Recent development of Fluorescence Polarization Immunoassays for food contaminants

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Introduction for contamination

The contamination environmental and food samples with contaminants maybe vary from low or "zero" levels up to very high concentration up to μg/ml.

The number of contaminants and their metabolites presented in samples may range from one to dozen and their distribution in different types of sample may also be very variable.
Food contaminants

Chemical
- Pesticides
- Drugs
- Detergents
- Toxins

Biological
- Bacteria
- Microorganisms

Bisphenol A

Melamine

Polychlorinated dibenzo-\(p\)-dioxins (PCDDs)

Polychlorinated dibenzofurans (PCDFs)

Polychlorinated biphenyls (PCBs)

Polybrominated diphenyl ethers (PBDEs)
Requirements for detection of chemical contaminants

- Simple
- Quick
- Precise
- Sensitive
- Multi detection
Tendency in Analytical Detection from Chromatography to Immunoassay

- HPLC
- GC
- GC-MS
- Immunoassay

- Labor intensive
- Complicated cleanup
- Expensive equipment
- Time consuming

High sensitivity
High specificity
Rapid and simple
High Throughput Screening
Immunoassay

Immunochemical methods are based on the reversible binding of an antigen to specific antibodies, which are specially prepared for a given analyte:

\[
\text{Ag} + \text{Ab} \rightleftharpoons \text{Ag} : \text{Ab}
\]

where \(\text{Ab}\) is a specific antibody, \(\text{Ag}\) is an antigen, \(\text{Ag} : \text{Ab}\) is an antigen–antibody immune complex, and \(K_{as}\) is the constant of formation of the complex.
"The advantage of Immunoassays is that they provide fast and low cost analyses of many samples" – Ulrich Panne
Director, Federal Institute for Materials Research and Testing (BAM), Berlin, Germany
Immunoassay

enzyme-linked immunosorbent assay (ELISA)
lateral flow immunoassay (strip-test)
fluorescence polarization immunoassay (FPIA)
In the immunoassay method, fluorescence polarization immunoassay (FPIA) is the most extensively used homogeneous technique, which meets the requirements of a simple, reliable, fast and cost-effective analysis.
Detection of Fluorescence Polarization

\[ P = \frac{I_v - I_\eta}{I_v + I_\eta} \]

1 Perrin (8) showed in his equation that the $P$ value can be expressed as a function of such factors as temperature and viscosity together with molecular parameters as follows.

$$\frac{1}{P} = \frac{1}{P_0} + \left(\frac{1}{P_0} - \frac{1}{3}\right) \times \left(\frac{RT}{V}\right) \frac{\tau}{\eta}$$

(2)

$P$ observed value of fluorescence polarization
$P_0$ a constant (maximal value of $P$ obtained in a rigid medium)
$R$ gas constant
$T$ absolute temperature
$\eta$ viscosity (poise)
$\tau$ relaxation time of fluorescence excitation(s)
$V$ molecular volume
Principle of Fluorescence Polarization

Fast rotation
Low Fluorescence Polarization

Slow rotation
High Fluorescence Polarization

\[ P = \frac{I_v - I_h}{I_v + I_h} \]
Principle of FPIA
competitive immunoassay
with separation of free and bond tracer

Ag – antigen, Ab - antibody

\[ Ag + Ab \rightleftharpoons AgAb \]

hv, plane polarized light

High value of fluorescence polarization
Performance of FPIA

Detection of FP
# Fluorescence Polarization ImmunoAssay (FPIA)

## Advantages
- No separation steps
- No washing steps
- Simple and quick
- Cost effective
- Stable tracer
- High precision
- Stability of the standard curve

## Limitations
- Only for small molecules
- Lower sensitivity than ELISA
- Matrix dependent
- Special instrument
TDxFLx Analyzer (Abbott Lab., USA)
Automated method for FPIA using TDxFLx Abbott (USA)

1. Insert reagent pack

2. Load carousel with samples (100-500 ul)

3. Push button and receive results after 5-20 min
Portable FP instrument – Sentry-200
Distributor in China - Wang Dong

ebiotek@gmail.com 133-8113-8788

Sentry FP
(Diachemix Corp, & Cape Cod Assoc.)

