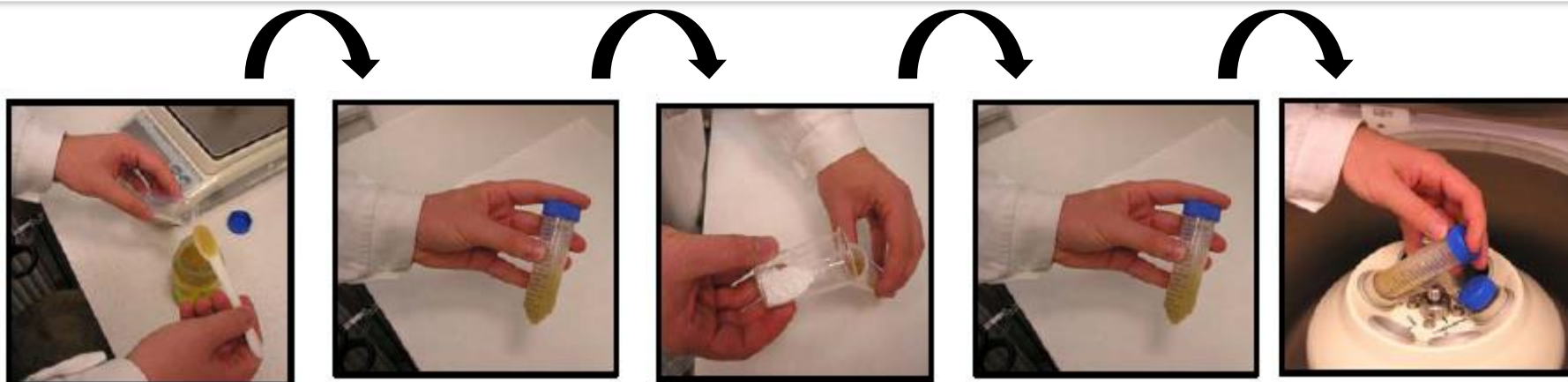
Partitioning of phases
removing of acetonitrile layer

