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Targeted methods for on-site testing of chemical hazards

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Introduction

- Currently, **2.4 billion people in the world do not have regular access to safe, nutritious and sufficient food**.

- One of the **17 Sustainable Development Goals** is to '*end food hunger, achieve food security and improved nutrition, and promote sustainable agriculture*'.

- **Banned or illegal chemicals** may be **re-introduced** into agricultural procedures to improve production rates.

- Recent report suggests **that ~30% of our food contains a** *"cocktail' of pesticides"* (EFSA, 2018).

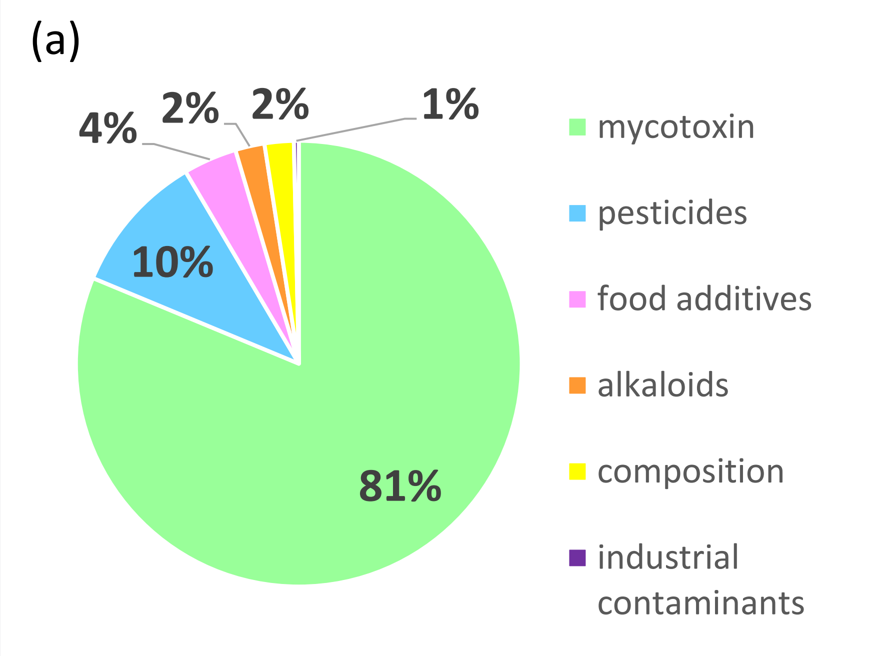
- The **majority of maize crop is contaminated with at least one mycotoxin**, and approximately 50% is co-contaminated with multiple mycotoxins.

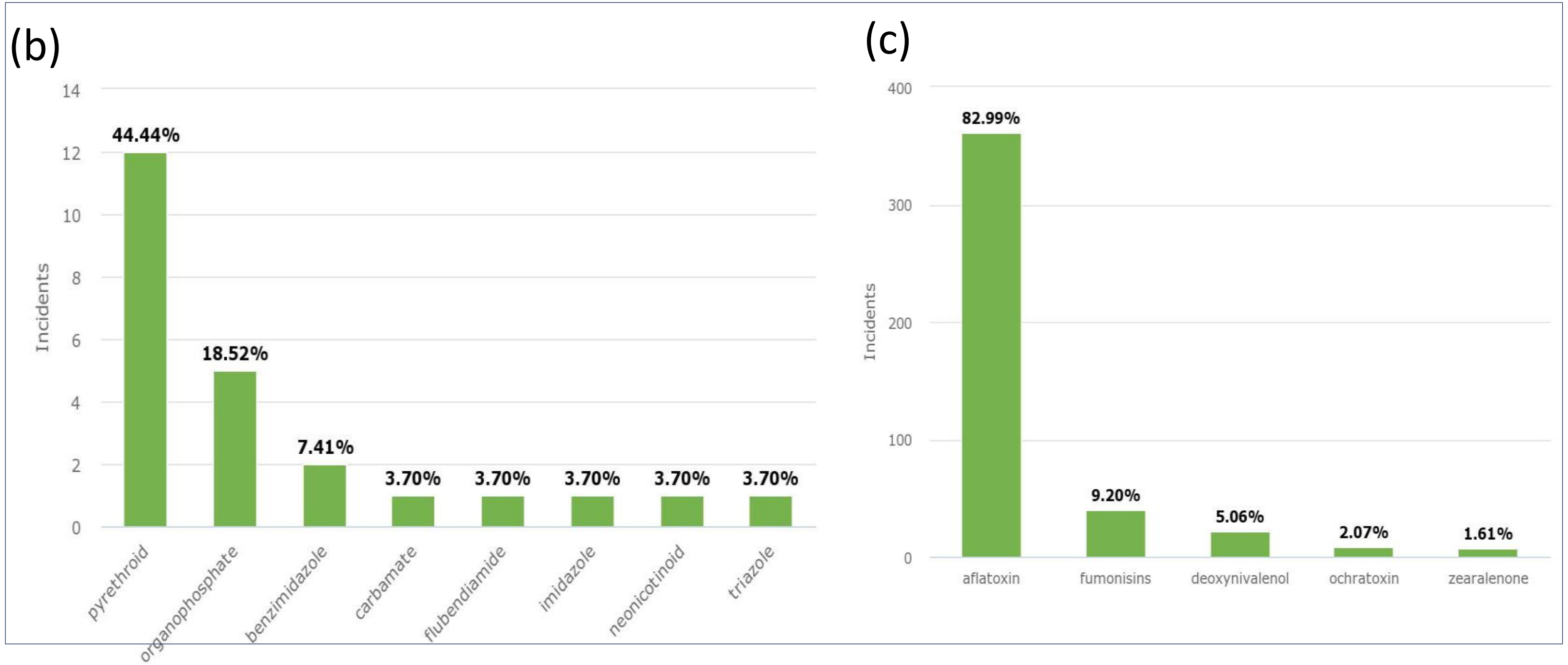
- **Climate change will influence mycotoxin occurrence** and promote the production of **emerging toxins**.

References:

- United Nations (2015) 2030 Agenda for Sustainable Development. https://sdgs.un.org/sites/default/files/publications/21252030%20Agenda%20for%20Sustainable%20Development%20web.pdf
- Tridge – Global Food Sourcing & Data Hub. https://www.tridge.com/
- EFSA, 2020. The 2018 European Union report on pesticide residues in food. https://doi.org/10.2903/j.efsa.2020.6057

Identification of chemical contamination in maize (2013-2023)





(a) Incidents of chemical contamination in maize.

Most commonly occurring **(b)** pesticides **(c)** mycotoxins in maize (FOODAKAI 2013-2023)

In the last decade:

- ❖ Mycotoxins, particularly **aflatoxins**, have been a **major problem in maize**.
- ❖ **Climate change** will **increase** the presence of **AFs** from low to moderate in food from Europe (EFSA, 2020).
- ❖ **Banned/illegal pesticides** have also **been frequently detected** in **maize** due to their re-introduction or persistence in the environment.

References: EFSA, 2020. Outcome of a public consultation on the draft risk assessment of aflatoxins in food. EFSA supporting publication 2016: 17(3): EN-1798. 52 pp.

