Online SPE-LC-APCI-MS/MS for the Determination of Steroidal Hormones in Drinking Water

presented by

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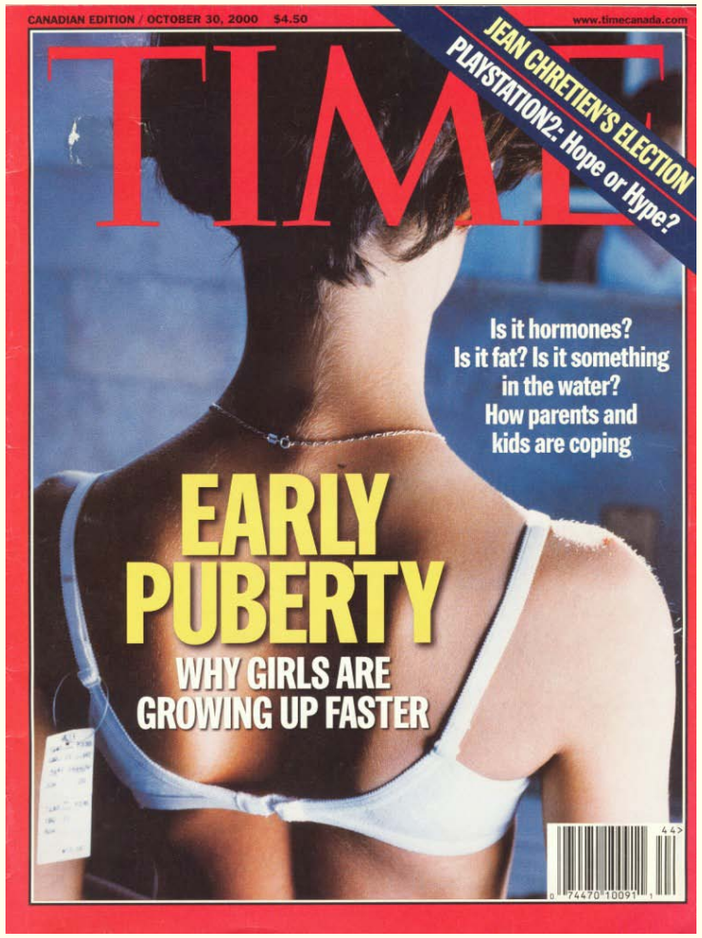
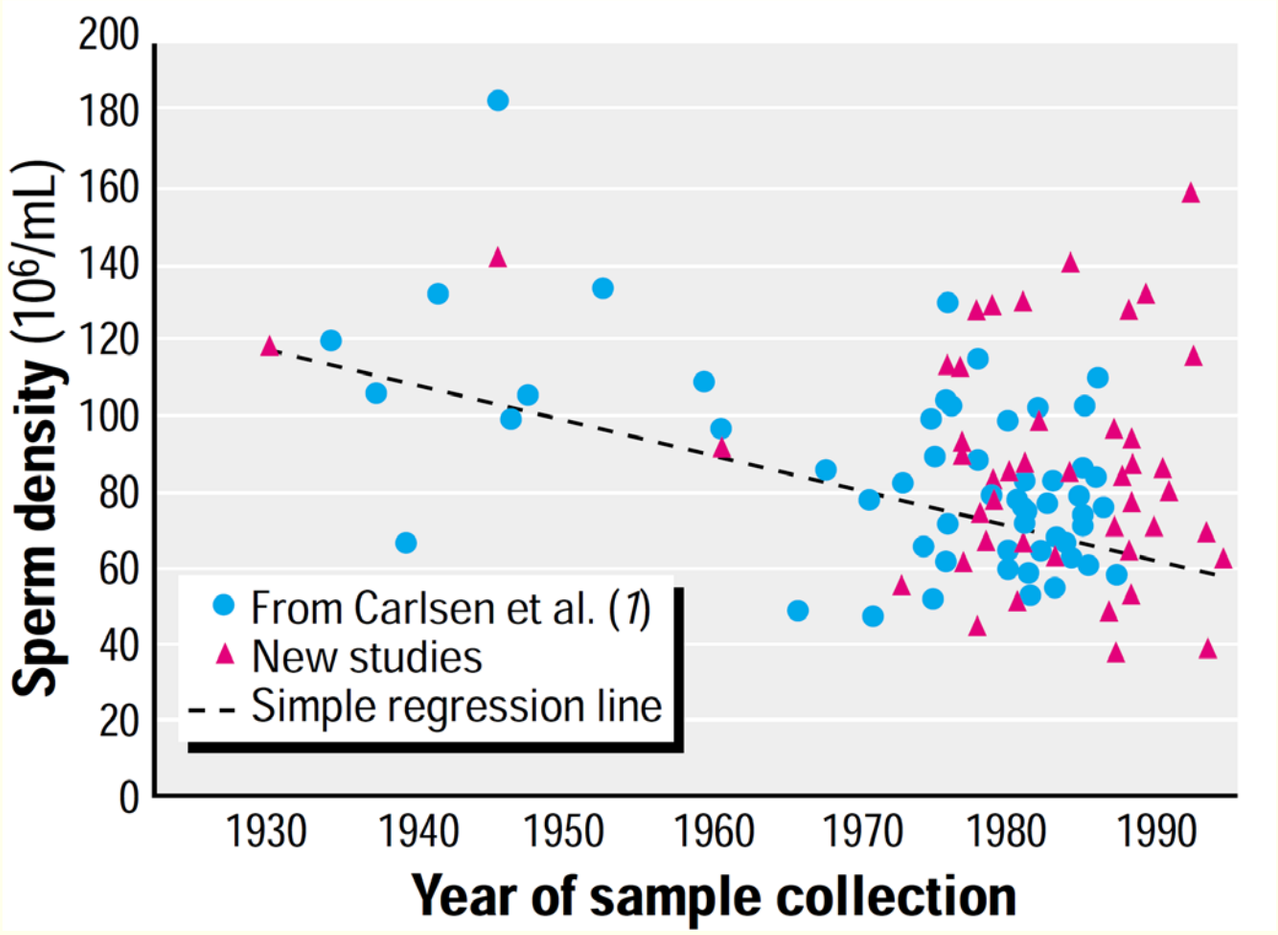
Exploring the problematic: context

