Rapid Detection of Aflatoxin M1 in Milk: Analytical Challenges and Validation Aspects Under EC Perspective

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Presentation Outline

- Concept of **rapid/screening method**

- Overview of **available rapid methods for AFM$_1$** detection in milk
  - Commercial kits
  - Promising research methods

- Evaluating performance characteristics of screening methods: overview of the Regulation **519/2014/EU**

- **A case study**

- Conclusions

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Rapid detection vs Conventional methods

Conventional methods (AOAC 2000.08)

- SAMPLE PREPARATION
  - Centrifugation to separate the fat layer

- IMMUNOAFFINITY COLUMN CLEAN UP

- HPLC determination with Fluorescence Detection

  Time of analysis: 4 h/sample

Rapid/emerging methods

- SAMPLE PREPARATION
  - (not always needed)

- DETECTION
  - (immunoassays - sensors)

  Time of analysis: 5 - 20 min

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Purpose of a rapid method

- Screening test against target levels
  - To determine legal compliance
  - To achieve operational and logistical goals (process management)

- Concept of screening method:
  - Negative samples are classified as such and NOT further analysed.
  - Positive samples need to be re-analysed using confirmatory methods

- False positive samples of truly negative samples have no impact on consumer safety... BUT...a high number of false positive results may question the benefit of such a screening test

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Available rapid methods for AFM₁ detection

Mostly based on:
- **Competitive immunoassay** formats
- **Surface** based formats

The **competitor** (AFM₁-protein conjugate) or the **receptor** (antibody or aptamer) is immobilized onto a surface (microtiter plate, membrane, electrode)
Enzyme-linked immunosorbent assay (ELISA)

- Coating of Ab onto a microtiter plate
- Add sample extract
- Add AFM$_1$-enzyme conjugate

Competition between free and enzyme-conjugate AFM$_1$

Add enzyme substrate for colour development

The signal is inversely proportional to AFM$_1$ concentration
## Commercially available ELISA kits


<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Test Kit</th>
<th>Test kit Range</th>
<th>Incubation time (min)</th>
<th>Intended Matrices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beacon Analytical Systems Inc.</td>
<td>Aflatoxin M1 Plate Kit</td>
<td>0.3 – 300 ng/kg</td>
<td>75</td>
<td>milk and milk powder</td>
</tr>
<tr>
<td>Bioo Scientific Corporation</td>
<td>MaxSignal Aflatoxin M1 ELISA Test Kit</td>
<td>&gt; 0.1 µg/kg</td>
<td>30</td>
<td>milk</td>
</tr>
<tr>
<td>EuroProxima</td>
<td>Aflatoxin M1 ELISA</td>
<td>&gt;6 ng/kg</td>
<td>60</td>
<td>milk, milk powder, cheese and butter</td>
</tr>
<tr>
<td>MP Biomedicals</td>
<td>Fast-TOX Aflatoxin M1 ELISA Kit</td>
<td>N/A</td>
<td>N/A</td>
<td>milk and milk powder</td>
</tr>
<tr>
<td>Neogen Corporation</td>
<td>Veratox for Aflatoxin M1</td>
<td>5-100 ng/kg</td>
<td>45</td>
<td>milk, milk powder, butter and cheese</td>
</tr>
<tr>
<td>R-Biopharm</td>
<td>RIDASCREEN Aflatoxin M1</td>
<td>&gt; 5 ng/kg</td>
<td>75</td>
<td>milk, milk powder and cheese</td>
</tr>
<tr>
<td>R-Biopharm</td>
<td>RIDASCREEN FAST Aflatoxin M1</td>
<td>&gt; 125 ng/kg</td>
<td>15</td>
<td>milk and milk powder</td>
</tr>
<tr>
<td>Reagen LLC</td>
<td>Aflatoxin M1 ELISA Test Kit</td>
<td>&gt;0.05 µg/kg</td>
<td>30</td>
<td>milk and milk powder</td>
</tr>
<tr>
<td>Romer Labs</td>
<td>AgraQuant Aflatoxin M1 Sensitive</td>
<td>25-500 ng/kg</td>
<td>80</td>
<td>milk, milk powder and cheese</td>
</tr>
<tr>
<td>Romer Labs</td>
<td>AgraQuant Aflatoxin M1 fast</td>
<td>100-2000 ng/kg</td>
<td>30</td>
<td>milk, milk powder and cheese</td>
</tr>
</tbody>
</table>

**US FDA action level** 500 ng/kg  
**EU maximum limit** 50 ng/kg

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Strip tests and Lateral Flow Immunoassays