LC-MS



ANALYSIS OF MYCOTOXINS

Multiple mycotoxin analysis



**Weighing
of sample**

**H₂O +
MeCN addition,
shaking**

**NaCl and
MgSO₄
addition**

Shaking

Centrifugation

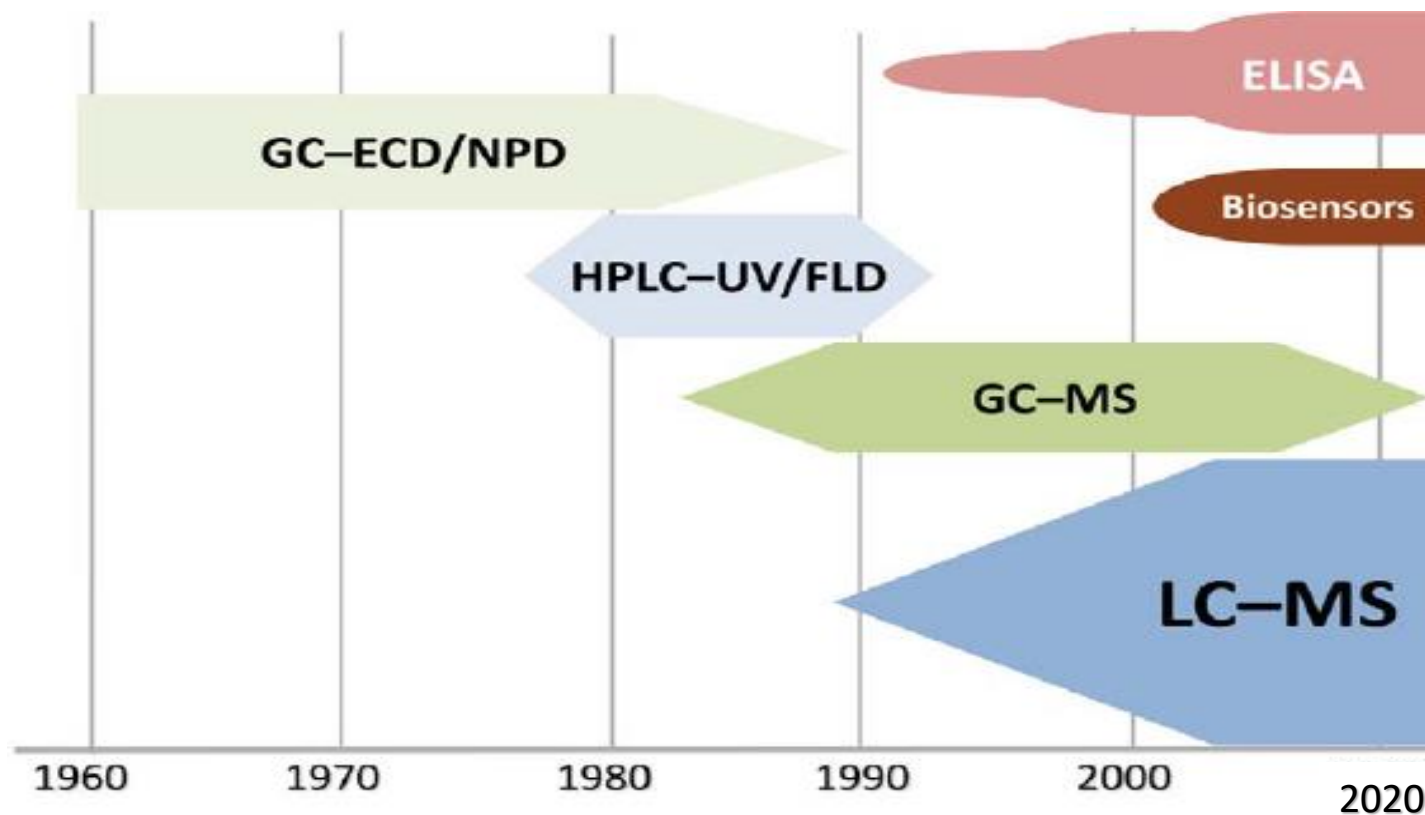


LC-MS

ANALYSIS OF MYCOTOXINS

Single and/or multiple mycotoxin analysis

INSTRUMENTAL ANALYSIS



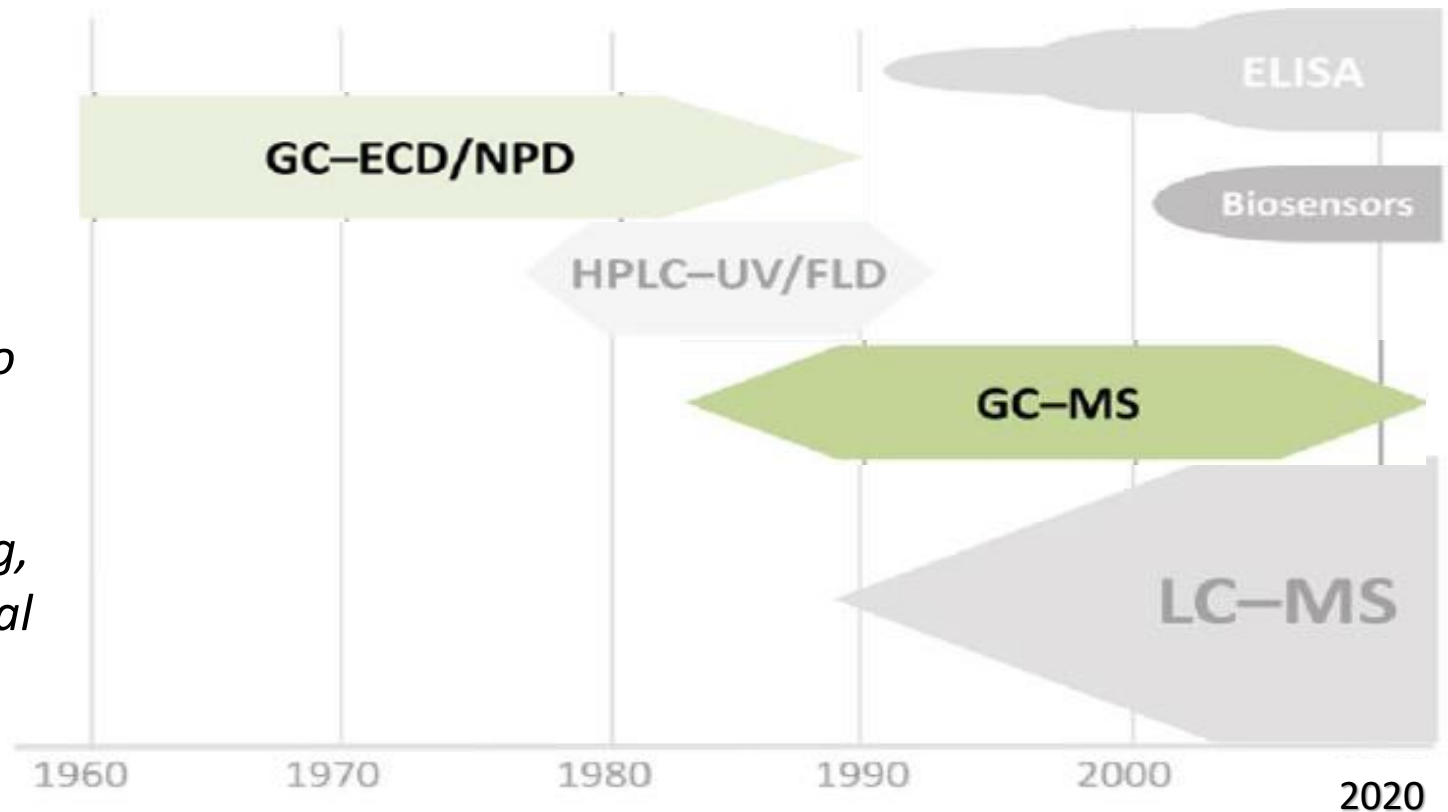
ANALYSIS OF MYCOTOXINS

Single and/or multiple mycotoxin analysis

INSTRUMENTAL ANALYSIS

o *GC-based methods:*

- *For laboratories owning the GC-system, and not having LC-MS*
