EASI-EXTRACT® VITAMIN B12

Product Code: P80 / P80B

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

P80/V23/04.08.21

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R-BIOPHARM
RHÔNE LTD

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 vitamin of interest. Following extraction of the vitamin the sample extract is diluted with buffer and filtered before being passed slowly through the immunoaffinity column. Any vitamin which is present in the sample is retained by the antibody within the gel suspension. The column is washed to remove any unbound material and the vitamin 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 2 hours to perform. The result is improved clean-up and concentration of the vitamin 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
- Solvents (HPLC Grade Methanol and Acetonitrile)
- Vitamin B12 Standard (Please refer to Preparation of Standards section)
- Sodium Acetate
- Pepsin*
- Trifluoroacetic Acid (TFA)
- Potassium Cyanide or Sodium Cyanide
- Taka Diastase from Aspergillus oryzae (α-amylase)*
- Acetic Acid

* 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.

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

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.

For optimal column performance, taking into account the LOQ of a typical HPLC system, aim to load sample containing a quantity of 0.01 - 0.5 µg of vitamin B12 onto the column. Do note exceed a quantity of 1.0 µg as this is close to the capacity.

Recoveries

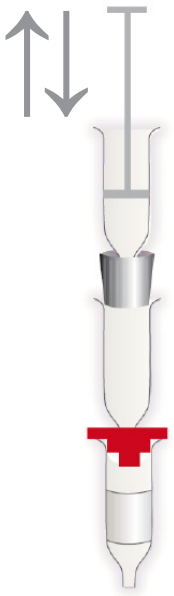
If an analyst wishes to account for losses during extraction it is recommended a spiked sample of the same commodity type as the material being tested is analysed following the complete procedure as a reference standard. The recoveries obtained with the spiked sample can be used to correct the results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

Backflushing

Backflushing is carried out to increase the time the solvent is in contact with the antibody within the gel suspension ensuring that all of the vitamin is eluted. 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 eluant. This should be repeated 3 times.



Preparation of Solution A (Water Containing 0.025 % TFA)

The solution should be prepared fresh on day of analysis.

1. Add 2 litres of water to a flask.
2. Remove 500 µl to waste.
3. Add 500 µl of trifluoroacetic acid (TFA).

Preparation of 50 mM Sodium Acetate Buffer

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

1. Weigh 4.1 g of sodium acetate into a flask.
2. Add 950 ml of water.
3. Adjust the pH to 4.0 using acetic acid.
4. Make up to 1 Litre with water and check that the pH is still 4.0.

Sample Preparation

• Infant Formula, Food and Energy Bars

1. Dependent on the commodity being analysed weigh the appropriate amount of ground sample into an amber glass screw cap bottle.

Commodity	Volume of Ground Sample
Infant formula and food (e.g. cereal, dairy, meat homogenate, baby food composite)	5 - 30 g
Energy bars	10 g

2. Add 50 ml of 50 mM sodium acetate buffer (pH 4.0).

3. Place on a magnetic stirrer and add 0.5 g of α-amylase and 2 g of pepsin. Leave stirring for 10 minutes.

4. Add 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 30 minutes. Remove the sample and allow to cool to room temperature.

7. Transfer the extract into a 100 ml amber volumetric flask and fill to the mark with 50 mM sodium acetate buffer.

8. Filter the sample through a Whatman S&S 597½ filter paper.

9. Dependent on the commodity being analysed pass the appropriate volume of filtrate through the column according to the table below. Pass the filtrate 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 vitamin by the antibody.

Commodity	Volume of Filtrate
Infant formula	5 - 10 ml
Food (e.g. cereal, dairy, meat homogenate, baby food composite)	15 - 30 ml
Energy bars	20 - 25 ml

10. 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.

11. Elute the vitamin from the column at a flow rate of 1 drop per second using 3 ml of 100 % methanol and collect in a glass tube. Backflushing is recommended. Please refer to Backflushing section for further information.

12. Evaporate the eluate to dryness under air at 60 - 70 °C.

13. Reconstitute with 300 µl of solution A. Vortex for 20 seconds.

14. Inject 100 µl onto the HPLC system.

Sample Preparation

• Milk

1. Measure 30 ml of sample into an amber glass screw cap bottle.
2. Add 50 ml of 50 mM sodium acetate buffer (pH 4.0).
3. Place on a magnetic stirrer and add 0.25 g of α-amylase and 1 g of pepsin. Leave stirring for 10 minutes.
4. Add 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 30 minutes. Remove the sample and allow to cool to room temperature.
7. Transfer the extract into a 100 ml amber volumetric flask and fill to the mark with 50 mM sodium acetate buffer.
8. Filter the sample through a Whatman S&S 597½ filter paper.
9. Dependent on the commodity being analysed pass the appropriate volume of filtrate through the column according to the table below. Pass the filtrate 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 vitamin by the antibody.

Commodity	Volume of Filtrate
Cow's milk, soya milk	10 ml
Long life UHT milk	20 ml

10. 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.
11. Elute the vitamin from the column at a flow rate of 1 drop per second using 3 ml of 100 % methanol and collect in a glass tube. Backflushing is recommended. Please refer to the Backflushing section for further information.
12. Evaporate the eluate to dryness under air at 60 - 70 °C.
13. Reconstitute with 300 µl of solution A. Vortex for 20 seconds.
14. Inject 100 µl onto the HPLC system.

Preparation of Standards

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

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.