CTRL line
Test line
AFM₁-Pr

NEGATIVE sample
Test Lines darker than CTRL line

POSITIVE sample
Test Lines lighter than CTRL line

Labelled antibodies

Optical Reading

Ratio T/C
# Commercially available Dipstick/Lateral Flow Test


<table>
<thead>
<tr>
<th>Manufacturer</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Charm</td>
<td>SLAFMQ</td>
<td>Quantitate 0 - 750 ng/kg</td>
<td>8</td>
<td>milk</td>
</tr>
<tr>
<td>Bioo Scientific Corporation</td>
<td>AuroFlow™ Aflatoxin M₁ Strip Test Kit</td>
<td>Screens @ 500 ng/kg</td>
<td>10</td>
<td>milk</td>
</tr>
<tr>
<td>Neogen Corporation</td>
<td>Reveal for Aflatoxin M₁</td>
<td>Screens @ 500 ng/kg</td>
<td>5</td>
<td>milk, milk powder, butter and cheese</td>
</tr>
<tr>
<td>Reagen LLC</td>
<td>Aflatoxin M₁ Strip Test</td>
<td>Screens @ 500 ng/kg</td>
<td>10</td>
<td>milk</td>
</tr>
<tr>
<td>Unisensor</td>
<td>Aflasensor</td>
<td>Semiquantitative 30-100 ng/kg</td>
<td>10</td>
<td>milk</td>
</tr>
<tr>
<td>Vicam</td>
<td>Afla M₁-V</td>
<td>Semiquantitative 25-750 ng/kg</td>
<td>12</td>
<td>milk</td>
</tr>
</tbody>
</table>
**Immunoaffinity column + Florometer**

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<thead>
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<th>Intended Matrices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waters Vicam</td>
<td>Afla M\textsubscript{1} FL+</td>
<td>Quantitate 12 – 200 ng/kg</td>
<td>25</td>
<td>Milk</td>
</tr>
</tbody>
</table>

**Flowchart:**
- MILK sample
- **IAC clean up**
- **Elute AFM\textsubscript{1} with methanol**
- Add Developer solution
- Measure in Fluorometer
Rapid methods based on Emerging Technologies

-Electrochemical affinity biosensors

(reviewed in Vidal et al, 2013, Biosensor and Bioelectronics, 46:146-158)
Example: Paniel et al, 2010, sensors, 10:9439-9448

microfluidic approaches -> miniaturization, on line monitoring, full automation

- Aptamer-based biosensors
- Dynamic light scattering
Flow Based Impedimetric Immunosensors

Measurement of **impedance** change resulting from **antigen-antibody** interaction

*Chiriacò et al. 2010, Lab on Chip, 11:658-663*

INTEGRATED MICROFLUIDIC PLATFORM

**Lab on chip**

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Aptasensors

Nguyen et al. Materials Science and Engineering C 33 (2013) 2229-2234

- **Aptamers** are single-stranded oligonucleotides (DNA or RNA) that bind with **high affinity** and **specificity** to specific targets.
- Aptamers, like antibodies, have potential in a broad range of **applications** including **biosensors**

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**Aptasensors**

**APT probe**

**Fe₃O₄/PANi film**

**micro electrode**

**AFM₁-Aptamer complexation**

**Electrochemical detection**

**Signal OFF**

**AFM₁-Aptamer Decomplexation**

**In APT reach solution**

**Electrochemical detection**

**Signal ON**

**LOD 2 ng/L**

**Standard solution**

**Applicability to real milk samples not yet demonstrated**

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Dynamic light scattering (DLS)


Direct competitive immunoassay

DLS is used to determine the concentration of unattached nanoprobes that is directly proportional to AFM\(_1\) concentration in the sample.

**LOD 27.5 ng/L in milk samples**

Analysis time 15 min
Rapid Methods for AFM$_1$ detection in Milk

**CHALLENGES**

- To achieve **low detection limits**
  (target levels: EC 50 ng/L – 25 ng/L baby food – US FDA 500 ng/L)
  ∴ To obtain appreciable and reproducible signal changes generated by small changes in analyte concentration
- To make them **reliable in on site conditions**
  ∴ To develop robust analysis protocols: e.g. by use of incubators, use of buffer diluents to manage matrix to matrix variations
  ...keeping as simple as possible the sample preparation step

**How to evaluate performances and fitness-for-purpose of rapid test kits?**
- AOAC Research Institute (Performance Tested Methods$^\text{SM}$)
- USDA-GIPSA (Performance Verified Rapid Test)
- **EU regulation 2014/519/EU**

**NO** AOAC or GIPSA Performance Tested Methods for AFM$_1$ in milk available today

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This concept is described in detail with an example in Analytical and Bioanalytical Chemistry DOI 10.1007/s00216-013-6922-1.

