Instruction of the Clean-up Process Using *Vitamin B12 IAC (3mL format)*

Content Determination Vitamin-B_{12} (Cyanocobalamin) in Vitamin Tablets, Liquid Vitamin Preparations, Cell Culture Extracts etc. by Combination of Immunoaffinity Column and HPLC

Principle:

Many methods of Vitamin B_{12} determination based on HPLC-UV detection show low selectivity if problematic matrices are applied.

This method of content determination of Vitamin B_{12} combines the high selectivity of immunoaffinity columns with its potential to concentrate elute and of purification by HPLC column.

Protocol:

Sample preparation:

Vitamin B_{12} 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_{12} 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 Vitamin B12 IAC 3ml 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 underpressure from the bottom.

Elution:

The sample reservoir on top of the Vitamin B12 IAC 3ml 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 elute.

The column elute may be injected into the HPLC directly or, if concentrations are very low, concentrated by evaporation, 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 B_{12} pro injection (R2=0.9999). The limit of detection is 3ng of vitamin B_{12} per injection (three times of signal/noise ratio). If the given dilution steps are obeyed, the vitamin B_{12} 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_{12} in the sample applying this protocol.

Recovery rates are >85% when vitamin B_{12} in buffer mixtures is analysed in **the range of 0.1 to 5µg per IAC**.

Example Sample Calculation content:

A) Calculation of Sample Gram Equivalents per HPLC injection:

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

B) Calculation of Vitamin B12 of examined commodity in µg/g:

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

Buffer, Chemicals, Apparatus and Literature:

Phosphate Buffered Saline pH 7.4 (= PBS):

1.24g KH_2PO_4
7.27g K_2HPO_4
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_2PO_4 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:

Vitamin B_{12} IAC (3ml column)

Standard:

- Vitamin B_{12} (Cyanocobalamin), 99%

Evaporation:
•nitrogen gas 5.0 (to evaporate IAC-eluate)

Apparatus:
• HPLC; Shimadzu; pump: LC-6A (2 pieces); auto sampler: SIL 6B; absorbance detector: SPD-10A; data handling: CLASS LC10

• Evaporator

• Vacuum SPE Manifold

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