

Natural and Synthetic Estrogens at Low PPQ Levels in Surface Water and Final Effluent Water by LC-ESI- MSMS

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Natural and Synthetic Estrogens

Some Background

- Estrogens are routinely used either as contraceptive medicines or in hormone replacement therapy and can enter aquatic environments via the discharge of final effluent waters.

- Compounds can include:
 - 17 alpha ethinyl estradiol (oral contraceptive and HRT)
 - 17 beta estradiol (postmenopausal drug)
 - estrone (perimenopausal and postmenopausal drug/natural hormone)

- Estrogens are believed to have a negative effect on aquatic environments by disrupting the hormonal systems of fish, which is thought to cause demasculation of aquatic animals.

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Official Journal of the European Union

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(Legislative acts)

DIRECTIVES

DIRECTIVE 2013/39/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL

of 12 August 2013

amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy

(Text with EEA relevance)

EU Water Framework Directive

Overview

- Addition of 12 new substances to the list of 33:
 - Plant protection product substances: aconifin, bifenoxy, cypermethrin, dicofol*, heptachlor*, quinoxyfen*
 - Substances used in biocide products: cybutryne, dichlorvos, terbutryn
 - Industrial Chemicals: PFOS*, hexabromocyclododecane* (HBCDD)
 - Combustion by-products: dioxin* and dioxin-like PCB's*
- Watch list
 - Pharmaceuticals: 17 beta-estradiol, 17 alpha-ethinyl estradiol, diclofenac
- Development of a specific strategy for pharmaceuticals
- It lowers the EQS values of certain substances:
 - Brominated diphenyl ethers, fluoranthene, nickel and PAH's

*priority hazardous substances

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DIRECTIVES

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Article 8b

Watch list

1. The Commission shall establish a watch list of substances for which Union-wide monitoring data are to be gathered for the purpose of supporting future prioritisation exercises in accordance with Article 16(2) of Directive 2000/60/EC, to complement data from, inter alia, analyses and reviews under Article 5 and monitoring programmes under Article 8 of that Directive.

The first watch list shall contain a maximum of 10 substances or groups of substances and shall indicate the monitoring matrices and the possible methods of analysis not entailing excessive costs for each substance. Subject to the availability of methods of analysis not entailing excessive costs, the maximum number of substances or groups of substances that the Commission is allowed to include in the list shall increase by one at each update of the list in accordance with paragraph 2 of this Article, up to a maximum number of 14. The substances to be included in the watch list shall be selected from amongst those for which the information available indicates that they may pose a significant risk at Union level to, or via, the aquatic environment and for which monitoring data are insufficient.

Diclofenac (CAS 15307-79-6), 17-beta-estradiol (E2) (CAS 50-28-2) and 17-alpha-ethinylestradiol (EE2) (CAS 57-63-6) shall be included in the first watch list, in order to gather monitoring data for the purpose of facilitating the determination of appropriate measures to address the risk posed by those substances.

Challenges of Steroid Hormone Analysis

- Very low limits of detection required
 - Surface waters MAC
 - 17 α ethinyl estradiol 0.00003ug/l
 - 17 β estradiol 0.0004ug/l

- Compounds do not ionise well in ESI or APCI

- Ion Suppression from matrix and extraction process
 - Significant sample clean-up and concentration required
 - Chromatographic separation crucial

- Method has to be robust to allow analysis of both surface and effluent waters

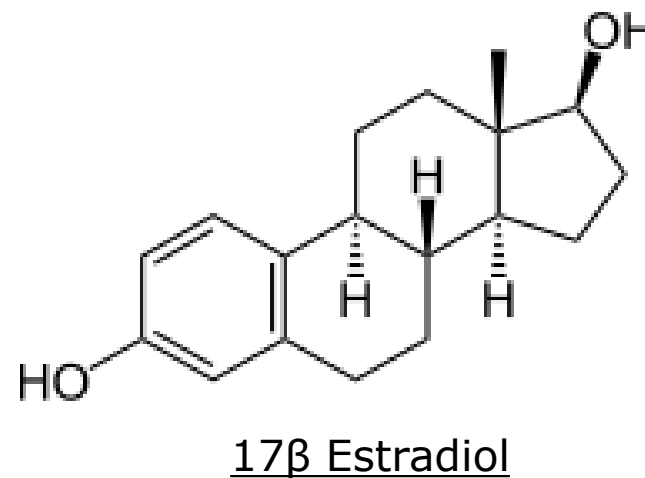
Good Sample Preparation Provides the Ability to...



- **Simplify the chromatographic separation**
 - Removing matrix and co-eluting species enables better, more consistent quantitation
- **Reduce analytical variability**
 - Higher, more consistent recovery
 - Minimize matrix effects
 - Less rework
- **Increase column lifetime**
 - Fewer columns need to be replaced
- **Reduce system downtime**
 - Less time spent with wrenches or waiting for service

