EASI-EXTRACT® MULTI-VIT B (LGE)

Product Code: P183 / P183B

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

P183/V5/01.03.2023

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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 antibodies specific to the vitamins of interest. The immunoaffinity columns are designed with an integrated reservoir and a tightly fitting top and bottom cap. The top frit is absent from the top of the antibody gel to enable more thorough mixing of the sample extract and the antibodies within the column. Following extraction of the vitamins the sample extract is filtered and the filtrate is added to the reservoir. The column is then placed on a rotary shaker where the sample mixes directly with the gel allowing binding to take place between the vitamins and the antibodies. After allowing the gel to settle the sample is drained slowly from the column. The column is washed to remove any unbound material and the vitamins are 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 extra contact between the sample and the antibodies results in a more uniform binding of the vitamins with the antibodies and leads to better repeatability and reproducibility.

The total extraction and clean-up time takes approximately 90 minutes to perform. The result is improved clean-up and concentration of the vitamins from food samples reducing ion suppression and removing the need to use matrix matched standards. This provides cleaner chromatography, improved sensitivity and greater accuracy.

Reagents Not Provided

- Distilled / Deionised Water (suitable for use with HPLC, e.g. MilliQ)
- Solvents (HPLC Grade Methanol and Acetonitrile)
- Vitamin Standards (Please refer to Preparation of Standards section)
- Formic Acid
- Sodium L-Ascorbate
- Ammonium Hydroxide
- Pepsin**
- Taka Diastase from Aspergillus oryzae (α-amylase)**
- Potassium Cyanide or Sodium Cyanide
- Sodium Phosphate Monobasic
- Sodium Phosphate Dibasic Heptahydrate
- Phosphate Buffered Saline (PBS) (RP202)*

** Please note that it is advised to check all enzymes for natural vitamin content prior to analysis as they may contain traces of Vitamin B12, Folic Acid or Biotin.

Accessory Products

- Whatman S&S 597½ 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.

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.

Hazards

Sodium cyanide and potassium cyanide are highly toxic and can be corrosive to the gastrointestinal tract, skin, nose and eyes. Only laboratories equipped to handle toxic materials and solvents should perform analyses. Any steps involving sodium cyanide or potassium cyanide should be performed in a ventilated fume hood. 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.

Decontamination

Glassware should be thoroughly washed and rinsed before use to avoid cross contamination.

Prior to disposal, excess sodium cyanide and potassium cyanide 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. It is recommended that the representative sample is finely ground and a portion (2 - 10 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.

For optimal column performance, taking into account the LOQ of a typical HPLC system, aim to load sample containing a quantity of vitamin according to the table below:

Vitamin	Aim to Load	Do Not Exceed
Biotin	0.3 - 0.6 µg	0.7 µg
Folic Acid	0.1 - 0.7 µg	1.0 µg
Vitamin B12	0.01 - 2.0 µg	4.0 µg

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 and bottom of the column and allow the storage buffer to drain by gravity. This will take approximately 5 minutes. Replace the lower cap and place the column in an immunoaffinity column rack or clamp stand.

Preparation of 0.1 M Sodium Phosphate Buffer

This buffer can be kept for 5 days if stored at room temperature.

1. Weigh 4.68 g of sodium phosphate monobasic (anhydrous) and 16.37 g of sodium phosphate dibasic heptahydrate into a flask.
2. Make up to 1 Litre with water.
3. Check that the pH is 7.0.

Preparation of 10 % Sodium Ascorbate Solution

The solution should be prepared fresh on day of analysis.

1. Weigh 10 g of sodium l-ascorbate into an amber flask.
2. Make up to 100 ml with water.

Preparation of 0.5 % Ammonium Hydroxide in Methanol

The solution can be kept for 1 month if stored at room temperature.

1. Add 1 litre of methanol to a flask.
2. Remove 5 ml to waste.
3. Add 5 ml of ammonium hydroxide.

Preparation of Mobile Phase A (0.0125 % Formic Acid in Water)

This solution should be prepared fresh on day of analysis.