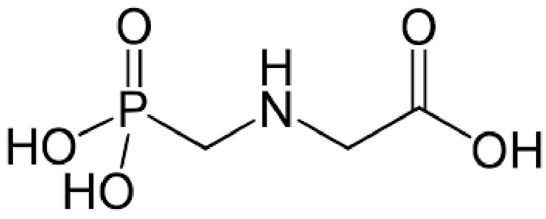
Activities carried out within HoliFood project (WP2, Task 2.6)

Targeted methods for on-site testing of pesticides, mycotoxins and biogenic amines

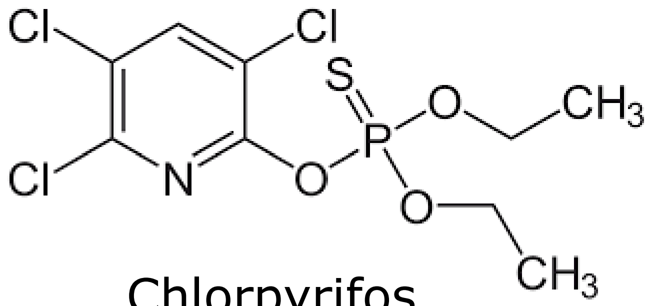
- ✓ Lateral flow device for **glyphosate** in **maize**
- ✓ SERS-based technology for **chlorpyrifos** in **maize**
- ✓ Aptamer-based lateral flow strip test for **aflatoxin B1** and **fumonisin B1** analysis in **maize**
- ✓ Aptamer-based lateral flow strip test for **tyramine** and **histamine** analysis in **poultry meat**




Pesticides



Glyphosate



Chlorpyrifos

Glyphosate and chlorpyrifos in maize

Glyphosate and chlorpyrifos

- **Pesticides** have been extensively used for decades **to protect** harvests and crops from **pests** such as **insects**.
- **Glyphosate** and **chlorpyrifos** are **organophosphate-based pesticides,** representing the **most dominant form of pesticides** in this sector, accounting for **nearly 40 %** of all pesticides produced.
- **Organophosphate pesticides** cause a **toxic reaction in the humans**, inhibiting the acetylcholinesterase enzyme resulting in **impaired respiratory tract** and **neuromuscular activity**.
- Due to the **expiration data of license (December 2023)** of glyphosate it has been assessed by Member States, the ECHA and the EFSA for **its controversial toxicity**. The EU Commission has **renewed its approval** up to 2033, by subjecting to certain new conditions and restrictions.
- The **use of chlorpyrifos** has been **banned** in EU since 2020 due to **adverse effects** on neurological development in children

Maximum Residue Level (MRL)

Glyphosate *(EC N. 396/2005, EC N. 293/2013)* 1 mg/kg

Chlorpyrifos The lowest limit detectable by the analytical method

Current methods for pesticides analysis

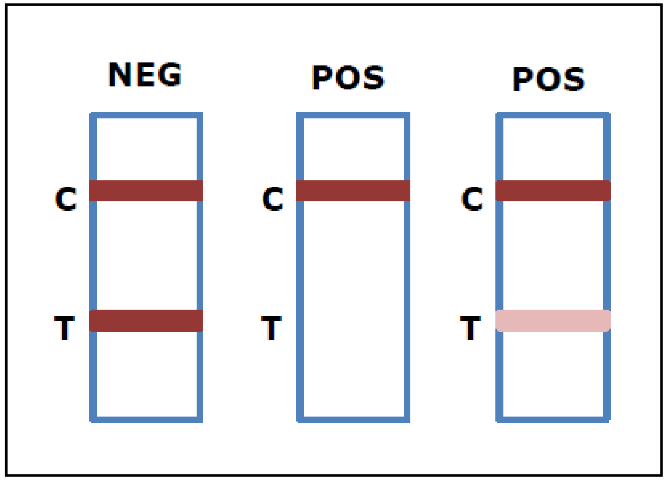
- Most of the **technologies require high-end equipment** and **resources** in **low throughput**, and **none of them** are adequate for **on-site and real-time field tests**, which may explain the lack of studies on occupational health associated with the chemical hazard.
- The **on-site** and **real-time detection** is a **highly demanded need** to **improve public policies.**
- Immunochromatographic test kit for **glyphosate** are commercially available but there are **not rigorous and scientific studies on their validation**.
- Commercial test kit based on enzyme inhibition and colorimetric **detection for the total content of organophosphate** residues are available *(and many only applicable to water)*.
  No available tests for individual pesticides (i.e., chlorpyrifos).

Rapid and fully automated assay for the detection of glyphosate in maize

Surface-Enhanced Raman Spectroscopy (SERS) for the detection of chlorpyrifos in maize

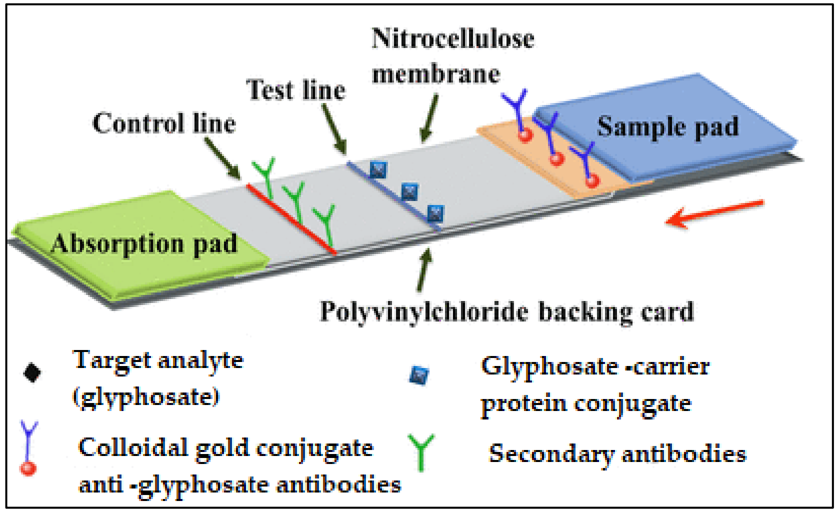
Rapid and fully automated assays for the detection of glyphosate in maize

Principle of test



Immunocromatographic test based on the indirect competition immunoassay approach

The **antigen** (gly–protein conjugate) is **immobilised** on the strip (TL) and the **gold labelled antibody** will compete between the free antigen in the sample, if presents, and the antigen immobilized on the strip



Negative Sample: two colored lines

(control line and test line)

In the absence of glyphosate, the **labelled antibody** binds to the gly-protein conjugate (test line) and the secondary antibody (control line)

Positive Sample: one colored line

(control line, test line is absent or slightly colored)

Labelled antibody binds **very poorly** to the **gly-conjugate** (test line), which is **already occupied with glyphosate**, and will be available to **bind to the secondary antibody**

Rapid and fully automated assays for the detection of glyphosate in maize

Assay protocol

Cereal (3 g),
addition of water (30 mL)
blending (2 min)

Sample
filtration

Sample transfer
(1 mL – 0.1 g of matrix)

Reagents A (buffer) and B
(derivatizing agent) addition
Incubation 5 min

Loading 0.1 mL of sample
to strip sample well
(development 5 min)