Sample Preparation for Estrogens in Water

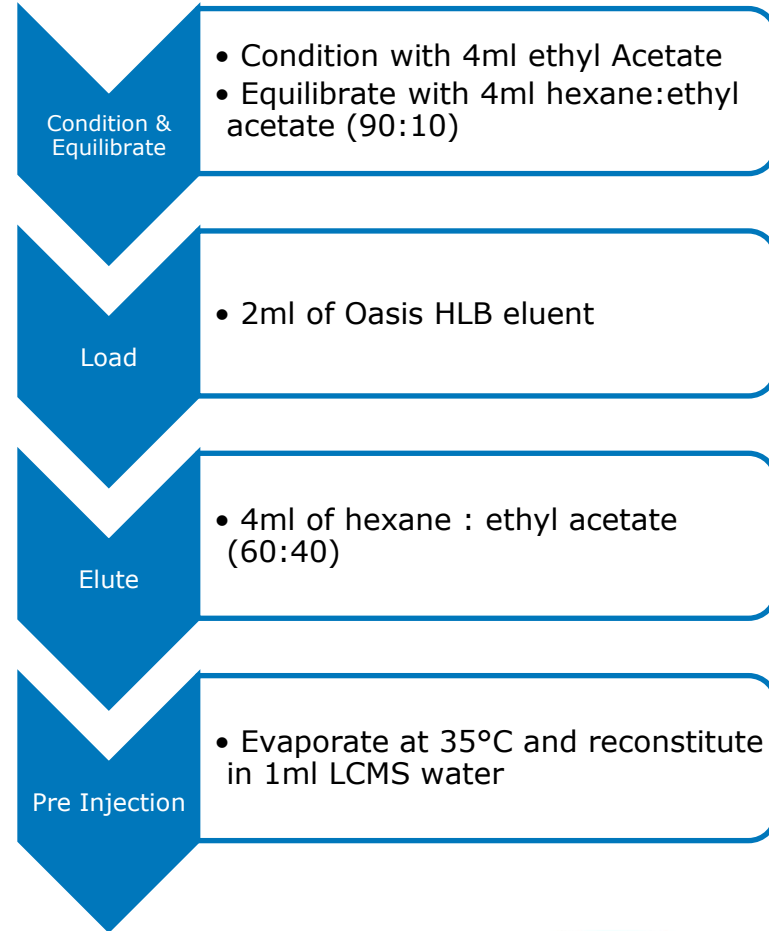
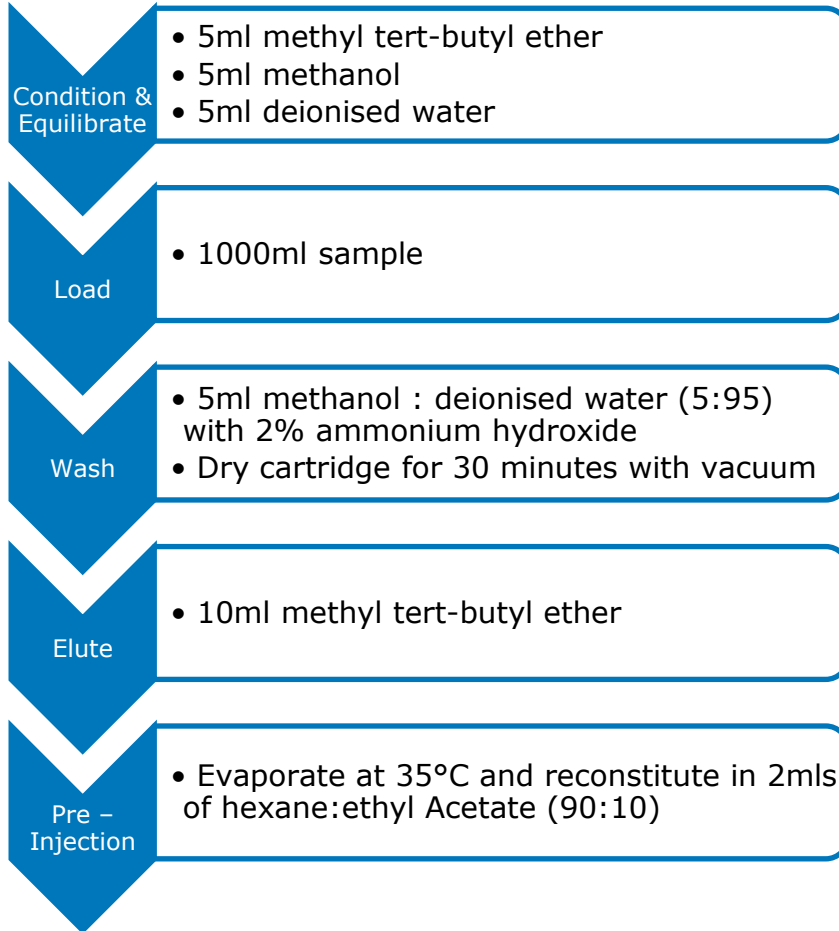
- **Characterize the analytes**
 - They are weak acids $pK_a > 10$
 - LogP 3.57 – 4.03 (hydrophobic)
 - -OH groups facilitates normal phase SPE
- **Select the appropriate sorbent**
 - Oasis HLB
 - Oasis MAX (strong anion exchange and HLB)
 - Sep-Pak Silica or alumina



SPE Method Details

Oasis® HLB 6cc, 150mg
(p/n 186003379)