1. Add 1 litre of water to a flask.
2. Remove 125 µl to waste.
3. Add 125 µl of formic acid.

Preparation of Mobile Phase B (0.0125 % Formic Acid in Acetonitrile)

This solution should be prepared fresh on day of analysis.

1. Add 1 litre of "S" Grade acetonitrile to a flask.
2. Remove 125 µl to waste.
3. Add 125 µl of formic acid.

Sample Preparation

• Infant Formula

1. Weigh 2 - 10 g of ground sample into a 100 ml amber glass screw cap bottle.

2. Add 50 ml of 0.1 M sodium phosphate buffer (pH 7.0).

3. Place on a magnetic stirrer and add 2 g of pepsin (and 1 g of α-amylase if the sample also contains starch). Leave stirring for 10 minutes.

4. Add 6 ml of sodium ascorbate and 1 ml of 1 % sodium cyanide solution or 1 ml of 1 % potassium cyanide solution and leave sample to stir for a further 5 minutes.

5. Incubate the sample in a shaking water bath at 37 °C for 30 minutes.

6. Transfer the sample to a second shaking water bath and incubate at 100 °C for 15 minutes. Remove the sample and allow to cool to room temperature.

7. Transfer the extract into a 100 ml amber volumetric flask and make up to the mark with 0.1 M sodium phosphate buffer.

8. Centrifuge the extract at 4,000 rpm for 10 minutes before filtering through a Whatman S&S 597½ filter paper.

9. Dependent on the commodity being analysed add the appropriate volume of filtrate (5 - 9 ml) to the pre-prepared column. Please refer to the Column Preparation section for further information. Replace the upper cap.

10. Invert the column end over end by hand to ensure the gel is thoroughly mixed and does not collect in the narrow part of the column. Place the column in a rotary shaker and mix slowly for 15 minutes.

11. Return the column to the column rack or clamp stand and allow the column to sit for 5 minutes before opening the caps to let the liquid drain by gravity.

12. Wash the column by passing 9 ml of phosphate buffered saline (PBS) solution through, followed by 9 ml of water using a pump unit at a flow rate of approximately 5 ml per minute. Pass air through the column to remove residual liquid. Dry the inside of the column with tissue paper without touching the gel to remove any residual water from the column.

13. Elute the vitamins from the column at a flow rate of 1 drop per second using 2 ml of 0.5 % ammonium hydroxide in methanol and collect in a glass tube. Pass approximately 40 ml of air though the column to remove residual liquid.

14. Following elution pass a further 1 ml of 0.5 % ammonium hydroxide in methanol through the column and collect in the same glass tube to give a 3 ml total volume. Pass air though the column to remove residual liquid.

15. Evaporate the eluate to dryness under nitrogen at 60 - 70 °C.

16. Reconstitute with 300 µl of mobile phase A. Vortex for 20 seconds.

17. Inject 100 µl onto the HPLC system.

Sample Preparation

- **Health Supplements**

This method has been tested on a number of samples including tablets, chewables and oil capsules.

Note: in certain chewable samples, if the level of pectin is high this can affect the folic acid recovery. Therefore, an alternative method may be required to release this vitamin from the matrix for analysis.

1. Place 1 whole tablet / chewable / capsule into a 100 ml amber glass screw cap bottle.

2. Add 50 ml of 10 % Tween 20 in 0.1 M sodium phosphate buffer (pH 7.0).

3. Add 1 ml of 1 % sodium cyanide or 1 ml of 1 % potassium cyanide solution and stir for 5 minutes.

4. Incubate the sample in a shaking water bath at 100 °C for 15 minutes. Remove the sample and allow to cool to room temperature in an ice bath.

5. Transfer the extract into a 100 ml amber volumetric flask and fill to the mark with 10 % Tween 20 in 0.1 M sodium phosphate buffer.

