Sample clean-up using immunoaffinity columns prior to detection with HPLC or LC-MS/MS

Speaker: Claire Milligan
Date: 29th September 2011
Principle of immunoaffinity columns

• Affinity chromatography is a method of separating mixtures based on a highly specific interaction between an antigen and an antibody.

• Immunoaffinity columns can be used prior to injection on an HPLC or LS-MS/MS system to selectively isolate and concentrate an antigen from a sample.

• R-Biopharm currently offers immunoaffinity columns for the detection of mycotoxins, vitamins and antibiotics in food and feed samples.
Principle of immunoaffinity columns

- An immunoaffinity column contains a gel suspension of antibody specific to the antigen of interest.

- Following extraction of the antigen the sample extract is diluted and passed through the immunoaffinity column.

- Any antigen which is present in the sample is retained by the antibody within the gel suspension.

- The column is washed to remove unbound material and the antigen is then released following elution with solvent.

- The eluate is collected prior to analysis by HPLC or LC-MS/MS.
Principle of immunoaffinity columns

**SAMPLE**
- The sample extract is passed through the column.

**WASHING**
- The antibody isolates and concentrates the antigen and retains it in the column.

**ELUTION**
- Passage of solvent through the column denatures the antibody and releases the antigen.

- ▲ Antigen
- ◆◆◆ Other material
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Principle of immunoaffinity columns

• The result is improved clean-up and concentration of the antigen from food and feed samples giving a much cleaner chromatogram and therefore providing more accurate and sensitive detection.

Chromatogram obtained without IAC clean-up for T-2 & HT-2

Chromatogram obtained without IAC clean-up for T-2 & HT-2
Introduction to mycotoxins

- More than 5 billion people in developing countries worldwide are at risk of chronic exposure to aflatoxins through contaminated food and medicinal plants.
- The presence of mycotoxins in food is unavoidable.
- Mycotoxins impair human health and cause economic losses in livestock due to the loss or reduction of livestock or in treating animals or humans suffering from the effects of mycotoxins.
- In 1988 the International Agency for Research on Cancer (IARC) placed aflatoxin B1 on the list of human carcinogens.
Introduction to mycotoxins

- FAO estimates that 25% of the world food crops are affected by mycotoxins each year.
- Crop loss due to aflatoxins contamination costs US producers more than $100 million per year on average including $26 million for peanuts alone.
Introduction to mycotoxins

- To ensure that food entering or being produced within a country contains little or no mycotoxins, there is the need to set official legislative levels for mycotoxins.
- For mycotoxins there are already EU limits for total aflatoxins, aflatoxins B1, aflatoxin M1, ochratoxin A, zearalenone, DON, fumonisn and patulin. EU legislative levels for T-2 & HT-2 are pending.
Introduction to mycotoxins

- Over 70 countries worldwide currently have legislation in place for mycotoxins.

Introduction to mycotoxins

- To reduce the chance of mycotoxins from entering the food chain it is important to test not only raw commodities but also processed food and feed.
- To achieve accurate results it is necessary to ensure correct sampling, extraction and to use high quality, validated test kits for screening and for quantitative analysis.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

Option of results required:

**Quantitative analysis**
(result given in form X ppb)
with HPLC and fluorescence detection

**Semi quantitative analysis**
(result given in form >X <Y ppb)
visual reading of fluorescence, TLC, fluorometry

**Qualitative analysis**
(result given in form < or > X ppb)
visual reading of cards
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

Option of results required:

- **Quantitative analysis**
  
  (result given in form $X$ ppb)
  
  with HPLC and fluorescence detection

- **Semi quantitative analysis**
  
  (result given in form $>X <Y$ ppb)
  
  visual reading of fluorescence, TLC, fluorometry

- **Qualitative analysis**
  
  (result given in form $<\text{ or } >X$ ppb)
  
  visual reading of cards
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

Immunoaffinity columns suit laboratories with highly trained personnel and well equipped facilities that need to meet the following requirements -

- Quantitative kits which comply with EU and international legislative limits.
- Accurate results.
- Ability to analyse different food commodities.
- Suitable for use with a range of methods in order to give optimum results.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