Assays for:
Deoxynivalenol
Fumonisins
Aflatoxin B₁
Zearalenone
POLARstar (DMG, Germany)

**POLARstar+ OPTIMA**
Brighter Solutions in Reading Technology

- **Fluorescence**
  - Ca²⁺, Enzyme Kinetics, Cell Viability
  - DNA/RNA/Protein Quantification

- **Fluorescence Polarization**
  - Homogeneous Ligand Binding Assays,
    Binding Kinetics, Protease/Kinase Assays

- **Time-Resolved Fluorescence**
  - High Sensitivity Detection, FRET Assay

- **Luminescence**
  - Luciferase, Dual Luciferase, Aequorin,
    BRET Assay

- **Absorbance**
  - ELISA, Enzyme Kinetics,
    DNA/RNA Quantification
Method FPIA

Load:
10 μL sample
0.1 mL tracer solution
0.1 mL antibody
mix and measure mP

Total time for assay 1 plate
Low than 10 min
FP instrumentet

Quote

Date  Quote No.
6/10/2014  160

Bill To: Sergei Eremim
Chemistry Department
Moscow State University
119991 Moscow Russia
Phone: 1(495) 330-5571

Ship To: Sergei Eremim
Chemistry Department
Moscow State University
119991 Moscow Russia
Phone: 1(495) 330-5571

<table>
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<th>Description</th>
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<th>Rate</th>
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Total $49,400.00

Sentry 2000S

Fluorescence polarization reader for 12-well strips

- Fast accurate reader for 12 samples
- Read time less than one second per well
- Equipped with an injector for automated operation
- Injector precision highest in the industry with CV of 1% when delivering 10 microliter tracer.
- Easy software operation directly from Microsoft® Excel®
- Adjustable read precision
- USB connectivity
- Lower than 1 mP standard deviation between readings
- Sensitivity 1 pM
- Built-in LCD Polarizer switch for fast polarization switching and fast operation

Ordering Information

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<tr>
<th>Catalog #</th>
<th>Description</th>
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<td>S2000S</td>
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<tr>
<td>S2000SI</td>
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<td>1 ml black 12-well strips</td>
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<td>STRIPS8</td>
<td>8-well microplate strips</td>
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<td>STRIPS12</td>
<td>12-well microplate strips</td>
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<td>13-well microplate strips</td>
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<td>STRIPS10</td>
<td>Tubing for reagent dispensing with needle</td>
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<tr>
<td>STRIPS12</td>
<td>Internal tubing set for S2000G</td>
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<tr>
<td>SNSERT1B</td>
<td>Aluminum adapter for 8-well microplate strips</td>
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<td>SNSERT12</td>
<td>Aluminum adapter for 12-well microplate strips</td>
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<tr>
<td>SCOMP</td>
<td>Sentry Laptop for instrument control</td>
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Sentry 2000S works with black strips with 12 wells which are custom made and hold 1 ml of liquid or with standard 12-well or 8-well microplate strips.

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FPIA development and optimization

- Our work on FPIA was focused on improvement of FPIA characteristics mainly sensitivity

- FPIA consist of:
  Antibody (Ab), Antigen (Ag) and the Tracer (Ag-F)

- It is based on the competitive binding of antigen to the antibody:

  \[
  \text{Ab} + \text{Ag-F} \xleftrightarrow{K_{d1}} \text{Ab:Ag-F} \quad \text{Kd}_1 = \frac{[\text{Ab}] \cdot [\text{*A}]}{[\text{Ab} - \text{*A}]} \quad \text{Kaff}=1/\text{Kd} \\
  \text{Ab} + \text{Ag} \xleftrightarrow{K_{d2}} \text{Ab:Ag} \quad \text{Kd}_2 = \frac{[\text{Ab}] \cdot [\text{A}]}{[\text{Ab} - \text{A}]} 
  \]