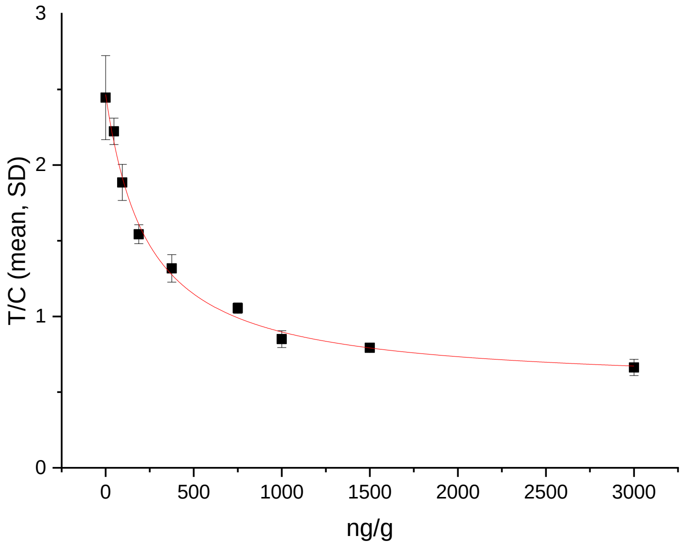
Photometric reading
(quantitative analysis)

- ✓ Assay at **room temperature**
- ✓ **Solvent free** extraction and analysis
- ✓ **Matrix specific calibration curve**
  (uploaded into the reader as QR code)
- ✓ Total **analysis time 12 minutes**

Rapid and fully automated assays for the detection of glyphosate in maize

Performances of the optimized prototype

Typical calibration curve in maize



WORKING RANGE: 0-3000 ng/g (0-3 MRL)

Maize sample (Lot D)	IC50	IC30
Maize (equivalent to 0.0083 g on test)	234 ± 40 ng/g	60 ± 10 ng/g

IC, Inhibitory concentration

Parameter derived from the calibration curve

Half maximum inhibitory concentration, IC50

Analyte concentration for which 50% of the test signal is switched off

Calibration curve specific for the commodity and the lot of strips is **saved as QR code.**

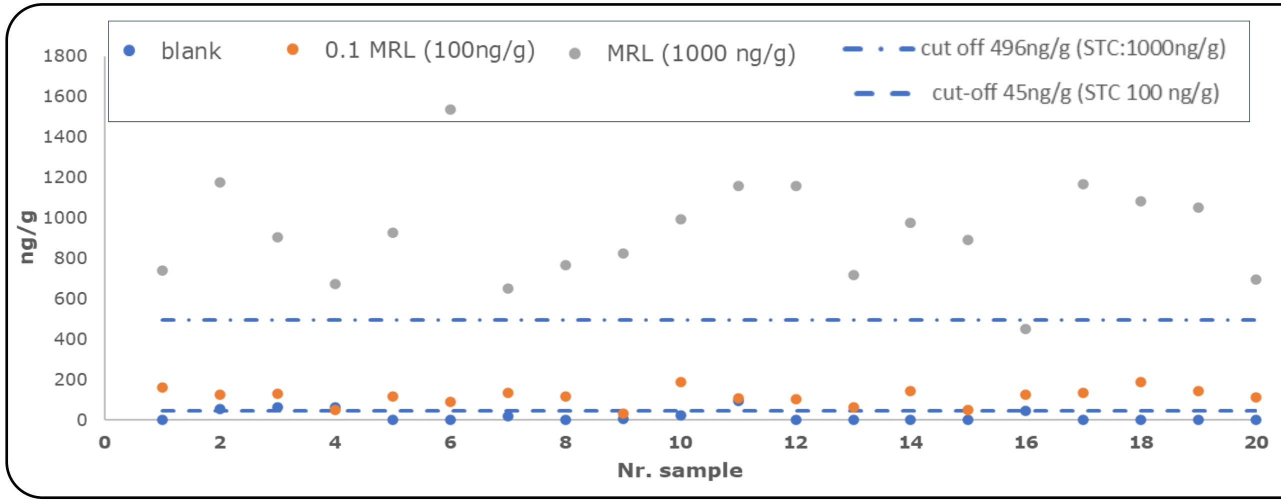
Glypho 23021SW.0

Satisfactory **repeatibility** and **discrimination** bewteen maize samples contaminated at 1 MRL (1000 ng/g), 0.5 MRL (500 ng/g) and in blank samples

Prototype suitable for validation experiments in maize

Rapid and fully automated assays for the detection of glyphosate in maize

Evaluation of the method precision (spiked maize samples)



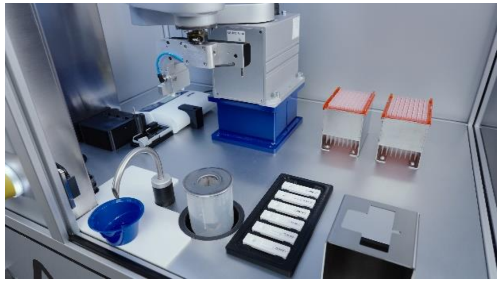
EU Commission Regulation 519/2014

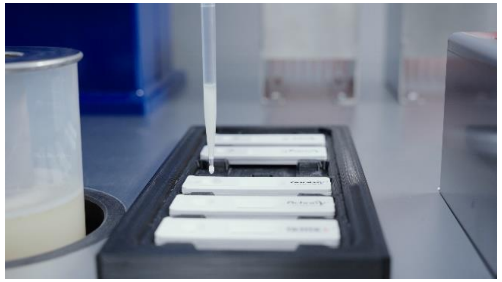
Satisfactory repeatability and discrimination bewteen blank maize samples and those contaminated at 1 MRL

False negative rates : 0% (samples contaminated at 1000 ng/g) – 27% samples contaminated at 100 ng/g

Ongoing & future work

- **Evaluation** of **cross-reactivity** towards glyphosate metabolites.
- **Full method validation.**
- Development of a **fully automated procedure**.
- **Prototype for the automatization** (at CNR-ISPA in July 2024).





- ✓ Automatization of sample preparation and analysis
- ✓ Calibration and traceability of the measurements
- ✓ Online data transfer and management
- ✓ Real samples analysis for monitoring purposes

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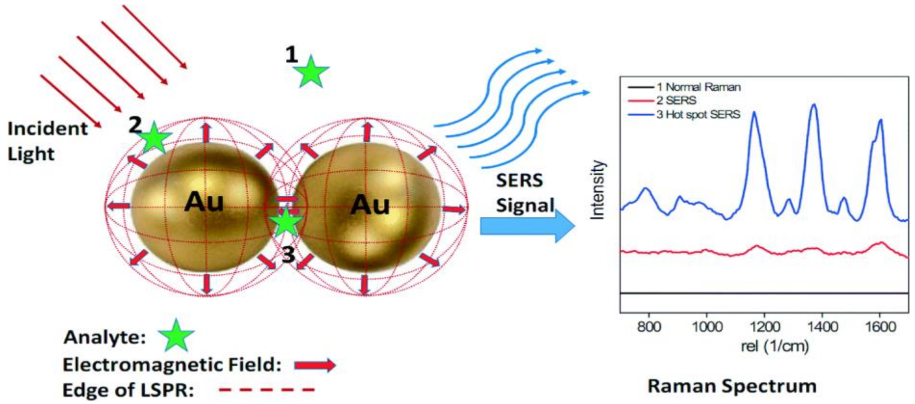
Surface-Enhanced Raman Spectroscopy (SERS) for the detection of chlorpyrifos in maize