7. Centrifuge the sample at 4,000 rpm for 10 minutes before filtering through a Whatman S&S 597½ filter paper.

Note: dilute an aliquot of the filtrate with 10 % Tween 20 in 0.1 M sodium phosphate buffer to obtain a concentration which will not exceed 700 ng biotin, 1000 ng folic acid and 4000 ng vitamin B12 when applied to the column.

8. **Tablets and capsules:** Dilute 0.5 ml of filtrate with 39.5 ml of 10 % Tween 20 in 0.1 M of sodium phosphate buffer.
Chewables: Dilute 1 ml of filtrate with 39 ml of 10 % Tween 20 in 0.1 M of sodium phosphate buffer.

9. Dependent on the commodity being analysed add the appropriate volume of filtrate (5 - 9 ml) to the pre-prepared column. Please refer to the Column Preparation section in the Instructions For Use for further information.

10. Invert the column end over end by hand to ensure the gel is thoroughly mixed and does not collect in the narrow part of the column. Place the column in a rotary shaker and mix slowly for 15 minutes.

11. Return the column to the column rack or clamp stand and allow the column to sit for 5 minutes before opening the caps to let the liquid drain by gravity.

HPLC:

12. Wash the column by passing 9 ml of phosphate buffered saline (PBS) followed by 9 ml of water.

13. Elute the vitamins from the column at a flow rate of 1 drop per second using 2 ml of 0.5 % ammonium hydroxide in methanol and collect in a glass tube. Pass air though the column to remove residual liquid.

14. Following elution pass a further 1 ml of 0.5 % ammonium hydroxide in methanol through the column and collect in the same glass tube to give a 3 ml total volume.

15. Evaporate the eluate to dryness under nitrogen at 65 °C.

16. Reconstitute with 300 µl of water of mobile phase A.

17. Inject 100 µl onto the HPLC-UV system.

LC-MS/MS:

12. Wash the column by passing 9 ml of 20 mM ammonium acetate followed by an additonal 9 ml of 20 mM ammonium acetate at a flow rate of approximately 5 ml per minute. Pass air through the column to remove residual liquid. Dry the inside of the column with tissue paper without touching the gel to remove any residual water from the column.

13. Elute the vitamins from the column at a flow rate of 1 drop per second using 2 ml of 0.5 % ammonium hydroxide in methanol and collect in a glass tube. Pass air though the column to remove residual liquid.

14. Following elution pass a further 1 ml of 0.5 % ammonium hydroxide in methanol through the column and collect in the same glass tube to give a 3 ml total volume.

15. Evaporate the eluate to dryness under nitrogen at 65 °C.

16. Reconstitute with 1 ml of water. Vortex for 20 seconds.

17. Inject 25 µl onto the LC-MS/MS system.

Preparation of Standards

• Preparation of Biotin Stock Solutions

1. Powdered D-Biotin can be purchased. The powder is dissolved to give a concentration of 100 µg/ml.
2. Leave overnight at 2 - 8 ºC to give a stock solution. All standards should be prepared in amber glassware.

• Preparation of Folic Acid Stock Solutions

1. Powdered folic acid can be purchased. The powder is dissolved to give a concentration of 200 µg/ml.
2. Leave overnight at 2 - 8 °C to give a stock solution. All standards should be prepared in amber glassware.

• Preparation of Vitamin B12 Stock Solutions

1. Powdered cyanocobalamin can be purchased. The powder is dissolved to give a concentration of 1,000 µg/ml.
2. Leave overnight at 2 - 8 °C to give a stock solution. All standards should be prepared in amber glassware.
3. This is then used to prepare a 100 µg/ml vitamin B12 stock solution.