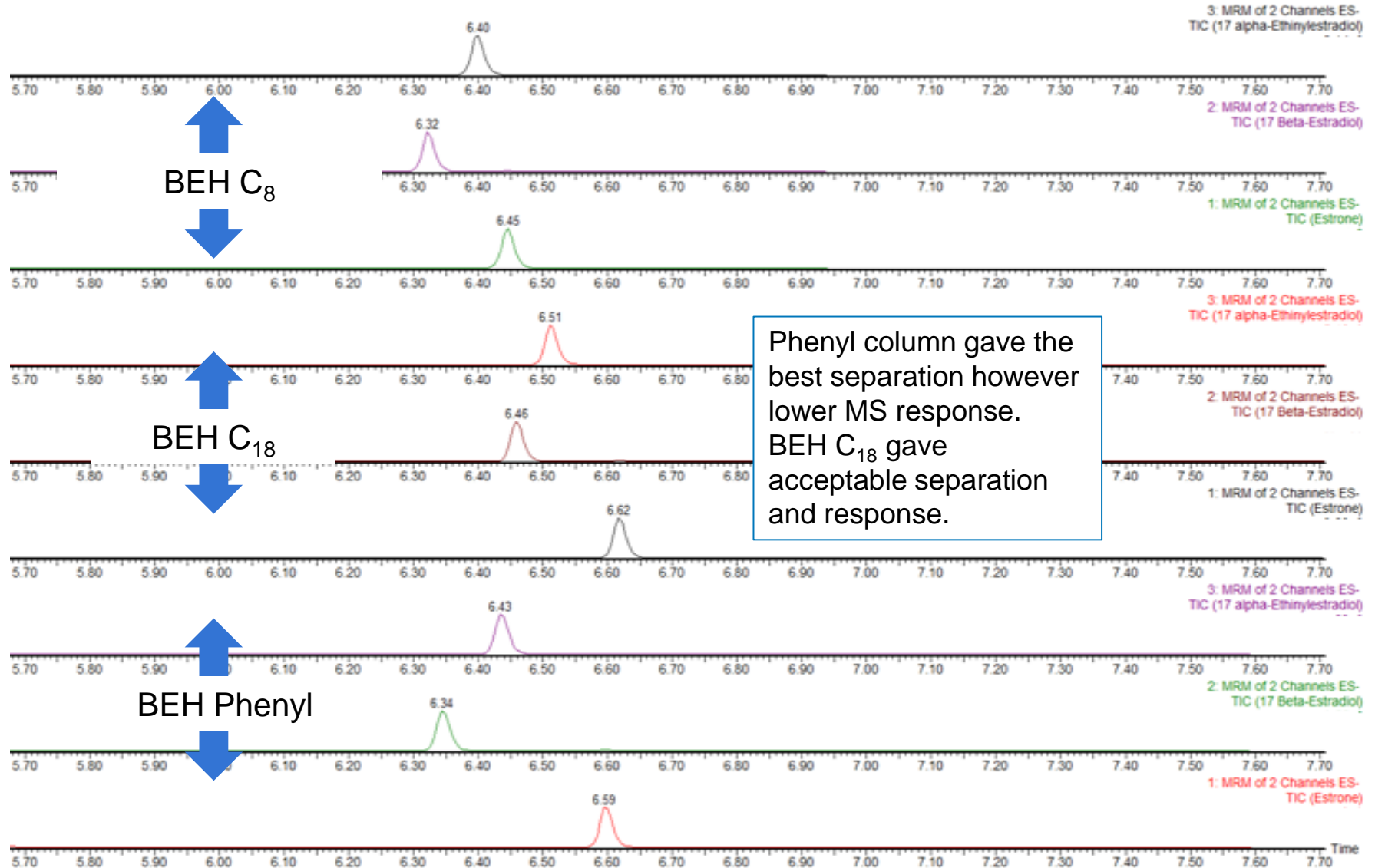
Sep-Pak® Silica 3cc, 500mg
(p/n 186004536)



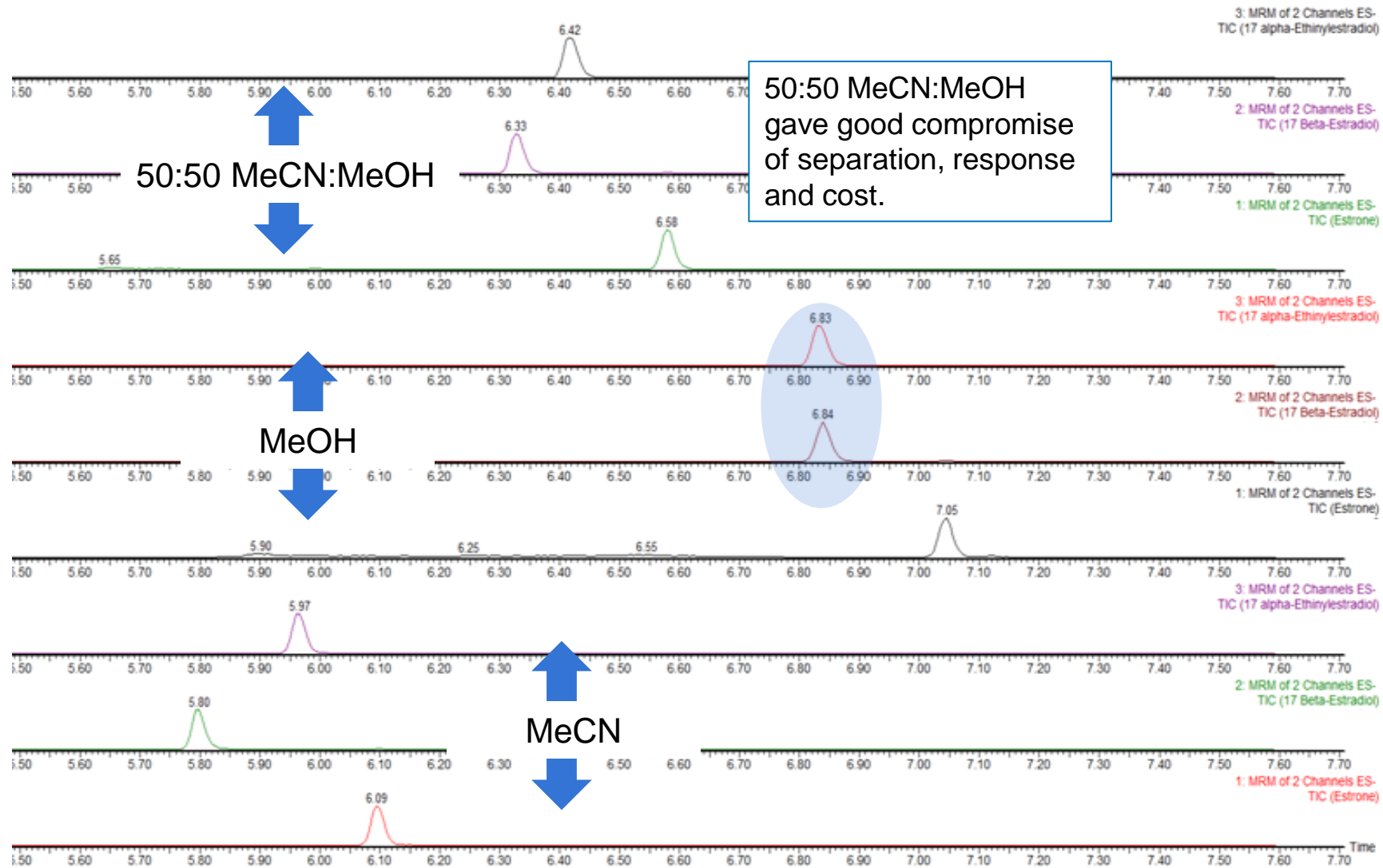
Solid phase extraction, clean-up and concentration methodology, provided by Scottish Water.



