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

Fast and Accurate Content Determination of Estrogens in Drinking Water and Waste Water by Immunoaffinity Chromatography and HPLC-FLD (or –MS)

Principle:
Many methods of Estrogens determination based on HPLC-UV detection show low selectivity if problematic matrices are applied.

This method of content determination of Estrogens combines the high selectivity of immunoaffinity columns with its high potential to concentrate elute and of purification by HPLC column.

The following instruction gives a optimized protocol for enrichment of very low contents in the pg/g range. In this case a mass detector might be best suited.

Please notice, that this instruction focuses on the handling with the IAC column. The given apparatus and detection (e.g. HPLC-FLD system) might serve as example among other possibilities.

Sample Preparation:
Sewage and drinking water samples are collected as described by Quintana et al[1]. Raw samples may be filtered through a 0.45µm pore size cellulose filter.

Example: 1000g sewage effluent sample (equals approximately 1L) firstly are filtered like mentioned before and then added to the filtrate 10ml **Buffer Solution** (see under Buffer and Chemicals) to maintain pH neutrality. Thus, the final sample contains 1% (v/v) Buffer Solution.

Enrichment Step IAC:
1.01L final sample (containing the quantity of estrogens from a 1000g sample if above-mentioned sample preparation is followed) are applied thorugh a reservoir on top of the ***B-TeZ Estradiol IAC*** Column. The optimal flow rate through the gel is between 1 to 3ml/min.

According to application and contents expected the applied sample volumes could vary. In case estrogen contents lie in the ng/g range, e.g. 1ml aliquots of samples may be diluted 1+9 with PBS and then applied on top of the column.

Wash:
After the whole sample has passed through the gel, the latter is washed with 5ml of **0.2M NH_4Ac** (see Buffer and Chemicals below). Remaining liquids in the gel are removed by applying either pressure from top of the column or depression from the bottom. Alternatively, you can wash with 5ml PBS if MS analysis is not due.

Elution:
The sample reservoir on top of the ***B-TeZ Estradiol IAC*** is removed, and an appropriate vial is placed below the affinity column. The bounded estrogens are 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 either slight low or over pressure. 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), redissolved in HPLC solvent and finally injected into the system. For the latter case, please see the sample calculation in which the sample concentrate has a final volume of 0.4ml of HPLC solvent.

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IAC Column Characteristics:

A) Working Range and Recovery Rates of *B-TeZ IAC Estradiol 3ml* Column:

Working Range of Column:	1– 500ng 17β-Estradiol per IAC
Zero Contamination per Column:	<1ng (LOD of HPLC-FLD method)
Guaranteed Recovery Rates within the Working Range(*):	>85%

(*) Recovery rates are confined to solvent content of sample extract below 15% methanol or 15% acetonitrile.

B) Cross Reactivities(**) of *B-TeZ IAC Estradiol 3ml* Column:

17β-Estradiol:	100%
17α-Ethinylestradiol:	77%
Mestranol:	54%

(**) Recovery rates if a total quantity of 30ng of 17β-Estradiol, 17α-Ethinylestradiol, Mestranol (molar ratio of 1:1:1) in a test solution of 50ml PBS is analyzed

C) Capacity(***) of *B-TeZ IAC Estradiol 3ml* Column:

Maximum Column Capacity:	1.5µg 17β-Estradiol

(***) Extrapolated value. An excess of Estradiol, e.g. 3µg, in a small volume of 2ml PBS is incubated with the IAC for 5 minutes; then the IAC is washed with 2ml PBS and the nonbonded fraction is analyzed. The difference of added analyt and nonbonded analyt equals maximum column capacity.

Analytical Method:

Machine: Shimadzu; Column: Trentec Reprosil-Pur RP C18 120 ODS3 5µm; 125x3,0mm with guard column; Mobile Phase A: acetonitrile / water (90 / 10 v/v); Mobile Phase B: acetonitrile / 50mM sodium acetate, pH 8.6 (35 / 65 v/v); Gradient: 0.01min B 100%; 1min B 100%; 20min B 7%; 20.1min B 100%; Flow Rate: 0.7ml/min; Time of Analysis: 30min; Injector Volume: 100µl; Fluorescence-Detection: λ_{EX} [nm]: 280nm; λ_{EM} [nm]: 304nm. Temperature: Machine and eluents are at room temperature. Eluents are degassed with helium gas.