AFLAPREP®, EASI-EXTRACT® AFLATOXIN, AFLAPREP® M

OCHRAPREP®

AFLAOCHRA PREP®

EASI-EXTRACT® ZEARALENONE

DONPREP®

FUMONIPREP®

EASI-EXTRACT® T-2 & HT-2

DZT MS-PREP®
Sample clean-up using IAC prior to HPLC or LC-MS/MS

### Analysis of mycotoxins

<table>
<thead>
<tr>
<th></th>
<th>AFLAPREP®</th>
<th>EASI-EXTRACT® AFLATOXIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product code</td>
<td>P07, P07/500 (50 or 500 columns)</td>
<td>RP70, RP70N, RP70/500 (10 or 50 or 500 columns)</td>
</tr>
<tr>
<td>Format</td>
<td>1ml column</td>
<td>3ml column</td>
</tr>
<tr>
<td>Antibody</td>
<td>Monoclonal Antibody (B1, B2, G1, G2)</td>
<td>Monoclonal Antibody (B1, B2, G1, G2)</td>
</tr>
<tr>
<td>Extraction</td>
<td>Methanol / acetonitrile / chloroform</td>
<td>Methanol / acetonitrile</td>
</tr>
<tr>
<td>Wash solution</td>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>Elution</td>
<td>1ml of 100% methanol</td>
<td>1.5ml of 100% methanol</td>
</tr>
<tr>
<td>Evaporation</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Derivatisation</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Detection</td>
<td>Fluorescence HPLC</td>
<td>Fluorescence HPLC</td>
</tr>
</tbody>
</table>
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

<table>
<thead>
<tr>
<th></th>
<th><strong>AFLAPREP® M</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Product code</strong></td>
<td>P04 (25 columns)</td>
</tr>
<tr>
<td><strong>Format</strong></td>
<td>1ml column</td>
</tr>
<tr>
<td><strong>Antibody</strong></td>
<td>Monoclonal Antibody (M1)</td>
</tr>
<tr>
<td><strong>Extraction</strong></td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Wash solution</strong></td>
<td>PBS</td>
</tr>
<tr>
<td><strong>Elution</strong></td>
<td>1.25ml of 40% methanol: 60% acetonitrile</td>
</tr>
<tr>
<td><strong>Evaporation</strong></td>
<td>No</td>
</tr>
<tr>
<td><strong>Derivatisation</strong></td>
<td>No</td>
</tr>
<tr>
<td><strong>Detection</strong></td>
<td>Fluorescence HPLC</td>
</tr>
</tbody>
</table>
Analysis of mycotoxins

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OCHRAPREP®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product code</td>
<td>P14, P14B, P14/500 (10 or 50 or 500 columns)</td>
</tr>
<tr>
<td>Format</td>
<td>3ml column</td>
</tr>
<tr>
<td>Antibody</td>
<td>Monoclonal Antibody (OTA)</td>
</tr>
<tr>
<td>Extraction</td>
<td>Methanol / sodium bicarbonate</td>
</tr>
<tr>
<td>Wash solution</td>
<td>PBS</td>
</tr>
<tr>
<td>Elution</td>
<td>1ml of 98% methanol : 2% acetic acid</td>
</tr>
<tr>
<td>Evaporation</td>
<td>No</td>
</tr>
<tr>
<td>Derivatisation</td>
<td>No</td>
</tr>
<tr>
<td>Detection</td>
<td>Fluorescence HPLC</td>
</tr>
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</table>
### Analysis of mycotoxins

<table>
<thead>
<tr>
<th></th>
<th>DONPREP®</th>
<th>EASI-EXTRACT® ZEARALENONE</th>
<th>FUMONIPREP®</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Product code</strong></td>
<td>P50, P50B, P50/500 (10, 50 or 500 columns)</td>
<td>RP90, RP90N, RP90/500 (10, 50 or 500 columns)</td>
<td>P31, P31/500 (25 or 250 columns)</td>
</tr>
<tr>
<td><strong>Format</strong></td>
<td>3ml column</td>
<td>3ml column</td>
<td>3ml column</td>
</tr>
<tr>
<td><strong>Antibody</strong></td>
<td>Monoclonal antibody (DON)</td>
<td>Monoclonal antibody (ZON)</td>
<td>Monoclonal antibody (FUM B1, B2, B3)</td>
</tr>
<tr>
<td><strong>Extraction</strong></td>
<td>Water</td>
<td>Acetonitrile</td>
<td>Acetonitrile : methanol : water</td>
</tr>
<tr>
<td><strong>Wash solution</strong></td>
<td>H₂O</td>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td><strong>Elution</strong></td>
<td>1.5ml of 100% methanol</td>
<td>1.5ml of 100% acetonitrile</td>
<td>1.5ml of 100% methanol</td>
</tr>
<tr>
<td><strong>Evaporation</strong></td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Derivatisation</strong></td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Detection</strong></td>
<td>UV HPLC</td>
<td>Fluorescence HPLC</td>
<td>Fluorescence HPLC</td>
</tr>
</tbody>
</table>
## Analysis of mycotoxins

