Maximizing Sensitivity in the LC-MS/MS Determination of Estrone (E1), 17β-Estradiol (E2), Estriol (E3) and 17α-Ethynyl Estradiol in Natural Waters

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Male Fish Feminization linked to WW

Assessment of Feminization of Male Fish in English Rivers by the Environment Agency of England and Wales

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- Introduction
- The Environment Agency's Assessment of the Widespread Feminization of Fish
- Identification of Causative Substances
- The Environment Agency's Risk Management Strategy

Abstract

In recent years there has been considerable concern over the ability of substances discharged into the environment to disrupt the normal endocrine function of wildlife. In particular, the apparent widespread feminization of male fish in rivers has received significant attention from regulators in the United Kingdom, the United States, Europe, and Japan. The U.K. and European epidemiological data sets have demonstrated that the occurrence of feminized fish is associated with effluent discharges and that the incidence and severity is positively correlated with the proportion of treated sewage effluent in receiving waters. Although weakly estrogenic substances may contribute to the overall effect, studies have concluded that steroid estrogens are the principal and most potent estrogenic components of domestic sewage. Extensive laboratory data sets confirm that steroid estrogens are capable of eliciting the effects observed in wild fish at concentrations that have been measured in effluents and in the environment. Based on evaluation of the available information, the Environment Agency (England and Wales) has concluded that the weight of evidence for endocrine disruption in fish is sufficient to develop a risk management strategy for estrogenically active effluents that discharge to the aquatic environment. Key words: endocrine disruption, ethinyloestradiol, feminization, fish, estriadiol, estrone, risk assessment, steroid estrogen. Environmental Health Perspectives 114(suppl 1): 147-151 (2006). doi:10.1289/ehp.8068 available via http://dx.doi.org/ [Online 21 October 2005]
Estrogens

17β-Estradiol E2

Estriol E3

Estrone E1

17α-Ethynyl estradiol EE
Method Development Plan

1. Solid-phase extraction using Oasis HLB cartridges (~100x concentration)
2. Reverse-phase liquid chromatographic separation using C18 column
3. Mass Analysis using ESI(-) LC/MS/MS
4 Estrogens using ESI(-) [10 μg/L]
β-estradiol fragmentation under ESI(-)

Precursor Ion: (m/z 271) 95%
Fragment Ion: (m/z 145) 5%
10 ppb ESI(−) Parent Ion

**E1 E2 E3 EE 10ppb_50Meoh_50h20_10uLNH4OH_100uL**

April 14, 07 Sample 20

**E1**

Area: 40173

**E2**

Area: 48187

**E3**

Area: 47525

**EE**

Area: 38123

**E1**

Area: 684

**E2**

Area: 1334

**E3**

Area: 3127

**EE**

Area: 3127
ESI(-) TIC (10 ppb)

April 14, 07, Sample 20

1. MRM of 2 Channels ESI TIC
   - Area: 4.87e5

2. MRM of 2 Channels ESI TIC
   - Area: 4.56e5

3. MRM of 2 Channels ESI TIC
   - Area: 3.08e5

4. MRM of 2 Channels ESI TIC
   - Area: 3.33e5

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E1: 52851
E2: 50461
E3: 47741
EE: 39569
Ethynyl Estradiol (EE) present in effluent
Amount of Estrogens Excreted

- Estradiol
- Estrone
- Estriol
- Ethinyl E*

ug/day

Pregnant
Menstruating
Menopausal
Male

• Only for those taking ethinyl
  (=0 for males, pregnant)
Ethynyl Estradiol (EE) present in effluent
Summary of ESI(-) LC/MS/MS

• 10.0 μg/L instrumental detection limit
• Sum of daughter ion and parent ion traces increases sensitivity but costs specificity
• 100μL injection volume increases sensitivity but reduces column life
• Method detection limit = 100 ng/L based on 100x conc. from SPE)
Where do we go from here?

- ESI(-) LC/MS/MS provided evidence of ethynyl estradiol (EE) in Saskatoon wastewater effluent at ~ 4000 ng/L
- Biological activity threshold for estrogens in the environment is ~ 1 ng/L
- Need lower method detection limit to assess environmental impact of EE
Challenge: connect all 9 dots using continuous straight-lines
Puzzle?
Eureka!