Example Sample Calculation:

A) Calculation of Sample Gramm Equivalents per HPLC injection:

$$\frac{\text{1000g Sample}}{\text{1010ml Diluted Sample}} \times \frac{\text{1010ml Diluted Sample}}{\text{0.4ml}} \times \text{0.1ml injector volume} = \text{250g Sample Equivalents}$$

B) Calculation of Estradiol content of examined commodity in ng/g:

$$\frac{\text{\# ng injected Estradiol}}{\text{Sample Equivalents [g]}} = \text{ng/g Estradiol in e.g. sewage effluent water}$$

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Buffer and Chemicals:

10X PBS Buffer:
12.4g KH_2PO_4,
72.7g K_2HPO_4 and
87.6g NaCl

Dissolve in 1L deionisized water. If necessary adjust pH to 7.2 (± 0.2) with 1N NaOH or 1N HCl

Buffer Solution:
1L 10X PBS Buffer and
37.2g Na_2EDTA

Dissolve in 1L 10X PBS Buffer

PBS:

Dilute 100ml 10X PBS with deionized water to a final volumen of 1L. Control pH to 7.4 (± 0.2)

0.2M NH_4Ac:

Dissolve 15.4g NH_4Ac in 1L deionisized water.

HPLC-Solvent
acetonitrile-50mM sodium acetate, pH 8.6 (35/65 v/v) (=B)

Dissolve 6.8g NaAc x $3H_2O$ in 650ml deionised water. Control pH 8.6. Add 350ml acetonitrile. Degas with helium.

acetonitrile-water (90:10 v/v) (=A)

Mix 900ml acetonitrile and 100ml deionised water. Degas with helium.

Chemicals:
- acetonitrile, HPLC grade
- methanol, HPLC grade
- deionised water
- dipotassium hydrogenphosphate, >98%
- potassium dihydrogenphosphate, >98%
- sodium chloride
- sodium acetate trihydrate
- ammonium acetate [Sigma-Aldrich A7262]
- Na_2EDTA [Fluka 03682], M=372.24g/mol

Consumables:
- ***B-TeZ Estradiol IAC*** Column [BTES320005]

Standard:
- β-Estradiol [Sigma 8875]
- 17α-Ethinylestradiol [Fluka 02463],
- Mestranol [Aldrich 855871]

Evaporation:
- nitrogen gas 5.0 [Air Liquide M55763810] (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 (with tripod) [VLM EVA EC1-S]
- Vacuum SPE Manifold (BAKER spe-24G Column Processor – process up to 24 samples) [J.T. Baker 7208]

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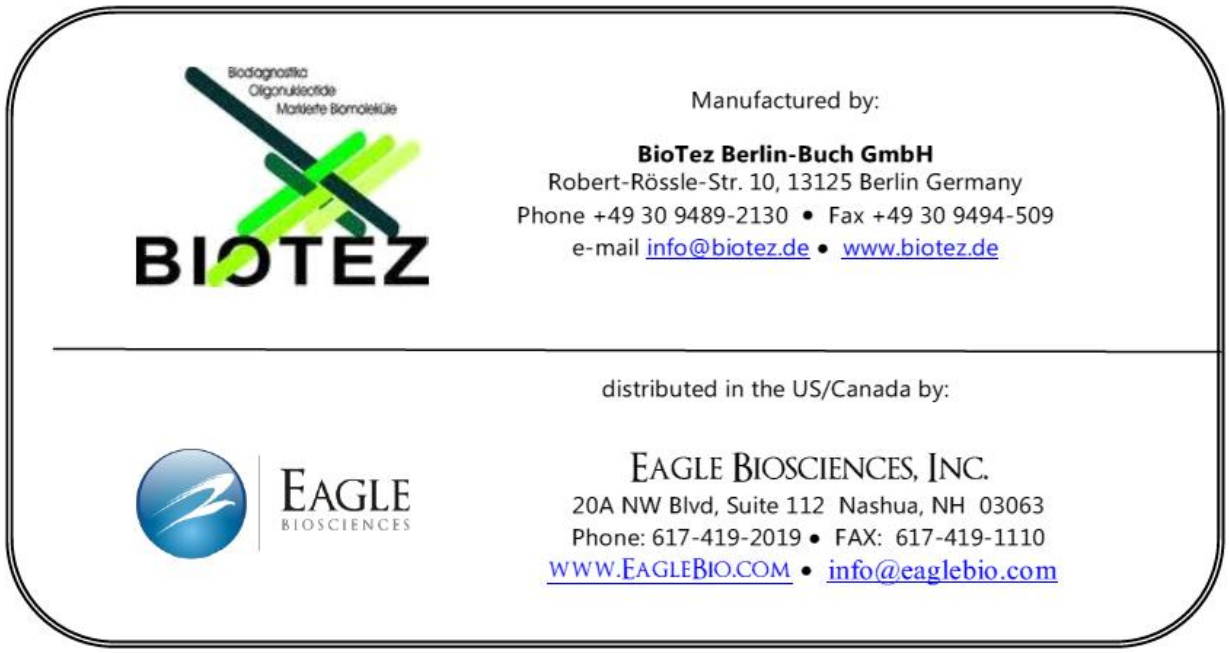
[1] "Determination of natural and synthetic estrogens in water by gas chromatography with mass spectrometric detection" J.B. Quintana, J. Carpinteiro, I. Rodriguez, R.A. Lorenzo, A.M. Carro, R. Cela Journal of Chromatography A, **1024** (2004) 177–185



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