<table>
<thead>
<tr>
<th></th>
<th>EASI-EXTRACT® T-2 &amp; HT-2</th>
<th>AFLAOCHRA PREP®</th>
<th>DZT MS-PREP®</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Product code</strong></td>
<td>P43, P43B (10 or 50 columns)</td>
<td>P89, P89B (10 or 50 columns)</td>
<td>P73, P73B (10 or 50 columns)</td>
</tr>
<tr>
<td><strong>Format</strong></td>
<td>3ml column</td>
<td>1ml column</td>
<td>1ml column</td>
</tr>
<tr>
<td><strong>Antibody</strong></td>
<td>Monoclonal antibodies (T-2, HT-2)</td>
<td>Monoclonal Antibodies (B1, B2, G1, G2, OTA)</td>
<td>Monoclonal antibodies (DON, ZON, T-2, HT-2)</td>
</tr>
<tr>
<td><strong>Extraction</strong></td>
<td>Methanol</td>
<td>Methanol</td>
<td>Methanol</td>
</tr>
<tr>
<td><strong>Wash solution</strong></td>
<td>H₂O</td>
<td>PBS</td>
<td>H₂O</td>
</tr>
<tr>
<td><strong>Elution</strong></td>
<td>1.5ml of 100% methanol</td>
<td>1ml of 100% methanol</td>
<td>1ml of 100% methanol</td>
</tr>
<tr>
<td><strong>Evaporation</strong></td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Derivatisation</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Detection</strong></td>
<td>Fluorescence HPLC</td>
<td>Fluorescence HPLC</td>
<td>LC-MS/MS</td>
</tr>
</tbody>
</table>
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- Various methods have been developed for the analysis of aflatoxins in a wide range of food and feed samples.
  - Cereals
  - Nuts
  - Spices
  - Cocoa
  - Rice
  - Coconut
  - Animal feed
  - and many more…….
Analysis of mycotoxins

- The extraction method and solvent varies depending on the commodity of interest.

- For example, for extracting aflatoxin from peanuts a methanol extraction is more efficient while an acetonitrile extraction for cocoa is preferred.

- The antibody contained in the gel of an immunoaffinity column will have a particular tolerance to the level of solvent applied. If too much solvent is applied to the immunoaffinity column this can affect recoveries.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- Solvent tolerance information for AFLAPREP® and EASI-EXTRACT® AFLATOXIN –

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solvent Tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>30 %</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>2.5 %</td>
</tr>
</tbody>
</table>
## Analysis of mycotoxins

- Recovery information for EASI-EXTRACT® AFLATOXIN –

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Extraction Solvent</th>
<th>Spike Level</th>
<th>AFT B1 Recovery</th>
<th>AFT B2 Recovery</th>
<th>AFT G1 Recovery</th>
<th>AFT G2 Recovery</th>
<th>TOTAL AFT Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanuts</td>
<td>60% MeOH</td>
<td>10ppb</td>
<td>90 %</td>
<td>93 %</td>
<td>90 %</td>
<td>78 %</td>
<td>90 %</td>
</tr>
<tr>
<td>Maize</td>
<td>80% MeOH</td>
<td>4ppb</td>
<td>98 %</td>
<td>88 %</td>
<td>87 %</td>
<td>91 %</td>
<td>92 %</td>
</tr>
<tr>
<td>Paprika</td>
<td>80% MeOH</td>
<td>10ppb</td>
<td>94 %</td>
<td>102 %</td>
<td>93 %</td>
<td>102 %</td>
<td>98 %</td>
</tr>
<tr>
<td>Figs</td>
<td>80% MeOH</td>
<td>10ppb</td>
<td>81 %</td>
<td>80 %</td>
<td>79 %</td>
<td>75 %</td>
<td>79 %</td>
</tr>
<tr>
<td>Animal feed</td>
<td>60 % A CN</td>
<td>50ppb</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>102 %</td>
</tr>
</tbody>
</table>
Analysis of mycotoxins

• Example chromatogram for the analysis of paprika using EASI-EXTRACT® AFLATOXIN –
Analysis of mycotoxins

- Aflatoxins B2 and G2 fluoresce brightly under UV light and can easily be detected by HPLC with a fluorescence detector.
- Aflatoxins B1 and G1 do not fluoresce naturally to such a high degree and must be derivatised using iodine or bromine.
- During derivatisation the chemical structures of aflatoxin B1 and G1 is changed to a more fluorescent form, increasing the fluorescent signal in each case for detection by HPLC.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- Derivatisation of aflatoxin B1 and G1 can be carried out using various derivatising procedures and agents in order to enhance their fluorescence –
  - Trifluoracetic acid – derivatisation occurs prior to HPLC column.
  - Iodine – derivatisation occurs after the HPLC column but before the detector.
  - Pyridium bromide perbromide – derivatisation occurs after the HPLC column but before the detector.
  - KOBRA® CELL
  - PHRED / UVE System.
Analysis of mycotoxins

