

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 and Infant/Nutritional Formula by Combination of Immunoaffinity Chromatography and HPLC-UV

Principle:

Many methods of Vitamin B₁₂ determination based on HPLC-UV 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 of purification by HPLC column.

Sample Preparation:

A) Vitamin tablets, liquid vitamin preparations etc.

Vitamin B₁₂ samples of vitamin tablets, liquid vitamin preparations etc. are to be extracted and analysed with the method of Li et al. [H.-B. Li, F. Cheng, Y. Jiang *J. Chromatogr. 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.

B) Infant and nutritional formula.

Vitamin B₁₂ samples of infant and nutritional formula are reconstituted as cyanocobalamin and extracted as described by the AOAC method [Vitamin B₁₂ (Cyanocobalamin) in Infant Formula and Adult/Pediatric Nutritional Formula, AOAC *Official Method* **2014.02**, 2018 AOAC International] with small modifications as follows:

1. The clear extract of the extraction process prior to the IAC enrichment process which pH is 4.0 must be pH adjusted with 5.0M sodium hydroxide solution at pH 6.5 ± 0.5.
2. The IAC is washed instead of 10ml water in the AOAC protocol with 10ml PBS.

A) Vitamin tablets, liquid vitamin preparations etc.

Enrichment Step IAC:

4ml extract (containing the quantity of Vitamin B₁₂ from a 1g sample if 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 even extract volumes of 200ml may be applied without significant loss of analyte as long as resulting pH is 7.0 ± 0.5 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 either pressure from top of the column or under-inflation 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 using a total volume of 3ml of HPLC grade 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 eluate.

The column eluate may be injected into the HPLC directly or, if concentrations are very low, 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.

For **B) samples** it is recommended to redissolve in 0.3ml solvent by AOAC protocol.

Caution: The AOAC protocol has to be modified regarding pH and washing buffer

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Caution: Carefully
adjust pH of extract!

B) Infant and nutritional formula.

Enrichment Step IAC:

9ml extract is pH adjusted with small portions (e.g. 0.2ml) of 5.0M sodium hydroxide at pH 6.5 ± 0.5 (a total volume of ca. 1ml is required) 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.

Wash:

After the whole sample has passed through the gel, the latter is washed with 10ml of PBS. Remaining liquids in the gel are removed by applying either pressure from top of the column or under-inflation 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 using a total volume of 3ml of HPLC grade methanol.

The elution process is performed in two steps. Please proceed as described under A) Elution above.

A) and B) samples:

Analytical Method:

Machine: Shimadzu; Column: Altmann Hypersil 120 BDS C18 3 μ m, 100x4.6mm; with guard column; Mobile Phase A: methanol /water (85:15 v/v) Mobile Phase B: 0.03M potassium phosphate, pH 7.0-methanol (80/20 v/v); Gradient: 0.01min B 95% (isocratic); Flow Rate: 0.8ml/min; Time of Analysis: 10min; Injector Volume: 100 μ l; Detection: λ_{ABS} [nm]: 361nm (PDA, see below).

A) samples:

Characteristics:

The measuring range is linear of 10 to 1000ng/ml Vitamin B₁₂ ($R^2=0.9999$). The limit of detection (LOD) is 1ng of Vitamin B₁₂ per column (S/N = 3).

If the given dilution steps are obeyed, the Vitamin B₁₂ contents of 0.01 to 1 μ g/g lie within the linear working range of the method. If the contents of used samples are higher than cited range, extracts or IAC eluates should be diluted in a suitable manner. The lower limit of quantification is 4ng/g of Vitamin B₁₂ in the sample applying this protocol.

Recovery rates are >85% when Vitamin B₁₂ in buffer mixtures is analysed in **the range of 0.004 to 5 μ g per IAC**.

B) samples:

Characteristics:

The measuring range is linear of 1 to 50ng/ml Vitamin B₁₂ ($R^2=0.9999$). The limit of detection (LOD) is 1ng of vitamin B₁₂ per column (S/N = 3).

The vitamin B₁₂ contents of 0.002 to 0.1 μ g/g lie within the linear working range of the method. The lower limit of quantification is 2ng/g of vitamin B₁₂ in the sample applying this protocol.

Recovery rates are >85% when Vitamin B₁₂ in infant formula is analysed in **the range of 0.001 to 0.05 μ g per IAC** (higher quantities were not tested in matrix).

