

DONPREP[®]

Product Code: P50 / P50B

Immunoaffinity columns for use in conjunction with HPLC or LC-MS/MS.
For in vitro use only.

P50/V18/27.05.22

www.r-biopharm.com



R·BIOPHARM
RHÔNE LTD

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Test Principle

The procedure is based on monoclonal antibody technology, which makes the test highly specific, sensitive, rapid and simple to perform.

The columns contain a gel suspension of monoclonal antibody specific to the toxin of interest. Following extraction of the toxin the sample extract is filtered, diluted and passed slowly through the immunoaffinity column. Any toxin 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 toxin is then released from the column following elution with solvent. The eluate is collected, evaporated and reconstituted prior to analysis by HPLC or LC-MS/MS.

The total extraction and clean-up time takes approximately 20 minutes to perform. The result is improved clean-up and concentration of the toxin from food and feed samples giving a much cleaner chromatogram and therefore providing more accurate and sensitive detection. The columns also have the added advantage that they can be automated for large scale analysis of samples.

Reagents Not Provided

- Distilled / Deionised Water (suitable for use with HPLC, e.g. MilliQ)
- Solvents (HPLC Grade Methanol and Acetonitrile)
- Deoxynivalenol Standard (Please refer to Preparation of Standards section)
- Sodium Hydroxide (to pH filtrate if required)
- Sodium Chloride

Accessory Products

- Whatman No. 113 or No. 4 Filter Paper
- Glass Microfibre Filter Paper
- Immunoaffinity Column Rack (CR1)*
- Immunoaffinity Column Accessory Pack (AP01)*

* Available from R-Biopharm. Please contact your local R-Biopharm distributor for further information.

Hazards

Mycotoxins are very hazardous substances. Only laboratories equipped to handle toxic materials and solvents should perform analyses. Suitable protective clothing, including gloves, safety glasses and lab coats should be worn throughout the analysis.

Flammable solvents should be stored in an explosion-proof cabinet. Use a chemical hood and protective equipment as applicable.

Contact your local R-Biopharm distributor for a Material Safety Data Sheet for further information if required.

Recommended Methods and Application Notes

Methods are available for all matrices covered by legislation as well as additional commodities. Deviation from the methods described in our Instructions for Use and Application Notes may not achieve optimum results. Please contact your local R-Biopharm distributor for further information.

Decontamination

Prior to disposal, excess standard solutions should be treated with at least one-tenth their volume of 5 % sodium hypochlorite. Labware and contaminated waste should be immersed in 5 % sodium hypochlorite solution for 30 minutes followed by the addition of 5 % acetone for 30 minutes. Flush with copious amounts of water before disposal. After decontamination labware should be thoroughly washed. Incinerate waste if regulations permit.

Storage & Shelf Life

The columns expire 18 months from date of manufacture if stored at 2 - 8 °C or 12 months from date of manufacture if stored at 21 - 25 °C. Do not freeze.

Ensure the column has not dried out and contains buffer above the gel. It is important to note the antibody included in the immunoaffinity column can be denatured by extreme temperature or pH change.

Sampling

A representative sample should be obtained by following one of the officially recognised sampling procedures. It is recommended that a minimum of 1 kg of representative sample is finely ground and a portion (5 - 50 g dependent on method used) of this is removed and extracted.

Sensitivity

The sensitivity is dependent on the final detection system employed by the analyst. However the test sensitivity may be improved if required by increasing the volume of sample passed through the immunoaffinity column. Please note the ratio of solvent to phosphate buffered saline (PBS) should be maintained.

Recoveries

If an analyst wishes to account for losses during extraction it is recommended a spiked sample of the same commodity type as the material being tested is analysed following the complete procedure as a reference standard. The recoveries obtained with the spiked sample can be used to correct the results obtained with the test sample.

Column Preparation

Immunoaffinity columns should be at ambient temperature before use. Remove the cap from the top of the column and discard. Firmly attach the column to a glass syringe barrel using an adapter and place in an immunoaffinity column rack or clamp stand.

Elution

In order to fully elute the toxin/s from the immunoaffinity column it is vital that the solvent is in contact with the antibody within the gel suspension for a sufficient period of time. This ensures that all of the bonds between the antibody and the toxin are broken, ultimately releasing all of the toxin from the column for analysis with the detection system of choice

To ensure that the solvent is in contact with the antibody gel for a sufficient period of time any of the following elution methods can be used: -

Backflushing (this is the preferred method of choice at R-Biopharm): backflush by gently raising and lowering the syringe plunger during passage of the solvent through the column. This process will reverse the direction of flow of the eluate through the gel. This should be repeated 3 times before collecting the eluate. Proceed to the next step in the method.

Application of small volumes of solvent: apply the volume of solvent required for elution in two or three smaller aliquots. Allow each aliquot to remain in contact with the gel suspension for a minimum of 30 seconds before allowing each to pass fully through the gel suspension for collection. Proceed to the next step in the method.

Incubation with solvent: apply the full volume of solvent required for elution and allow 2-3 drops of the solvent to pass through the column for collection. Allow the remainder of the solvent to remain in contact with the gel suspension for a minimum of 60 seconds before allowing it to pass through the gel suspension for collection. Proceed to the next step in the method.



Sample Preparation

• Cereal

This method has been tested on a number of cereals including wheat, barley, maize and cereal based products.

1. Weigh 25 g of ground sample and 5 g of sodium chloride into a 1 litre capacity, solvent resistant blender jar.
2. Add 200 ml of water and blend at high speed for 2 minutes.
3. Filter the sample through Whatman No. 113 or No. 4 filter paper, or centrifuge at 4,000 rpm for 10 minutes.
4. Filter through glass microfibre filter paper.
5. Pass 2 ml of the filtrate (equivalent to 0.25 g of sample) through the column at a flow rate of 2 ml per minute (or the sample can be allowed to pass through the column by gravity if preferred). A slow, steady flow rate is essential for the capture of the toxin by the antibody.
6. Wash the column by passing 10 ml of water through at a flow rate of approximately 5 ml per minute. Pass air through the column to remove residual liquid.
7. Elute the toxin from the column at a flow rate of 1 drop per second using 1.5 ml of 100 % methanol and collect in an appropriate vessel. Please refer to the Elution section for further information.

HPLC:

8. Evaporate the eluate to dryness under air at 60 - 70 °C.
9. Reconstitute with 1 ml of 15 % methanol. Vortex for 20 seconds.
10. Inject 100 µl of reconstituted eluate onto the HPLC system.

LC-MS/MS:

8. Following elution pass 1.5 ml of water through the column and collect in the same vessel to give 3 ml total volume.
9. Inject 10 µl of eluate onto the LC-MS/MS system.

Sample Preparation

• Animal Feed

1. Weigh 25 g of ground sample and 5 g of sodium chloride into a 1 litre capacity, solvent resistant blender jar.
2. Add 200 ml of water and blend at high speed for 2 minutes.
3. Centrifuge the sample at 4,000 rpm for 10 minutes.
4. Adjust pH to around 7.4 using 2 M sodium hydroxide.
5. Filter the supernatant through a glass microfibre filter paper.
6. If analysing at levels of 6,000 ppb or below, pass 2 ml of the filtrate (equivalent to 0.25 g of sample) through the column at a flow rate of 2 ml per minute (or the sample can be allowed to pass through the column by gravity if preferred). A slow, steady flow rate is essential for the capture of the toxin by the antibody.

or

If analysing at levels of greater than 6,000 ppb, dilute 2 ml of the filtrate with 2 ml of water.

