Instruction of the Clean-up Process Using *Biotin (Vitamin B8) IAC (3mL format)*

Fast and Accurate Content Determination of Biotin in Vitamin Tablets, Liquid Vitamin Preparations, Cell Culture Extracts, etc. by Combination of Affinity Chromatography, HPLC-FLD and Post Column Derivatisation with Fluorescein-Streptavidin Conjugate as Fluorophor

Principle:

Many methods of biotin determination based on HPLC-UV detection show either low sensitivity or low selectivity if problematic matrices are applied depending on the factor of dilution of the matrices.

This method of content determination of Biotin combines the high selectivity of affinity columns with its potential to concentrate elute and the very sensitive detection of biotin by post column labelling with fluorescein-streptavidin conjugate.

Protocol:

Sample preparation:

Biotin samples are to be extracted and analysed with the method of Bachas et al. [N.G. Hentz, L.G. Bachas *Methods Enzymol.* **1997**; 279:275-86], 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 Biotin 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 Biotin (Vitamin B8) IAC. 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 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 Biotin (Vitamin B8) IAC is removed, and an appropriate vial is placed below the affinity column. The bounded biotin 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 flow rate should lie below 3ml/min. The remaining methanolic solutions are 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 at 50°C for 1h (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: methanol /water (85:15 v/v) (use only for cleaning purposes at the beginning and at the end of analytical series); Mobile Phase B: 0.1M potassium phosphate, pH 7.0-methanol (85/15 v/v); Gradient: 0.01min B 100%; 12min B 100%; Flow Rate: 0.4ml/min; Time of Analysis: 12min; Injector Volume: 100µl; Detection: λ_{EX} [nm]: 495nm; λ_{EM} [nm]: 518nm. Post column derivatization: A solution of Fluorescein (FITC)-

Streptavidin-Conjugate of protein concentration of 2µg/ml in 0.1M potassium phosphate, pH=8.2; flow rate: 0.2ml/min.

Characteristics:

The HPLC measuring range is linear of 5ng to 40ng Biotin per injection (R2=0.994). The limit of detection is 2ng of biotin per injection (three times of signal/noise ratio). If the given dilution steps are obeyed, the Biotin contents of **20 to 160 ng/g** lie within the linear working range of the method. If the contents of used samples are higher than the cited range, extracts or the IAC column elutes should be diluted in a suitable manner. The lower limit of quantisation is 10ng/g of Biotin in the sample.

Recovery rates are >85% when Biotin contents in buffer mixtures are 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 Biotin of examined commodity in µg/g:

$$\frac{\text{\# µg injected Biotin}}{\text{Sample Equivalents [g]}} = \text{µg/g Biotin in e.g. multivitamin tablets}$$

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

0.1M potassium phosphate, pH 7.0-methanol (85/15 v/v)

Disolve 13.6g KH_2PO_4 in 850ml deionised water. Adjust to pH 7.0 with 1M NaOH. Add 150ml methanol. Degas with helium.

0.1M potassium phosphate, pH 8.2

Take 200ml 0.1M potassium phosphate, pH 7.0. Adjust to pH 8.2 with 1M NaOH. Degas with helium.

methanol /water (85:15 v/v)
(HPLC Column Cleaning)

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

Chemicals:

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

Consumables:

- Bitoin (Vitamin B8) IAC (3ml column)

Standard:

- D(+)-Biotin, 99%

Flourescence Label for Biotin:
•Streptavidin-FITC-Conjugate (contact-us)

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

Apparatus:
- HPLC; Shimadzu; pump: LC-6A (2 pieces); auto sampler: SIL 6B; fluorescence detector: RF-10AXL; data handling: CLASS LC10
- Evaporator
- Vacuum SPE Manifold

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