- *Great disadvantage – necessity to produce **volatile derivatives** (e.g. trimethylsilyl ethers of trichothecenes) – time consuming, increasing probability of analytical bias*



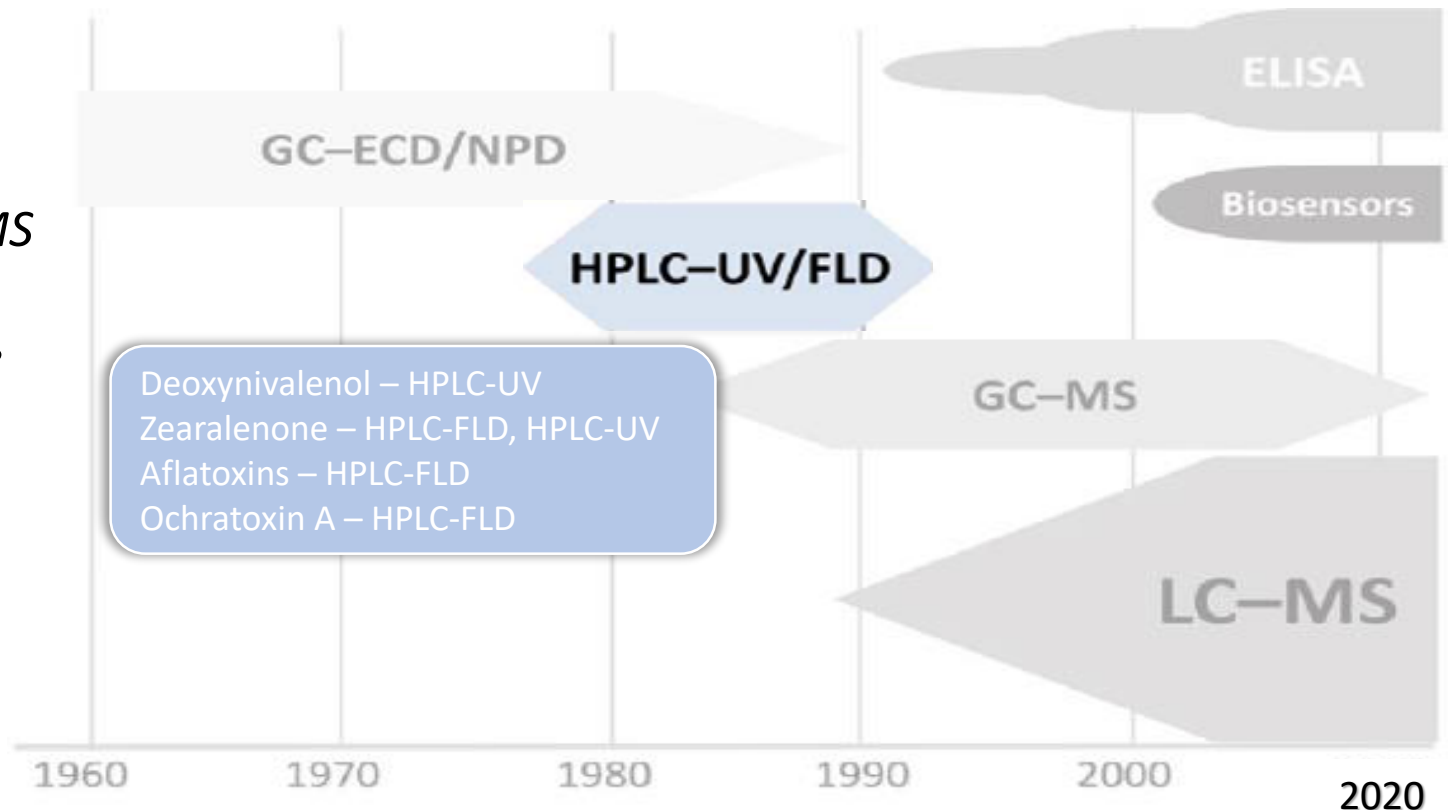
ANALYSIS OF MYCOTOXINS

Single and/or multiple mycotoxin analysis

INSTRUMENTAL ANALYSIS

o *HPLC-UV/FLD methods:*

- *For laboratories not owning LC-MS systems*
- *„conventional“ detectors lack the selectivity (selectivity usually assured by appropriate clean-up)*
- *Not convenient for multi-mycotoxins methods*