- The improvement in sensitivity demands:
  - The use of low antigen concentration, requires high affinity constant antibodies (Kaff)
  - The antigen must compete binding of tracer. The two constants \(\text{Kd}_1\) and \(\text{Kd}_2\) should be similar
Fluorescein derivatives with active group and Synthesis of tracers (fluorescein labeled antigen)

**FITC**

\[ \text{Ag-NH}_2 + \text{FITC} \rightarrow \text{Ag-NH-CS-NH-Fluorescein} \]

\[ \text{NH}_2-\text{CH}_2\text{CH}_2-\text{NH}_2 + \text{FITC} \rightarrow \]

\[ \text{NH}_2-\text{CH}_2\text{CH}_2-\text{NH-CS-NH-Fluorescein (NH}_2\text{-EDF)} \]

**EDF**

\[ \text{Ag-COOH} \rightarrow \text{Ag-CO-Z} \]

\[ \text{Ag-CO-Z} + \text{NH}_2\text{-EDF} = \text{Ag-CO-NH-EDF} \]
Amino-Fluorescein derivatives

$\text{NH}_2-(\text{CH}_2)_n-\text{Fluorescein}$

$n = 6, 4, 2, 0$

HDF
BDF
AF
EDF
TLC purification for tracer
FPIA for DiBP (Di-iso-Buthyl-Phthalate)

GDUT, 15 July 2015

The binding of 5B8 MAb with the BVA–AMF tracer in BB working solution

FPIA calibration curve and linear fitting for BPA using the 5B8 MAb and BVA-AMF tracer
The measurement of an individual sample can be carried out within 4 min.
Carbamazepine main drug contaminants in river water

CBZ FPIA calibration curves (black solid lines), precision profiles (blue dashed lines) and measurement ranges (intersection points at 30% relative error of concentration, dotted red lines) determined on MTP (A) and in tubes (B).

Figure 1. Synthesis process of three tracers (BFNB-AF, BFNB-EDF and BFNB-HDF).

Figure 5. The standard curve of three tracers.
The limit of detection (10% inhibition) of the FPIA was 9.3 ng/mL

The maximum amount of melamine allowed is 1 mg kg⁻¹ in powdered infant formula.
Linlin Ren, Meng Meng, Peng Wang, Zhihuan Xu, Sergei A. Eremin, Junhong Zhao, Yongmei Yin, Rimo Xi. Determination of sodium benzoate in food products by fluorescence polarization immunoassay. Talanta, 121, 136-143 (2014).

The IC50 value for sodium benzoate is 2.48 μg/mL.
Conclusion

- Careful selection of immunogens for Ab production and tracers with different bridge between analyte and fluorescein could be useful for development sensitive, specific or group-specific.
Acknowledgements

The research was supported by Grant of Russian Foundation for Basic Research (14-03-00753)
Mycotoxins in corn
(Maximum Permit Limit – MPL)

- Zearalenone (ZEN)
  MPL 20 - 200 ng/g
- Ochratoxin (OTA)
  MPL 0.5 - 10 ng/g
- Deoxynivalenol (DON)
  MPL 20 - 1750 ng/g
- Aflatoxin B1 (AFB1)
  MPL 0.1 - 20 ng/g
Synthesis of fluorescein labelled antigens (tracers) for Zearalenone

Ag-COOH + NH2-Z-Fluorescein → Ag-CO-NH-Z-Fluorescein
Derivatives of Fluorescein with NH2-group for ZEA-tracers
Structure of tracer for Zearalenone (ZEN-CMO-EDF)
Preliminary FPIA standard curves for Zearalenone (ZEN) with different tracers

- IC$_{50}$ 15 ng/mL
- IC$_{50}$ 55 ng/mL
- IC$_{50}$ 36 ng/mL
- IC$_{50}$ 67 ng/mL
- IC$_{50}$ 15 ng/mL

ZEN, ng/mL

- ZEN-EDF
- ZEN-PIP
- ZEN-GMF
- ZEN-GAF
- ZEN-AMF

mP/mP$_0$

ZEN, ng/mL
FPIA standard curve for Zearalenone (ZEN)

