AO ZON PREP®

Product Code: P112 / P112B

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

P112/V15/16.10.24

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R-BIOPHARM RHÔNE LTD

www.r-biopharm.com

CO2 NEUTRAL

Contents

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. Recycle decontaminated plastic waste if local regulations permit.

Storage & Shelf Life

The columns expire 2 years 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 though 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 though 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**

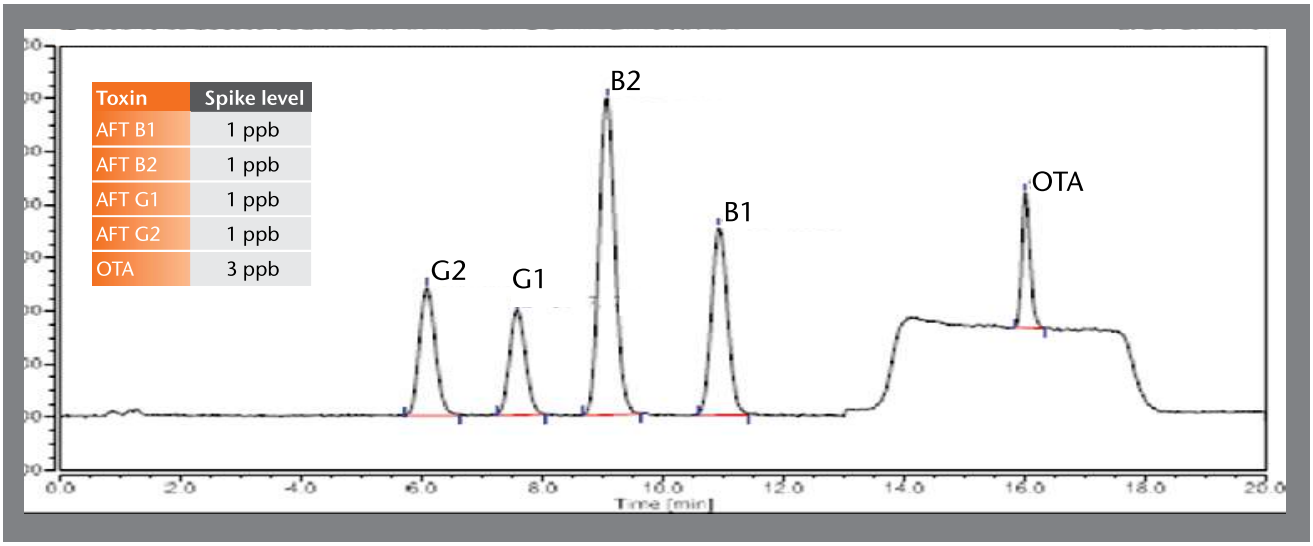
HPLC Conditions				
Analytical Column	Inertsil ODS-3V 5 µm, 4.6 mm x 150 mm (Hichrom) or equivalent			
Mobile Phase	**Mobile Phase A:** Water **Mobile Phase B:** Methanol Add 119 mg of potassium bromide and 350 µl 4 M Nitric Acid to 1 litre of mobile phase A and B. Prepare fresh on day of analysis			
Gradient Conditions	Time (min)	% Mobile Phase A	% Mobile Phase B	Flow Rate (ml/min)
	0	62.5	37.5	1.4
	4	62.5	37.5	1.4
	12	57.5	42.5	1.6
	12.5	30	70	1.8
	16	30	70	1.8
	16.5	62.5	37.5	1.4
	20	62.5	37.5	1.4
HPLC Pump	To deliver mobile phase			
Flow Rate	1.4 - 1.8 ml per minute			
Fluorescence Detector	Time (min)	Excitation (nm)	Emission (nm)	
	0 – 13	365	442	
	13 – 20	333	463	
Column Heater	Maintain analytical columns at 50 °C			
Integrator / Data Control System	From preferred supplier			
Injector	Autosampler / Rheodyne valve			
Injection Volume	100 µl			