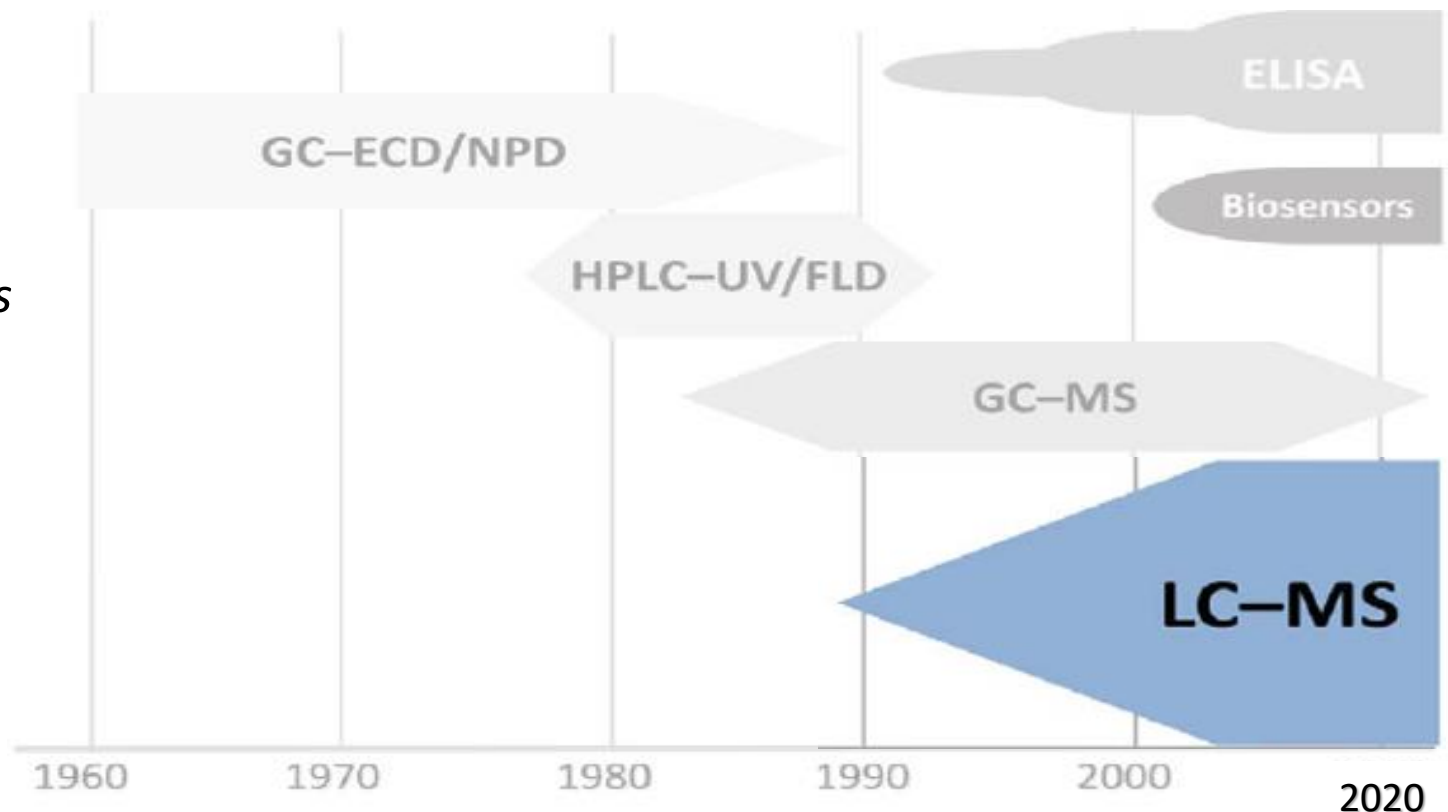
ANALYSIS OF MYCOTOXINS

Single and/or multiple mycotoxin analysis

INSTRUMENTAL ANALYSIS

o *LC-MS methods:*

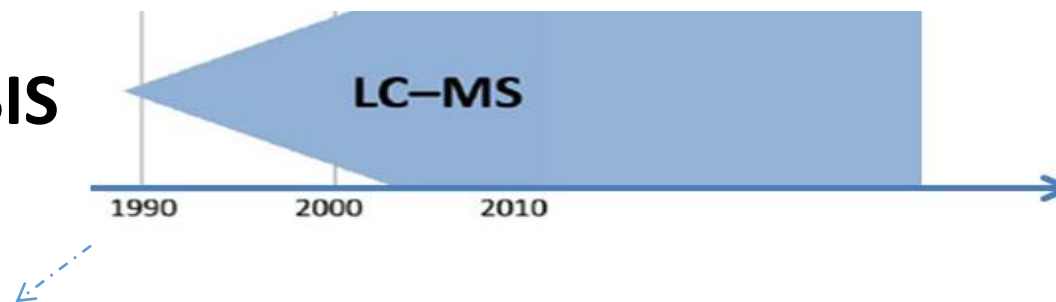
- *Most widely used separation-detection approach in mycotoxins analysis*
- *High **selectivity**, high **sensitivity***
- *Good potential for confirmation*
- *Suitable for **multi-mycotoxins methods***