Example of Separation on Different Column Ligands



Example of Separation on Using Different Organic Modifiers

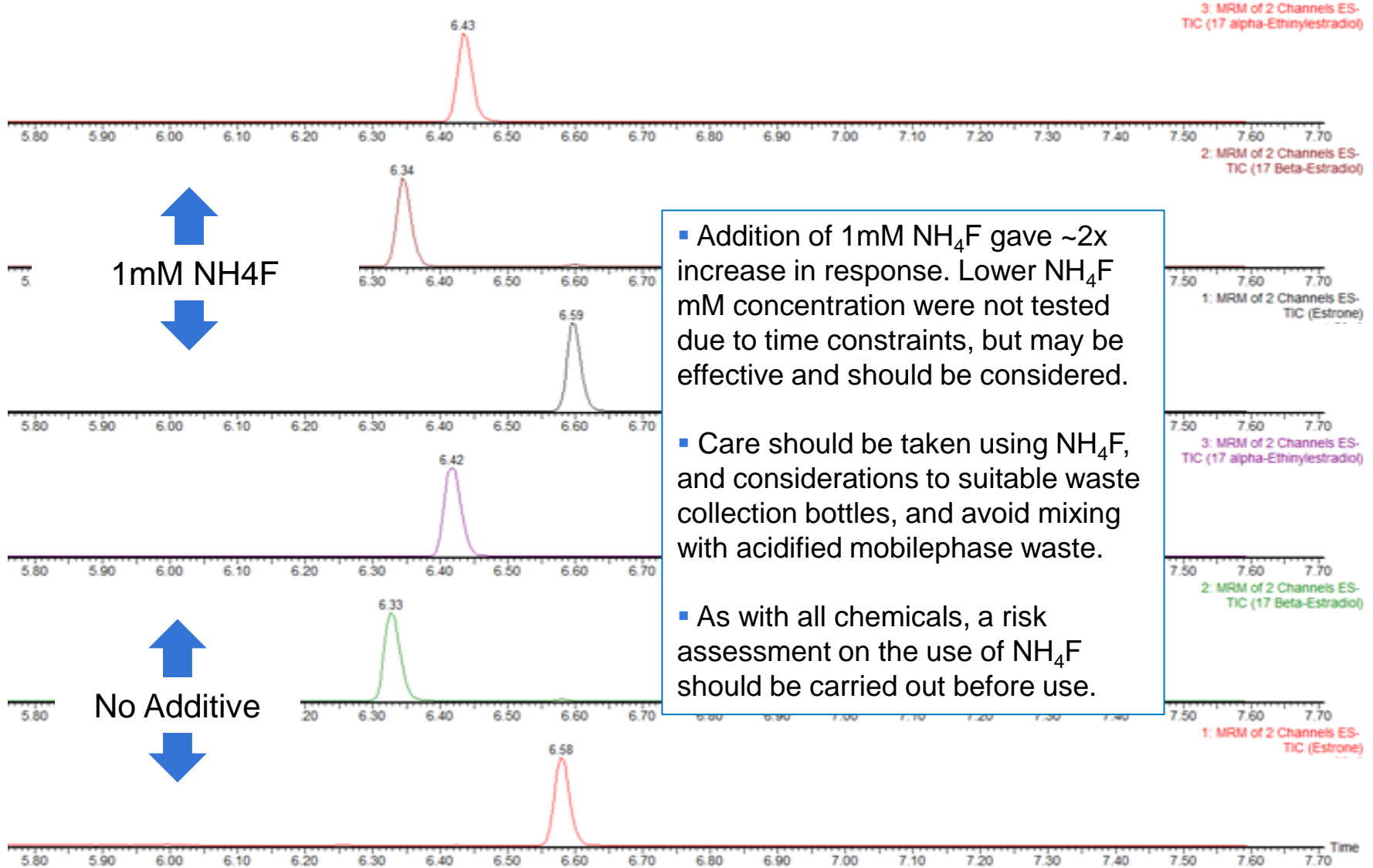


Mobilephase Modifier Options

- Ionisation of steroid hormones can be improved using suitable additives
- Some suggestions from literature include:
 - Ammonium Hydroxide
 - Waters Technology Brief 720005626en utilizes 0.05% ammonium hydroxide, sub ppt detection limits were achieved using 2D UPLC-MS/MS
 - Post column addition of ammonia
 - Addition of 0.1% ammonia solution at 0.1 ml/min has been reported in literature to give 3-4 x response increase
 - Ammonium Fluoride
 - Has been reported to give up to 5x increase in response in ESI (–) for some compounds
 - Increased deprotonation as a result of the fluoride anion in the gas phase*