- Derivatisation by Trifluoracetic acid (TFA)
Analysis of mycotoxins

• Derivatisation by Trifluoracetic acid (TFA)

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-column derivatisation method only requires one HPLC pump</td>
<td>Highly manipulative and numerous steps means that this method requires experience to obtain good derivatisation</td>
</tr>
<tr>
<td>Traditional derivatisation method for aflatoxins</td>
<td>TFA is a harmful, corrosive chemical therefore regular handling should be discouraged</td>
</tr>
<tr>
<td></td>
<td>The incorporation of an evaporation step leads to aflatoxin losses</td>
</tr>
</tbody>
</table>
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- Derivatisation by Iodine
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- Derivatisation by Iodine

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Official and widely used method</td>
<td>Requires a second HPLC pump and water bath or column jacket which increases the capital cost</td>
</tr>
<tr>
<td>Reliable and accurate results</td>
<td>The saturated iodine solution needs to be freshly prepared daily because it is unstable</td>
</tr>
<tr>
<td>Requires no additional sample preparation following elution from immunoaffinity column</td>
<td>Maintenance and cleaning is important in order to avoid iodine crystallising in-line and blocking flow</td>
</tr>
</tbody>
</table>
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins
- Derivatisation by Pyridium bromide perbromide (PBPB)
# Analysis of mycotoxins

- Derivatisation by Pyridium bromide perbromide (PBPB)

## Advantages

<table>
<thead>
<tr>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Publications demonstrating reliable and accurate results</td>
<td>Requires a second HPLC pump which increases the capital cost</td>
</tr>
<tr>
<td>Requires no additional sample preparation following elution from immunoaffinity column</td>
<td>The PBPB solution can be difficult to dissolve</td>
</tr>
<tr>
<td>Short reaction time (only 30cm reaction coil required) at ambient temperature</td>
<td>Maintenance and cleaning is important in order to avoid PBPB crystallising in-line and blocking flow</td>
</tr>
<tr>
<td>No water bath is required</td>
<td>PBPB is hazardous to handle</td>
</tr>
<tr>
<td>Peak area is larger than with iodine derivatisation</td>
<td>Mostly used in the UK, not an internally used method</td>
</tr>
</tbody>
</table>
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- **KOBRA® CELL = KOk BRomine**

  Apparatus

- The KOBRA® CELL is a unique system which offers a popular alternative derivatisation method with testing aflatoxins in conjunction with HPLC.
The KOBRA® CELL is an electrochemical cell consisting of a platinum working electrode and a stainless steel auxiliary electrode separated from another by a membrane. These layers are sandwiched between a rigid plastic housing.

- The CELL is fitted between the HPLC column and the detector that generates the derivatisation agent, bromine, on-line from potassium bromide and nitric acid present in the mobile phase.
- The derivatisation results in a significant increase in the fluorescent signals of the modified forms of aflatoxin B1 and G1.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

• KOBRA® CELL structure.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- KOBRA® CELL structure.

Analysis of mycotoxins

- Analysis of vitamins
- Analysis of antibiotics

Conclusions

**PLEASE NOTE:**
fit all parts as illustrated otherwise the KOBRA® CELL may fail to operate correctly

R-Biopharm Group
Argentina • Australia • Austria • Brazil • Canada • China • France • Germany • Italy • Netherlands • Spain • Switzerland • UK • USA

September 2011
Analysis of mycotoxins

- Derivatisation by KOBRA® CELL

The length and diameter of tubing between CELL and the detector is critical to allow derivatisation to occur.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- KOBRA® CELL fitting guide
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

• Chromatograms of aflatoxin standards obtained with and without the KOBRA® CELL.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- Advantages of a KOBRA® CELL compared to traditional methods –
  - No daily preparation of derivatising reagents.
  - No second pump required.
  - No water bath or column heater required.
  - Comparable detection limit to other derivatisation methods.
  - Low maintenance electrochemical cell.
  - Easy to install.
  - Sharp peaks, no risk of peak broadening normally associated with the introduction of a derivatisation agent.
  - No odour, non-hazardous derivatising agent.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

• There are direct competitor products to the KOBRA® CELL available on the market –
  • Cobra Zelle or Coring Cell.
  • CK by BK or Le Cell Kerbaje.
  • Romer Cell or Romer Derivatisation Unit.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- All are based on the KOBRA® CELL and are made by Coring systems.

