AOAC Official Final Action Method 2014.02

Product referenced

EASI-EXTRACT® VITAMIN B12 (LGE)

Product Code: P88

Immunoaffinity columns for use in conjunction with HPLC.
For in vitro use only.

P88/V18/10.03.22

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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. 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 antibody within the column. Following extraction of the vitamin 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 vitamin and the antibody. 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 vitamin is then released from the column following elution with solvent. The eluate is collected, evaporated and reconstituted prior to analysis by HPLC.

The extra contact between the sample and the antibody results in a more uniform binding of the vitamin with the antibody and leads to better repeatability and reproducibility.

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.

Reagents Not Provided

- Distilled / Deionised Water (suitable for use with HPLC, e.g. MilliQ)
- Solvents (HPLC Grade Methanol and Acetonitrile)
- Vitamin B12 Standard
- Sodium Acetate
- Trifluoroacetic Acid (TFA)
- Sodium Cyanide or Potassium Cyanide
- Taka Diastase from Aspergillus oryzae (α-amylase)*
- Acetic Acid
- Skimmed Milk Powder

* 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

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.

Please note that these immunoaffinity columns are suitable for use with AOAC Official Methods 2011.09 and 2014.02 . Please refer to these for further information or contact your local R-Biopharm distributor.

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 and bottom of the column and allow the storage buffer to drain by gravity. This should take approximately 5 minutes however a syringe pump can be used to aid the removal of the storage buffer from the column. Replace the lower cap and place the column in an immunoaffinity column rack or clamp stand.

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

This method is based on the AOAC Official Method 2014.02 vitaman B12 (cyanocobalamin) in instant formula and adult/pediatric nutritional formula.

• Powder Samples - infant formula and adult/pediatric formulas

1. Weigh 25 g of ground sample into a 250 ml amber glass screw cap bottle.
2. Place on a magnetic stirrer and add 200 ml of water at 35 - 45 °C. Leave stirring for 10 minutes. Alternatively, mix with a glass rod until the suspension is homogenous.
3. Weigh 60 g of sample suspension into a 250 ml amber glass screw cap bottle.
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. If the sample contains starch add 0.05 g of α-amylase, mix thoroughly and incubate in a shaking water bath at 35 - 45 °C for 30 minutes.
6. Add 25 ml of sodium acetate buffer and mix.
7. Transfer the sample to a second shaking water bath and incubate at boiling point for 30 minutes. Alternatively, autoclave at 100 °C for 30 minutes. Remove the sample and allow to cool in an ice bath.
8. Transfer the extract into a 100 ml amber volumetric flask and make up to the mark with water.
9. Filter the sample through a Whatman S&S 597½ filter paper.
10. Add 9 ml of filtrate to the pre-prepared column. Please refer to the Column Preparation section for further information. Replace the upper cap.
11. 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.
12. 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.
13. Wash the column by passing 10 ml of water through 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.
14. 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. Pass approximately 40 ml of air though the column to remove residual liquid.
15. Following elution pass a further 0.5 ml of 100 % methanol through the column and collect in the same glass tube to give a 3.5 ml total volume. Pass approximately 20 ml of air though the column to remove residual liquid.
16. Evaporate the eluate to dryness under nitrogen at 50 - 60 °C.
17. Reconstitute with 300 µl of solution A. Vortex for 20 seconds.
18. Inject 100 µl onto the HPLC system.

Sample Preparation

This method is based on the AOAC Official Method 2014.02 vitaman B12 (cyanocobalamin) in instant formula and adult/pediatric nutritional formula.

• Liquid Samples - infant formula and adult/pediatric formulas

1. Mix the sample well to ensure homogeneity.
2. Weigh 60 g of sample suspension into a 250 ml amber glass screw cap bottle.
3. 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.
4. If the sample contains starch add 0.05 g of α-amylase, mix thoroughly and incubate in a shaking water bath at 35 - 45 °C for 30 minutes.
5. Add 25 ml of sodium acetate buffer and mix.
6. Transfer the sample to a second shaking water bath and incubate at boiling point for 30 minutes. Alternatively, autoclave at 100 °C for 30 minutes. Remove the sample and allow to cool in an ice bath.
7. Transfer the extract into a 100 ml amber volumetric flask and make up to the mark with water.
8. Filter the sample through a Whatman S&S 597½ filter paper.
9. Add 9 ml of filtrate 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 10 ml of water through 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 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. Pass approximately 40 ml of air though the column to remove residual liquid.
14. Following elution pass a further 0.5 ml of 100 % methanol through the column and collect in the same glass tube to give a 3.5 ml total volume. Pass approximately 20 ml of air though the column to remove residual liquid.
15. Evaporate the eluate to dryness under nitrogen at 50 - 60 °C.
16. Reconstitute with 300 µl of solution A. Vortex for 20 seconds.
17. Inject 100 µl onto the HPLC system.