Pass 2 ml of the diluted filtrate (equivalent to 0.125 g of sample) through the column at a flow rate of 2 ml per minute (or the sample can be allowed to pass through the column by gravity if preferred). A slow, steady flow rate is essential for the capture of the toxin by the antibody.

7. Wash the column by passing 10 ml of water through at a flow rate of approximately 5 ml per minute. Pass air through the column to remove residual liquid.
8. Elute the toxin from the column at a flow rate of 1 drop per second using 1.5 ml of 100 % methanol and collect in an appropriate vessel. Please refer to the Elution section for further information.

HPLC:

9. Evaporate the eluate to dryness under air at 60 - 70 °C.
10. Reconstitute with 1 ml of 15 % methanol. Vortex for 20 seconds.
11. Inject 100 µl of reconstituted eluate onto the HPLC system.

LC-MS/MS:

9. Following elution pass 1.5 ml of water through the column and collect in the same vial to give 3 ml total volume.
10. Inject 10 µl of eluate onto the LC-MS/MS system.

Preparation of Standards

It is advised to start with a 100,000 ng/ml deoxynivalenol stock solution.

Calibration Curve

It is recommended to run at least a 3 - 6 point calibration curve. In constructing a suitable curve the levels of the calibration standards should bracket or include the range of expected results. The diluted standard solutions should be prepared fresh on the day of analysis and used within a 24 hour period.

Example of how to prepare a six point calibration curve (can be modified according to legislative requirements or contamination levels):

HPLC:

1. Add 200 μ l of reconstituted crystalline standard to 800 μ l of 100 % acetonitrile (equivalent to 20,000 ng/ml).
2. Evaporate 200 μ l of the solution to dryness under air at 60 - 70 °C.
3. Standard 6: Reconstitute with 2 ml of 15 % methanol (equivalent to 2,000 ng/ml).
4. Standard 5: Take 1 ml of Standard 6 and add 1 ml of 15 % methanol (equivalent to 1,000 ng/ml).
5. Standard 4: Take 1 ml of Standard 5 and add 1 ml of 15 % methanol (equivalent to 500 ng/ml).
6. Standard 3: Take 1 ml of Standard 4 and add 1 ml of 15 % methanol (equivalent to 250 ng/ml).
7. Standard 2: Take 1 ml of Standard 3 and add 1 ml of 15 % methanol (equivalent to 125 ng/ml).
8. Standard 1: Take 1 ml of Standard 2 and add 1 ml of 15 % methanol (equivalent to 62.5 ng/ml).
9. Inject 100 μ l of each solution onto the HPLC system.

LC-MS/MS:

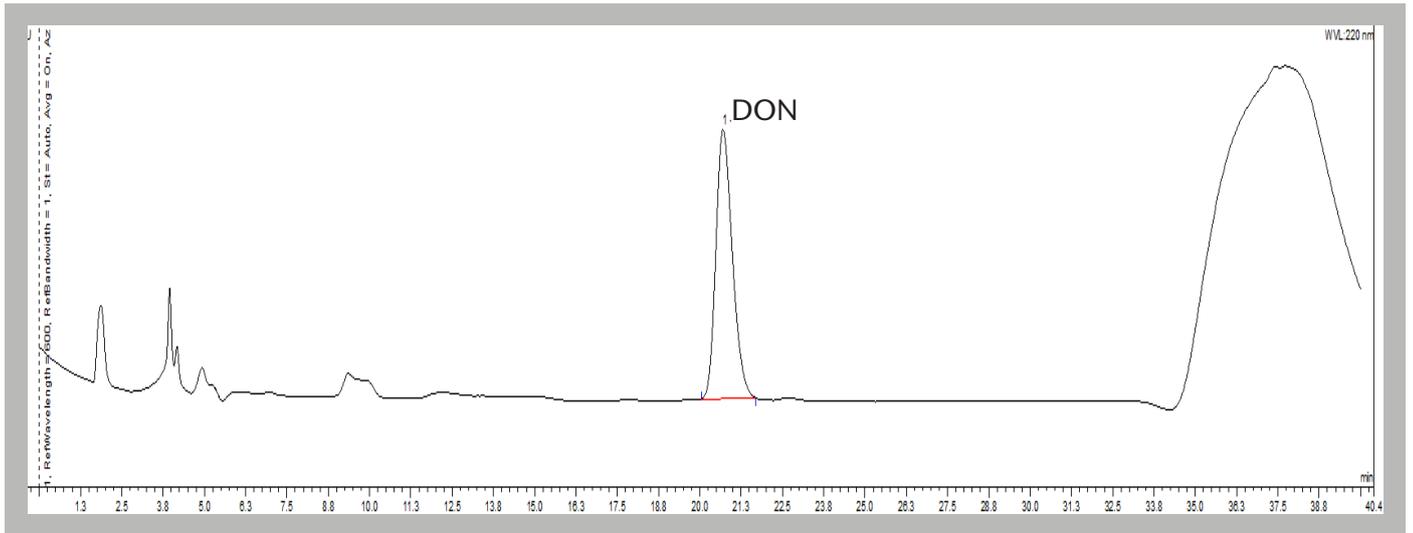
1. Standard 6: Take 10 ml of 50 % methanol and remove 66.7 μ l to waste. Add 66.7 μ l of 100,000 ng/ml deoxynivalenol standard to give a concentration of 666.7 ng/ml.
2. Standard 5: Take 2 ml of Standard 6 and add 2 ml 50 % methanol (equivalent to 333.3 ng/ml)
3. Standard 4: Take 2 ml of Standard 5 and add 2 ml 50 % methanol (equivalent to 166.7 ng/ml)
4. Standard 3: Take 2 ml of Standard 4 and add 2 ml 50 % methanol (equivalent to 83.3 ng/ml)
5. Standard 2: Take 2 ml of Standard 3 and add 2 ml 50 % methanol (equivalent to 41.7 ng/ml)
6. Standard 1: Take 2 ml of Standard 2 and add 2 ml 50 % methanol (equivalent to 20.8 ng/ml)
7. Inject 10 μ l of each solution onto the LC-MS/MS system.