Limit of Detection - 3 ng/mL

Range of linearity - 4 - 40 ng/mL

DON <0.1%
AFB1 <0.1%
OTA <0.1%
Derivatives of Fluorescein with NH2-group for DON-tracers

AF

de-EDF

AMF

H2N
(CH2)2

H2N
H2N
(S
HN
HN

H2N

H2N
FPIA standard curve for DON

Limit of Detection - 36 ng/mL

Range of linearity – 125-1000 ng/mL

AFB1 <0,1%
OTA <0,1%
ZEN <0,1%
NIV <0,1%
Derivatives of Fluorescein with NH2-group for AFB1-tracers

AF

EDF

H₂N
(CH₂)₂
Preliminary FPIA standard curves for Aflatoxin B1 (AFB1) with 5 different Antibodies and 2 different tracers
FPIA standard curves for Aflatoxin B1 (AFB1) with Mono- and Polyclonal Antibodies

Limit of Detection - 1.5 ng/mL

Range of linearity - 2 – 8 ng/mL

IC$_{50}$ = 4 ng/mL

IC$_{50}$ = 14 ng/mL

Monoclonal Ab anti-AFB1
Polyclonal Ab anti-AFB1

Limit of Detection - 1.5 ng/mL
Range of linearity - 2 – 8 ng/mL
Cross-reactivity for detection of AFB1 by FPIA

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>100%</td>
</tr>
<tr>
<td>AFB2</td>
<td>33.8%</td>
</tr>
<tr>
<td>AFG1</td>
<td>31.7%</td>
</tr>
<tr>
<td>AFG2</td>
<td>23.5%</td>
</tr>
<tr>
<td>DON</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>ZEN</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>OTA</td>
<td>&lt;0.1%</td>
</tr>
</tbody>
</table>

**Graph:**
- mP/mP₀, % vs. Mycotoxin, ng/mL
- Different symbols represent different mycotoxins.
- AFB1 shows 100% cross-reactivity, while others show varying degrees of cross-reactivity.
FPIA standard curves for AFB1 with monAb and recombinant Fab-fragment of MonAb

- **IC$_{50}$ = 1000 ng/mL**
- **IC$_{50}$ = 120 ng/mL**
Derivatives of Fluorescein with NH2-group for OTA-tracers

EDF
AMF
Lys-FITC
Preliminary FPIA standard curves for Ochratoxin A (OTA) with different Ab and tracers

IC$_{50}$ = 5 ng/mL

IC$_{50}$ = 14 ng/mL
FPIA standard curve for Ochratoxin A (OTA)

Limit of Detection – 2 ng/mL

Range of linearity - 4 – 70 ng/mL
Specificity of FPIA standard curve for Ochratoxin A (OTA)

- 100% specificity for OTA
- 100% specificity for other mycotoxins
- <0.1% specificity for other mycotoxins

Mycotoxin, ng/mL
### Parameters for FPIA of mycotoxins

<table>
<thead>
<tr>
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<th>Ab</th>
<th>Tracer</th>
<th>LOD, ng/mL</th>
<th>Range, ng/mL</th>
<th>MPL, ng/g</th>
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</thead>
<tbody>
<tr>
<td>ZEN</td>
<td>MAb</td>
<td>EDF</td>
<td>3</td>
<td>4-40</td>
<td>100</td>
</tr>
<tr>
<td>DON</td>
<td>MAb</td>
<td>AMF</td>
<td>36</td>
<td>125-1000</td>
<td>1250</td>
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<tr>
<td>AF</td>
<td>PAb</td>
<td>EDF</td>
<td>1.5</td>
<td>2.1-8.2</td>
<td>4</td>
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<tr>
<td>OTA</td>
<td>MAb</td>
<td>AMF</td>
<td>2</td>
<td>4-70</td>
<td>5</td>
</tr>
</tbody>
</table>
Sample pre-treatment for FPIA