Sample Preparation

This method is based on the AOAC Official Method 2014.02 vitaman B12 (cyanocobalamin) in instant formula and adult/pediatric nutritional formula.

• Amino Acid Based Products - infant formula and adult/pediatric formulas

1. Weigh 25 g of ground sample into a 250 ml amber glass screw cap bottle.
2. Place on a magnetic stirrer and add 190 ml of water at 35 - 45 °C and 10 g of skimmed milk powder. Leave stirring for 10 minutes. Alternatively, mix with a glass rod until the suspension is homogenous.

   Note: In the case of high-fat nutritional products, if recovery is low, samples can be diluted in water (e.g., 50 g sample and 50 g of water) before extraction to improve recovery.
3. Weigh 60 g of sample suspension into a 250 ml amber glass screw cap bottle.
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. If the sample contains starch add 0.05 g of α-amylase, mix thoroughly and incubate in a shaking water bath at 35 - 45 °C for 30 minutes.
6. Add 25 ml of sodium acetate buffer and mix.
7. Transfer the sample to a second shaking water bath and incubate at boiling point for 30 minutes. Alternatively, autoclave at 100 °C for 30 minutes. Remove the sample and allow to cool in an ice bath.
8. Transfer the extract into a 100 ml amber volumetric flask and make up to the mark with water.
9. Filter the sample through a Whatman S&S 597½ filter paper.
10. Add 9 ml of filtrate to the pre-prepared column. Please refer to the Column Preparation section for further information. Replace the upper cap.
11. 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.
12. 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.
13. Wash the column by passing 10 ml of water through 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.
14. 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. Pass approximately 40 ml of air though the column to remove residual liquid.
15. Following elution pass a further 0.5 ml of 100 % methanol through the column and collect in the same glass tube to give a 3.5 ml total volume. Pass approximately 20 ml of air though the column to remove residual liquid.
16. Evaporate the eluate to dryness under nitrogen at 50 - 60 °C.
17. Reconstitute with 300 µl of solution A. Vortex for 20 seconds.
18. Inject 100 µl onto the HPLC system.

Sample Preparation

• Validation Method

1. Using a pipette measure 180 ml of 50 mM sodium acetate buffer (pH 4) into an amber glass screw cap bottle. Remove 300 µl to waste. Add 300 µl of 10 µg/ml standard solution and mix. This gives a 0.0167 µg/ml solution of cyanocobalamin.

2. Take 5 immunoaffinity columns and close the lower caps. Add 9 ml of the prepared solution to each column and close the upper caps. This is equivalent to passing 0.15 µg of cyanocobalamin through the immunoaffinity column.

3. 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.

4. 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.

5. Wash the column by passing 10 ml of water through using a pump unit at a flow rate of approximately 5 ml per minute. Pass air though 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.

6. 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. Pass approximately 40 ml of air though the column to remove residual liquid.

7. Evaporate the eluate to dryness under nitrogen at 50 - 60 °C.

8. Reconstitute with 1 ml of solution A. Vortex for 20 seconds.

9. Inject 100 µl onto the HPLC system.

10. Calculate the µg/ml recovered from the 0.0167 µg/ml cyanocobalamin solution. Average recoveries should lie between 70 - 110 %.

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.