Recommended HPLC Conditions

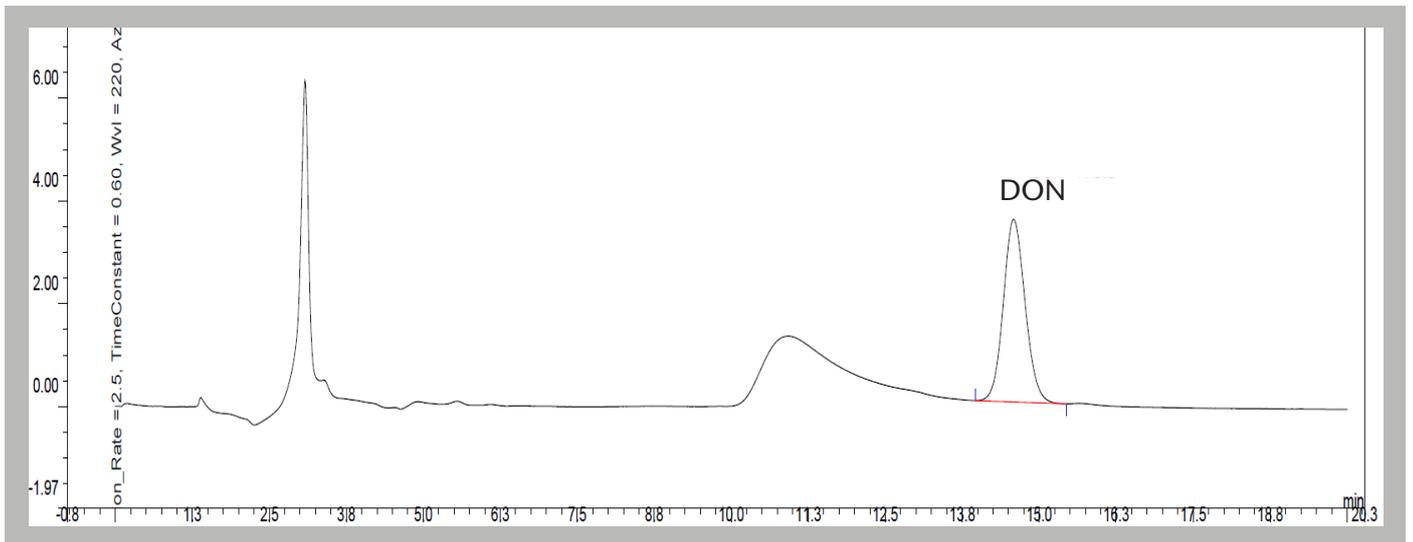
HPLC Conditions			
Guard Cartridge	AQUASIL C18 3 µm, 4 mm x 10 mm or equivalent		
Analytical Column	AQUASIL C18 3 µm, 4.6 mm x 150 mm or equivalent		
Mobile Phase	Mobile Phase A: Water Mobile Phase B: Methanol Prepare fresh on day of analysis		
Gradient Conditions	Time (min)	% Solution A	% Solution B
	0	85	15
	25	85	15
	26	50	50
	30	50	50
	31	85	15
	40	85	15
HPLC Pump	To deliver mobile phase		
Flow Rate	0.4 ml per minute		
UV Detector	220 nm		
Column Heater	Maintain guard and analytical column at 30 °C		
Integrator / Data Control System	From preferred supplier		
Injector	Autosampler / Rheodyne valve		
Injection Volume	100 µl		

Example HPLC Chromatograms

- Cereal (spiked at 1250 ppb)



- Animal Feed (spiked at 5000 ppb)



Recommended LC-MS/MS Conditions

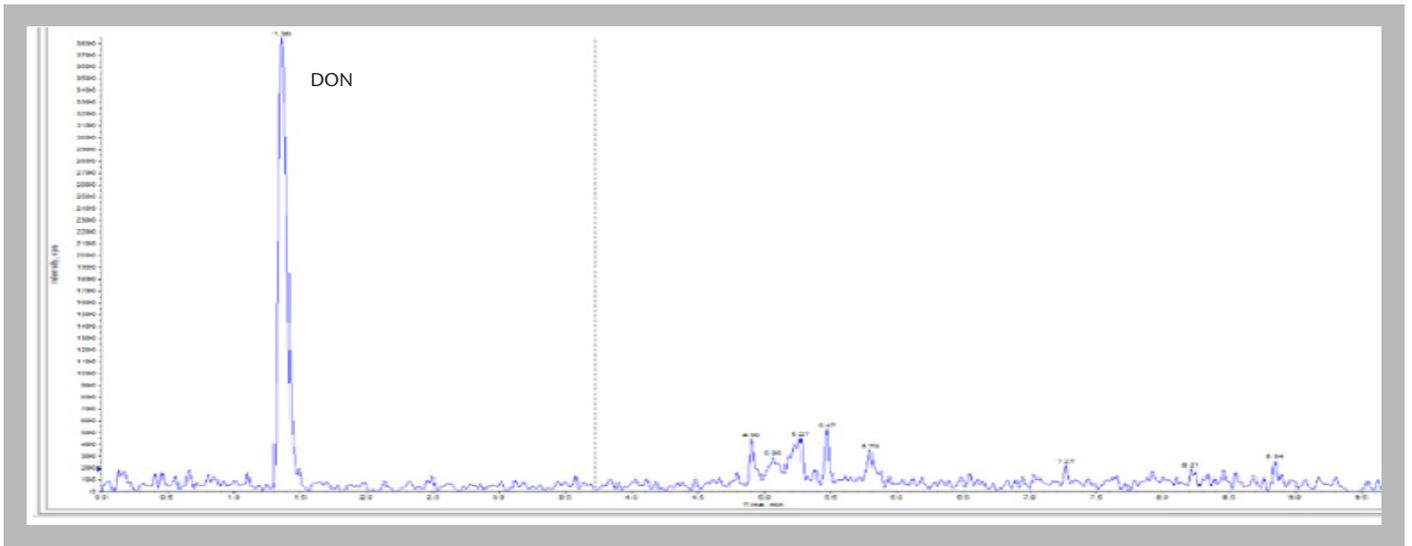
LC-MS/MS Conditions			
Analytical Column	Luna Omega Polar C18, 110 Å, 100 x 3 mm, 3 µm or equivalent		
Mobile Phase	Mobile Phase A: 1 mM Ammonium Formate and 0.1 % Formic Acid in water : methanol 95 : 5 (v/v) Mobile Phase B: 1 mM Ammonium Formate and 0.1 % Formic Acid in water : methanol 2 : 98 (v/v). Prepare fresh on day of analysis		
Gradient Conditions	Time (min)	% Solution A	% Solution B
	0	60	40
	0.5	60	40
	4.0	0	100
	6.0	0	100
	6.1	60	40
	10	60	40
HPLC Pump	To deliver mobile phase		
Flow Rate	0.6 ml per minute		
Column Heater	Maintain guard and analytical column at 40 °C		
Integrator / Data Control System	From preferred supplier		
Injection Volume	10 µl		

Mass Spectrometry Conditions	
Instrument	Sciex QTRAP 5500
Mode	Multiple Reaction Monitoring (MRM)
Source Temperature	450 °C
Ion Spray	3500 V
IonSource Gas 1	50 psi
IonSource Gas 2	55 psi
Curtain Gas	50 psi

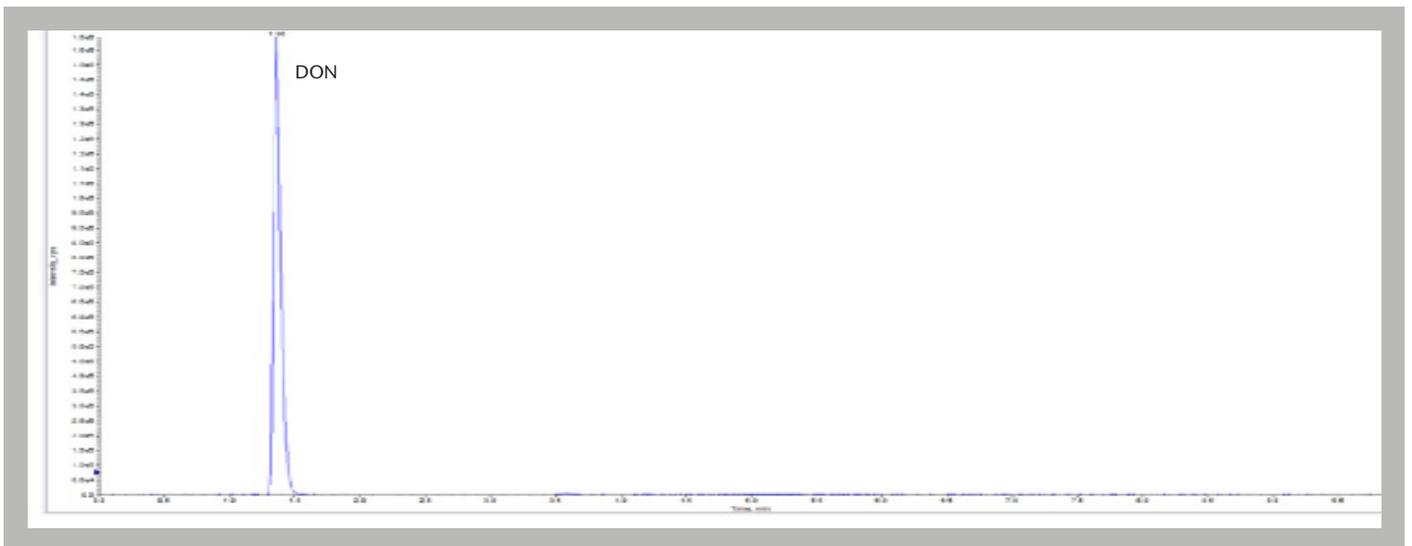
Instrument Setting						
Toxin	Time Segment (min)	Precursor Ion (m/z)	Product Ions (m/z)	Dwell Time (s)	Collision Energy (V)	Cell Exit Potential (V)
DON	1.36	297.1 [M+H] ⁺	249.00 (Quantifier) 231.00 (Qualifier)	20	15.00 17.00	16.00 15.00