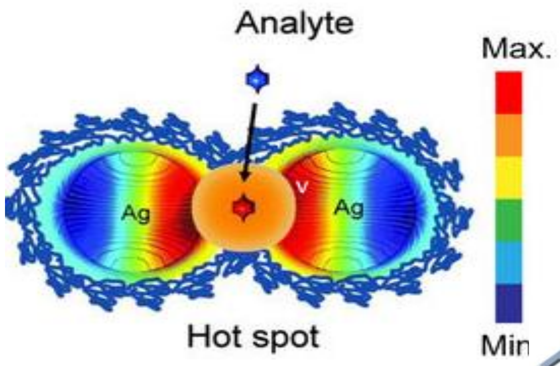
Principle of test

- **Surface-sensitive spectroscopy technique** improving **Raman scattering** using **metallic substrates** (e.g. Au or Ag).
- **Analytes are adsorbed** to the surface or close to the surface, **reducing inter-particle distance**.
- **Electromagnetic field enhancement** by the generation of '**hot-spots**'.
- Widely used for molecular identification, structural characterization and provides "**fingerprint-like**" spectra.



Scheme 1: Principle of SERS

Au, gold; Ag, silver



Advantages:

Rapid, portable, quantitative *(unique spectral information)*, detection limits and selectivity can be adjusted using recognition elements, cheap & stable substrate production

Surface-Enhanced Raman Spectroscopy (SERS) for the detection of chlorpyrifos in maize

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Main steps from fabrication to data analysis

Fabrication

SERS substrates

Synthesis

Characterisation

Determination of particle properties

Detection

Development

Colorimetric & SERS

Optimisation

Recognition elements

Five pesticides & aflatoxin

Portable detection

Evaluation

Validation

Spiked samples

Naturally contaminated (if available)

Sensitivity, specificity, accuracy, repeatability, precision

Suitability

Multiplexing

Potential to measure multiple contaminants in maize

Five pesticides

Multiple mycotoxins

Both contaminants

Analytics

Data analysis

Chemometrics

Machine learning algorithms

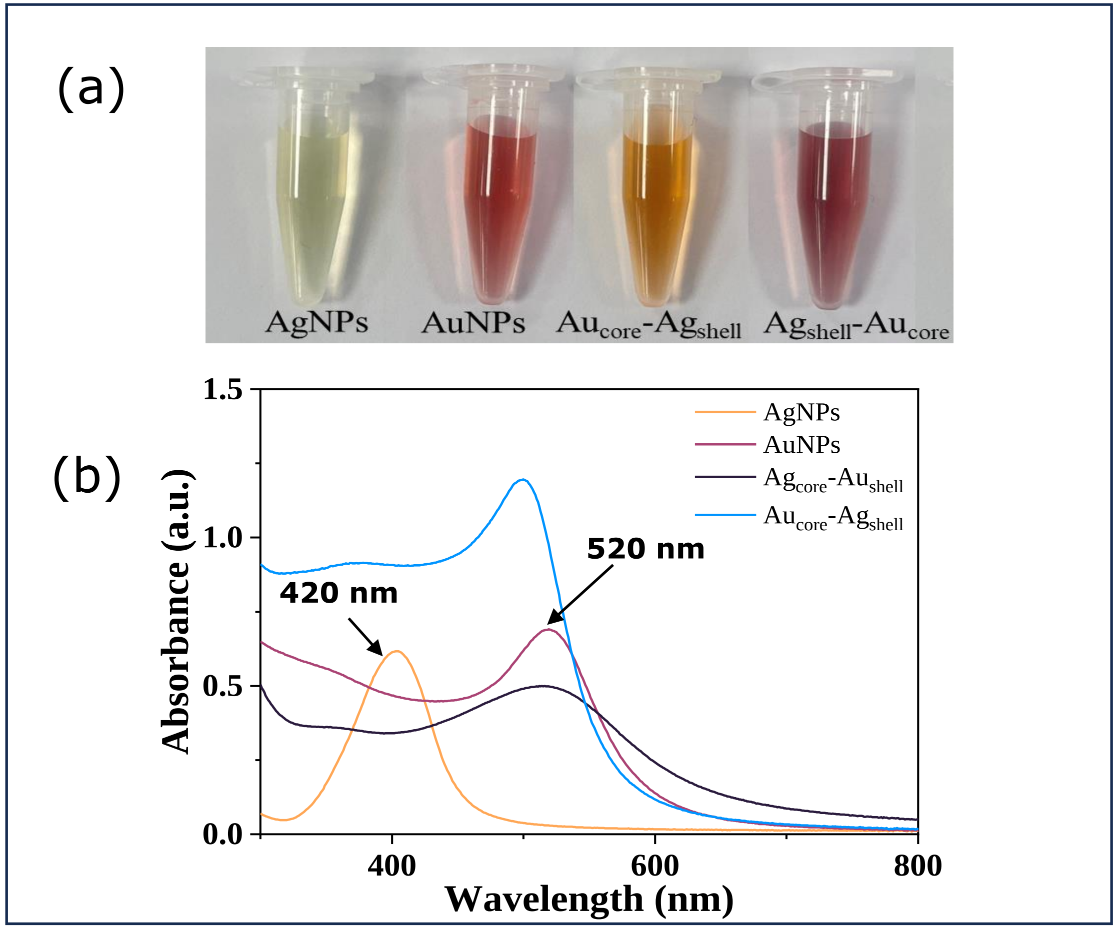
Assist with multiple contaminant detection in maize

Objective

Development of colorimetric & SERS-based technologies for five pesticides and aflatoxin in maize