Context

- ***Controversial*** effects of endocrine disrupting compounds (EDCs) in humans:
  - ✓ **Reduction of male births around the world** (Canada, Denmark)
  - ✓ **Increase cancer rates** (testicular, breast and prostate)
  - ✓ **Early puberty in young women** (7 and 8 years old!)
  - ✓ **Lower sperm counts/quality** (1992, 61 articles and 2000, 101 articles)



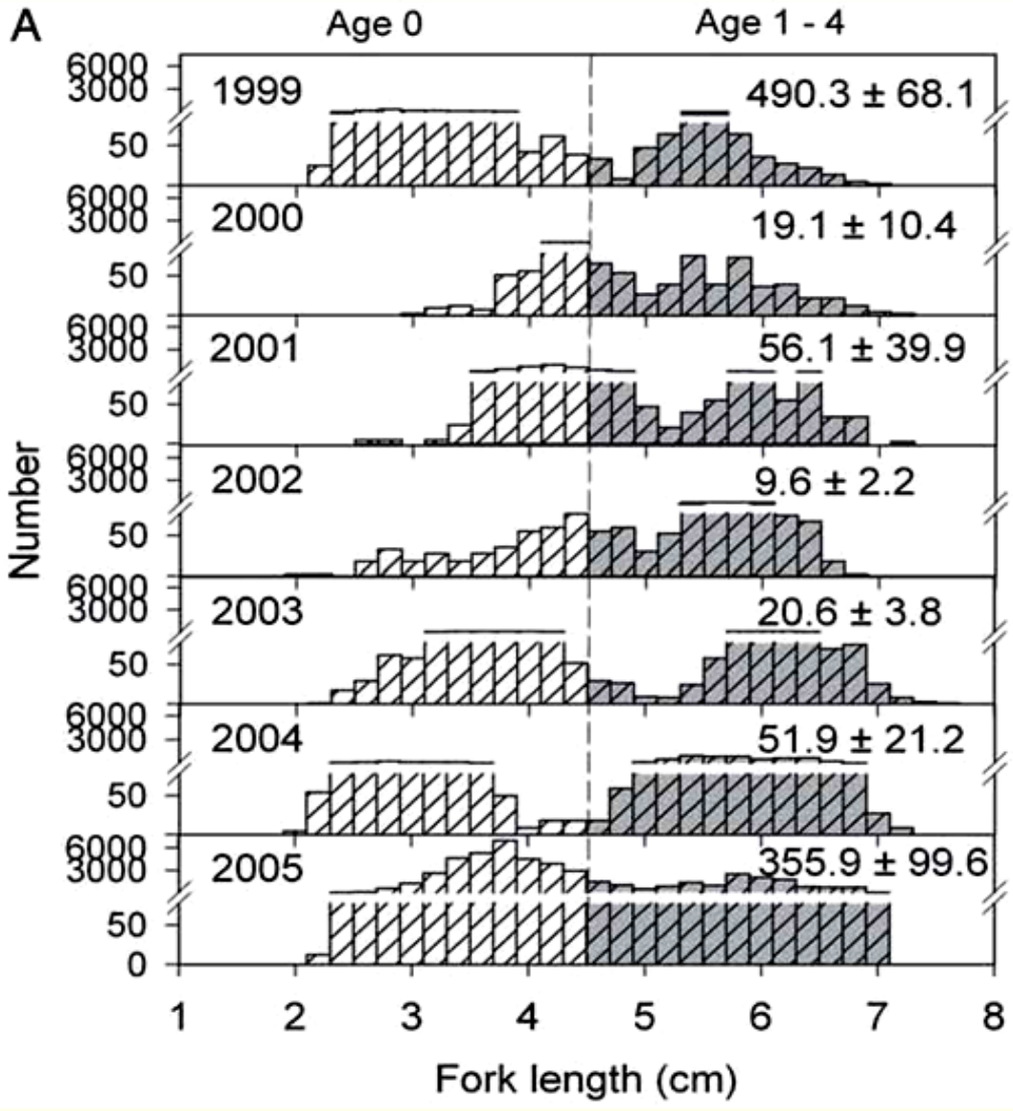


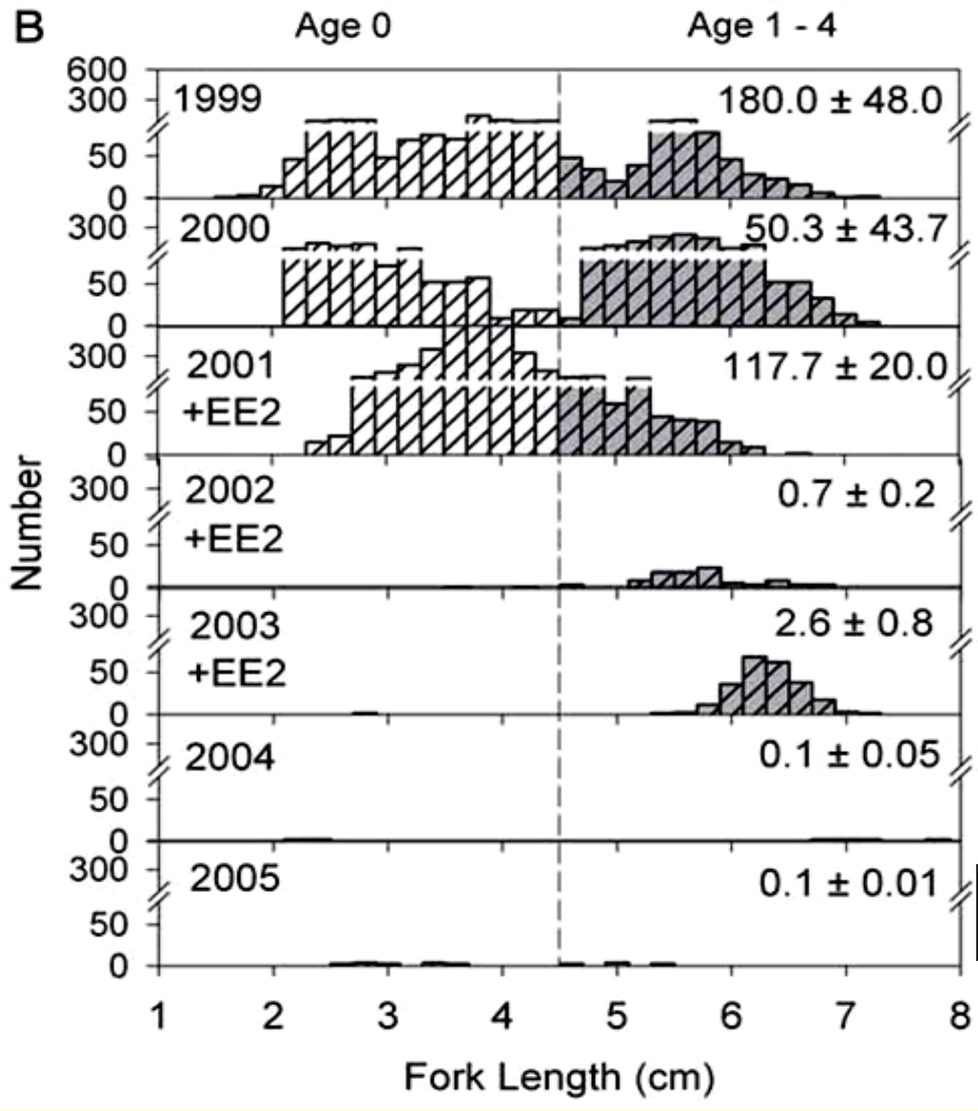
Landrigan P., *Environ. Health Perspect.*, 2003 (13), 1678; Mendes J.J.A., *Food Chemi.Toxicol.*, 2002 (40), 781; Swan S.H., *Environ. Health Perspect.*, 2000 (10), 961.