ANALYSIS OF MYCOTOXINS

Single and/or multiple mycotoxin analysis

INSTRUMENTAL ANALYSIS



MS/MS

Triple quadrupol, ion trap

- Unit resolving power
- Target analysis

657/2002 EC
SANTE 10178/2021

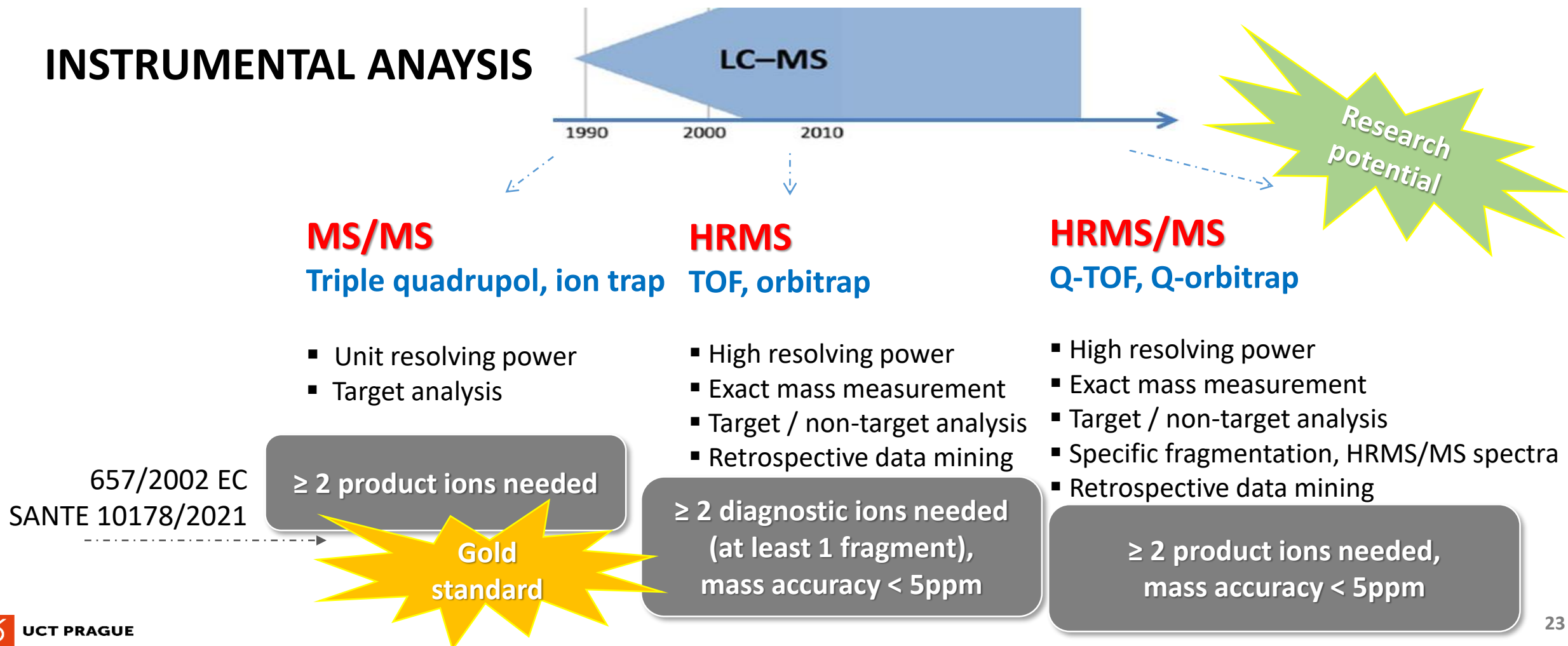
≥ 2 product ions needed

Gold
standard

ANALYSIS OF MYCOTOXINS

Single and/or multiple mycotoxin analysis

INSTRUMENTAL ANALYSIS