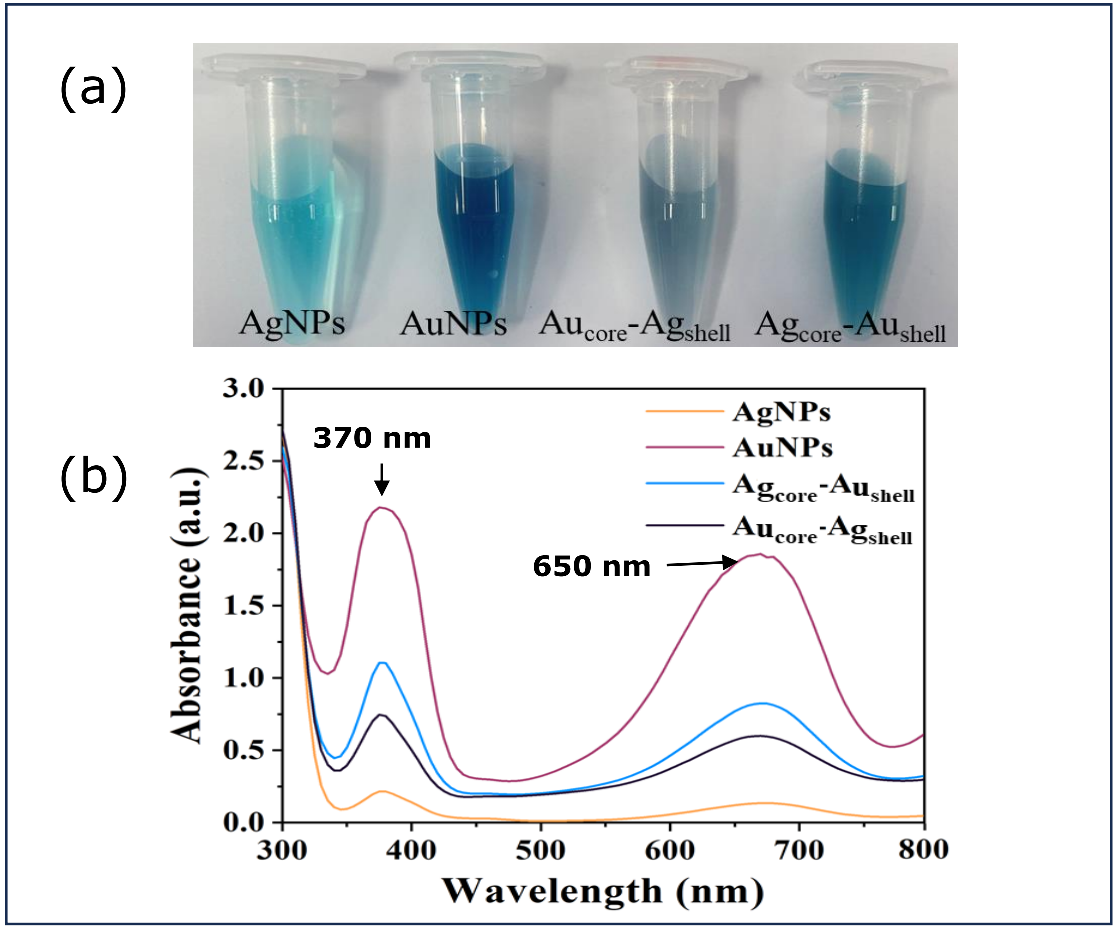
Surface-Enhanced Raman Spectroscopy (SERS) for the detection of chlorpyrifos in maize

Synthesis of SERS substrates



Gamma **cyclodextrin** (γ-CD) capped **gold** (Au), **silver** (Ag) and **core-shell nanoparticles.** CD are used as reducing agent and stabilizer.
(a) Coloured photograph of fabricated particles.
(b) UV-vis absorbance spectra

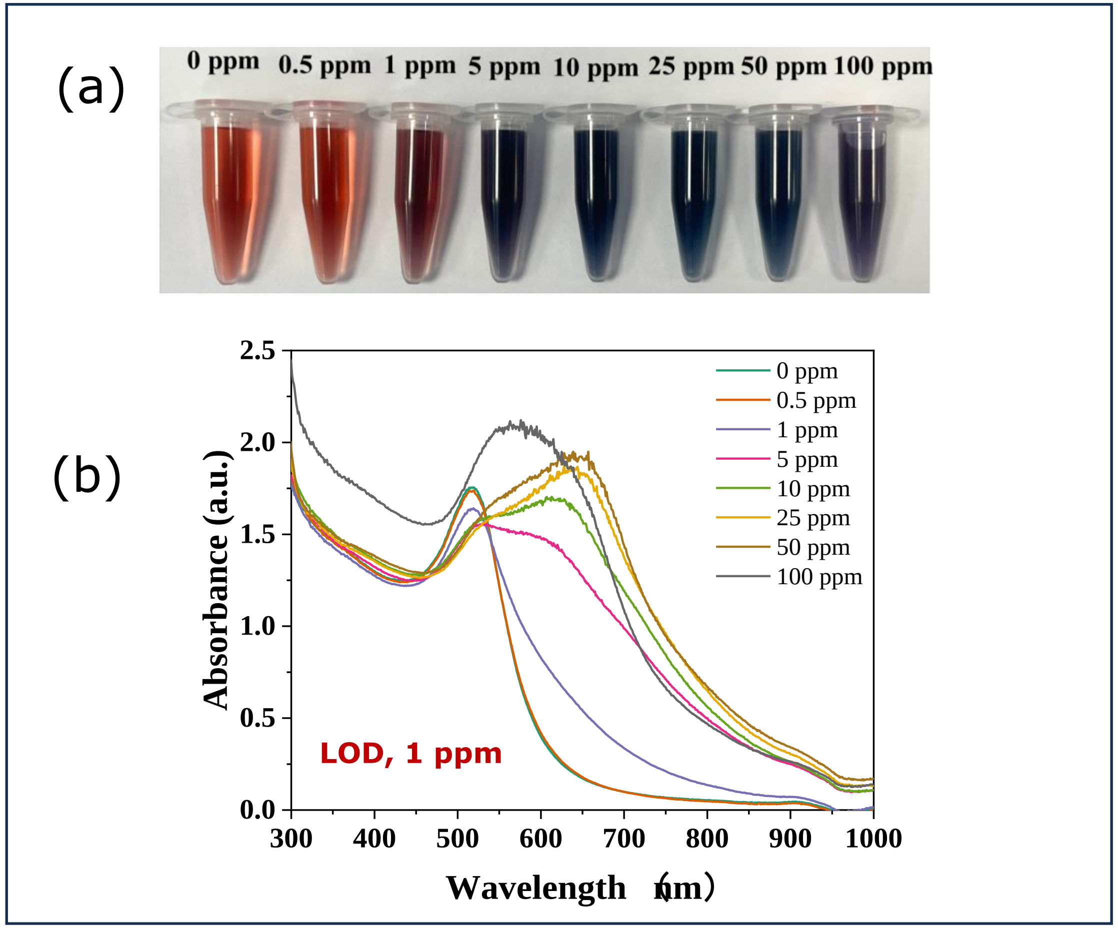
Characterisation of catalytic properties



Catalytic properties of synthesised γ-CD nanoparticles in the presence of TMB substrate and H_2O_2.
(a) Coloured photograph
(b) UV-vis absorbance spectra

Surface-Enhanced Raman Spectroscopy (SERS) for the detection of chlorpyrifos in maize

Visual detection of chlorpyrifos
(using cyclodextrine capped AuNPs)

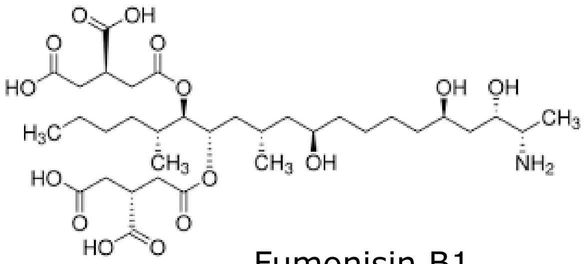


(a) Colorimetric detection of chlorpyrifos at different concentrations in the presence of ***γ-CD AuNPs (visual LOD=1ppm)***
(b) Corresponding UV-vis spectra

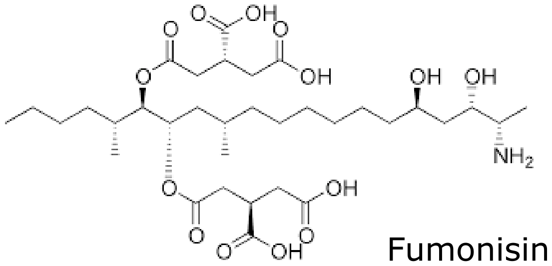
Ongoing & future work

- **Improvements of sensitivity assay** for **chlorpyrifos detection in maize**
- **Validation of the assay** on spiked and naturally contaminated maize
- Application of the assay to the **analysis of additional 4 pesticides** prior to multiplexed/chemometric analysis.
- Development of **a SERS-based competitive immunoassay** for **aflatoxin detection in maize**
- Evaluation of **performance parameters** of the assay **using colorimetric/visual properties of AuNPs**.
- SERS measurements & assessment of **assay sensitivity**.
- **Validation of assay** using spiked maize samples & naturally contaminated samples.