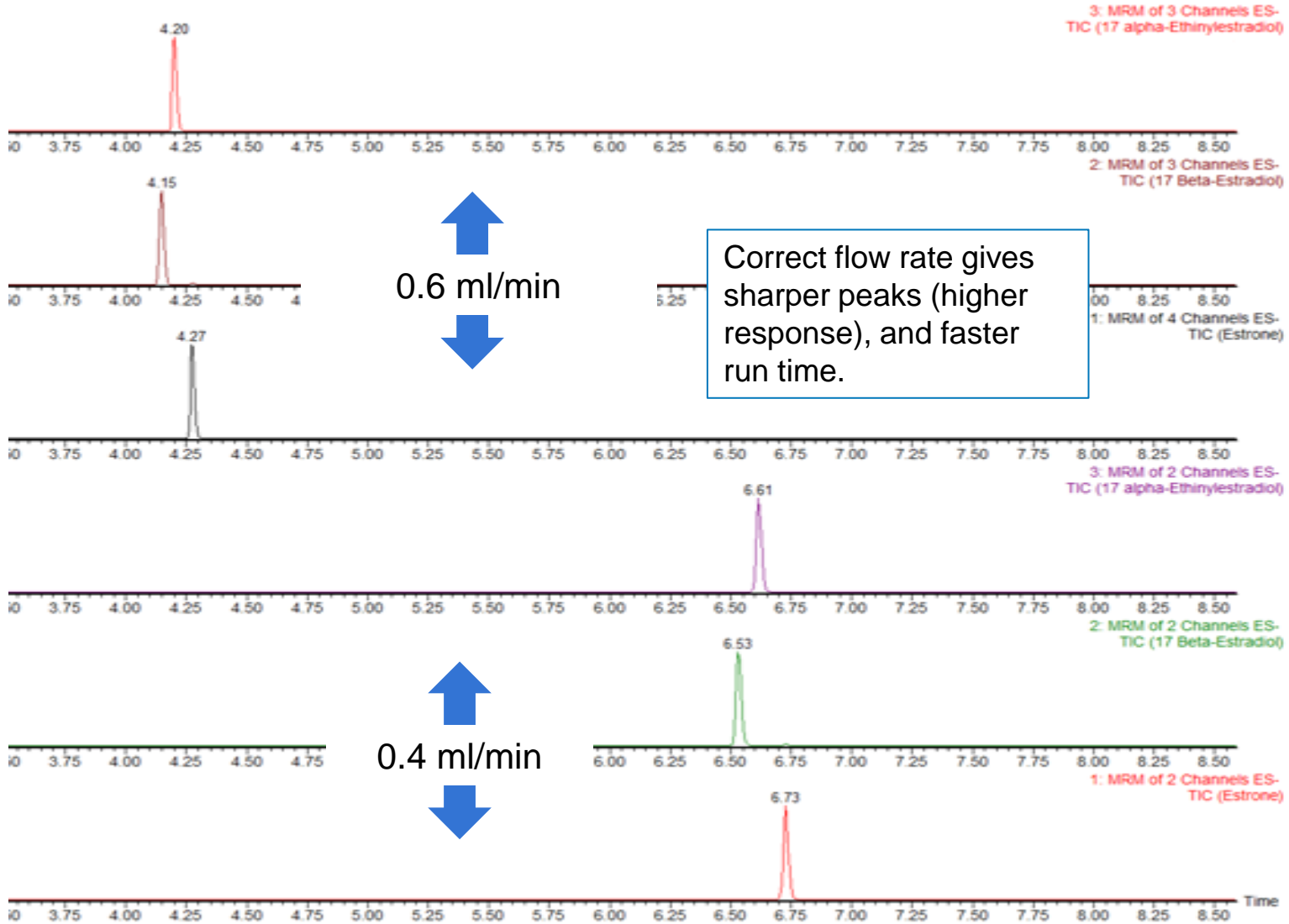
* Petrie, B., Youdan, J., Barden, R. and Kasprzyk-Hordern, B. (2016) "Multi-residue analysis of 90 emerging contaminants in liquid and solid environmental matrices by ultra-high-performance liquid chromatography tandem mass spectrometry", *Journal of Chromatography A*, 1431, pp. 64-78.

Impact of using NH₄F Modifier



- Addition of 1mM NH₄F gave ~2x increase in response. Lower NH₄F mM concentration were not tested due to time constraints, but may be effective and should be considered.
- Care should be taken using NH₄F, and considerations to suitable waste collection bottles, and avoid mixing with acidified mobilephase waste.
- As with all chemicals, a risk assessment on the use of NH₄F should be carried out before use.

Impact of Using Correct Flow Rate



Separation Conditions

UPLC CONDITIONS:

System: ACQUITY® UPLC® H-Class (with extension loop, needle and syringe to facilitate a large volume injection)

Column: ACQUITY® BEH C₁₈ (3 x 100 mm, 1.7 μm)

Mobile phase: A: LCMS Grade Water with 1mM NH₄F (analytical grade)

Mobile phase: B: 50:50 LCMS Grade Acetonitrile : Methanol with 1mM NH₄F (analytical grade)

Purge: 80: 20 water: methanol

Injection vol: 100 μL

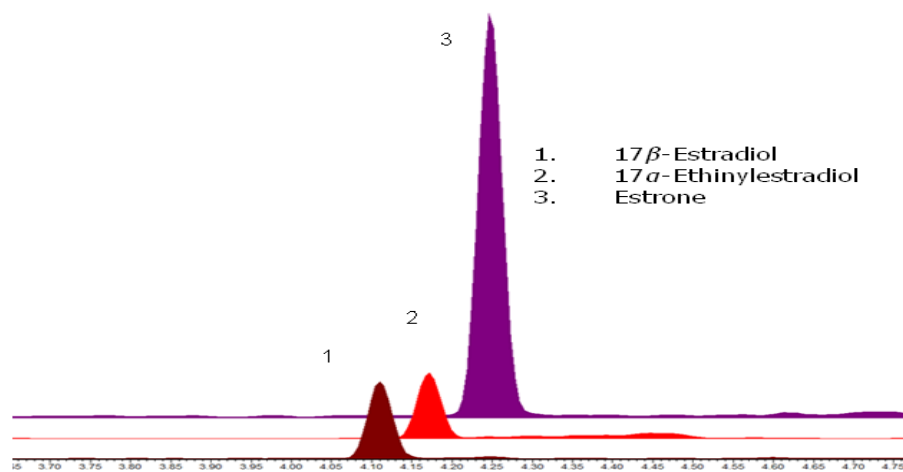
Column temp: 30 ° C

Sample temp: 10 ° C

Flow rate: 0.6ml/min

Gradient tablet:

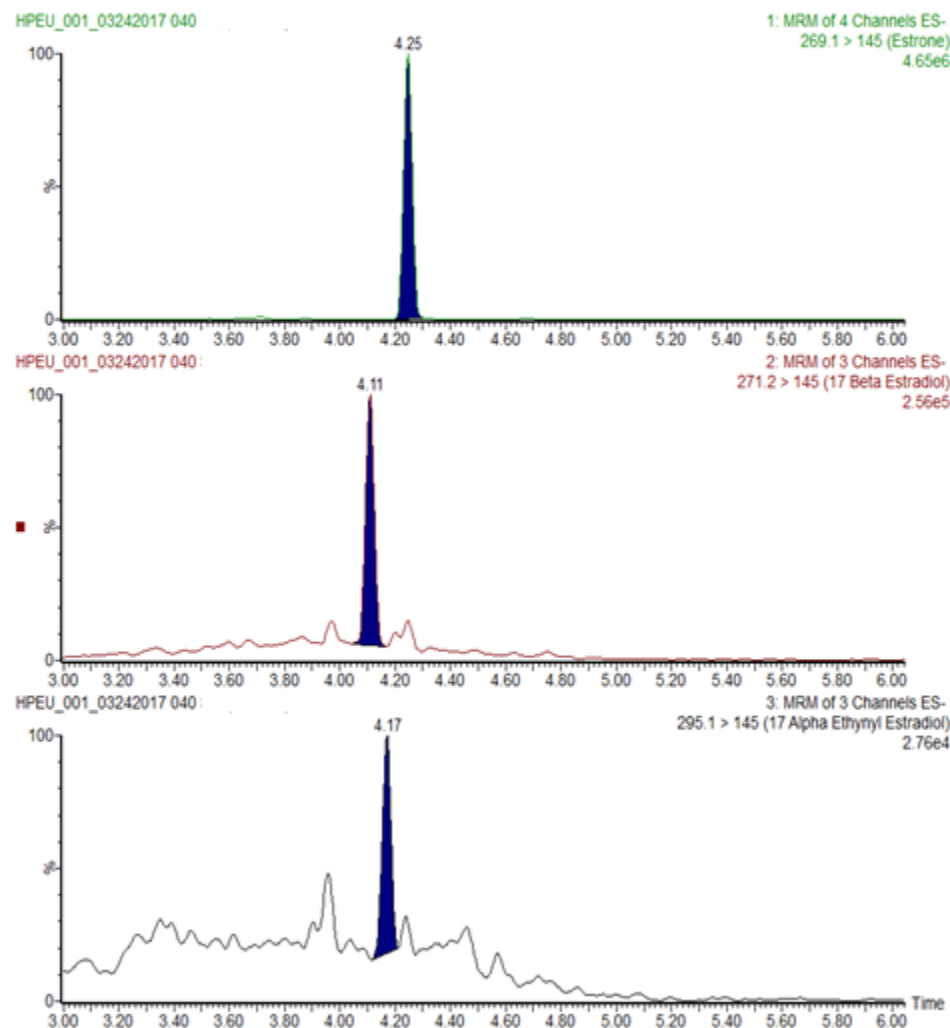
Time	% A	% B
Initial	70	30
1.00	70	30
3.50	5	95
5.50	5	95
5.60	70	30
8.60	70	30



MS Conditions

■ MS CONDITIONS:

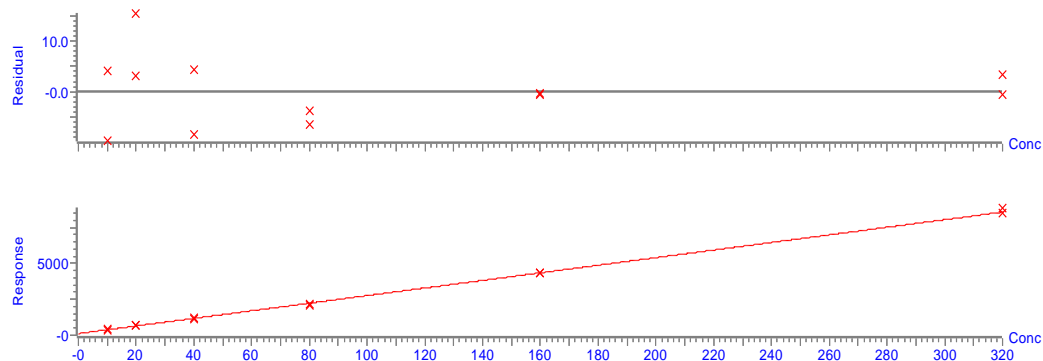
- Mass spectrometer: Xevo® TQ-XS
- Ionisation Mode: ESI –
- Acquisition mode: MRM
- Capillary voltage: 2.0 kV
- Source temp: 150 ° C
- Desolvation temp: 600 ° C
- Desolvation gas flow: 1200 L/Hr
- Cone gas flow: 150 L/Hr
- Data management: MassLynx® v4.1,
TargetLynx XS