- Vicam originally sold a Vicam Cell however Coring now only deal with Romer and Libios.

- The word ‘Cobra’ with ‘Cell’ can’t be used as a name in any form as it trades on the KOBRA® CELL registration mark.
Analysis of mycotoxins

- There are some competitor photochemical derivatisation units available on the market –
  - PHRED by Aura Industries.
  - UVE by LC Tech.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

• PHRED – PHotochemical Reactor for Enhanced Detection of aflatoxins.

• Consists of a lamp holder, a low pressure mercury lamp at 254nm and PTFE reaction coils.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

• The PTFE reaction coils are 5 – 20cm in length.

• Delay coils 30m for peak separation.

• The aflatoxin B1 is converted to B2a by hydroxylation of the double bond (water is added to the aflatoxin molecule and excited by the UV light).

• The run time is only 9 minutes ex/e = 365 / 415nm.

• AFT / OTA / ZEA multi-mycotoxin method run time is 21 minutes ex/e = 315 / 415nm.

• The end user prices is approximately 1500 US $.

• Some published references, albeit quite old however highly credible.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

• The UVE derivatisation module is similar to PHRED.

• It consists of a low pressure mercury UV lamp = 9W at 254nm.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- Reactor loop is fluorocarbon, longer life than PTFE / Teflon.
- Lamp and reactor loop cooled by fan which is meant to extend the shelf life.
- Leak proof due to drain tube.
- CE certification.
- The end user prices is approximately 1800 €.
- The quartz coil will need replaced after about 9 months (medium use) due to loss of signal. This costs about 700 – 900 €. This is not covered by warranty.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

The disadvantages of the photochemical derivatisation units compared to the KOBRA® CELL –

- UV wavelength shifts and UV light diminishes over time = loss of effectiveness. And reduction in derivatisation
- PTFE becomes blocked due to age & UV damage.
- Complex knitting of reaction coils creates blank zones where UV light cannot reach.
- Column heater/jacket is required.
- Leaking & damage may be a problem.
- Breakdown time is significant due to frequent leaking.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- The advantages of the KOBRA® CELL compared to the photochemical derivatisation units –
  - KOBRA® CELL is the only device listed by CEN reference method for import control.
  - KOBRA® CELL is an officially recognized AOAC method.
  - KOBRA® CELL is tested under ISO 9001 Quality Manufacturing Systems.
  - KOBRA® CELL is recommended by several competitors.
Analysis of mycotoxins

- In recent years we have seen a need for analysis of multiple mycotoxins and different mycotoxin combinations.
- There is also more pressure being placed on analysts to test more therefore there is a need for faster and less labour intensive methods.
- Multi-mycotoxin immunoaffinity columns, which can be used in conjunction with LC-MS/MS offers the solution to these problems.
- LC-MS/MS is one of the most advanced techniques for mycotoxin analysis and many analysts and official labs are moving towards this technique.
Analysis of mycotoxins

• LC-MS/MS is a hyphenated technique, combining the separation power of HPLC, with the detection power of mass spectrometry.

• Even with a very sophisticated MS instrument, HPLC is required to remove the interferences from a sample that would impact on ionisation.

• In all cases there is the need for an interface (ion source) between the HPLC and MS. The ion source will eliminate the solvent from the HPLC eluant and generate gas phase ions, which are then transferred to the optics of the mass spectrometer.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- LC-MS/MS is particularly suitable for analysis of multi parameters.
- One extraction method can be used for the analysis of different mycotoxins enabling the simultaneous detection of more than one mycotoxin.
- However, extraction and clean-up techniques are often necessary prior to analysis in order to ensure well separated peaks without interference from matrix components.
- Immunoaffinity columns can also help to remove interfering components from a range of products which can otherwise result in quenching of the ions.
Analysis of mycotoxins

- Example ion chromatogram for the analysis of Dried Distillers Grain with and without clean-up with DZT MS-PREP®.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

September 2011

Analysis of mycotoxins

- Example ion chromatogram for the analysis of Dried Distillers Grain with and without clean-up with DZT MS-PREP®.