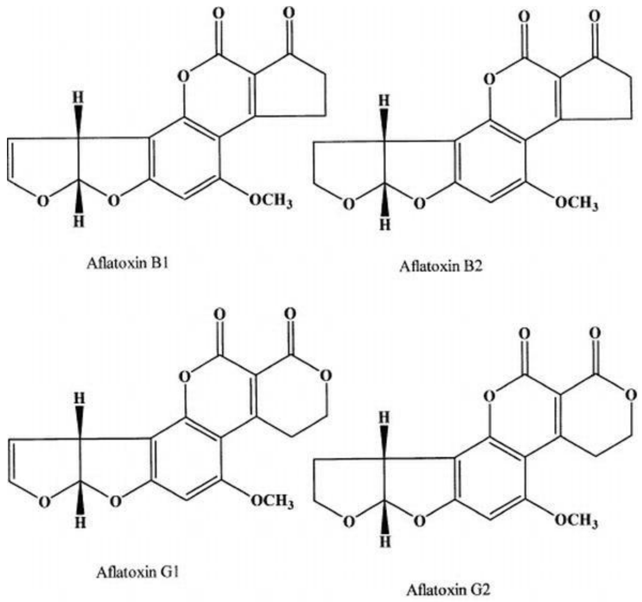
Mycotoxins



Fumonisin B1



Fumonisin B2



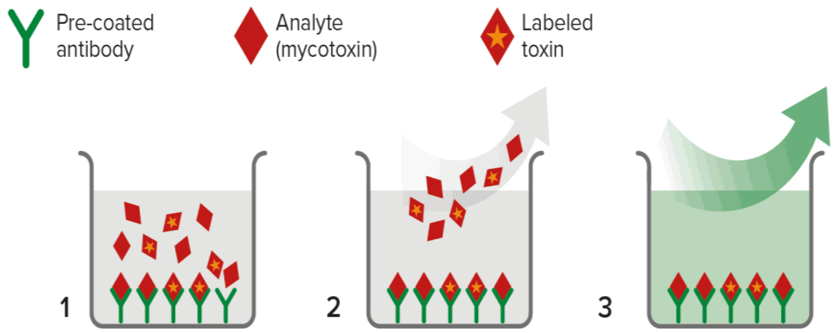
Aflatoxins and fumonisins in maize

Mycotoxins

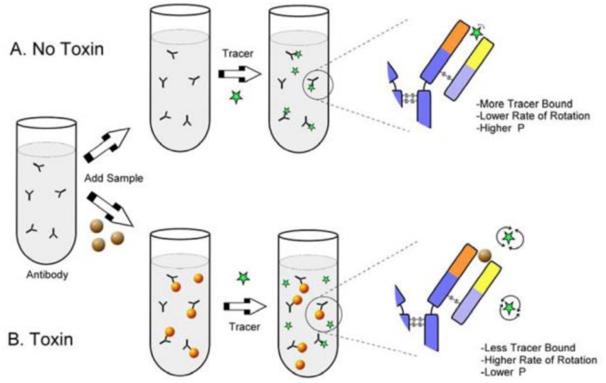
	Aflatoxins (B1, B2, G1, G2)	**Fumonisins (B1, B2)**
Fungal producer	*Aspergillus flavus,* *A. parasiticus*	*Fusarium graminearum,* *F. culmorum*
Commodity	**Maize**, peanuts, hazelnuts, spices, dried fruit	**Maize** and derived products, rice, sorghum, barley
Toxic effects on human	Association with liver cancer, acute poisoning (aflatoxicosis), impairment of child growth	Possible role in oesophageal cancer and neural tube defects
IARC Classification	Group 1: AFB1 carcinogenic to humans	Group 2B: FB1 possible carcinogenic to humans
Maximum permitted levels in unprocessed maize *(EC Reg. 915/2023)*	5 µg/kg (AFB1) 10 µg/kg (Total)	4000 µg/kg (FB1+FB2)

Rapid methods for mycotoxins analysis

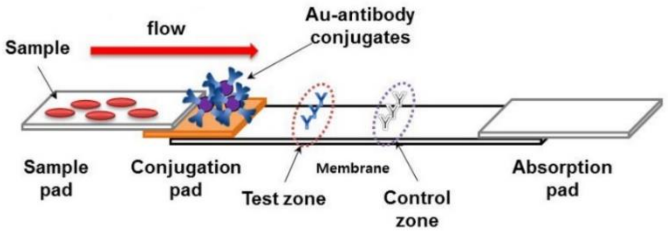
- **Rapid diagnostic kit market is very competitive**
- **Immunoassays/immunosensors**:
  - Enzyme Linked Immunosorbent Assay (ELISA)
  - Flow Through Immunoassay (FIA)
  - Lateral flow devices (LFD) or dipsticks
  - Fluorescence Polarization Immunoassay (FPIA)
  - Clean-up IAC and fluorimetric detection
  - Surface plasmon resonance (SPR)
  - Electrochemical immunosensors (ES)
- **Methods using alternative receptors**: aptamers, antibody fragments, molecularly imprinted polymers, peptides
- **Indirect screening methods**: Infrared spectroscopy (FT-IR), Electronic noses (E-noses)
- **Mass spectrometry-based screening method**: portable MS, DART-MS, LC-HRMS
- **Mainly used by:** grain importers and traders, food and feed manufacturers



Enzyme Linked Immunosorbent Assay (ELISA)



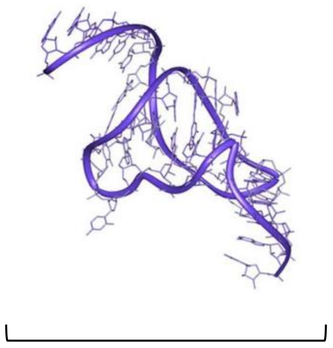
Fluorescence Polarization Immunoassay (FPIA)



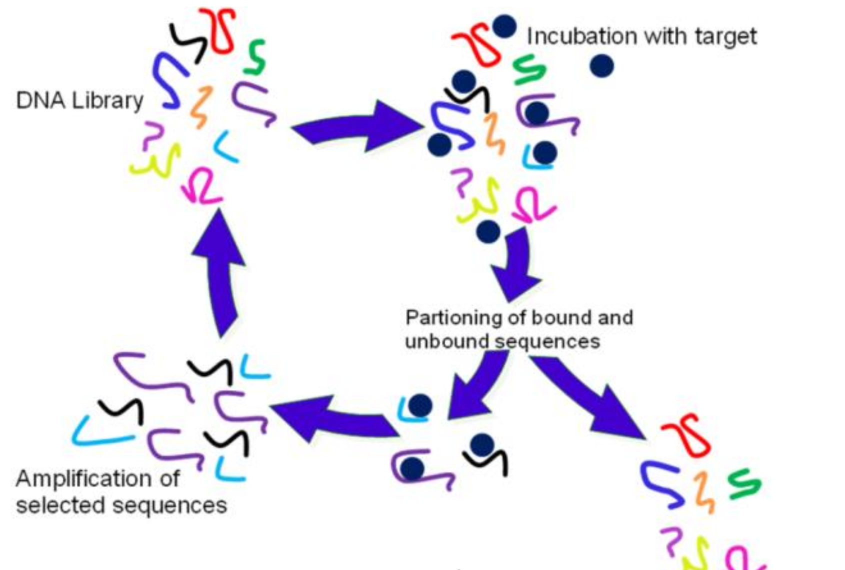
Lateral Flow Device (LFD)