• Combined Working Solution

1. Add the following volumes of stock solutions and make up to 10 ml with water to prepare combined working solution.

Vitamin	Stock Solution Concentration (µg/ml)	Volume of Stock Solution Added (µl)	Working Solution Concentration (µg/ml)
Biotin	100 µg/ml	300 µl	3 µg/ml
Folic Acid	200 µg/ml	500 µl	10 µg/ml
Vitamin B12	100 µg/ml	100 µl	1 µg/ml

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 requirements or expected range of vitamin):

To prepare a six point calibration curve:

1. Standard 6: Take 2 ml of combined working solution and make up to 10 ml with mobile phase A (equivalent to 0.6 µg/ml of biotin, 2 µg/ml of folic acid and 0.2 µg/ml of vitamin B12).

2. Standard 5: Take 1.5 ml of standard 6 and make up to 10 ml with mobile phase A (equivalent to 0.45 µg/ml of biotin, 1.5 µg/ml of folic acid and 0.15 µg/ml of vitamin B12).

3. Standard 4: Take 1 ml of standard 5 and make up to 10 ml with mobile phase A (equivalent to 0.3 µg/ml of biotin, 1 µg/ml of folic acid and 0.1 µg/ml of vitamin B12).

4. Standard 3: Take 0.5 ml of standard 4 and make up to 10 ml with mobile phase A (equivalent to 0.15 µg/ml of biotin, 0.5 µg/ml of folic acid and 0.05 µg/ml of vitamin B12).

5. Standard 2: Take 0.25 ml of standard 3 and make up to 10 ml with mobile phase A (equivalent to 0.075 µg/ml of biotin, 0.25 µg/ml of folic acid and 0.025 µg/ml of vitamin B12).

6. Standard 1: Take 0.125 ml of standard 2 and make up to 10 ml with mobile phase A (equivalent to 0.0375 µg/ml of biotin, 0.125 µg/ml of folic acid and 0.0125 µg/ml of vitamin B12).

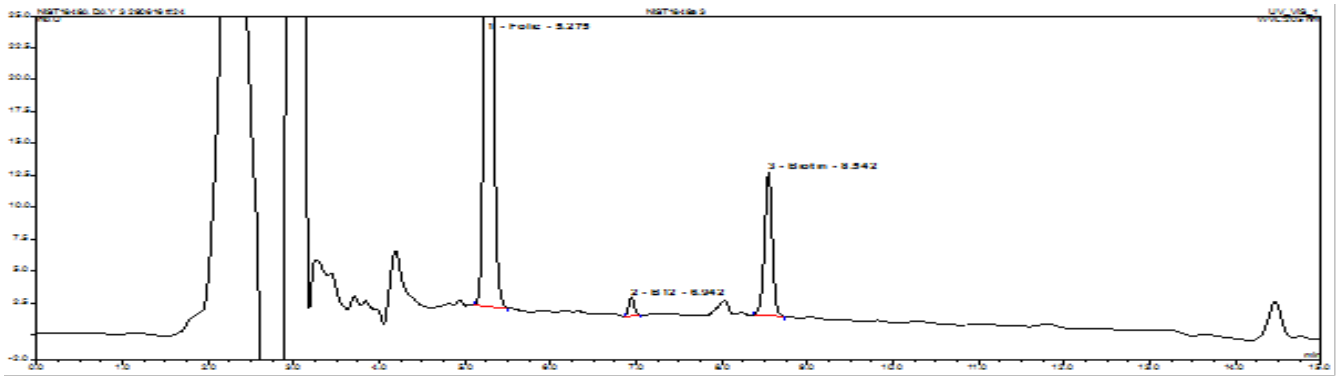
7. Inject 100 µl of each solution onto the HPLC system.