Example LC-MS/MS Chromatograms

- Cereal (spiked at 20 ppb)



- Animal Feed (spiked at 5000 ppb)



Quality

RBR products are developed, manufactured, tested and dispatched under an ISO 9001 registered Quality Management System, guaranteeing a consistent product, which always meets our performance specifications. Our products have been used in many collaborative studies to develop standard European and International Methods and are widely used by key institutions, food companies and government laboratories. Customer references for RBR products are available on request..

Technical Support

RBR understand that from time to time users of our products may need assistance or advice. Therefore, we are pleased to offer the following services to our customers:

- Analysis of problem samples.
- Application notes for difficult samples.
- References from the RBR library.
- Installation and support of the KOBRA® CELL.
- Advice on detection parameters.
- Advice on preparation and handling of standards.
- Updates on legislation, sampling and other news by e-mail.
- Provision of spiked samples.

Please contact your local R-Biopharm distributor for further information.

Warranty

R-Biopharm Rhône Ltd makes no warranty of any kind, express or implied, except that all products made by R-Biopharm Rhône Ltd are made with materials of suitable quality. If any materials are defective, R-Biopharm Rhône Ltd will provide a replacement product. The user assumes all risk and liability resulting from the use of R-Biopharm Rhône Ltd products and procedures. R-Biopharm Rhône Ltd shall not be liable for any damages, including special or consequential damages, loss or expense arising directly or indirectly from the use of R-Biopharm Rhône Ltd products or procedures.

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Product Code: P112 / P112B

Immunoaffinity columns for use in conjunction with HPLC or LC-MS/MS.
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P112/V12/27.05.22

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Test Principle

The procedure is based on monoclonal antibody technology, which makes the test highly specific, sensitive, rapid and simple to perform.

The columns contain a gel suspension of monoclonal antibody specific to the toxins of interest. Following extraction of the toxins the sample extract is filtered, diluted and passed slowly through the immunoaffinity column. Any toxins which are present in the sample are retained by the antibody within the gel suspension. The column is washed to remove unbound material and the toxins are then released from the column following elution with solvent. The eluate is collected prior to analysis by HPLC or LC-MS/MS. Aflatoxins are required to be derivatised when analysed by HPLC.

The total extraction and clean-up time takes approximately 20 minutes to perform. The result is improved clean-up and concentration of the toxins from food and feed samples giving a much cleaner chromatogram and therefore providing more accurate and sensitive detection. The columns also have the added advantage that they can be automated for large scale analysis of samples.

Reagents Not Provided

- Distilled / Deionised Water (suitable for use with HPLC, e.g. MilliQ)
- Solvents (HPLC Grade Methanol and Acetonitrile)
- Phosphate Buffered Saline (PBS) (RP202)*
- Mycotoxin Standards (Please refer to Preparation of Standards section)
- Sodium Chloride
- Sodium Hydroxide (to pH filtrate if required)
- Nitric Acid (only required when derivatising with a KOBRA® CELL)
- Potassium Bromide (only required when derivatising with a KOBRA® CELL)

Accessory Products

- Whatman No. 113 or No. 4 Filter Paper
- KOBRA® CELL (K01)*
- Immunoaffinity Column Rack (CR1)*
- Immunoaffinity Column Accessory Pack (AP01)*

* Available from R-Biopharm. Please contact your local R-Biopharm distributor for further information.

Hazards

Mycotoxins are very hazardous substances. Only laboratories equipped to handle toxic materials and solvents should perform analyses. Suitable protective clothing, including gloves, safety glasses and lab coats should be worn throughout the analysis.

Flammable solvents should be stored in an explosion-proof cabinet. Use a chemical hood and protective equipment as applicable.

Contact your local R-Biopharm distributor for a Material Safety Data Sheet for further information if required.

Recommended Methods and Application Notes

Methods are available for all matrices covered by legislation as well as additional commodities. Deviation from the methods described in our Instructions For Use and Application Notes may not achieve optimum results. Please contact your local R-Biopharm distributor for further information.

Decontamination

Prior to disposal, excess standard solutions should be treated with at least one-tenth their volume of 5 % sodium hypochlorite. Labware and contaminated waste should be immersed in 5 % sodium hypochlorite solution for 30 minutes followed by the addition of 5 % acetone for 30 minutes. Flush with copious amounts of water before disposal. After decontamination labware should be thoroughly washed. Incinerate waste if regulations permit.

Storage & Shelf Life

The columns expire 18 months from date of manufacture if stored at 2 - 8 °C or 12 months from date of manufacture if stored at 21 - 25 °C. Do not freeze.

Ensure the column has not dried out and contains buffer above the gel. It is important to note the antibody included in the immunoaffinity column can be denatured by extreme temperature or pH change.

Sampling

A representative sample should be obtained by following one of the officially recognised sampling procedures. It is recommended that a minimum of 1 kg of representative sample is finely ground and a portion (5 - 50 g dependent on method used) of this is removed and extracted.

Sensitivity

The sensitivity is dependent on the final detection system employed by the analyst. However the test sensitivity may be improved if required by increasing the volume of sample passed through the immunoaffinity column. Please note the ratio of solvent to phosphate buffered saline (PBS) should be maintained.

Recoveries

If an analyst wishes to account for losses during extraction it is recommended a spiked sample of the same commodity type as the material being tested is analysed following the complete procedure as a reference standard. The recoveries obtained with the spiked sample can be used to correct the results obtained with the test sample.

Column Preparation

Immunoaffinity columns should be at ambient temperature before use. Remove the cap from the top of the column and discard. Firmly attach the column to a glass syringe barrel using an adapter and place in an immunoaffinity column rack or clamp stand.

Elution

In order to fully elute the toxin/s from the immunoaffinity column it is vital that the solvent is in contact with the antibody within the gel suspension for a sufficient period of time. This ensures that all of the bonds between the antibody and the toxin are broken, ultimately releasing all of the toxin from the column for analysis with the detection system of choice

To ensure that the solvent is in contact with the antibody gel for a sufficient period of time any of the following elution methods can be used: -

Backflushing (this is the preferred method of choice at R-Biopharm): backflush by gently raising and lowering the syringe plunger during passage of the solvent through the column. This process will reverse the direction of flow of the eluate through the gel. This should be repeated 3 times before collecting the eluate. Proceed to the next step in the method.