Novel materials for mycotoxin analysis: Aptamers

Aptamers



~ 4 nm



SELEX procedure
from McKeague et al 2010, Int. J. Mol. Sci., 11, 4864-4881

Characteristics	Aptamers	Antibodies
Production	In vitro	In vivo (need to use animals at least for their initial production)
Batch-to batch variation	None	Possible
Target	Small molecules, macromolecules, cells	Must be immunogenic (not too much toxic)
Modification	Easy	Possible (heterogeneous products)
Selectivity/Affinity	Medium/High	High
Shelf life	Years at different temperatures	Weeks at 4°C
Application conditions	Physiological, non-physiological (medium)	Physiological, non-physiological (low)

- Aptamers are single-stranded oligonucleotides (DNA or RNA) that bind with **high affinity** and **specificity** to specific targets.
- Aptamers are produced by an ***in vitro* selection** process called **SELEX** (Systematic Evolution of Ligands by Exponential).
- Aptamers, like antibodies, have potential in a broad range of applications including **biosensors**, **affinity chromatography**, **lateral flow devices**.
- Aptamers for **OTA, FB1, AFB1, AFB2, AFM1, ZEA, T-2, HT-2** and **DON** have been produced.

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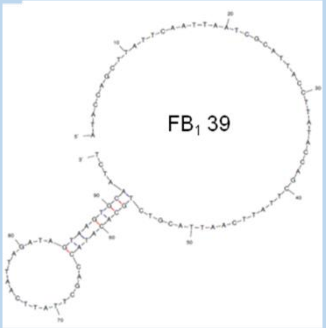
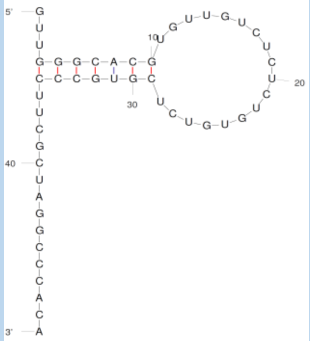
Aptamer-based LFD strip test: Simultaneous determination of AFB1 and FB1 in maize



Fumonisin B1 (FB1) aptamer

5’-AAT CGC ATT ACC TTA TAC CAG CTT ATT CAA TTA CGT CTG CAC ATA CCA GC TTA TTC AAT T-3’

Binding assay by:
- Magnetic beads *Kd 1.53 ± 0.67 nM*
- Microscale termophoresis *Kd 31 ± 22 nM*

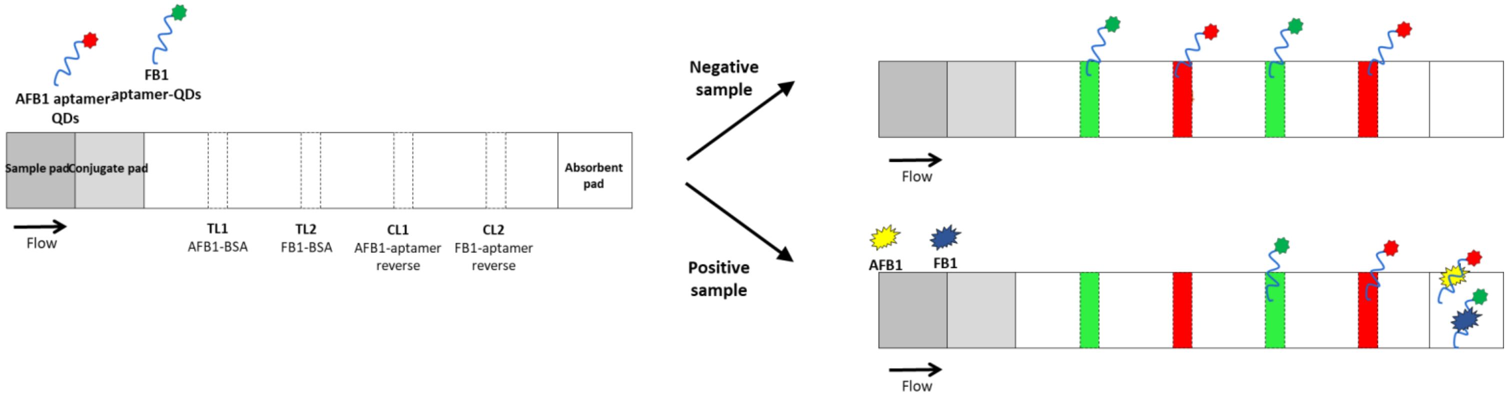


Aflatoxin B1 (AFB1) aptamer

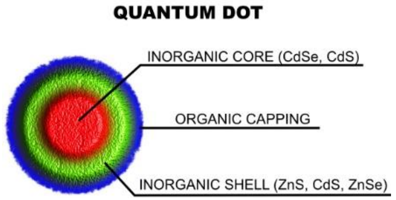
5’-AAT CGC ATT ACC TTA TAC CAG CTT ATT CAA TTA CGT CTG CAC ATA CCA GC TTA TTC AAT T-3’

Binding assay by:
- Micro dialysis *Kd 228 ± 27 nM*

DNA aptamer-based strip test design *(indirect competition)*



❖ **Conjugation of quantum dots (QDs)** with FB1 aptamer to increase the sensitivity of the strip test

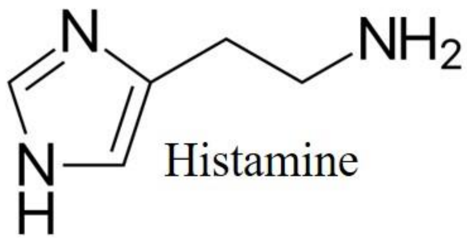


- ✓ High photostability and low photobleaching
- ✓ Size-tunable absorption and emission bands
- ✓ High intensity of luminescence
- ✓ Narrow, very specific, stable emission spectra

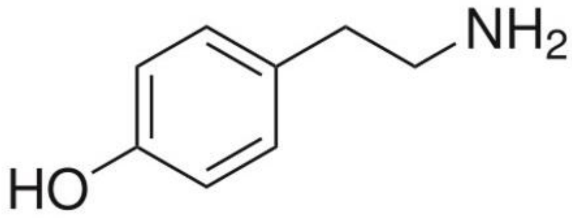
Ongoing & future work

- ❖ Conjugation and testing of AFB1 aptamer with QDs
- ❖ Synthesis of AFB1-BSA conjugates for test lines (TL)
- ❖ Development of an aptamer-based strip test assay