Experimental design for in-house validation of a screening immunoassay kit. The case of a multiplex dipstick for *Fusarium* mycotoxins in cereals

Veronica M. T. Lattanzio • Christoph von Holst • Angelo Visconti
Regulation 519/2014/EC
Specific requirements for semi-quantitative screening methods

**SCOPE**: includes bioanalytical methods based on immuno-recognition or receptor binding

Methods of which result of the measurement is a **numerical value**

**RESULT**

- **NEGATIVE**
- **SUSPECT** → Requires confirmatory analysis

Classification with respect to the **Screening Target Concentration (STC)**

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VALIDATION PROCEDURE

Determination of

- CUT OFF
- False suspect rate
- False negative rate

Include:
- Sensitivity
- Selectivity
- Precision

Validation

- Single Laboratory Validation
- Inter Laboratory Validation

Aim of the validation is to demonstrate the **fitness for purpose** of the screening method.
SINGLE LABORATORY VALIDATION

MATRICES

Each commodity group

At least one representative matrices for each commodity group

(when the method is known to be applicable to multiple commodities)

<table>
<thead>
<tr>
<th>Commodity group</th>
<th>Commodity Categories</th>
<th>Typical representative commodities included in the category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk and milk products</td>
<td>Milk</td>
<td>Cow, goat and buffalo milk</td>
</tr>
<tr>
<td></td>
<td>Cheese</td>
<td>Cow, goat cheese</td>
</tr>
<tr>
<td></td>
<td>Dairy products</td>
<td>Yogurt, cream</td>
</tr>
</tbody>
</table>
SINGLE LABORATORY VALIDATION

**Minimum Sample Set**

- 20 homogeneous negative samples*
- 20 homogeneous samples at STC

Intermediate precision conditions spread over 5 different days

Additional sets of 20 homogeneous samples at other levels can be added

**Negative sample:** sample known to be “free” of the mycotoxin of interest (1/5 of the Screening Target Concentration)

The kit responses for negative and positive samples are taken as basis for the calculation of required parameters

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CUT OFF level: the response, signal or concentration, obtained with the screening method, above which the sample is classified as “suspect”.

IMPORTANT: the cut off is determined during the validation and takes the variability of the measurement into account.

Cut Off: response value ensuring a rate of false negative results < 5%
CUT OFF DETERMINATION

Calculation of CUT OFF value: for screening methods with a response INVERSELY proportional with the mycotoxin concentration

1. Calculate of the mean of the results from the experiments of the samples with the analyte at target level ($R_{STC}$)
2. Use total standard deviation from the precision experiments ($SD_{STC}$)
3. Use one-sided t-value ($\beta = 5\%$) from a statistical table
4. Calculate cut-off value as follows:

$$ \text{Cut off value} = R_{STC} + \text{t-value (}\beta=0.05) \times SD_{STC} $$

By using this specific t-value for cut off calculation, the response value ensuring a rate of false negative results < 5%

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CUT OFF DETERMINATION

Calculation of CUT OFF value: For screening methods with a response proportional with the mycotoxin concentration

1. Calculate of the mean of the results from the experiments of the samples with the analyte at target level \( R_{\text{STC}} \)
2. Use total standard deviation from the precision experiments \( SD_{\text{STC}} \)
3. Use one-sided t-value \(( \beta = 5 \%) \) from a statistical table
4. Calculate cut-off value as follows:

\[
\text{Cut off value} = R_{\text{STC}} - t\text{-value (} \beta=0.05\text{)} \times SD_{\text{STC}}
\]
FALSE SUSPECT RATE

Calculation of false suspect rate for blank samples:
for screening methods with a response inversely proportional with the mycotoxin concentration

\[
t-value = \frac{\text{cut off} - \text{mean}_{\text{BLANK}}}{\text{SD}_{\text{BLANK}}}
\]

for screening methods with a response proportional with the mycotoxin concentration

\[
t-value = \frac{\text{mean}_{\text{BLANK}} - \text{cut off}}{\text{SD}_{\text{BLANK}}}
\]

From the obtained t-value, based on the degrees of freedom calculated from the number of experiments, the probability of false suspect samples for a one tailed distribution can either be calculated (e.g., spread sheet function “TDIST”) or taken from a table for t-distribution.

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VALIDATION REPORT:

The validation report shall contain:

— A statement on the **STC** (*Screening Target Concentration*)
— A statement on the obtained **cut-off**

— A statement on **calculated false suspect rate**
— A statement on how the false suspected rate was generated

*Note: The statement on the calculated false suspected rate indicates if the method is **fit-for-purpose** as it indicates the number of blank (or low level contamination) samples that will be subject to verification.*
RESULT REPORTING

The result of the screening shall be expressed as:

“Suspected to be non-compliant”
the sample exceeds the cut-off level and may contain the mycotoxin at a level higher than the STC.
Any suspect result triggers a confirmatory analysis for unambiguous identification and quantification of the mycotoxin.

“Compliant” means that the mycotoxin content in the sample is < STC with a certainty of 95 % (i.e. there is a 5 % chance that samples will be incorrectly reported as negative).
GENERAL CONCLUSIONS

By evaluating performances of screening methods according to EC guidelines it is possible to obtain:

• **Cut off** value with **rate of false positive** results

• **The precision profile** of the method

• **Ruggedness** of test – ANOVA results can provide useful suggestion for method improvement

Performance evaluation according to EC guidelines should be part of QC for each new kit lot
THANK YOU!

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