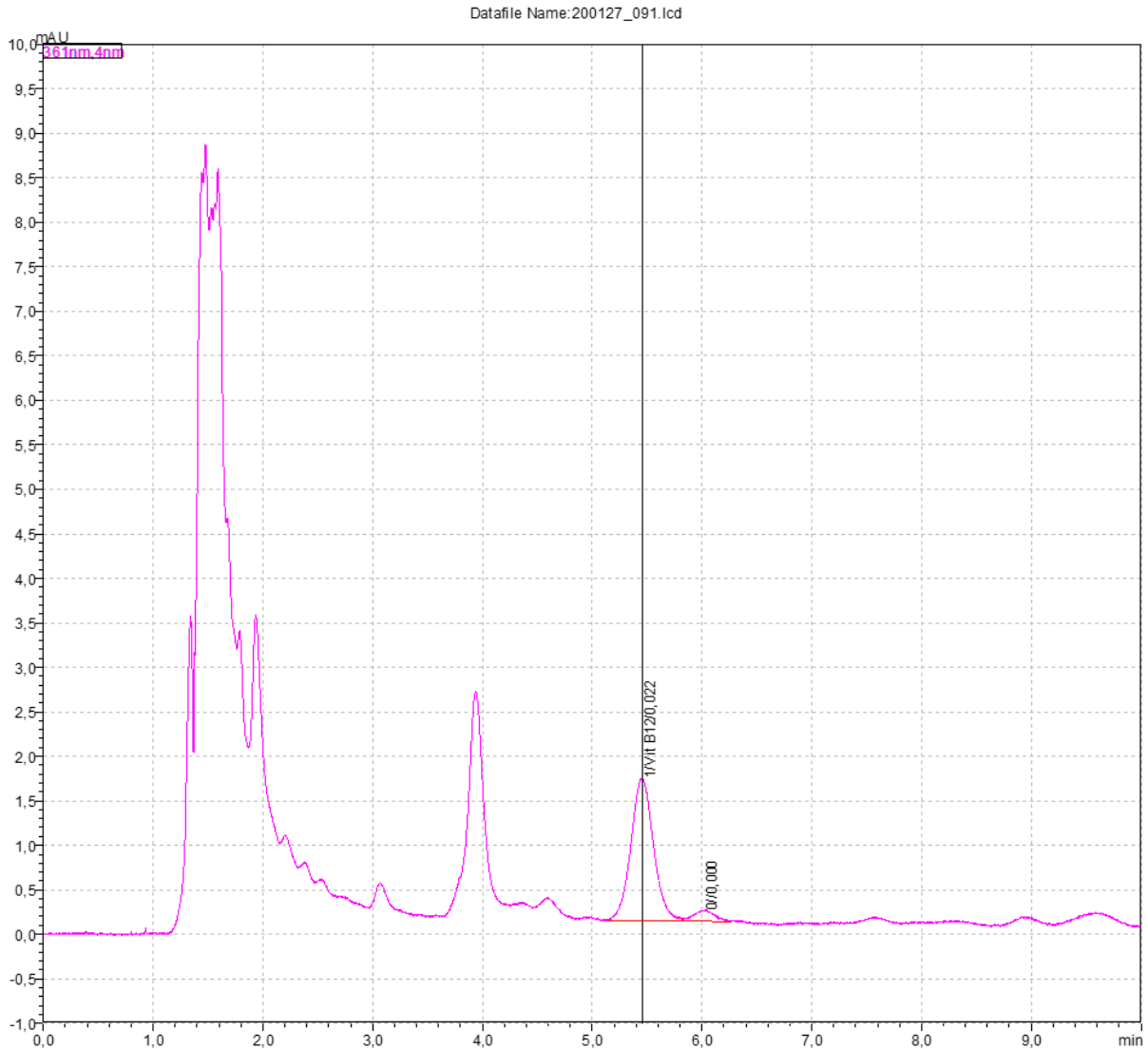
See Example chromatogram of Vitamin B₁₂ containing milk powder next page.

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B) samples: Example chromatogram.

200127_091.lcd: HPLC-UV Chromatogram of Infant formula - Milk powder (0.011µg/g Vitamin B₁₂)



A) Vitamin tablets, liquid vitamin preparations etc.

Example Sample Calculation:

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

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

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B) Infant and nutritional formula.

Sample Calculation according *AOAC Official Method 2014.02*, 2018 AOAC International.

Buffer and Chemicals:

For buffers and chemicals used in the *AOAC Official Method 2014.02* protocol, please refer to this.

5.0 M Sodium hydroxide solution:

20g NaOH

Dissolve in 0.1L deionised water.

Phosphate Buffered Saline pH 7.4 (= PBS):

1.24g KH₂PO₄

7.27g K₂HPO₄

8.76g NaCl

Dissolve in 1L deionised 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 deionised water. Adjust to pH 7.0 with 1M NaOH. Add 200ml methanol. Degas with Helium.

methanol / water (85:15 v/v)

Mix 85ml methanol and 15ml deionised water. Degas with helium.

Chemicals:

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

Consumables:

- B-TeZ IAC Vitamin-B₁₂-[BTCA318005]*

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; Photoarray detector: Shimadzu SPD-M30A with high sensitivity cell; Data handling: LabSolutions Vers. 5.93
- Vacuum SPE Manifold (BAKER spe-24G Column Processor – process up to 24 samples) [J.T. Baker 7208]
- Evaporator [VLM EVA EC1-S]

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