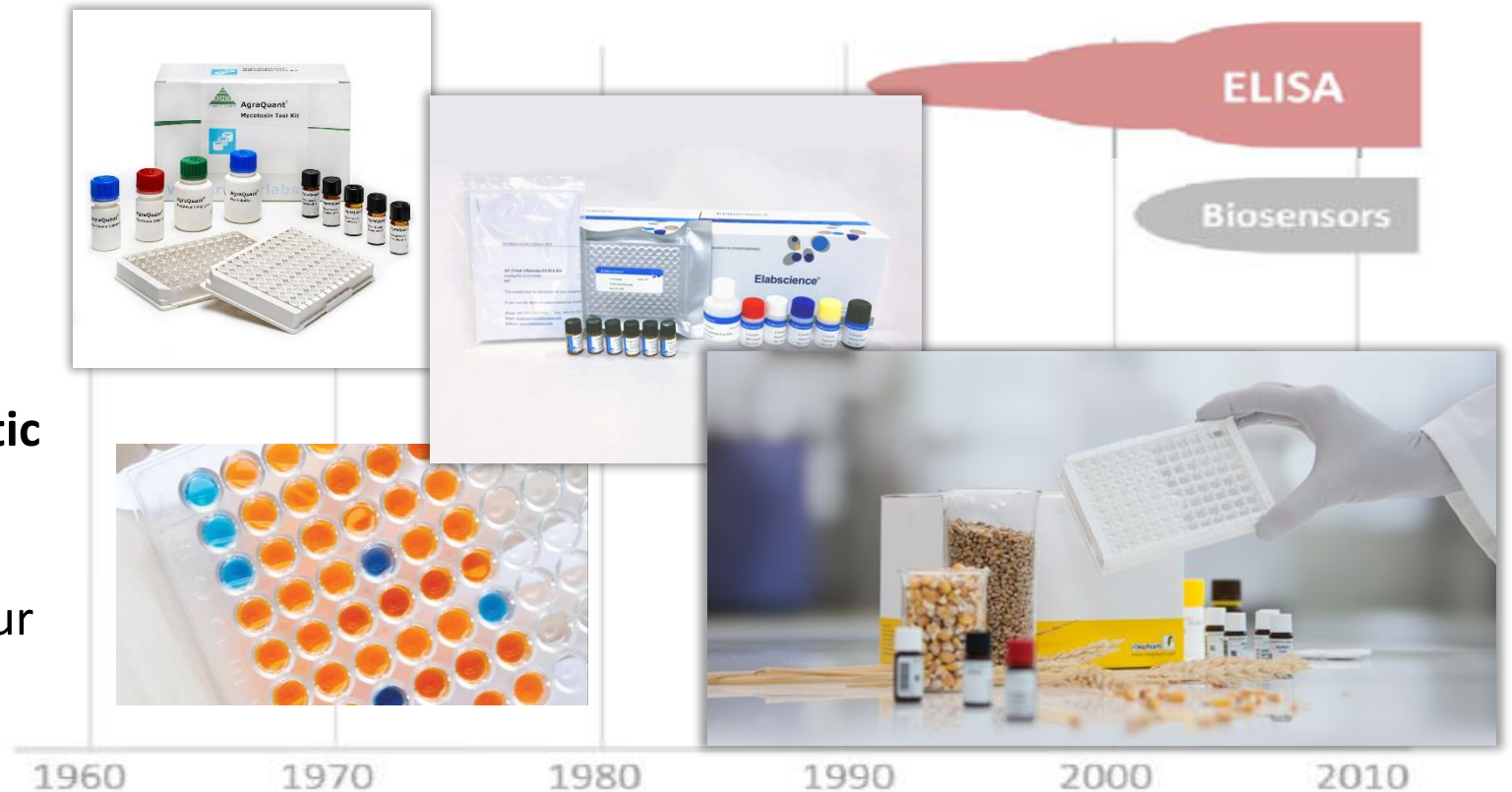
ANALYSIS OF MYCOTOXINS

Single mycotoxin screening

SCREENING APPROACHES

o **ELISA:**

- Enzyme-Linked Immunosorbent Assay
- Interaction between **mycotoxin (antigen)** and **antibody**; **enzymatic label** catalyses the reaction after addition of substrate, measurement of the signal (colour intensity)