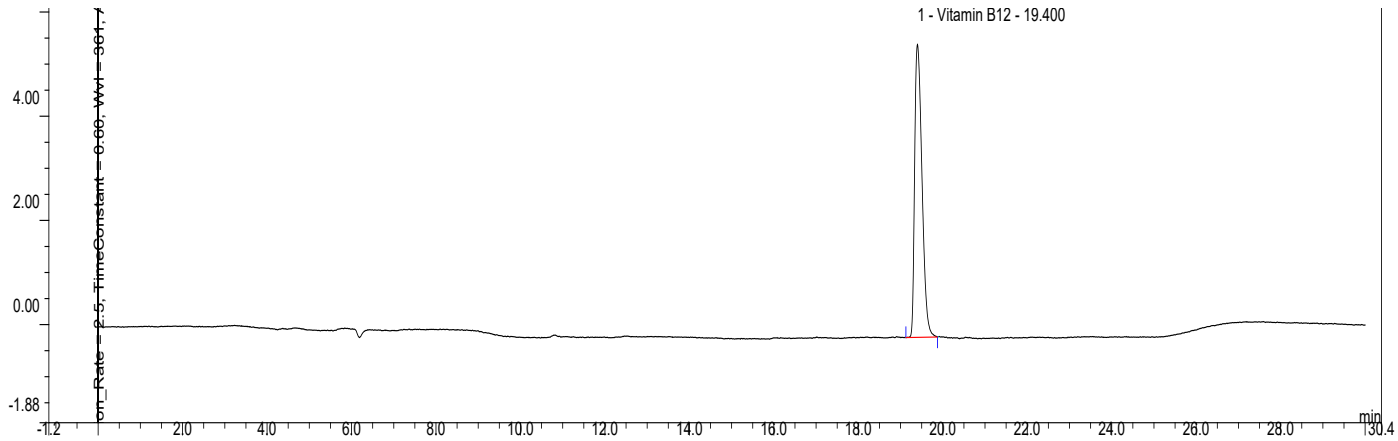
Example of how to prepare a four point calibration curve (can be modified according to expected vitamin content):

1. Take 100 ml of water and remove 1 ml to waste.
2. Add 1 ml of 1 mg/ml cyanocobalamin standard to give a 10 µg/ml cyanocobalamin solution.
3. Standard 4: Take 8 ml of solution A and remove 120 µl to waste. Add 120 µl of 10 µg/ml solution (equivalent to 0.15 µg/ml).
4. Standard 3: Take 1 ml of 0.15 µg/ml and add 1 ml of solution A (equivalent to 0.075 µg/ml).
5. Standard 2: Take 1 ml of 0.075 µg/ml and add 1 ml of solution A (equivalent to 0.0375 µg/ml).
6. Standard 1: Take 1 ml of 0.0375 µg/ml and add 1 ml of solution A (equivalent to 0.01875 µg/ml).
7. Inject 100 µl of each solution onto the HPLC system.

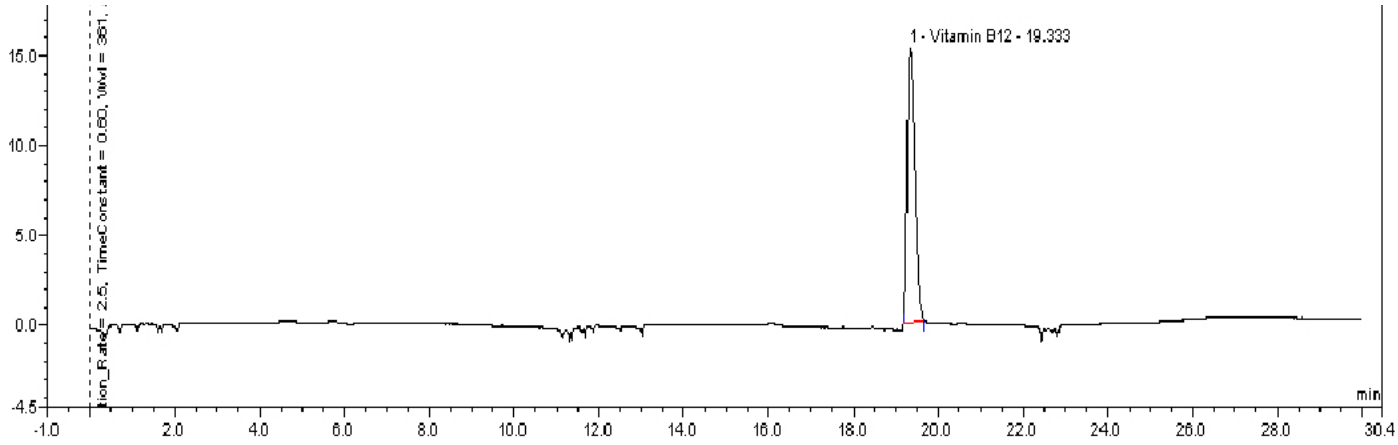
Recommended HPLC Conditions

HPLC Conditions			
Guard Cartridge	ACE 3 AQ 3 µm, 4 mm x 10 mm or equivalent		
Analytical Column	C18 ACE 3 AQ 3 µm, 3 mm x 150 mm or equivalent		
Mobile Phase	Solution A: 0.025 % TFA in Water (pH 2.6) Solution B: Acetonitrile Prepare fresh on day of analysis.		
Gradient Conditions	Time	% Solution A	% Solution B
	0	100	0
	0.5	100	0
	11	85	15
	19	75	25
	20	90	10
	26	100	0
	30	100	0
HPLC Pump	To deliver mobile phase		
Flow Rate	0.25 ml per minute		
UV Detector	361 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 Chromatogram for Baby Food Composite



Example HPLC Chromatogram for Cow's Milk



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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