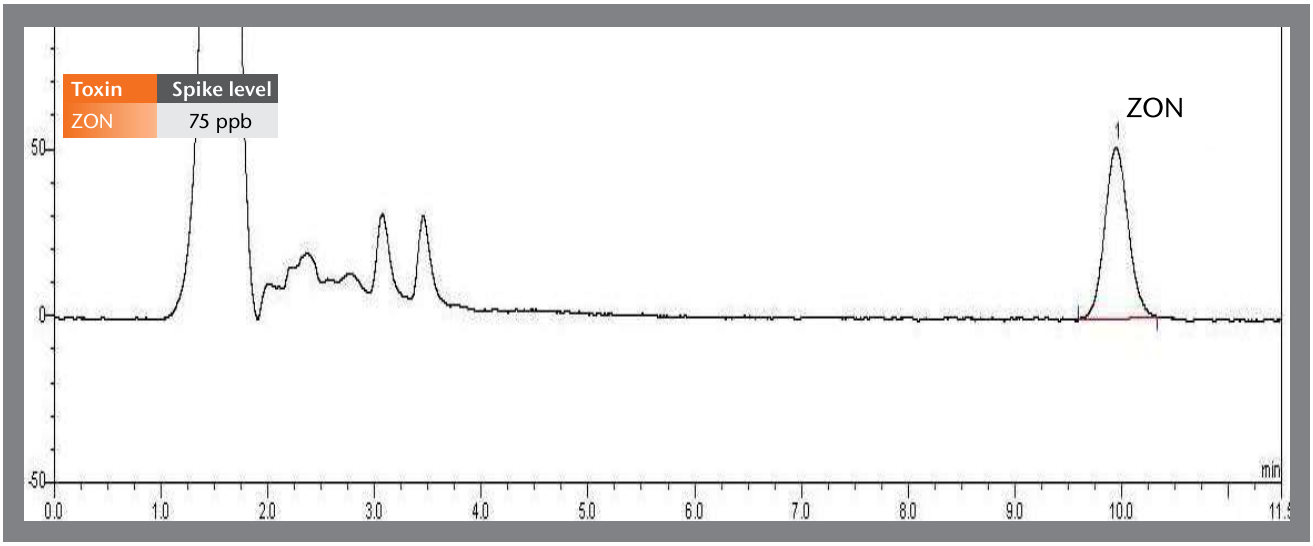
- **Zearalenone**

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

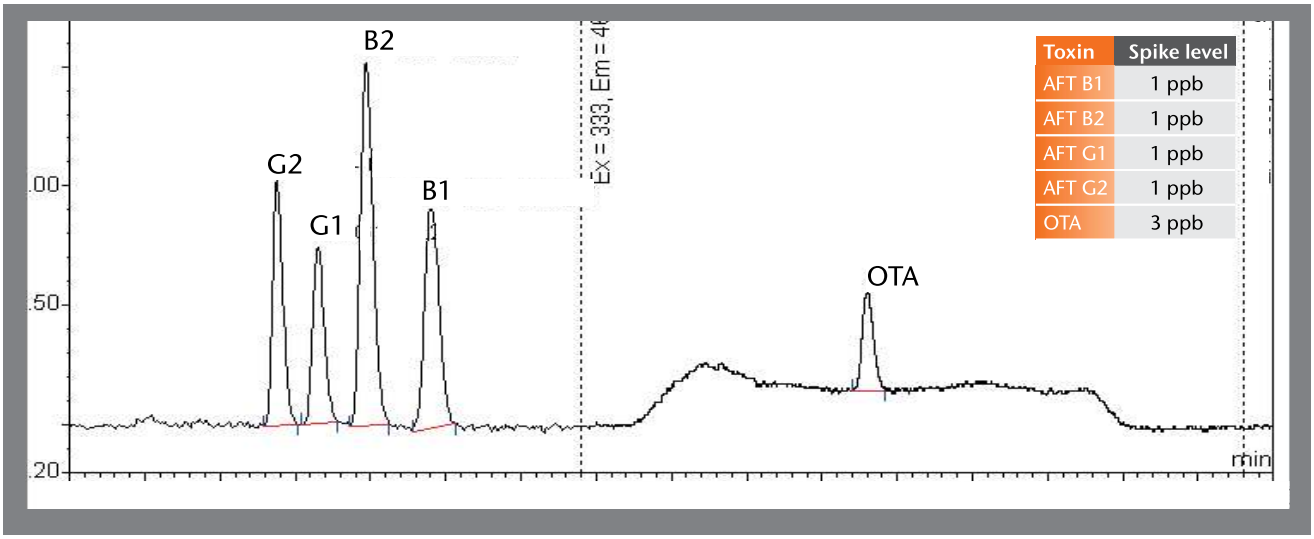
Example HPLC Chromatograms

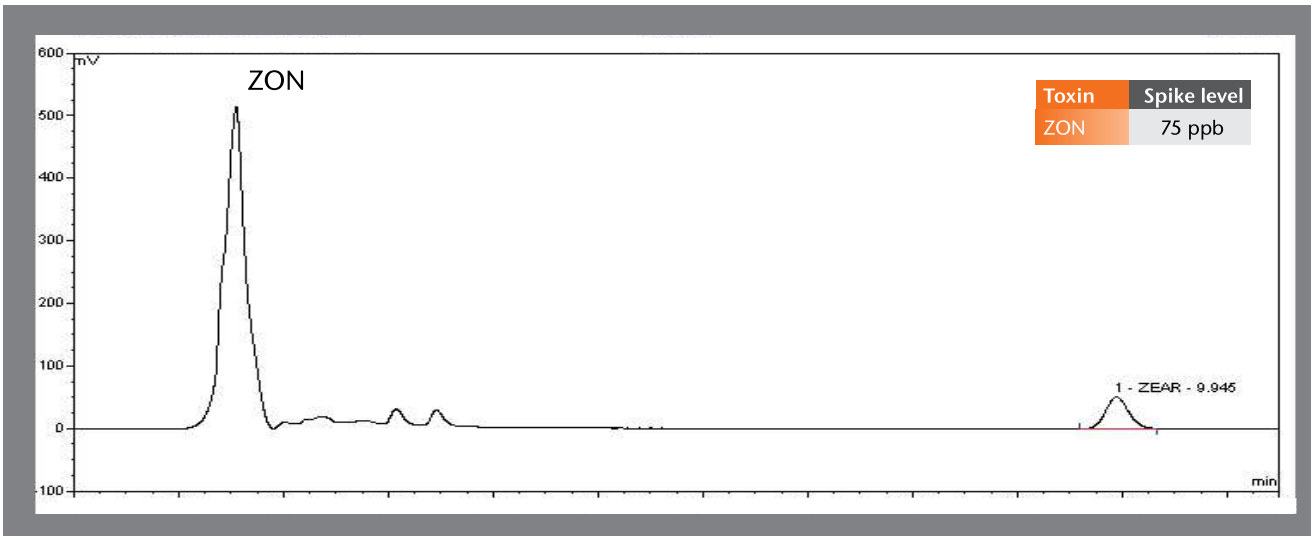
- **Maize**





- **Rye**


B2
G2
G1
B1
OTA
min
Toxin | Spike level
AFT B1 | 1 ppb
AFT B2 | 1 ppb
AFT G1 | 1 ppb
AFT G2 | 1 ppb
OTA | 3 ppb


mV
ZON
1 - ZEAR - 9.945
min
Toxin | Spike level
ZON | 75 ppb

Recommended LC-MS/MS Conditions

LC Conditions			
Analytical Column	Phenomenex Luna Omega 3 µm Polar C18 100 mm x 3 mm or equivalent		
Mobile Phase	Mobile Phase A: Water: Methanol: Formic Acid (95:5:0.1 v/v/v) containing 1 mM Ammonium