Application of small volumes of solvent: apply the volume of solvent required for elution in two or three smaller aliquots. Allow each aliquot to remain in contact with the gel suspension for a minimum of 30 seconds before allowing each to pass fully through the gel suspension for collection. Proceed to the next step in the method.

Incubation with solvent: apply the full volume of solvent required for elution and allow 2-3 drops of the solvent to pass through the column for collection. Allow the remainder of the solvent to remain in contact with the gel suspension for a minimum of 60 seconds before allowing it to pass through the gel suspension for collection. Proceed to the next step in the method.



Sample Preparation

- **Cereal**

This method has been tested on a number of commodities including wheat, maize.

1. Weigh 25 g of ground sample and 5 g of sodium chloride into a 1 litre capacity, solvent resistant blender jar.
2. Add 100 ml of 80 % methanol and blend at high speed for 2 minutes.
3. Filter the sample through Whatman No. 113 or No. 4 filter paper, or centrifuge at 4,000 rpm for 10 minutes.
4. Dilute 10 ml of the filtrate with 40 ml of phosphate buffered saline (PBS).
5. Filter the diluted extract through glass microfibre filter paper.
6. Pass 20 ml of the filtrate (equivalent to 1 g of sample) through the column at a flow rate of 2 ml per minute (or the sample can be allowed to pass through the column by gravity if preferred). A slow, steady flow rate is essential for the capture of the toxins by the antibody.
7. **HPLC:** Wash the column with 20 ml of PBS.
LC-MS/MS: Wash the column with 20 ml of water.
The column should be washed at a flow rate of approximately 5 ml per minute. Pass air through the column to remove residual liquid.
8. Elute the toxins from the column at a flow rate of 1 drop per second using 1.5 ml of 100 % methanol and collect in an amber glass vial. Please refer to the Elution section for further information.
9. Following elution pass 1.5 ml of water through the column and collect in the same vial to give a 3 ml total volume.
10. **HPLC:** Pipette 1 ml of eluate into two separate amber glass vials and inject 100 µl onto each HPLC system for analysis of zearalenone and combined analysis of total aflatoxin and ochratoxin A.
LC-MS/MS: Inject 25 µl onto the LC-MS/MS system

Sample Preparation

- **Baby Food**

1. Weigh 25 g of ground sample and 5 g of sodium chloride into a 1 litre capacity, solvent resistant blender jar.
2. Add 100 ml of 80 % methanol and blend at high speed for 2 minutes.
3. Filter the sample through Whatman No. 113 or No. 4 filter paper, or centrifuge at 4,000 rpm for 10 minutes.
4. Dilute 10 ml of the filtrate with 40 ml of phosphate buffered saline (PBS) and filter the diluted extract through glass microfibre filter paper.
5. **HPLC:** Pass 20 ml of filtrate (equivalent to 1 g of sample) through the column.
LC-MS/MS: Pass 45 ml of filtrate (equivalent to 2.25 g of sample) through the column.
The filtrate should pass through the column at a flow rate of 2 ml per minute (or the sample can be allowed to pass through the column by gravity if preferred). A slow, steady flow rate is essential for the capture of the toxins by the antibody.
6. **HPLC:** Wash the column with 20 ml of PBS.
LC-MS/MS: Wash the column with 20 ml of water.
The column should be washed at a flow rate of approximately 5 ml per minute. Pass air through the column to remove residual liquid.
7. **HPLC:** Elute the toxins from the column using 1.5 ml of 100 % methanol.
LC-MS/MS: Elute the toxins from the column using 1 ml of 100 % methanol.
Collect in an amber glass vial. Please refer to the Elution section for further information.
8. **HPLC:** Following elution pass 1.5 ml of water through the column and collect in the same vial to give a 3 ml total volume.
LC-MS/MS: Following elution pass 1 ml of water through the column and collect in the same vial to give a 2 ml total volume.
9. **HPLC:** Pipette 1 ml of eluate into two separate amber glass vials and inject 100 µl onto each HPLC system for analysis of zearalenone and combined analysis of total aflatoxin and ochratoxin A.
LC-MS/MS: Inject 25 µl onto the LC-MS/MS system.

Sample Preparation

- **Rye**

1. Weigh 25 g of ground sample into a 1 litre capacity, solvent resistant blender jar.
2. Add 100 ml of 60 % acetonitrile and blend at high speed for 2 minutes.
3. Filter the sample through Whatman No. 113 or No. 4 filter paper, or centrifuge at 4,000 rpm for 10 minutes.
4. Dilute 10 ml of the filtrate with 40 ml of phosphate buffered saline (PBS).
5. Filter the diluted extract through glass microfibre filter paper.
6. Pass 20 ml of the filtrate (equivalent to 1 g of sample) through the column at a flow rate of 2 ml per minute (or the sample can be allowed to pass through the column by gravity if preferred). A slow, steady flow rate is essential for the capture of the toxin by the antibody.
7. **HPLC:** Wash the column with 20 ml of PBS.
LC-MS/MS: Wash the column with 20 ml of water.
The column should be washed at a flow rate of approximately 5 ml per minute. Pass air through the column to remove residual liquid.
8. Elute the toxins from the column at a flow rate of 1 drop per second using 1.5 ml of 100 % methanol and collect in a 5 ml amber glass vial. Please refer to the Elution section for further information.
9. Following elution pass 1.5 ml of water through the column and collect in the same vial to give a 3 ml total volume.
10. **HPLC:** Pipette 1 ml of eluate into two separate amber glass vials and inject 100 µl onto each HPLC system for analysis of zearalenone and combined analysis of total aflatoxin and ochratoxin A.
LC-MS/MS: Inject 25 µl onto the LC-MS/MS system.

Preparation of Standards

- **Aflatoxin Stock Solution**

It is advised to start with a 1,000 ng/ml total aflatoxin stock solution.

Note: The ratio of B1, B2, G1 and G2 may vary in each standard. Please note the correct ratio for the standard purchased.

- **Total Aflatoxin Standard Dilution**

1. Measure 1 ml of 100 % methanol into an amber vial.
2. Remove 100 µl to waste.
3. Add 100 µl of 1,000 ng/ml total aflatoxin stock solution to give a 100 ng/ml total aflatoxin solution.

- **Ochratoxin Stock Solution**

It is advised to start with a 1,000 ng/ml ochratoxin A stock solution.

- **Ochratoxin A Standard Dilution**

1. Measure 1 ml of 100 % methanol into an amber vial.
2. Remove 100 µl to waste.
3. Add 100 µl of 1,000 ng/ml ochratoxin A stock solution to give a 100 ng/ml ochratoxin A solution.

- **Zearalenone Stock Solution**

It is advised to start with a 1,000 ng/ml zearalenone stock solution.

Calibration Curve

It is recommended to run at least a 3 - 6 point calibration curve. In constructing a suitable curve the levels of the calibration standards should bracket or include the range of expected results. The diluted standard solutions should be prepared fresh on the day of analysis and used within a 24 hour period.