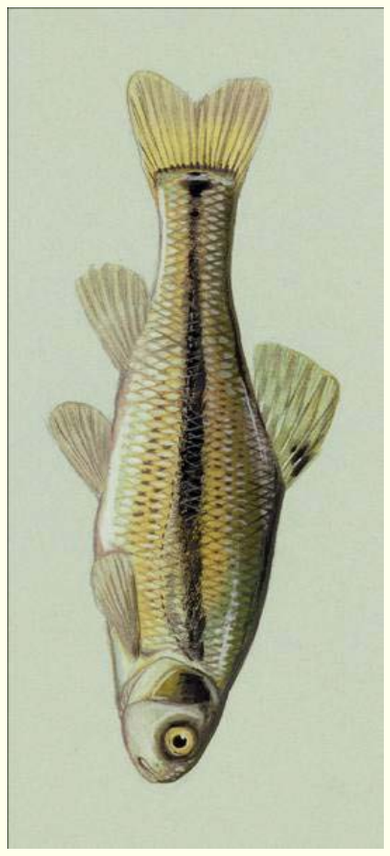
Context

- ***Demonstrated*** effects of EDCs in the aquatic environment :
  - ✓ **Reproduction decrease in fish species** (pulp and paper industries)
  - ✓ **Altered male/female ratios** (crocodiles and turtles)
  - ✓ **Increase cancer rates in fish** (testicular and liver)

Populations : fathead minnow fish in 2 ontarien experimental lakes (Kidd *et al.*, 2007)





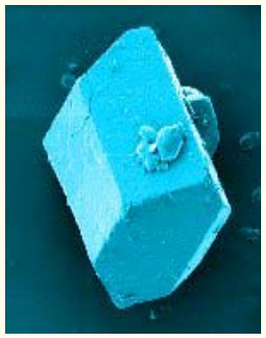


With 5-6ng/L E2

Snyder A., *Environ. Eng. Sci.*, 2003 (20), 240; (6) Tillmann M., *Ecotoxicol.* 2001 (10), 373; (7) Kidd K.A., *PNAS*, 2007 (104), 8897

Context

- EDCs (such as steroid hormones) concentrations that can cause these deleterious effects in the aquatic environment are very low, between 0.1 and 5 ng/L.



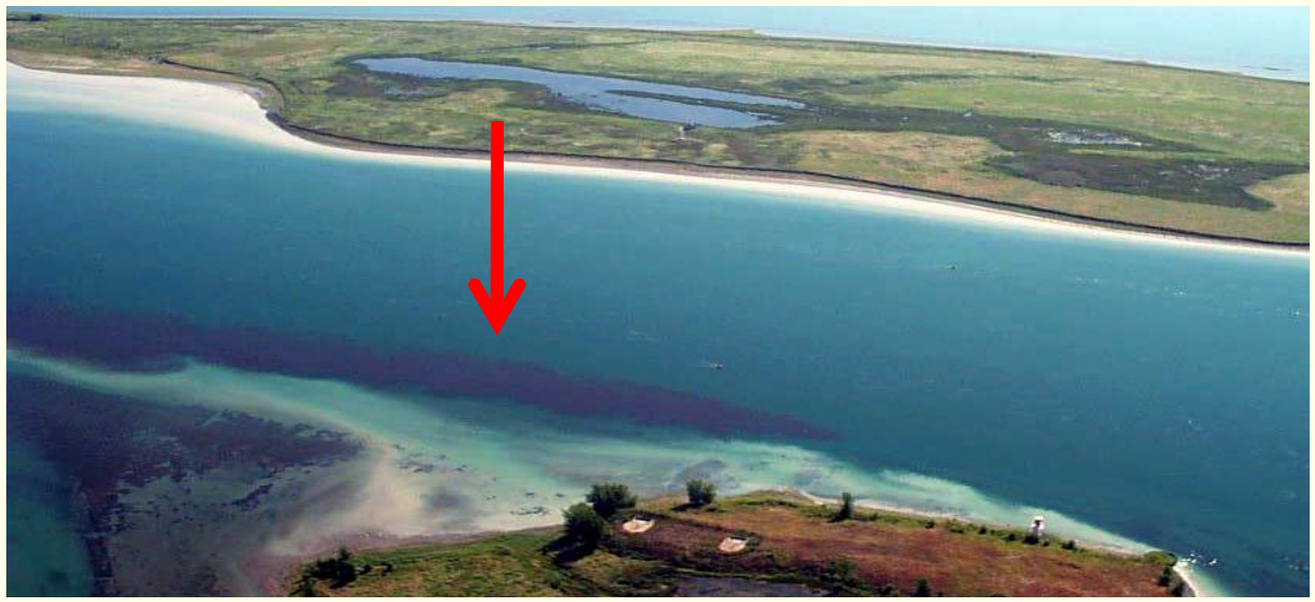
✓ **Therefore the development of analytical methods able to detect and quantify these EDCs, such as steroid hormones, is of importance, especially when considering their known effect on wildlife and potential impact on humans in the future.**