T-2 484.2 / 305.1  
DZT clean-up

T-2 484.2 / 305.1  
No clean-up

T-2 484.2 / 245.2  
DZT clean-up

T-2 484.2 / 245.2  
No clean-up
Sample clean-up using IAC prior to HPLC or LC-MS/MS

September 2011

Analysis of mycotoxins

- Example ion chromatogram for the analysis of Dried Distillers Grain with and without clean-up with DZT MS-PREP® -

**HT-2 442.1 / 263.1**

- **DZT clean-up**

- **HT-2 442.1 / 263.1**

- **No clean-up**

**HT-2 442.1 / 215.0**

- **DZT clean-up**

- **HT-2 442.1 / 215.0**

- **No clean-up**
Analysis of mycotoxins

- Example ion chromatogram for the analysis of Dried Distillers Grain with and without clean-up with DZT MS-PREP®.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Mass(es)</th>
<th>Comment</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDG IAC cleanup</td>
<td>ZON 317.1 / 131.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDG no cleanup</td>
<td>ZON 317.1 / 131.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDG IAC cleanup</td>
<td>ZON 317.1 / 175.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDG no cleanup</td>
<td>ZON 317.1 / 175.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

- Direct injection of complex matrices causes ion suppression therefore there is the requirement for some form of clean-up.
- Although solid phase clean-up columns are often used there is still some ion suppression, this is lowered when using immunoaffinity columns.
- There is a need to use matrix matched standards for direct injection or with solid phase clean-up however there is reduced requirement for matrix matched standards when using immunoaffinity columns.
## Analysis of mycotoxins

- Recovery information for DZT MS-PREP® –

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Extraction Solvent</th>
<th>Spike Level</th>
<th>DON Recovery</th>
<th>ZEA Recovery</th>
<th>T-2 Recovery</th>
<th>HT-2 Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>70% MeOH</td>
<td>800ppb DON, 80ppb ZEA, 100ppb T-2 &amp; HT-2</td>
<td>92%</td>
<td>94%</td>
<td>109%</td>
<td>106%</td>
</tr>
<tr>
<td>Wheat</td>
<td>70% MeOH</td>
<td>800ppb DON, 80ppb ZEA, 100ppb T-2 &amp; HT-2</td>
<td>98%</td>
<td>74%</td>
<td>103%</td>
<td>103%</td>
</tr>
<tr>
<td>Oats</td>
<td>82%</td>
<td>82%</td>
<td>82%</td>
<td>84%</td>
<td>99%</td>
<td>97%</td>
</tr>
<tr>
<td>Animal feed</td>
<td>80% ACN</td>
<td>5ppm DON, 1ppm ZEA, T-2 &amp; HT-2</td>
<td>87%</td>
<td>101%</td>
<td>104%</td>
<td>104%</td>
</tr>
<tr>
<td>Baby food</td>
<td>80% MeOH</td>
<td>5ppm DON, 1ppm ZEA, T-2 &amp; HT-2</td>
<td>91%</td>
<td>89%</td>
<td>106%</td>
<td>104%</td>
</tr>
<tr>
<td>Beer</td>
<td>N/A</td>
<td>20ppb DON, 2ppb ZEA, T-2 &amp; HT-2</td>
<td>89%</td>
<td>89%</td>
<td>99%</td>
<td>99%</td>
</tr>
</tbody>
</table>
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of mycotoxins

• Example ion chromatogram for the analysis of a cereal sample using DZT MS-PREP® –
Analysis of vitamins

- Vitamins cannot be synthesized in sufficient quantities and therefore must be obtained from the diet.
- Thirteen vitamins are currently universally recognized.
Analysis of vitamins

- The analysis of vitamins is carried out to check that foods have been fortified with the correct amount of vitamin.

- It should however, be noted that foods are generally over fortified with vitamin to account for losses due to degradation over time.

- Analysis of vitamins can often be problematic due to the very small levels of vitamin present and the complexity of the sample so method sensitivity and sample preparation are particularly important.

- Enzymes are used to cleave the protein and peptides from the food and release the vitamin.

- Immunoaffinity columns can then be used to selectively isolate and concentrate the vitamin from a sample.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of vitamins

EASI-EXTRACT® VITAMIN B12

EASI-EXTRACT® VITAMIN B12 (LGE)

EASI-EXTRACT® FOLIC ACID

EASI-EXTRACT® BIOTIN

- These immunoaffinity columns were produced since the detection of the above vitamins can be particularly problematic by HPLC or LC-MS/MS.
## Analysis of vitamins