Example of how to prepare a four point calibration curve (can be modified according to legislative requirements or contamination levels):

1. Standard 4:
 - Measure 3 ml of 100 % methanol into an amber vial and discard 780 µl to waste.
 - Add 240 µl of 100 ng/ml total aflatoxin standard, 240 µl of 100 ng/ml ochratoxin A standard and 300 µl of 1,000 ng/ml of zearalenone standard.
 - Add 3 ml of water (equivalent to 4 ng/ml total aflatoxin, 4 ng/ml ochratoxin A and 50 ng/ml zearalenone). Vortex for 20 seconds.
2. Standard 3: Take 3 ml of Standard 4 and add 3 ml of 50 % methanol (equivalent to 2 ng/ml total aflatoxin, 2 ng/ml ochratoxin A and 25 ng/ml zearalenone).
3. Standard 2: Take 3 ml of Standard 3 and add 3 ml of 50 % methanol (equivalent to 1 ng/ml total aflatoxin, 1 ng/ml ochratoxin A and 12.5 ng/ml zearalenone).
4. Standard 1: Take 3 ml of Standard 2 and add 3 ml of 50 % methanol (equivalent to 0.5 ng/ml total aflatoxin, 0.5 ng/ml ochratoxin A and 6.25 ng/ml zearalenone).
5. **HPLC:** Dispense 1 ml of each standard into 2 amber vials and inject 100 µl of each solution onto the HPLC system.
LC-MS/MS: Dispense 1 ml of each standard into an amber vial and inject 25 µl of each solution onto the LC-MS/MS system.

Recommended HPLC Conditions

- **Total Aflatoxin and Ochratoxin A**

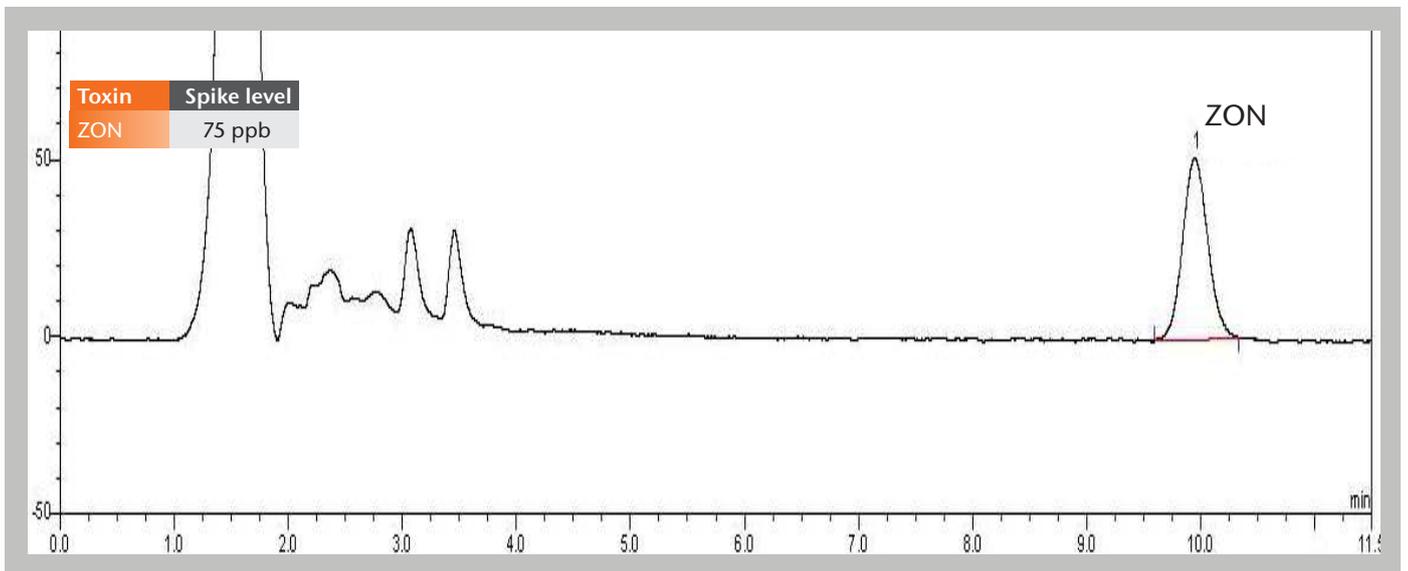
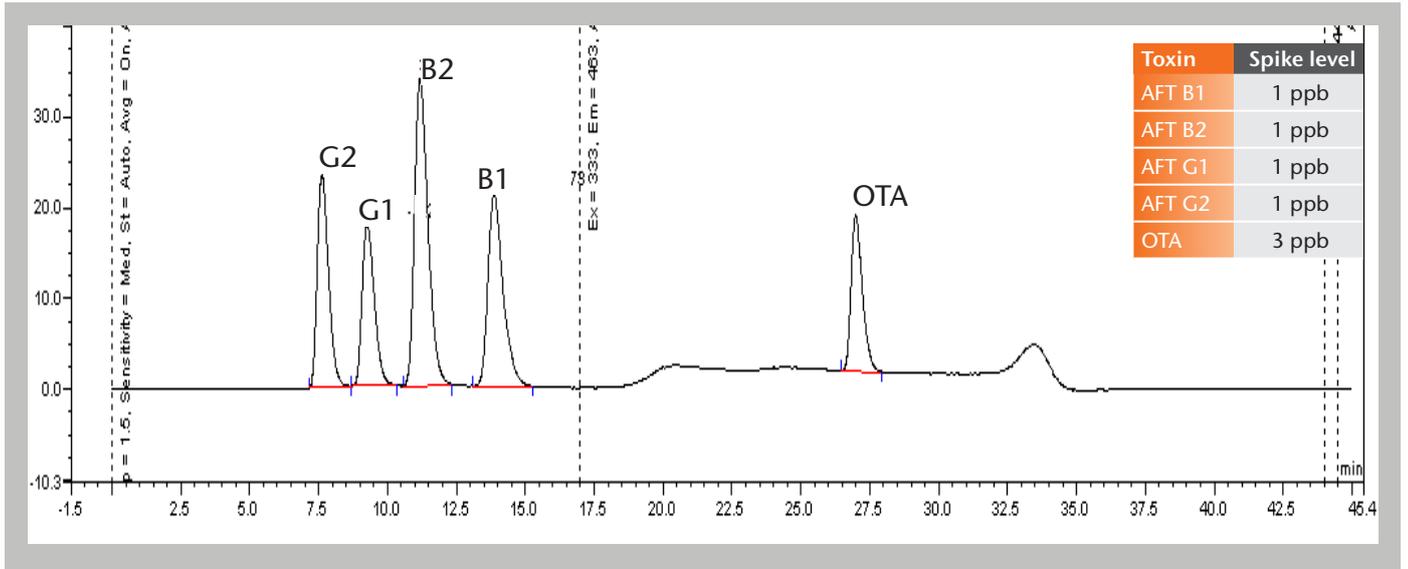
HPLC Conditions			
Derivatisation	KOBRA® CELL at 100 µA setting		
Guard Cartridge	Inertsil ODS-3 5 µm, 4 mm x 10 mm or (Hichrom) equivalent		
Analytical Column	Inertsil ODS-3V 5 µm, 4.6 mm x 150 mm (Hichrom) or equivalent		
Mobile Phase	Mobile Phase A: Water : Methanol (55 : 45 v/v) Mobile Phase B: Water : Methanol (20 : 80 v/v) Add 119 mg of potassium bromide and 350 µl 4 M Nitric Acid to 1 litre of mobile phase A and B. Prepare fresh on day of analysis.		
Gradient Conditions	Time (min)	% Solution A	% Solution B
	0	100	0
	14	100	0
	16	35	65
	30	35	65
	31	100	0
	40	100	0
HPLC Pump	From preferred supplier		
Flow Rate	0.8 ml per minute		
Fluorescence Detector	Time (min)	Excitation (nm)	Emission (nm)
	0	365	442
	17	333	463
Column Heater	Maintain guard and analytical columns at 40 °C		
Integrator / Data Control System	From preferred supplier		
Injector	Autosampler / Rheodyne valve		
Injection Volume	100 µl		
Elution Order	G2, G1, B2, B1, ochratoxin A		