Recommended HPLC Conditions

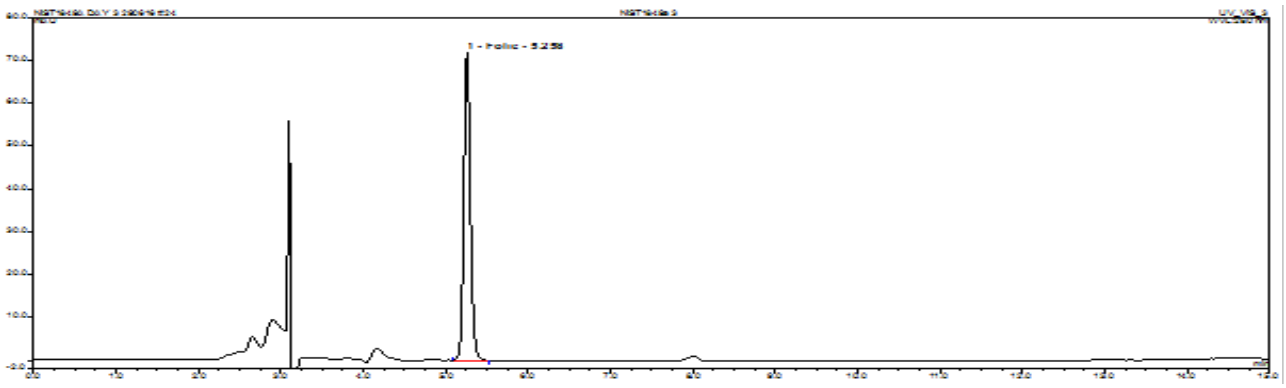
HPLC Conditions			
Guard Cartridge	KrudKatcher 0.5 µm depth filter or equivalent		
Analytical Column	Hypersil Gold 3 µm, 4.6 mm x 150 mm or equivalent		
Mobile Phase	**Mobile Phase A:** 0.0125 % Formic Acid in Water **Mobile Phase B:** 0.0125 % Formic Acid in Acetonitrile ("S" Grade) Prepare fresh on day of analysis.		
Gradient Conditions	Time (min)	% Solution A	% Solution B
	0	92.5	7.5
	0.1	92.5	7.5
	10.0	80	20
	15.0	80	20
	15.1	92.5	7.5
	25.0	92.5	7.5
HPLC Pump	To deliver mobile phase		
Flow Rate	0.8 ml per minute		
UV Detector	Biotin:	205 nm	
	Folic Acid:	280 nm	
	Vitamin B12:	361 nm	
Column Heater	Maintain guard and analytical column at 40 °C		
Integrator / Data Control System	From preferred supplier		
Injector	Autosampler / Rheodyne valve		
Injection Volume	100 µl		

Example HPLC Chromatograms

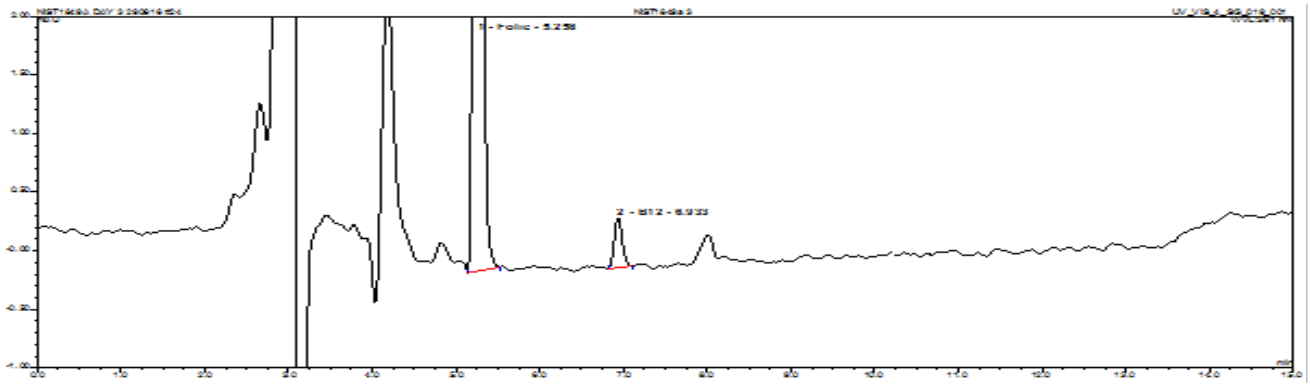
- **Milk Formula for Biotin at 205 nm containing 0.786 µg/ml of Biotin**