- Early literature suggested using dansyl chloride derivatization to boost selectivity and sensitivity in LC/UV methods for estrogens.
- Recent literature indicated a 1000x increase in sensitivity for dansyl-derivatives using tandem mass spectrometry in ESI(+) over ESI(-).
Derivatization of ethynyl estradiol

Dansyl chloride + 17α-Ethynyl estradiol

60°C (100mM carbonate pH 11)

dansyl-ethynyl estradiol
Derivatization Procedure

- Add 150uL dansyl chloride (in acetone) and 50uL sodium carbonate buffer (pH 11) to the dry residue obtained after either liquid/liquid or solid-phase extraction.
- Heat in water bath for 3-5 min. at 40-60 °C.
- Evaporate under a stream of N₂ and reconstitute in 1.0 mL of 50/50 (50% MeOH/50% H₂O).
10ppb dansyl-estrogen derivatives ESI(+)
ESI(+) dansyl-EE fragmentation

(+) m/z 530  
Precursor Ion

(+) m/z 171  
Fragment Ion
Dansyl-derivatized 100mL Effluent Extract

April17_07_Sample17

100mL_WWTP_beforeChlorination_der_50/50

MRM of 5 Channels ES+
530 > 171
2.16e7
Area

Area5

2.82
2320271

522 > 171
4.07e5
Area

2.27

506 > 171
8.57e4

504 > 171
1.08e5

2.62 2.69 2.97 3.02
3.14 3.19 3.47 3.72
3.92 4.05 4.17 4.30
4.50
4.57 4.70 4.82

3.04
3.39 3.57 3.65 3.82
3.97 4.15 4.25 4.50
4.57
Summary of ESI(+)  

- Increased sensitivity due to higher yield of the daughter ion  
- Decreased column degradation due to smaller injection volume (20uL) with increased confidence in identification  
- Adduct formation, derivatization yield variability and lack of available standards
Conclusions

• Ethynyl estradiol (EE) was identified in Saskatoon wastewater discharge by:
  a) using ESI(-) LC/MS/MS = 4000ng/L
  b) using ESI(+) LC/MS/MS = 1600ng/L

• Discharge of EE in wastewater effluent could potentially disrupt endocrine function of fish and other species downstream
The Future

• Determine SPE extraction efficiency
• Increase sensitivity of LOD to 1 ng/L:
  a) by improving chromatography
  b) by optimizing dansylation yield
• Monitor fate of estrogens in aquatic ecosystems and their biologic effect on bacteria, algae and invertebrates
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- Agriculture and Agri-Food Canada
- 2007 WCTOW organizing committee
- You for your attention!
The End
ESI(-) fullscan Estrone
ESI(-) Fragment Ion Formation

1. Estrone (25%)
2. β-estradiol (4%)
3. Estriol (5%)
4. Ethynyl estradiol (4%)
Liquid Chromatographic Separation

LC Instrument: Waters 2695 Alliance HPLC System
Column: Waters Xterra: C\textsubscript{18}: 2.1mm x 100mm
Particle size: 3.5u
Column temp: 30\textdegree C
Mobile phase: Solvent A: 10\% H\textsubscript{2}O
Solvent B: 90\% MeOH
Flow rate: 200uL/min
Separation mode: isocratic
Injection volume: 100uL
Natural Estrogens

17β-Estradiol

Estriol

Estrone
Synthetic hormone

17β-Estradiol

17α-Ethynyl estradiol
Synthetic estrogenic compound

17β-Estradiol

17α-Ethynyl estradiol

orally active 50 μg/day
Matrices for Method Development

1. Non-chlorinated sewage effluent
2. Chlorinated sewage effluent
3. De-ionized (18.3MΩ) water
MS/MS Parameters

1. Instrument: Micromass Quattro Ultima
2. Source temp: 90ºC
3. Electrospray Interface: (+) ion mode
4. Cone Voltage: 47 V
5. Capillary voltage setting: 2.95 kV
6. Hexapole 1: 8.6 V
7. Hexapole 2: 0V
8. Aperture Voltage: 0V
9. Desolvation temp: 220ºC
100ppb Estrogens by ESI (+)