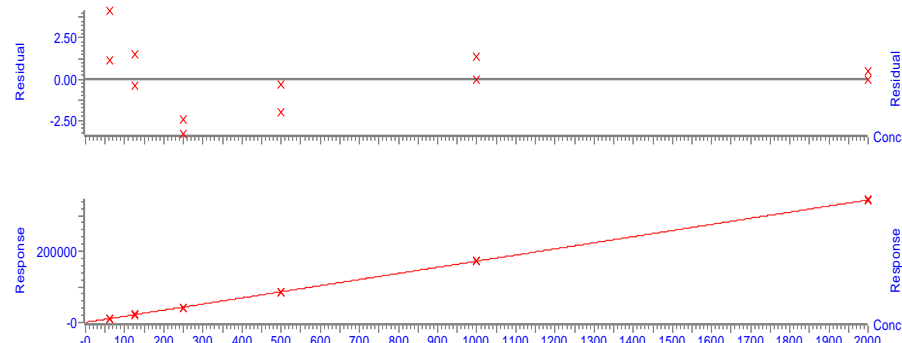
Sample pre-spiked (prior to extraction) with 17 α -ethynylestradiol at 30pg/l , 17 β -estradiol 120pg/l and estrone 400 pg/l in surface water

Surface Water Calibration Curves (post spiked curve)

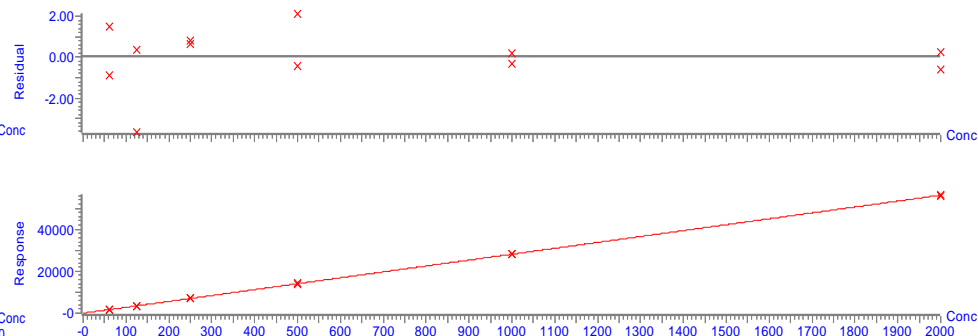
Compound name: 17 Alpha Ethynyl Estradiol
Correlation coefficient: $r = 0.999001$, $r^2 = 0.998004$
Calibration curve: $26.4155 * x + 120.806$
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Compound name: Estrone
Correlation coefficient: $r = 0.999910$, $r^2 = 0.999819$
Calibration curve: $171.91 * x + 336.28$
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Compound name: 17 Beta Estradiol
Correlation coefficient: $r = 0.999950$, $r^2 = 0.999901$
Calibration curve: $28.1779 * x + -59.3078$
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Surface Water SPE % Recovery and Precision

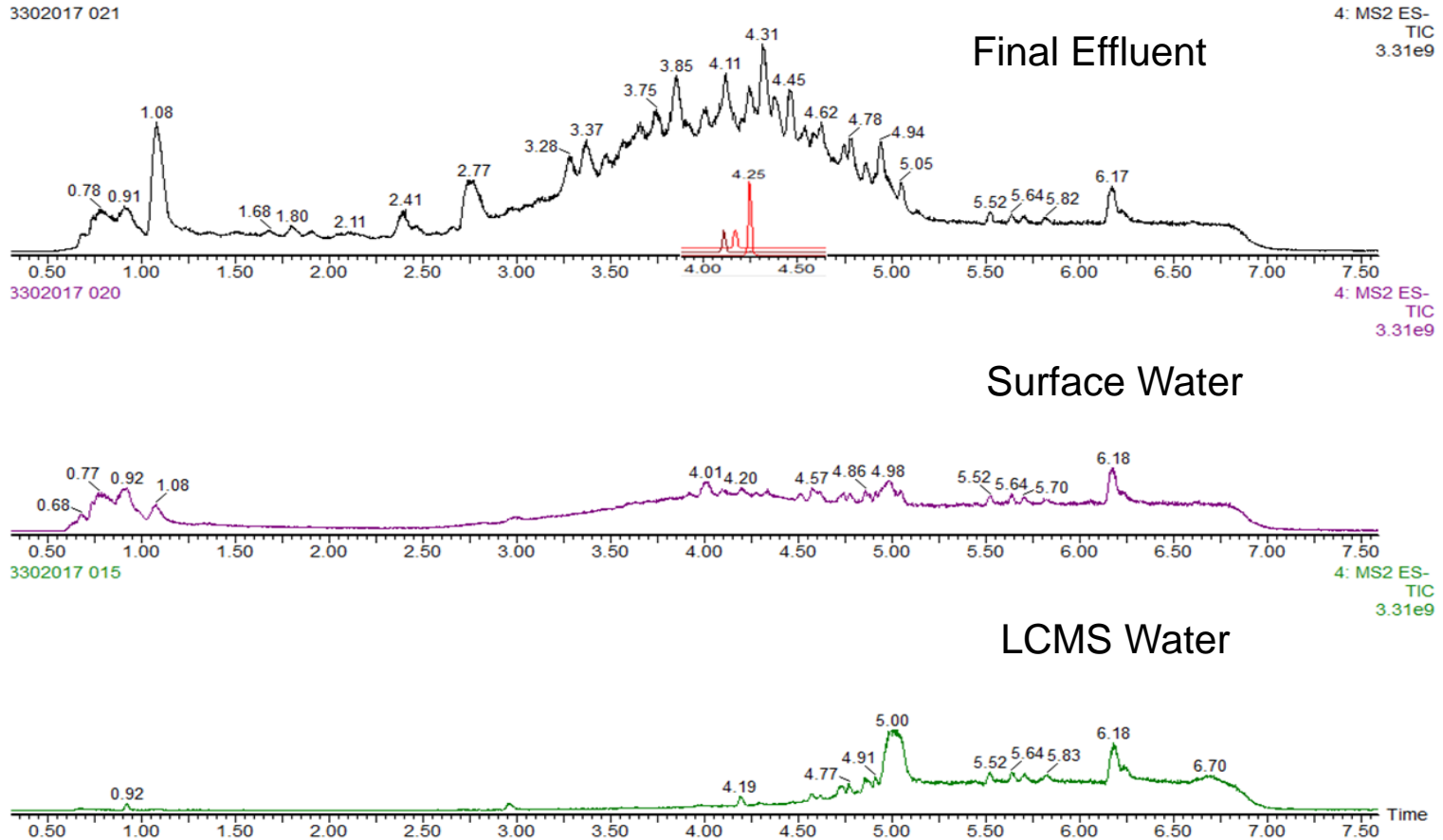
Compound	17α-ethinylestradiol	17β-estradiol	estrone
Spike Level 1 (pg/l) (surface water)	30.0	120.0	400.0
% Recovery (n=3)	80.0	99.5	92.2
% RSD (n=3)	10.8	4.0	1.6
Spike Level 2 (pg/l) (surface water)	60.0	300.0	1000
% Recovery (n=3)	71.3	100.6	92.6
% RSD (n=3)	12.3	4.6	1.9