- **Zearalenone**

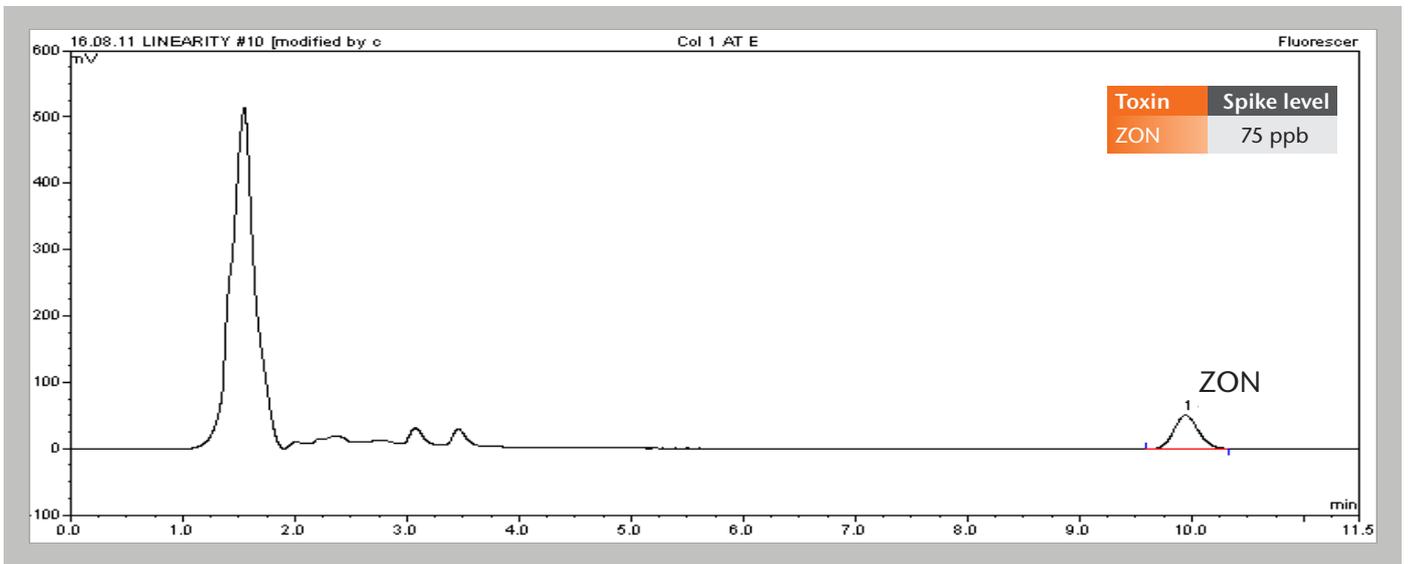
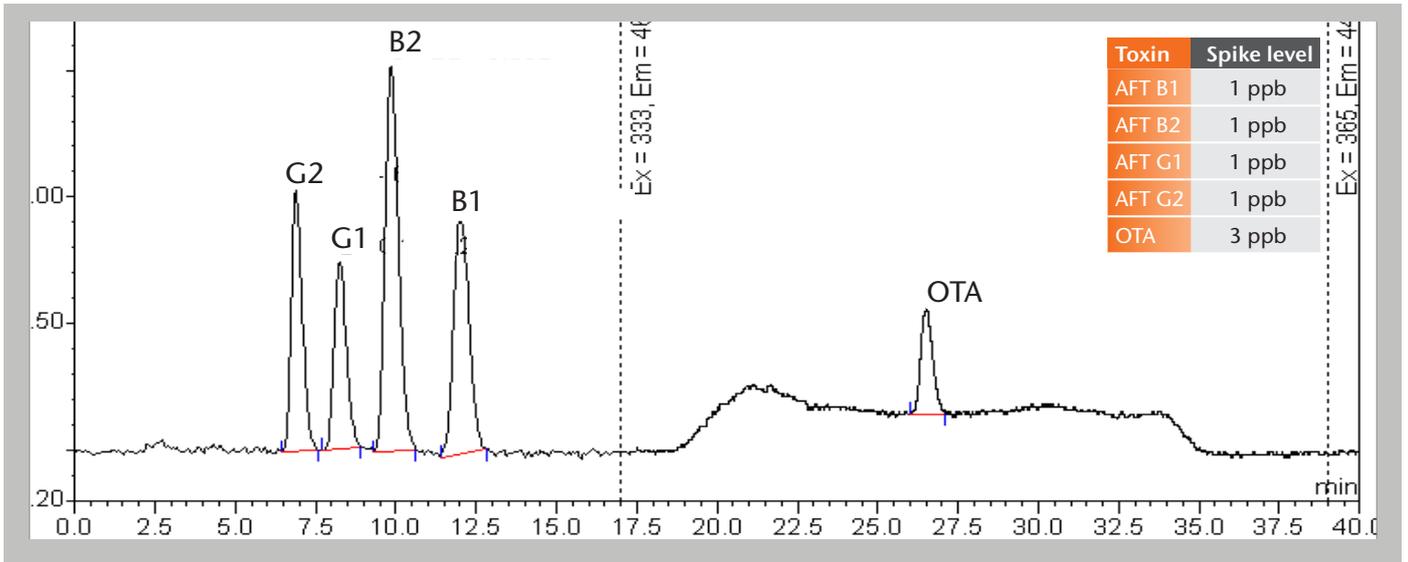
HPLC Conditions	
Guard Cartridge	Inertsil ODS-3 5 µm, 4 mm x 10 mm or (Hichrom) equivalent
Analytical Column	Inertsil ODS-3V 5 µm, 4.6 mm x 150 mm (Hichrom) or equivalent
Mobile Phase	Acetonitrile : Water : Methanol (46 : 46 : 8 v/v) Prepare fresh on day of analysis.
HPLC Pump	To deliver mobile phase
Flow Rate	1.0 ml per minute
Fluorescence Detector	Excitation: 274 nm Emission: 455 nm
Column Heater	Maintain guard and analytical columns at 40 °C
Integrator / Data Control System	From preferred supplier
Injector	Autosampler / Rheodyne valve
Injection Volume	100 µl