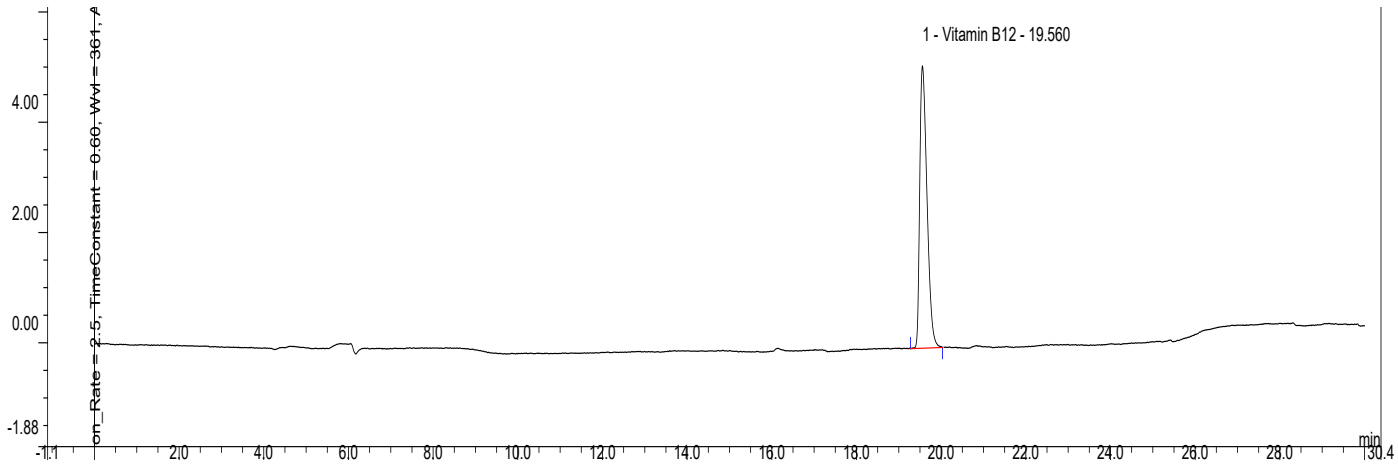
Example of how to prepare a four point calibration curve (can be modified according to expected vitamin content):

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: Add 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.

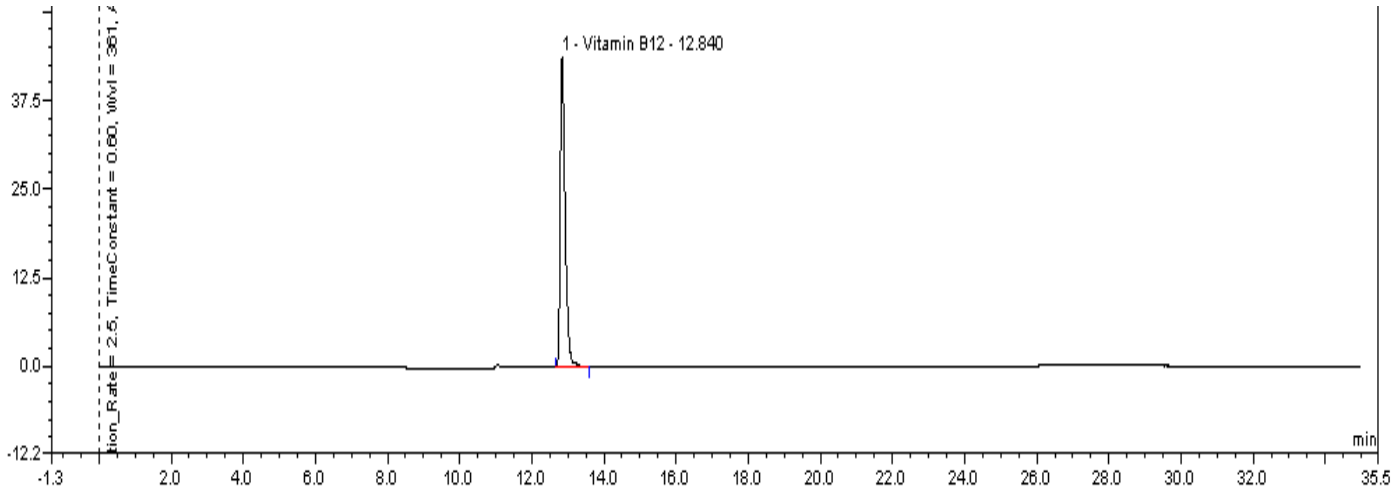
Recommended HPLC Conditions

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 (min)	% 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 Powdered Samples



Example HPLC Chromatogram for Validation Method



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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