- Corn sample

- 3 g corn + 15 mL eluent.
- Centrifugation

- Eluent: Methanol:water = 6:4

- FPIA for supernatant
Multi-FPIA

1 sample

OTA  AFB1   ZEN  DON

4 mycotoxins
Parameters for FPIA of mycotoxins in corn samples

<table>
<thead>
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<th></th>
<th>Ab</th>
<th>Tracer</th>
<th>LOD, ng/mL</th>
<th>LOD, ng/g</th>
<th>Range, ng/mL</th>
<th>MPL ng/g</th>
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<td>EDF</td>
<td>3</td>
<td>15</td>
<td>20-200</td>
<td>100</td>
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<tr>
<td>DON</td>
<td>MAb</td>
<td>AMF</td>
<td>36</td>
<td>180</td>
<td>625-5000</td>
<td>1250</td>
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<tr>
<td>AF</td>
<td>PAb</td>
<td>EDF</td>
<td>1.5</td>
<td>7.5</td>
<td>10-40</td>
<td>4</td>
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<tr>
<td>OTA</td>
<td>MAb</td>
<td>AMF</td>
<td>2</td>
<td>10</td>
<td>20-350</td>
<td>5</td>
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## Recovery of mycotoxins in corn samples by FPIA

<table>
<thead>
<tr>
<th>Spiked sample 1</th>
<th>Mycotoxin</th>
<th>Added, ng/mL</th>
<th>Founded, ng/mL</th>
<th>Recovery, %</th>
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<tr>
<td>ZEN</td>
<td>100</td>
<td>91±5</td>
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<td>91</td>
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<tr>
<td>DON</td>
<td>1000</td>
<td>960±50</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>AFB1</td>
<td>20</td>
<td>18.5±0.9</td>
<td></td>
<td>93</td>
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<tr>
<td>OTA</td>
<td>100</td>
<td>97±5</td>
<td></td>
<td>97</td>
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<td>Spiked sample 2</td>
<td>ZEN</td>
<td>150</td>
<td>140±7</td>
<td>93</td>
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<tr>
<td>DON</td>
<td>3000</td>
<td>2850±150</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>AFB1</td>
<td>30</td>
<td>28±1</td>
<td></td>
<td>94</td>
</tr>
<tr>
<td>OTA</td>
<td>300</td>
<td>280±10</td>
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<td>95</td>
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<tr>
<td></td>
<td>ZEN</td>
<td>DON</td>
<td>AFB1</td>
<td>OTA</td>
</tr>
<tr>
<td>----------------</td>
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<td>--------</td>
<td>--------</td>
<td>--------</td>
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<tr>
<td>Wheat 1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>7.5±0.5</td>
<td>n.d.</td>
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<tr>
<td>Wheat 2</td>
<td>38±1</td>
<td>750±40</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>Wheat 3</td>
<td>n.d.</td>
<td>280±20</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>n.d.</td>
<td>n.d.</td>
<td>13±1</td>
<td>n.d.</td>
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<tr>
<td>Wheat 6</td>
<td>100±5</td>
<td>n.d.</td>
<td>17.5±2</td>
<td>n.d.</td>
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Principle of Fluorescence Polarization Immunoassay

Standard FPIA

\[ \text{Ab} \xrightarrow{\text{Ag}} \text{Ab-Ag-F} \xrightarrow{\text{Ag-F}} \text{Ab-Ag} \]

Single-Reagent FPIA

\[ \text{Ab} \xrightarrow{\text{Ag-F}} \text{Ab-Ag-F} \xleftarrow{\text{Ag}} \text{Ab-Ag-F} \]
Kinetic of association (Ag* + Ab = Ag*:Ab) and dissociation of immuno-complex (Ag*:Ab + Ag = Ag:Ab + Ag*) for Chloramphenicol
Displacement of tracer from SR

Chloramphenicol (ug/mL)
- 0
- 1
- 10
- 100
- 1000

mP

t (min)
Single-Reagent Standard Curve for FPIA of Chloramphenicol

Chloramphenicol (mg/mL)
Thanks for attention
Fluoroquinolones
Enrofloxacin (ENR)  Norfloxacin (NOR)
Ofloxacin (OFL)  Ciprofloxacin (CIP)

Specific and group-Specific detection
Specific and group-specific immunoassays for FQ