Weber L.P., *Aqua.Toxicol.*, 2003 (63), 431

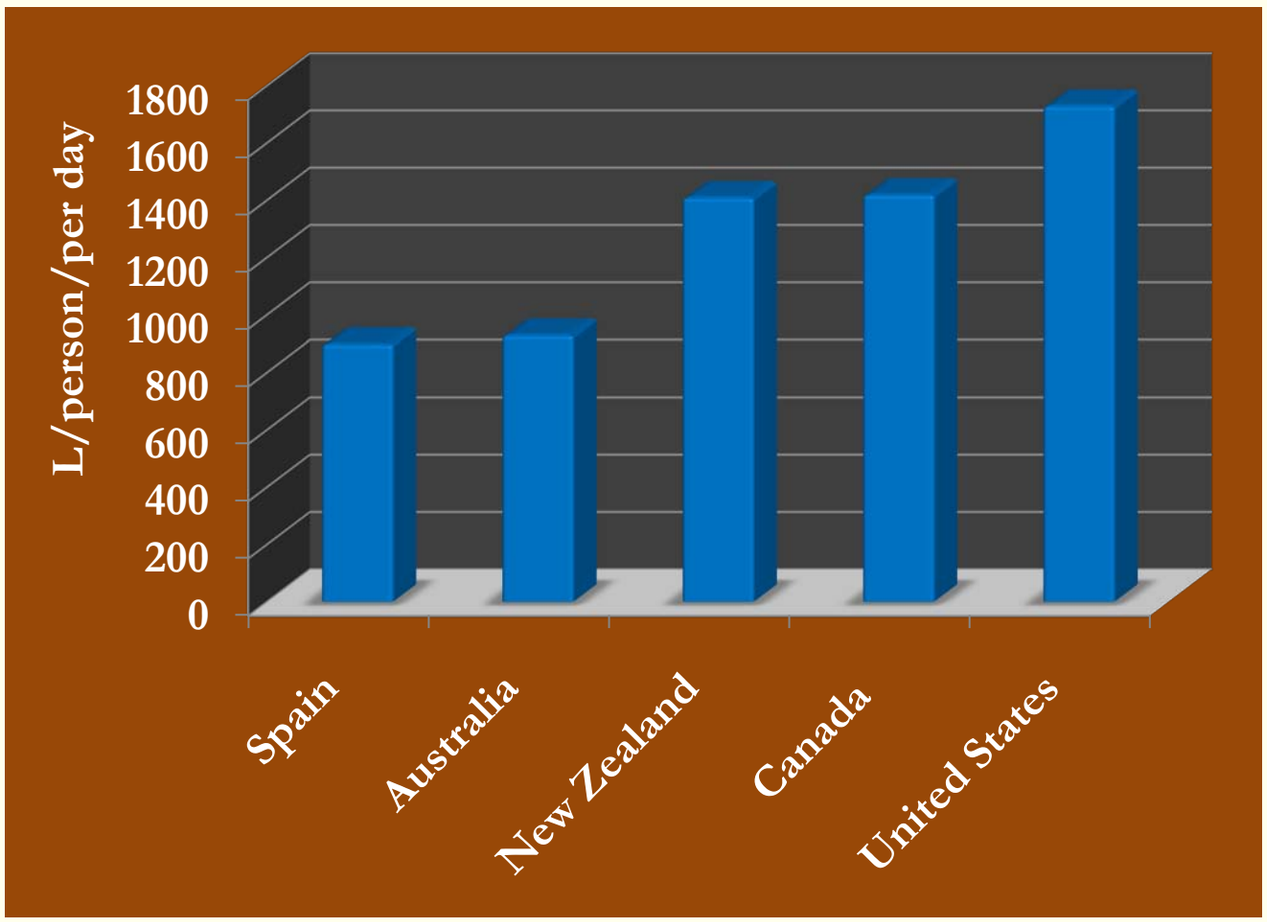
Analytical challenges: dilution and interferences