Formate Mobile Phase B: Water: Methanol: Formic Acid (2:98:0.1 v/v/v) containing 1 mM Ammonium Formate. 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
	8.0	60	40
HPLC Pump	To deliver mobile phase		
Flow Rate	0.6 ml per minute		
Column Heater	Maintain analytical column at 40 °C		
Integrator / Data Control System	From preferred supplier		
Injector	Autosampler / Rheodyne valve		
Injection Volume	25 µl		

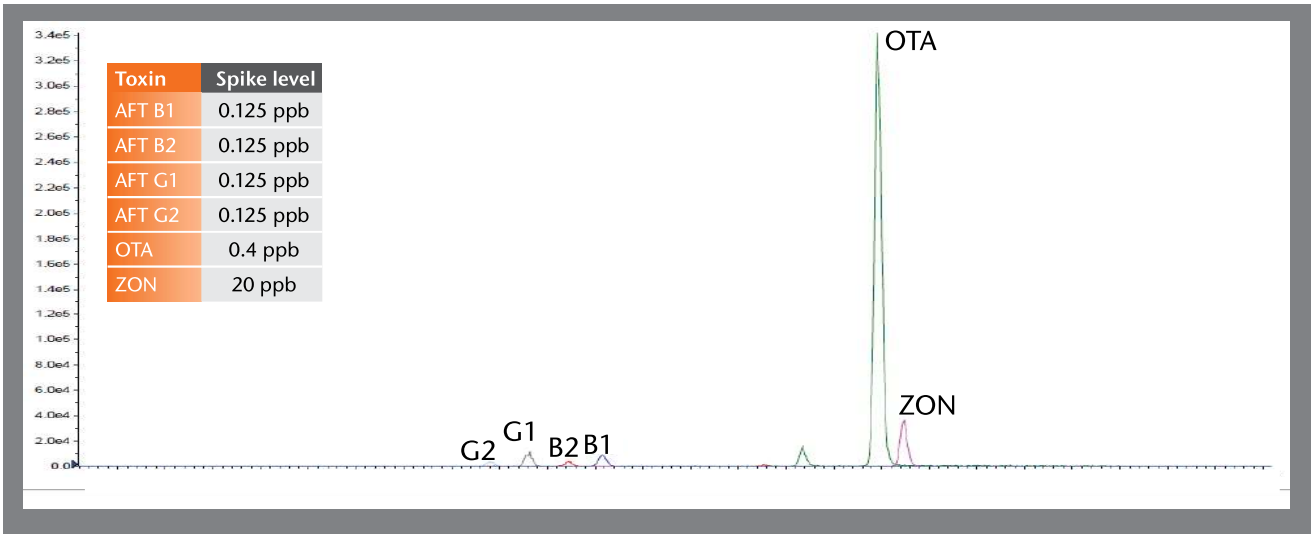
Mass Spectrometry Conditions	
Instrument	Sciex QTRAP 3500
Mode	Multiple Reaction Monitoring (MRM) Mode with positive polarity
Source Temperature	500 °C
IonSpray	4500 V
IonSource Gas 1	40 psi
IonSource Gas 2	50 psi