SPE method recovery and repeatability data for all analytes, spiked at two levels in triplicate into surface water matrix, pre-spiked levels are indicated in bold.

Repeatability Surface Water Post Spiked Extracted Matrix n=8

Compound	Estrone	Beta Estradiol	EthynylEstradiol
Spike Level (ng/l)	20	20	20
SD n=8	115.38	39.08	33.57
% RSD n=8	1.50	5.26	4.26

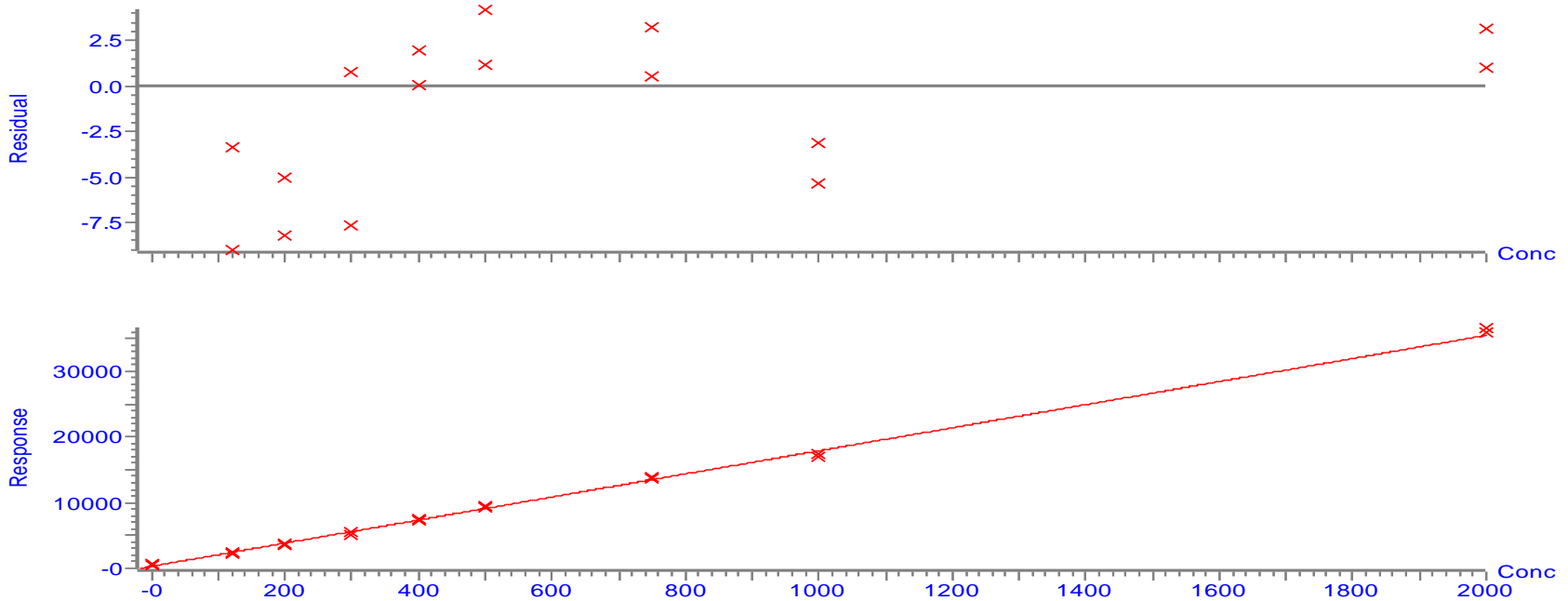
RADAR Scan of Different Extracted Water Matrices



RADAR scan (full scan m/z 100 to 1000) , on matrix samples after clean-up and concentration by SPE. Example peak for the target compounds are shown against the final effluent RADAR scan indicate the area they elute.

Use of Standard Addition for Final Effluent

Compound name: 17 Alpha Ethynyl Estradiol
Correlation coefficient: $r = 0.998732$, $r^2 = 0.997467$
Calibration curve: $17.5798 * x + 296.967$
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: $1/x$, Axis trans: None
Standard Addition Concentration : 16.8925



Standard addition method used to calculate low level concentration of 17 α -ethynylestradiol in final effluent (n=2).

Conclusions

- Method highlights the analysis of low ppq levels of synthetic estrogens in surface and final effluent waters.
- Utilising Oasis® SPE and Sep-Pak® SPE sample preparation technologies, the optimised extraction method was found to provide the required concentration and clean-up, given acceptable recoveries and repeatability in spiked surface water samples.
- The use of a large volume injection in combination with the ACQUITY® UPLC H-Class and XEVO® TQ-XS, allowed for the challenging detection requirements of this analysis to be achieved in surface water.
- The use of standard addition allowed for accurate quantification of trace residues in final effluent samples.



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