Dilution

- The total volume of treated wastewater by a treatment plant in Canada is evaluated at 42214 million m^3, i.e. ~1420 L per person per day.



Source: Picture takin by Environnement Canada (2001) of St-Lawrence river in Montreal (Qc, Canada)



Source: Adapted from OECD Factbook 2007: Economic, Environmental and Social Statistics - ISBN 92-64-02946-X

Dilution

Interference

- The second analytical challenge is matrix type and interfering compounds

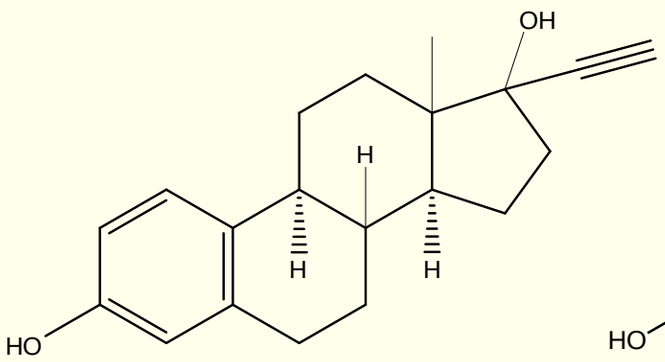
1. **Influent**
2. **Effluent (not filtered)**
3. **Effluent (filtered at 0.45µm)**
4. **HPLC grade water**

✓ **To overcome these analytical challenges as well as quantify low ng/L levels of steroid hormones, the use of solid phase extraction (SPE) is used prior to analysis by LC-MS/MS.**

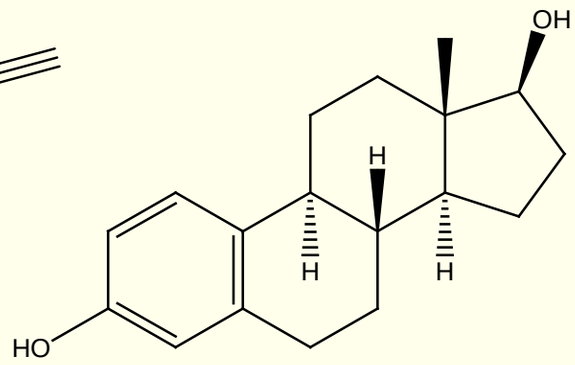
Objective

- **Develop a rapid, sensitive and selective analytical method to detect and quantify eight selected steroid hormones, using an on-line SPE method coupled to an LC-MS/MS.**

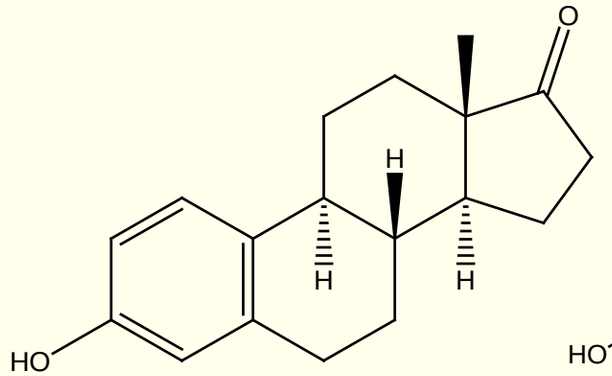
Estrogens



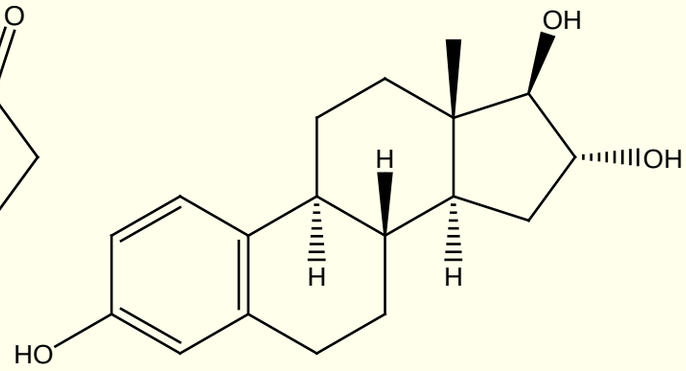
Ethynilestradiol (EE2)



