

# Instruction of the Clean-up Process using **B-TeZ IAC Vitamin B<sub>12</sub> 3ml**



## Fast and Accurate Content Determination of Vitamin-B<sub>12</sub> (Cyanocobalamin) in Vitamin Tablets, Liquid Vitamin Preparations, Cell Culture Extracts etc. by Combination of Immunoaffinity Chromatography and HPLC

### **Principle:**

Many methods of Vitamin B<sub>12</sub> determination based on HPLC-UV detection show low selectivity if problematic matrices are applied.

This method of content determination of Vitamin B<sub>12</sub> combines the high selectivity of immunoaffinity columns with its potential to concentrate elute and of purification by HPLC column.

### **Sample Preparation:**

Vitamin B<sub>12</sub> samples 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.

### **Enrichment Step IAC:**

4ml extract (containing the quantity of Vitamin B<sub>12</sub> 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 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 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 B<sub>12</sub> 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 elute.

The column elute 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.

### **Analytical Method:**

**Machine:** Shimadzu; **Column:** Trentec Reprisil-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<sub>ABS</sub> [nm]: 361nm.

### **Characteristics:**

The measuring range is linear of 25ng to 1250ng Vitamin B<sub>12</sub> pro injection (R<sub>2</sub>=0.9999). The limit of detection is 3ng of vitamin B<sub>12</sub> per injection (three times of signal/noise ratio). If the given dilution steps are obeyed, the vitamin B<sub>12</sub> contents of **0.1 to 5µg/g** lie within the linear working range of the method. If the contents of used samples are higher than cited range, extracts should be diluted in a suitable manner. The lower limit of quantification is 10ng/g of vitamin B<sub>12</sub> in the sample applying this protocol.

**Recovery rates are >85%** when vitamin B<sub>12</sub> in buffer mixtures is analysed in **the range of 0.1 to 5µg per IAC**.

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## Example Sample Calculation:

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

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

## Buffer and Chemicals:

### **Phosphate Buffered Saline pH 7.4 (= PBS):**

1.24g KH<sub>2</sub>PO<sub>4</sub>  
7.27g K<sub>2</sub>HPO<sub>4</sub>  
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 KH<sub>2</sub>PO<sub>4</sub> in 800ml deionised 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 deionised water. Degas with helium.

### **Chemicals:**

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

### **Consumables:**

- B-TeZ IAC Vitamin-B<sub>12</sub>-[BTCA318005]

### **Standard:**

- Vitamin B<sub>12</sub> (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]