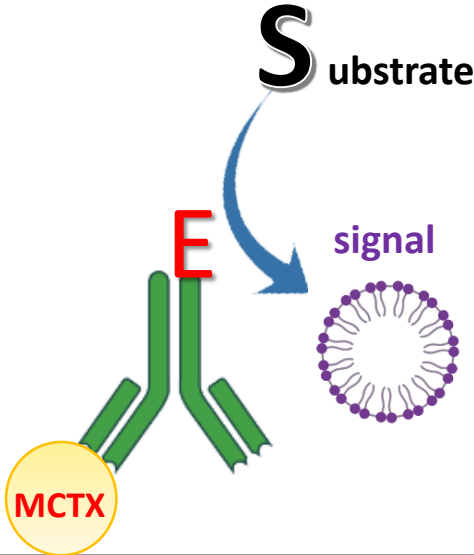
Possible arrangements of ELISA

Most widely used in mycotoxins analysis

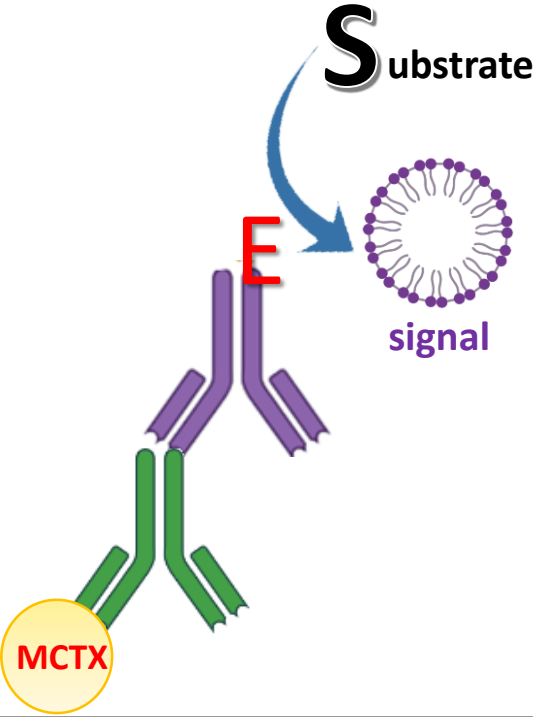


Primary Ab

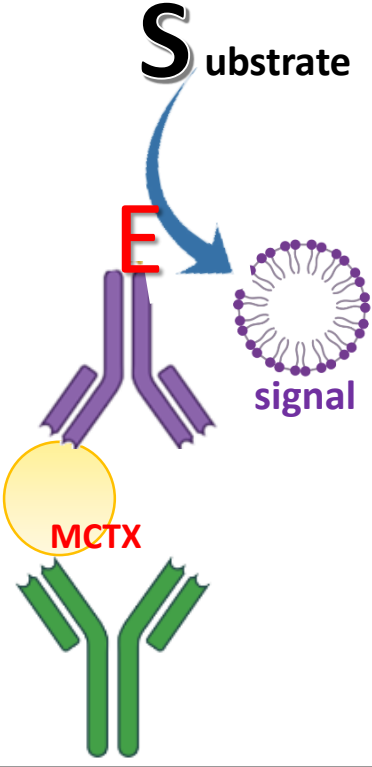
Secondary Ab



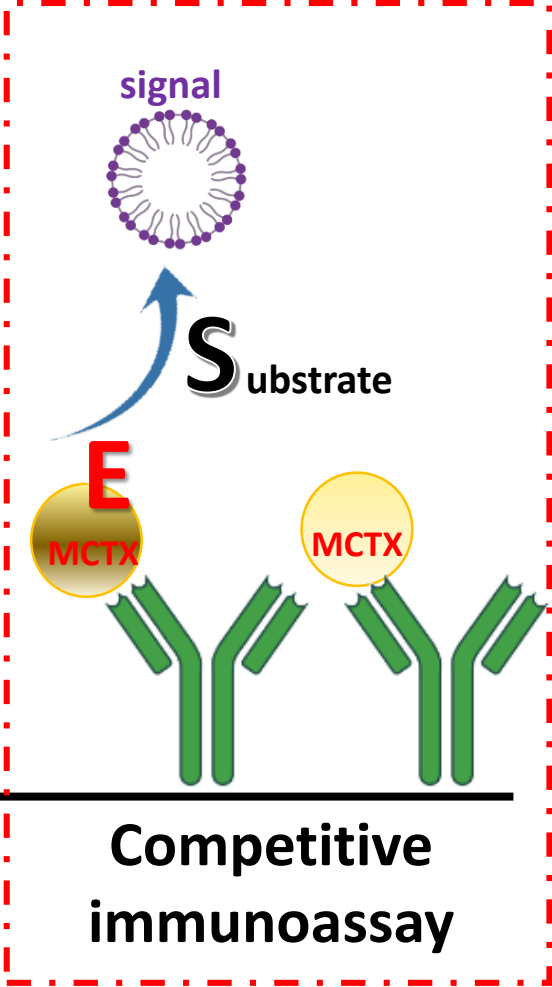
Direct immunoassay



Indirect immunoassay



Sandwich immunoassay



Competitive immunoassay

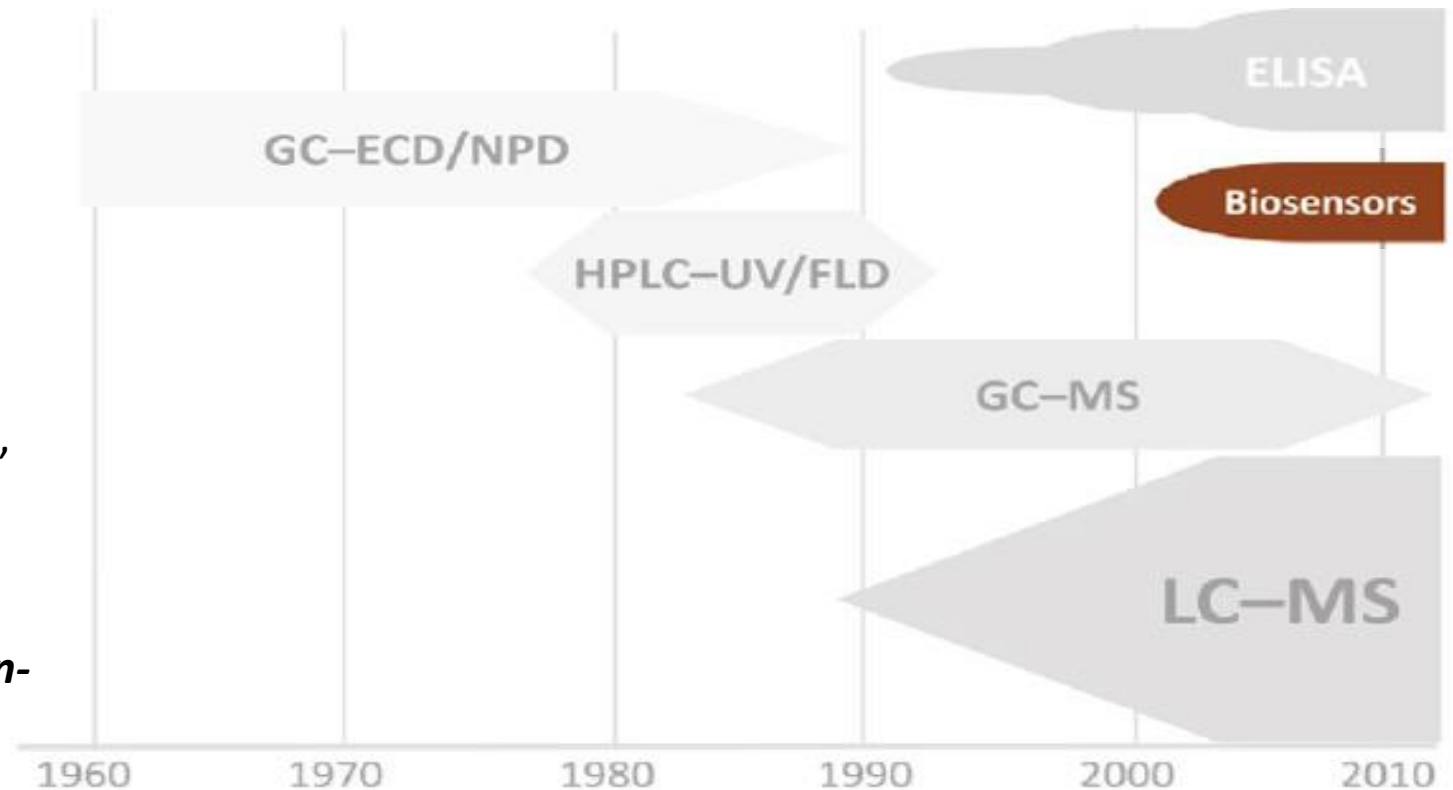
ANALYSIS OF MYCOTOXINS

Single mycotoxin screening

SCREENING APPROACHES

o **Biosensors:**

- Analytical device that uses specific **biochemical reactions** mediated by enzymes, immunosystems, tissues, cells etc., to **detect mycotoxins by physico-chemical detector** (electrical, thermal or optical signals)
- Not commercially available
- **Rapid measurement of mycotoxins *in-situ***



CONCLUSION - SUMMARY

Single mycotoxin analysis

- Extraction
- Extract purification
- Analysis (LC-MS, LC-UV/FLD, ...)

↑ Good sensitivity
Low LODs/LOQs

↓ Laborious sample preparation
Limited information about contamination by multiple mycotoxins

Multiple mycotoxin analysis

- Extraction
- Minimal / no extract clean-up
- Analysis (LC-MS)

↑ Fast and easy sample preparation
Selectivity of detection

↓ Higher demands on skilled MS-operators
High cost of LC-MS systems

Single mycotoxin screening

- Extraction
- No extract purification
- Analysis (ELISA)

↑ Easy protocols, fast procedures
Minimum demands on laboratory instruments

↓ Risk of false positive results caused by rather low specificity (cross-reactions)
Higher LOQs

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