Estradiol (E2)

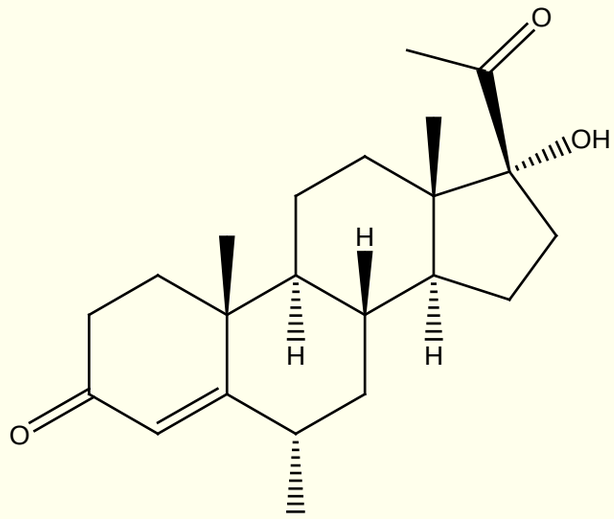


Estrone (E1)

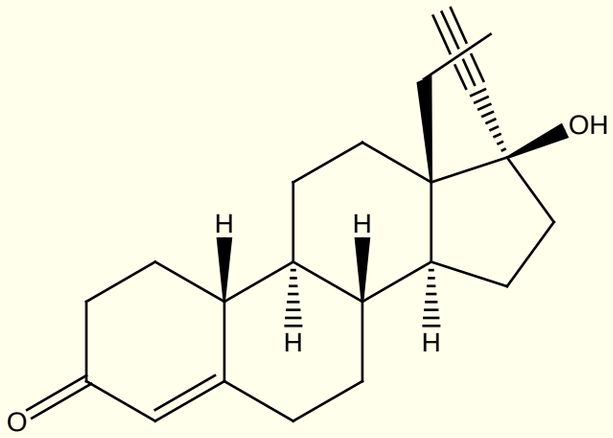


Estriol (E3)

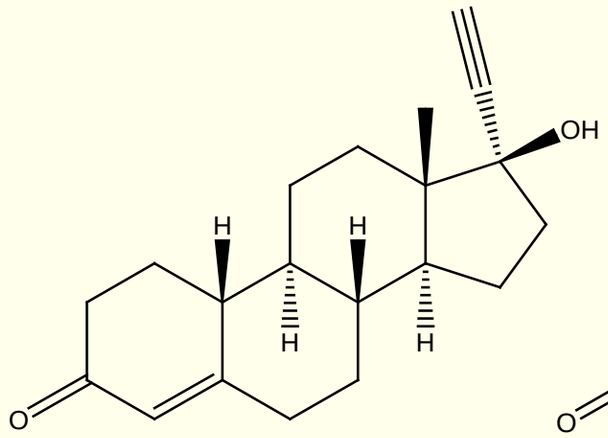
Progestagens



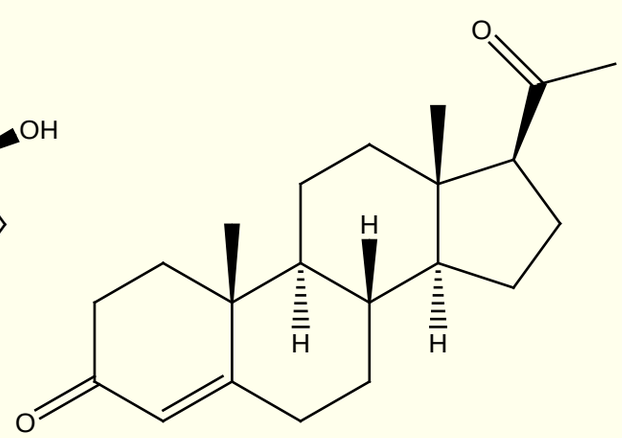
Medroxyprogesterone (MEDRO)



Levonorgestrel (LEVO)