<table>
<thead>
<tr>
<th></th>
<th>EASI-EXTRACT® VITAMIN B12</th>
<th>EASI-EXTRACT® VITAMIN B12 (LGE)</th>
<th>EASI-EXTRACT® FOLIC ACID</th>
<th>EASI-EXTRACT® BIOTIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product code</td>
<td>P80, P80B (10 or 50 columns)</td>
<td>P88, P88B (10 or 50 columns)</td>
<td>P81, P81B (10 or 50 columns)</td>
<td>P82, P82B (10 or 50 columns)</td>
</tr>
<tr>
<td>Format</td>
<td>3ml column</td>
<td>10ml column</td>
<td>3ml column</td>
<td>3ml column</td>
</tr>
<tr>
<td>Antibody</td>
<td>Monoclonal</td>
<td>Monoclonal</td>
<td>Monoclonal</td>
<td>Monoclonal</td>
</tr>
<tr>
<td>Extraction buffer</td>
<td>50 mM sodium acetate</td>
<td>50 mM sodium acetate</td>
<td>0.1M Phosphate buffer</td>
<td>0.1M Phosphate buffer</td>
</tr>
<tr>
<td>Enzymes</td>
<td>Pepsin, α-amylase, 1% KCN</td>
<td>Pepsin, α-amylase, 1% KCN</td>
<td>Pancreatin, 10% sodium ascorbate</td>
<td>Pancreatin, 10% sodium ascorbate</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>Water</td>
<td>Water</td>
<td>Water</td>
<td>PBS</td>
</tr>
<tr>
<td>Elution</td>
<td>100% methanol</td>
<td>100% methanol</td>
<td>Water containing 0.2% TFA : Acetonitrile (70 : 30 v/v)</td>
<td>Water containing 0.2% TFA : Acetonitrile (70 : 30 v/v)</td>
</tr>
<tr>
<td>Evaporation</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Detection</td>
<td>UV HPLC</td>
<td>UV HPLC</td>
<td>UV HPLC</td>
<td>UV HPLC</td>
</tr>
</tbody>
</table>
## Analysis of vitamins

- Recovery information for EASI-EXTRACT® VITAMIN 12 –

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Vitamin B12 Claim</th>
<th>Vitamin B12 Concentration Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B12 Tablets</td>
<td>2000 µg / 0.849 g</td>
<td>1990 µg / 0.849 g</td>
</tr>
<tr>
<td>Energy Drink</td>
<td>0.1 µg / 100ml</td>
<td>0.107 µg / 100ml</td>
</tr>
<tr>
<td>Milk Based Infant Feed</td>
<td>1.25 µg / 100 g</td>
<td>1.47 µg / 100 g</td>
</tr>
<tr>
<td>Protein Shake</td>
<td>5.26 µg / 100g</td>
<td>1.17 µg / 100 g</td>
</tr>
<tr>
<td>Yoghurt</td>
<td>3.5 µg / 100 g</td>
<td>5.42 µg / 100 g</td>
</tr>
<tr>
<td>Animal Feed</td>
<td>2 µg / 100g</td>
<td>3.08 µg / 100 g</td>
</tr>
</tbody>
</table>
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of vitamins

- In response to a series of customer requests,
  RBR have developed a protocol for Vitamin B12 determination in cocoa powder.
- Cocoa powder is a particularly difficult matrix to analyse.

Chromatogram obtained without sufficient clean-up
Analysis of vitamins

1. Weigh 5g of sample into an amber glass bottle & add 50ml 50mM sodium acetate buffer (pH4). Place on magnetic stirrer & begin stirring.
2. Add 2g pepsin, 0.5g alpha-amylase & 2ml 1% KCN solution & stir for 10 min.
3. Place sample in shaking water bath at 37°C for 30 min, then transfer to a 100°C shaking water bath for 30 min. Cool sample to room temperature.
4. Transfer sample to a 100ml volumetric flask & fill to mark with 50mM sodium acetate buffer and centrifuge the sample at 4000rpm for 15 minutes.
5. Apply 30ml of sample to EASI-EXTRACT® VITAMIN B12.
6. Wash with 20ml of 2% Tween 20 followed by a 10ml water wash.
7. Elute column with 3ml MeOH & evaporate to dryness at 60-70°C.
8. Reconstitute samples in 300µl 0.025% TFA & inject 100 µl onto HPLC.
Analysis of vitamins

- Example chromatogram for analysis of cocoa powder using EASI-EXTRACT® VITAMIN B12 –

![Chromatogram](image-url)
Analysis of antibiotics

- In some countries antibiotics are used to promote animal growth and to treat sick animals caused by poor hygiene conditions on farms.
- Antibiotic residue levels in edible tissues indicate drug misuse, are contrary to regulations and may have health and economic implications.
- Due to the toxicity of chloramphenicol and resistance to this antibiotic, it is no longer used as a first line agent.
- In most countries the drug is banned for use in food producing animals.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of antibiotics

• Surveillance and testing of antibiotics has increased therefore there is a need for a rapid, easy to perform and inexpensive test.