Biogenic amines



Histamine

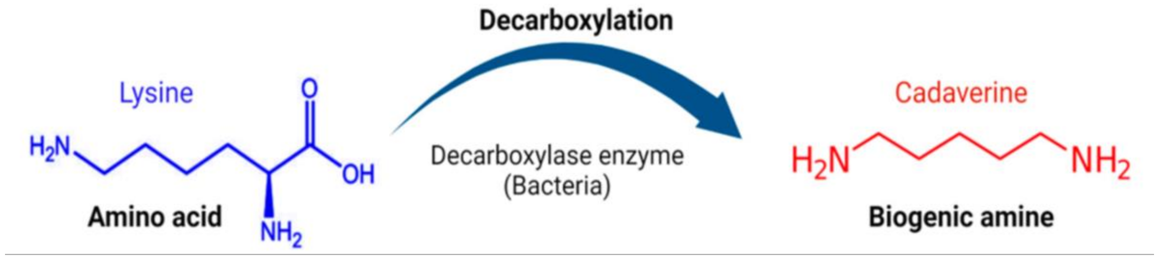


Tyramine

Tyramine and histamine in poultry meat

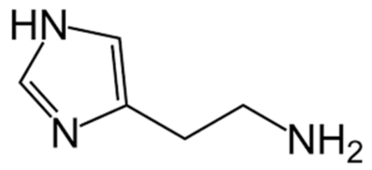
Biogenic amines

- **Biogenic amines (BAs)** are organic, basic, **nitrogenous compounds** of low molecular weight, mainly formed by the **decarboxylation of amino acids** and can be found in any group of **protein-containing foods**.

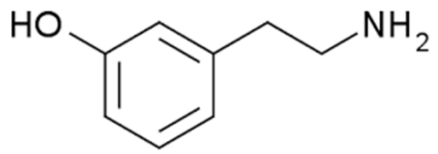


- **BAs** are determined in food as **quality markers** because are related to the **decay of food**.
- The **consumption** of a **large amount** of foods rich of BAs may cause **serious health problems**.
- **BAs most commonly found** in poultry meat are **histamine** and **tyramine** as well as cadaverine and putrescine.
- **Symptoms of histamine poisoning:** urticaria, fall in blood pressure, tachycardia, nausea, vomiting, diarrhea, headache, and convulsions, occurring within a few hours of food intake.
- **Symptoms of tyramine poisoning:** headache, palpitations, nausea and vomiting, a rise in blood pressure, sweating, and stiffness in the neck.

Histamine (HIS)



Tyramine (TYR)



Biogenic amines

- **Tyramine and histamine levels increase** during **poultry meat storage** and in **modified atmosphere packaging**.
- International **maximum limits of BAs** in food are **absent.**
- The **daily consumption** should not exceed **50 mg for histamine** and **600 mg for tyramine** (FDA report, 2014).
- **Monitoring of BAs** in food samples is of high importance.
- **Risk assessment** is a scientific approach to **assess food safety** and to provide scientific criteria for **decision making** in risk management.



Current methods for biogenic amines

- **Control measures** to **prevent mycotoxins and BAs formation** in foods and/or **reduce their levels** are needed.
- The **determination of mycotoxins and BAs** is most commonly performed by means of chromatographic methods (HPLC, GC and LC-MS).
- These **methods are often time-consuming** with long and tedious sample pretreatment and require **skilled personnel**.

Rapid/screening methods for the determination of these contaminants **are highly demanded.**

Aptamer-based LFD strip test: Simultaneous determination of TYR and HIS in maize

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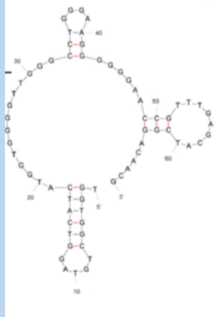
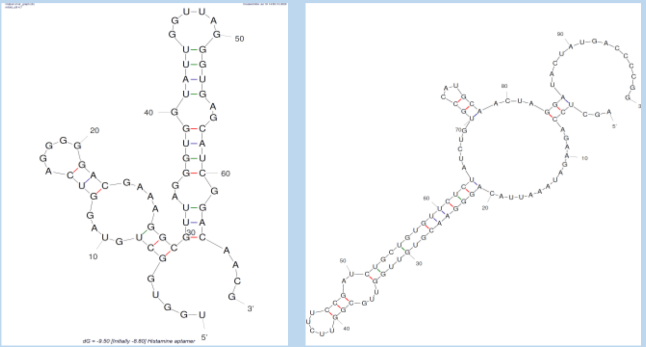


Aptamer Tyramine (TYR)

5′-AAT CGC ATT ACC TTA TAC CAG CTT ATT CAA TTA CGT CTG CAC ATA CCA GCT TAT TCA ATT-3′

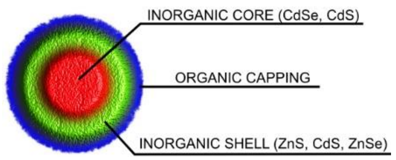
Binding assay by:
- Microscale termophoresis *Kd 97 ± 37 nM*



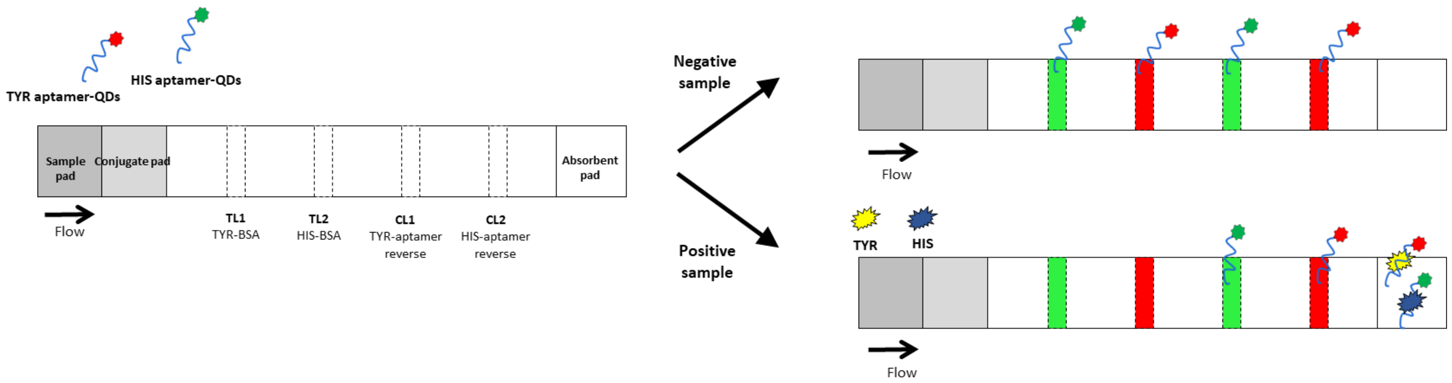
Aptamers Histamine (HIS)

Binding assays to be performed for two sequences

QUANTUM DOT



DNA aptamer-based strip test design *(indirect competition)*



TL, test line
CL, control line
QDs, quantum dots

Ongoing & future work

- Binding studies on HIS aptamers (Kd measurements)
- Conjugation of TYR and HIS aptamers with QDs
- Synthesis of BAs-BSA conjugates for TL
- Synthesis of reverse aptamer conjugates for CLs
- Development of an aptamer-based strip test assay

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Thank you for the attention

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