

Instruction of the Clean-up Process using **B-TeZ IAC Vitamin B₁₂ 3ml**

Fast and Accurate Content Determination of Vitamin-B₁₂ (Cyanocobalamin) in Vitamin Tablets, Liquid Vitamin Preparations, Cell Culture Extracts etc. by Combination of Immunoaffinity Chromatography and HPLC

Principle:

Many methods of Vitamin B₁₂ determination are based on HPLC-UV (High Performance Liquid Chromatography-ultraviolet) detection show low selectivity if problematic matrices are applied. This method of content determination of Vitamin B₁₂ combines the high selectivity of immunoaffinity columns with its potential to concentrate, elute, and purify with the HPLC column.

Sample Preparation:

Vitamin B₁₂ samples are to be extracted and analyzed with the method of Li et al. [H.-B. Li, F. Cheng, Y. Jiang *J. Chromatography. A* **2000**; 891:243-247], e.g. vitamin tablets, liquid vitamin preparations, cell culture extracts.

Example: 25g vitamin containing tablets are dissolved in 100ml PBS. The resulting extract may be filtered through a 0.45µm membrane filter.

Enrichment Step

Immunoaffinity Chromatography (IAC):

4ml extract (containing the quantity of Vitamin B₁₂ from a 1g sample of above-mentioned sample preparation is followed) is diluted with a total volume of 20ml PBS and then applied in a reservoir on top of the BioTeZ-Immunoaffinity Column. The optimal flow rate through the gel is between 1 to 3ml/min.

According to application and contents expected, the applied extract volumes could vary. E.g. extracts may be diluted 1+1 with PBS or 1+4 as mentioned above. In case of very low contents, extract volumes of 200ml may be applied without significant loss of analyte as long as resulting pH is fairly neutral and alcohol or acetonitrile content lies under 15%.

Wash:

After the whole sample has passed through the gel, the latter is washed with 5ml of PBS. Remaining liquids in the gel are removed by applying pressure from either the top of the column or pressure from the bottom.

Elution:

The sample reservoir on top of the BioTeZ-Immunoaffinity Column is removed, and an appropriate vial is placed below the affinity column. The bounded vitamin B₁₂ is eluted by

methanol. The elution process is performed in two steps. First, an amount of 1ml methanol is applied. Once this amount has passed through the column, there should be a waiting time of 30 seconds. After that, the second portion of 2ml of methanol is eluted through the column. The remaining methanolic solutions should be eluted by application of slight under- or overpressure. All methanolic fractions are unified to give the column elute.

The column elute may be injected into the HPLC directly or if concentrations are very low, the elute can be concentrated by evaporation (e.g. using VLM evaporator), re-dissolved in HPLC solvent, and finally injected into the system. For the latter case, please see the sample calculation in which the sample concentrate is re-dissolved in 0.4ml HPLC solvent.

Analytical Method:

- Machine: Shimadzu;
- Column: Trentec Reprosil-Pur RP C18 120 ODS3 5µm; 125x3,0mm with guard column
- Mobile Phase A: acetonitrile /water (70:30 v/v) (use only for cleaning purposes at the beginning and at the end of analytical series)
- Mobile Phase B: 0.03M potassium phosphate, pH 7.0-methanol (80/20 v/v)
- Gradient: 0.01min B 100%; 30min B 100% (isocratic)
- Flow Rate: 0.5ml/min
- Time of Analysis: 30min
- Injector Volume: 100µl
- Detection: λ_{ABS} [nm]: 361nm.

Characteristics:

The measuring range is linear of 25ng to 1250ng Vitamin B₁₂ pro injection (R²=0.9999). The limit of detection is 3ng of vitamin B₁₂ per injection (three times of signal/noise ratio). If the given dilution steps are followed, the vitamin B₁₂ contents of 0.1 to 5µg/g lie within the linear working range of the method. If the contents of the samples used are higher than the cited range, extracts should be diluted in a suitable manner. The lower limit of detection is 10ng/g of vitamin B₁₂ in the sample.

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Recovery rates are >85% when vitamin B₁₂ in buffer mixtures is analyzed in the range of 0.1 to 5µg per IAC.

Example Sample Calculation:

$$\frac{25\text{g Sample}}{100\text{ml Extraction Solvent}} \times \frac{4\text{ml Extract}}{0.4\text{ml}} \times \frac{0.1\text{ml}}{\text{injector volume}} = 0.25\text{g Sample Equivalents}$$

$$\frac{\# \mu\text{g injected Vitamin B}_{12}}{\text{Sample Equivalents [g]}} = \mu\text{g/g Vitamin B}_{12} \text{ in e.g. vitamin tablet}$$

Buffer and Chemicals:

Phosphate Buffered Saline pH 7.4 (= PBS):

1.24g KH₂PO₄
7.27g K₂HPO₄
8.76g NaCl

Dissolve in 1L deionized water. If necessary adjust pH to 7.4

HPLC-Solvent

0.03M potassium phosphate, pH 7.0-methanol (80/20 v/v)

Dissolve 4.1g KH₂PO₄ in 800ml deionized water. Adjust to pH 7.0 with 1M NaOH. Add 200ml methanol. Degas with helium.

acetonitrile / water (70:30 v/v)
(HPLC Column Cleaning)

Mix 70ml acetonitrile and 30ml deionized water. Degas with helium.

Chemicals:

- acetonitrile, HPLC grade
- deionised water
- dipotassium hydrogenphosphate, >98%
- potassium dihydrogenphosphate, >98%
- sodium chloride

Consumables:

- B-TeZ-Vitamin-B₁₂-Immunoaffinity Column

Standard:

- Vitamin B₁₂ (Cyanocobalamin), 99% [Sigma V-2876]

Evaporation:

- nitrogen gas 5.0 [Air Liquide M55763810] (to evaporate IAC-eluate)

Apparatus:

- HPLC; Shimadzu; pump: LC-6A (2 pieces); auto sampler: SIL 6B; absorbance detector: SPD-10A; data handling: CLASS LC10
- Vacuum SPE Manifold (BAKER spe-24G Column Processor – process up to 24 samples) [J.T. Baker 7208]
- Evaporator (with tripod) [VLM EVA EC1-S]

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Manufactured by:

BioTez Berlin-Buch GmbH

Robert-Rössle-Str. 10, 13125 Berlin Germany
Phone +49 30 9489-2130 • Fax +49 30 9494-509
e-mail info@biotez.de • www.biotez.de

distributed in the US/Canada by:



EAGLE
BIOSCIENCES

EAGLE BIOSCIENCES, INC.

20A NW Blvd, Suite 112 Nashua, NH 03063
Phone: 617-419-2019 • FAX: 617-419-1110
WWW.EAGLEBIO.COM • info@eaglebio.com

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