Example HPLC Chromatograms

- Maize



- Rye



Recommended LC-MS/MS Conditions

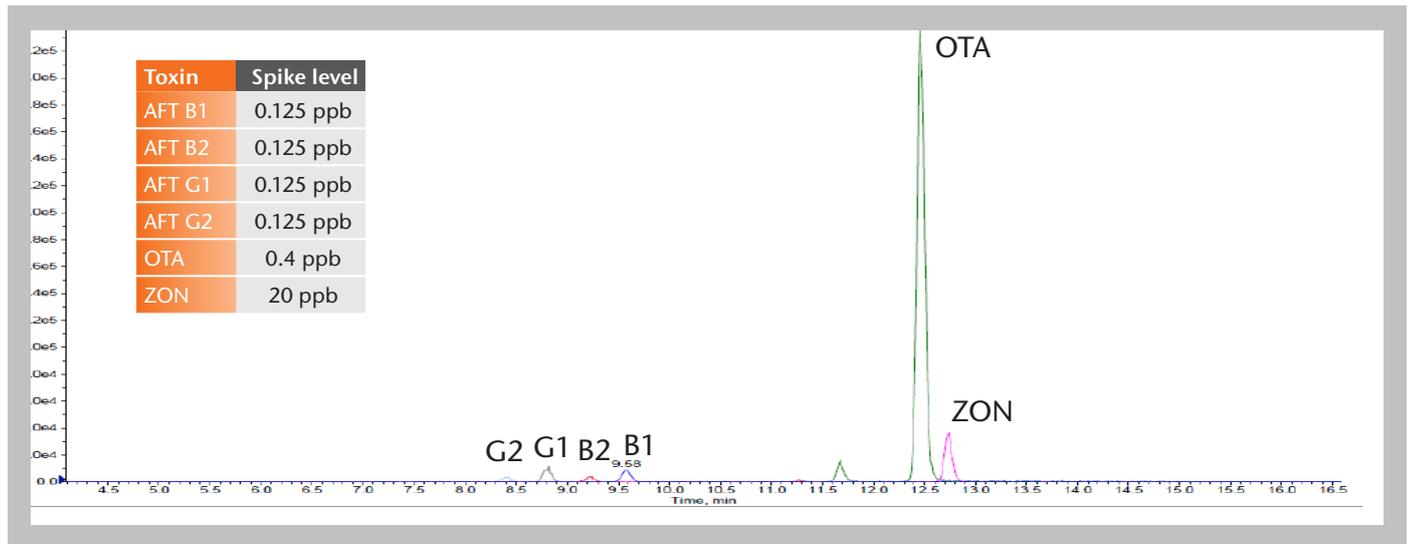
LC Conditions			
Analytical Column	Phenomenex Gemini C18, 110 Å, 150 x 3 mm, 5 µm		
Mobile Phase	Mobile Phase A: 1 mM Ammonium Formate and 0.1 % Formic Acid in Water : Methanol (95 : 5 v/v) Mobile Phase B: 1 mM Ammonium Formate and 0.1 % Formic Acid in Water : Methanol (2 : 98 v/v)		
Gradient Conditions	Time (min)	% Solution A	% Solution B
	0	80	20
	0.1	80	20
	10	10	90
	15	10	90
	15.1	80	20
	20	80	20
HPLC Pump	To deliver mobile phase		
Flow Rate	0.3 ml per minute		
Column Heater	Maintain guard and analytical columns at 40 °C		
Integrator / Data Control System	From preferred supplier		
Injection Volume	25 µl		

Mass Spectrometry Conditions	
Instrument	Sciex QTRAP 4500
Mode	Multiple Reaction Monitoring (MRM)
Source Temperature	450 °C
Ion Spray	3500 V
IonSource Gas 1	50 psi
IonSource Gas 2	50 psi
Curtain Gas	50 psi

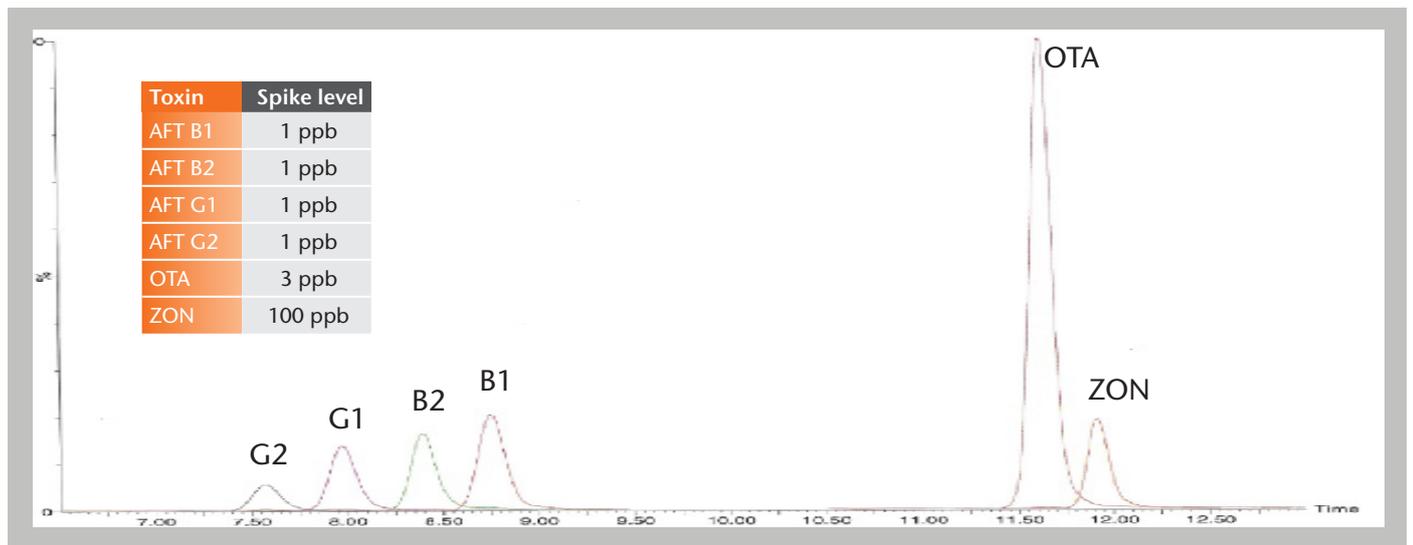
Instrument Setting						
Toxin	Time Segment (min)	Precursor Ion (m/z)	Product Ions (m/z)	Dwell Time (s)	Collision Energy (V)	Cell Exit Potential (V)
AFT G2	8.81	331.1 [M+H] ⁺	256.90 (Quantifier)	20	40.90	20.00
			189.10 (Qualifier)		54.40	14.00
AFT G1	8.81	329.1 [M+H] ⁺	243.00 (Quantifier)	20	36.60	15.00
			199.90 (Qualifier)		53.70	15.00
AFT B2	9.24	315.2 [M+H] ⁺	287.10 (Quantifier)	20	34.90	17.00
			259.00 (Qualifier)		38.60	17.00
AFT B1	9.59	313.2 [M+H] ⁺	285.10 (Quantifier)	20	30.70	17.00
			241.00 (Qualifier)		48.20	17.00
OTA	12.74	404.1 [M+H] ⁺	239.00 (Quantifier)	20	31.20	15.00
			358.00 (Qualifier)		19.10	18.00
ZON	12.47	319.1 [M+H] ⁺	283.00 (Quantifier)	20	16.40	17.00
			187.00 (Qualifier)		25.90	12.00

Example LC-MS/MS Chromatograms

- Baby Food**



- Rye**



Quality

RBR products are developed, manufactured, tested and dispatched under an ISO 9001 registered Quality Management System, guaranteeing a consistent product, which always meets our performance specifications. Our products have been used in many collaborative studies to develop standard European and International Methods and are widely used by key institutions, food companies and government laboratories. Customer references for RBR products are available on request.

Technical Support

RBR understand that from time to time users of our products may need assistance or advice. Therefore, we are pleased to offer the following services to our customers:

- Analysis of problem samples.
- Application notes for difficult samples.
- References from the RBR library.
- Installation and support of the KOBRA® CELL.
- Advice on detection parameters.
- Advice on preparation and handling of standards.
- Updates on legislation, sampling and other news by e-mail.
- Provision of spiked samples.

Please contact your local R-Biopharm distributor for further information.

Acknowledgement

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Warranty

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