• To ensure compliance appropriate analytical methods are required.

• Analysis of antibiotics can often be problematic due to the very small levels present so methods which improve sensitivity and limits of detection are particularly important.
Analysis of antibiotics

- With certain matrices there can sometimes be problems with false positives results in ELISAs due to matrix effects.
- Complex matrices can also contain pigments making it difficult to detect low levels of chloramphenicol by HPLC or LC-MS/MS.
- Therefore, clean-up with immunoaffinity columns prior to HPLC or LC-MS/MS help to eliminate these issues and to improve detection.
## Analysis of antibiotics

<table>
<thead>
<tr>
<th>EASI-EXTRACT® CHLORAMPHENICOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product code</td>
</tr>
<tr>
<td>Format</td>
</tr>
<tr>
<td>Antibody</td>
</tr>
<tr>
<td>Extraction buffer</td>
</tr>
<tr>
<td>Wash buffer</td>
</tr>
<tr>
<td>Elution</td>
</tr>
<tr>
<td>Evaporation</td>
</tr>
<tr>
<td>Detection</td>
</tr>
</tbody>
</table>
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of antibiotics

- Weigh 5g of sample into a centrifuge tube.
- Add 5ml of McIlvanie buffer (pH 2.5).
- Vortex for 20 seconds or mix for 30 minutes on an orbital shaker.
- Double filter the extract through Whatman No. 113.
- Pass 2ml of extract through the column.
- Wash the column with 10ml of water.
- Elute the antibiotic from the column using 100% methanol (2ml if analysing by HPLC or 1ml if analysing by LC-MS/MS).
- For HPLC analysis, evaporate and reconstitute in mobile phase.
## Analysis of antibiotics

- Recovery information for EASI-EXTRACT® CHLORAMPHENICOL –

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Spike Level</th>
<th>HPLC Recovery</th>
<th>LC-MS/MS Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese Honey</td>
<td>48 ppb</td>
<td>104 %</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.48 ppb</td>
<td>-</td>
<td>96 %</td>
</tr>
<tr>
<td>Brazilian Honey</td>
<td>48 ppb</td>
<td>106 %</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.51 ppb</td>
<td>-</td>
<td>100 %</td>
</tr>
<tr>
<td>Bee Pollen</td>
<td>0.51 ppb</td>
<td>-</td>
<td>83 %</td>
</tr>
<tr>
<td>Royal Jelly</td>
<td>0.54 ppb</td>
<td>-</td>
<td>85 %</td>
</tr>
<tr>
<td>Prawn</td>
<td>0.15 ppb</td>
<td>-</td>
<td>85 %</td>
</tr>
<tr>
<td>Crab</td>
<td>0.3 ppb</td>
<td>-</td>
<td>95 %</td>
</tr>
</tbody>
</table>
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of antibiotics

• Example chromatogram for HPLC analysis of honey using

EASI-EXTRACT® CHLORAMPHENICOL—
Analysis of antibiotics

• Example chromatogram for LC-MS/MS analysis of prawn using EASI-EXTRACT® CHLORAMPHENCIOl–
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of antibiotics

• RBR took part in a recent FAPAS round (02169 for prawns) with the EASI-EXTRACT® CHLORAMPHENICOL columns.

• RBR were lab number 67 and we obtained a Z-score of 0.

• We reported a value of 1.89ppb, the assigned value of the sample was also 1.89ppb.
Analysis of antibiotics

• Overview of Z-scores for FAPAS round 02169 -

Figure 1: Z-Scores for Chloramphenicol
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Analysis of antibiotics

• For HPLC analysis chloramphenicol is detected using UV detector set at a low wavelength of 280 nm. Due to the low wavelength there can be a lot of background noise on the chromatogram.
• The LOD of the honey HPLC method is 20 ng/ml.
• The LOQ of the honey HPLC method is 60 ng/ml.
• The use of immunoaffinity columns in conjunction with LC-MS/MS offers lower detection limits compared to HPLC analysis.
• The LOD of the LC-MS/MS method is 0.2 ng/ml.
• The LOQ of the LC-MS/MS method is 0.6 ng/ml.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Conclusions

- Columns are manufactured to ISO 9001 Quality Standards and are accompanied by Certificate of Conformance.
- Columns can be stored at room temperature.
- Technical support and training is available from an experienced team.
- RBR also provide a range of application notes to aid analysis.
Sample clean-up using IAC prior to HPLC or LC-MS/MS

Thank you for your Attention