- **Milk Formula for Folic Acid at 280 nm containing 0.954 µg/ml of Folic Acid**



- **Milk Formula for Vitamin B12 at 361 nm Containing 0.025 µg/ml of Vitamin B12**



Recommended LC-MS/MS Conditions

LC Conditions			
Analytical Column	Hypersil Gold 3 µm, 4.6 mm x 150 mm or equivalent		
Mobile Phase	**Mobile Phase A:** 0.1 % Acetic Acid in Water **Mobile Phase B:** 0.1 % Acetic Acid in Acetonitrile (LC-MS/MS Grade) Prepare fresh on day of analysis.		
Gradient Conditions	Time (min)	% Solution A1	% Solution B1
	0	92.5	7.5
	0.1	92.5	7.5
	10.0	80	20
	11.0	80	20
	11.1	92.5	7.5
	13.0	92.5	7.5
Flow Rate	0.8 ml per minute		
Column Heater	Maintain guard and analytical column at 40 °C		
Integrator / Data Control System	From preferred supplier		
Injector	Autosampler / Rheodyne valve		
Injection Volume	25 µl		

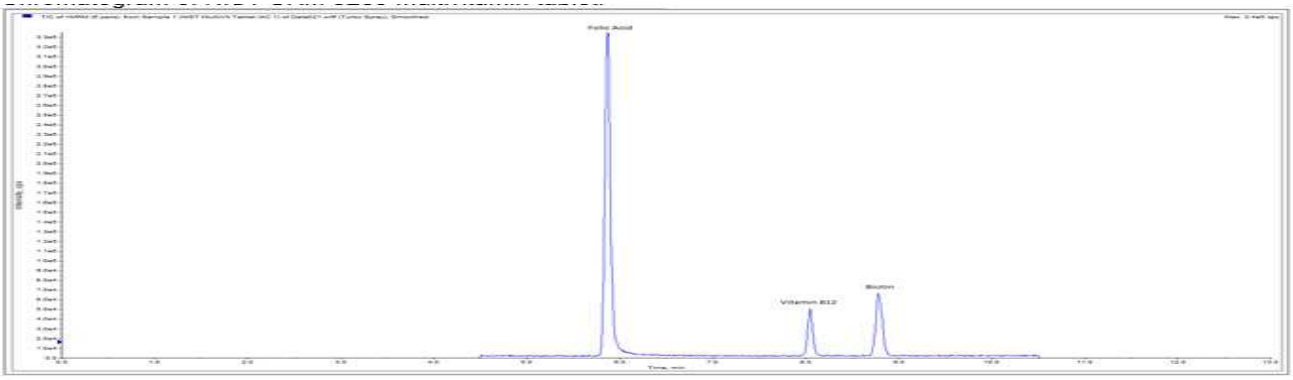
Mass Spectometry Conditions	
Instrument	Sciex QTRAP 4500
Mode	MRM
Curtain Gas	40 psi
Collision Gas	6 psi
Temperature	600 °C
Ion Spray Voltage	2000 V
Ion Source Gas 1	40 psi
Ion Source Gas 2	60 psi
Entrance Potential	10 V

Instrument Settings

Analyte	RT (min)	Precursor Ion (m/z)	Product Ions (m/z)	Dwell Time (ms)	Declustering Potential	Collision Energy (V)	Cell Exit Potential (V)
B7	8.9	245.0	227.1 97.0	20	50	9 24	15 15
B9	6.0	442	176.0 120.0	20	85	36 30	12 12
B12	8.15	678.4	147.0 359.2	20	100	34 42	11 11

Example LC-MS/MS Chromatogram

- **Vitamin tablet**



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.

R-Biopharm Rhône Ltd
Block 10 Todd Campus
West of Scotland Science Park
Acre Road, Glasgow G20 0XA
www.r-biopharm.com