Instrument Setting

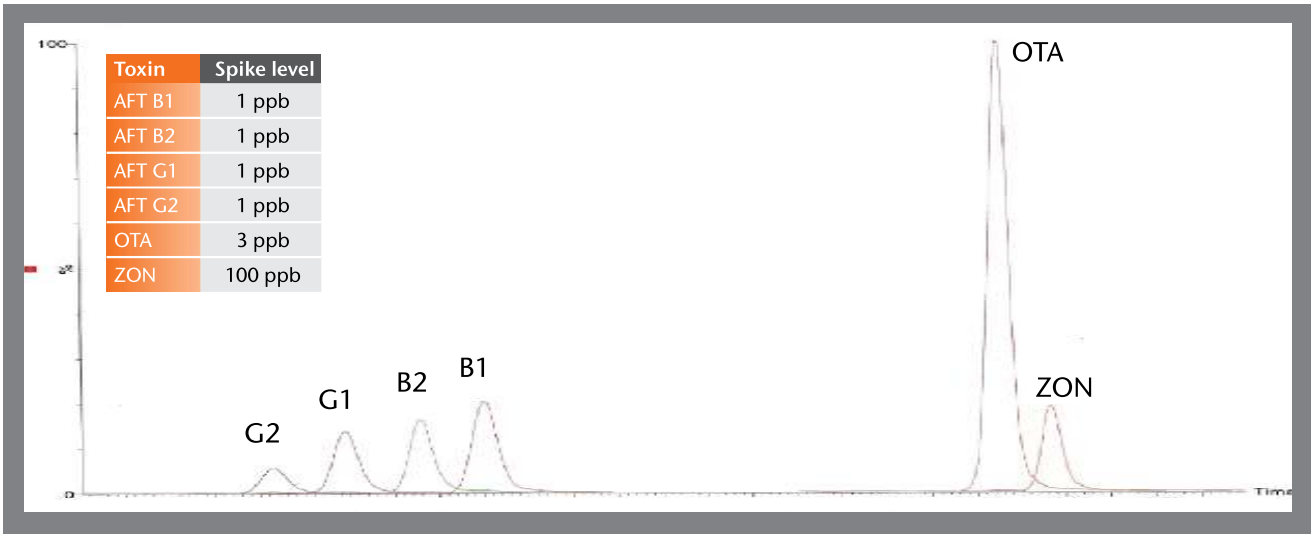
Toxin	Precursor Ion (m/z)	Declustering Potential (V)	Product Ions (m/z)	Dwell Time (s)	Collision Energy (V)	Cell Exit Potential (V)
AFT G2	331.1 [M+H]+	140	189.0 (Quantifier) 256.9 (Qualifier)	20	54.4 40.9	14 20
AFT G1	329.1 [M+H]+	140	243.0 (Quantifier) 199.9 (Qualifier)	20	38.6 53.7	15 15
AFT B2	315.2 [M+H]+	170	287.1 (Quantifier) 259.0 (Qualifier)	20	34.9 38.6	17 17
AFT B1	313.2 [M+H]+	170	285.1 (Quantifier) 189.0 (Qualifier)	20	30.7 48.2	17 17
OTA	404.1 [M+H]+	78	239.0 (Quantifier) 358.0 (Qualifier)	20	31.2 19.1	15 18
ZON	319.1 [M+H]+	90	283.0 (Quantifier) 187.1 (Qualifier)	20	16.4 25.9	17 12

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

RBR would like to acknowledge the invaluable help of Peter Mann, Campden BRI, Brewing Division for his assistance during the development of this product.

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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