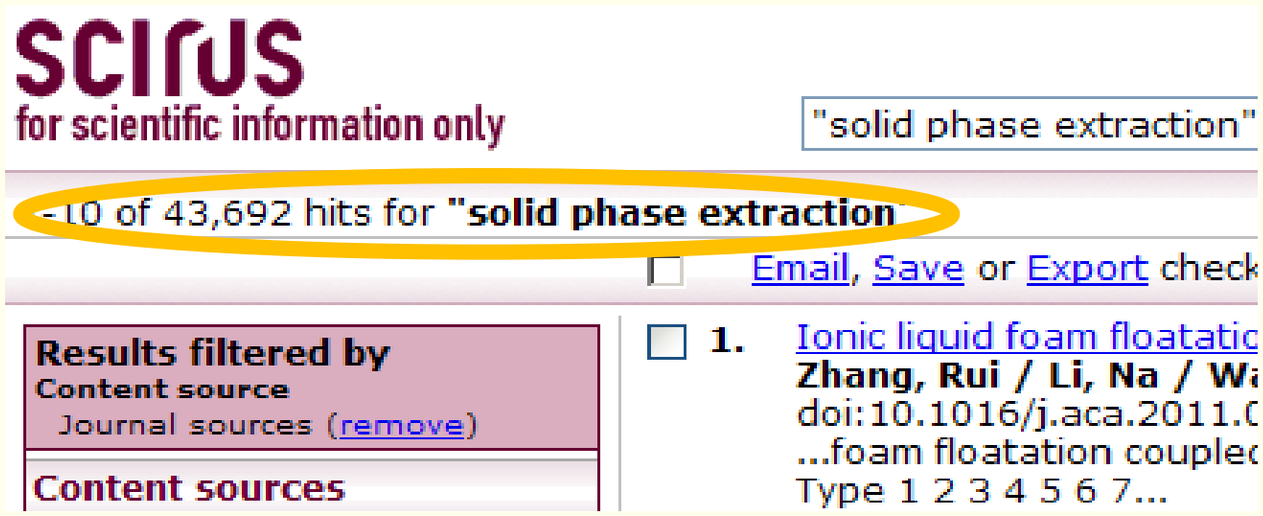
19-Norethindrone (NORE)

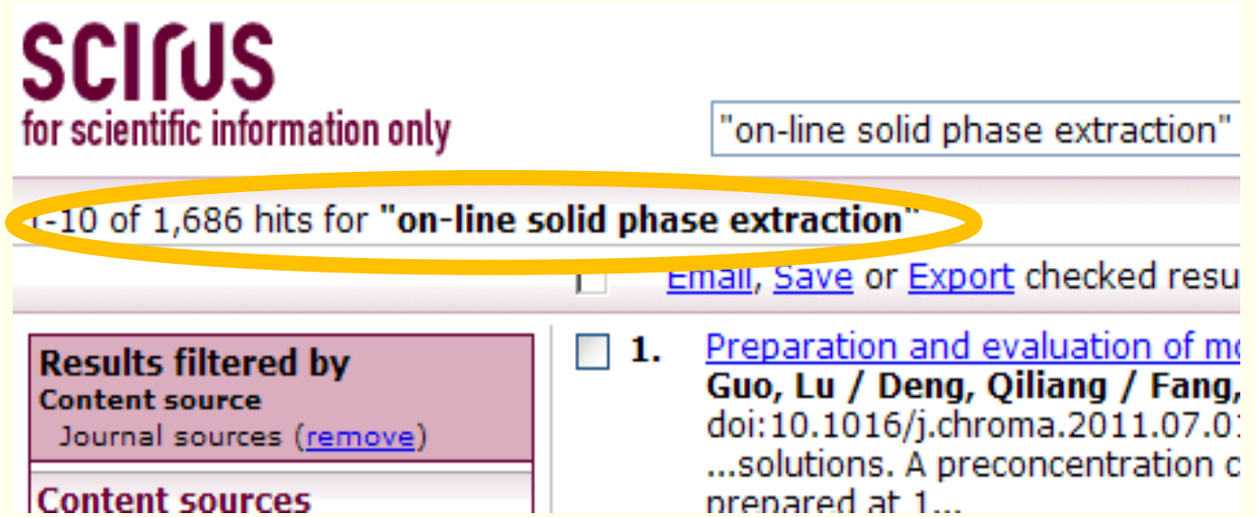


Progesterone (PROG)

Analytical method: on-line SPE-LC-APCI-MS/MS

Off-line SPE

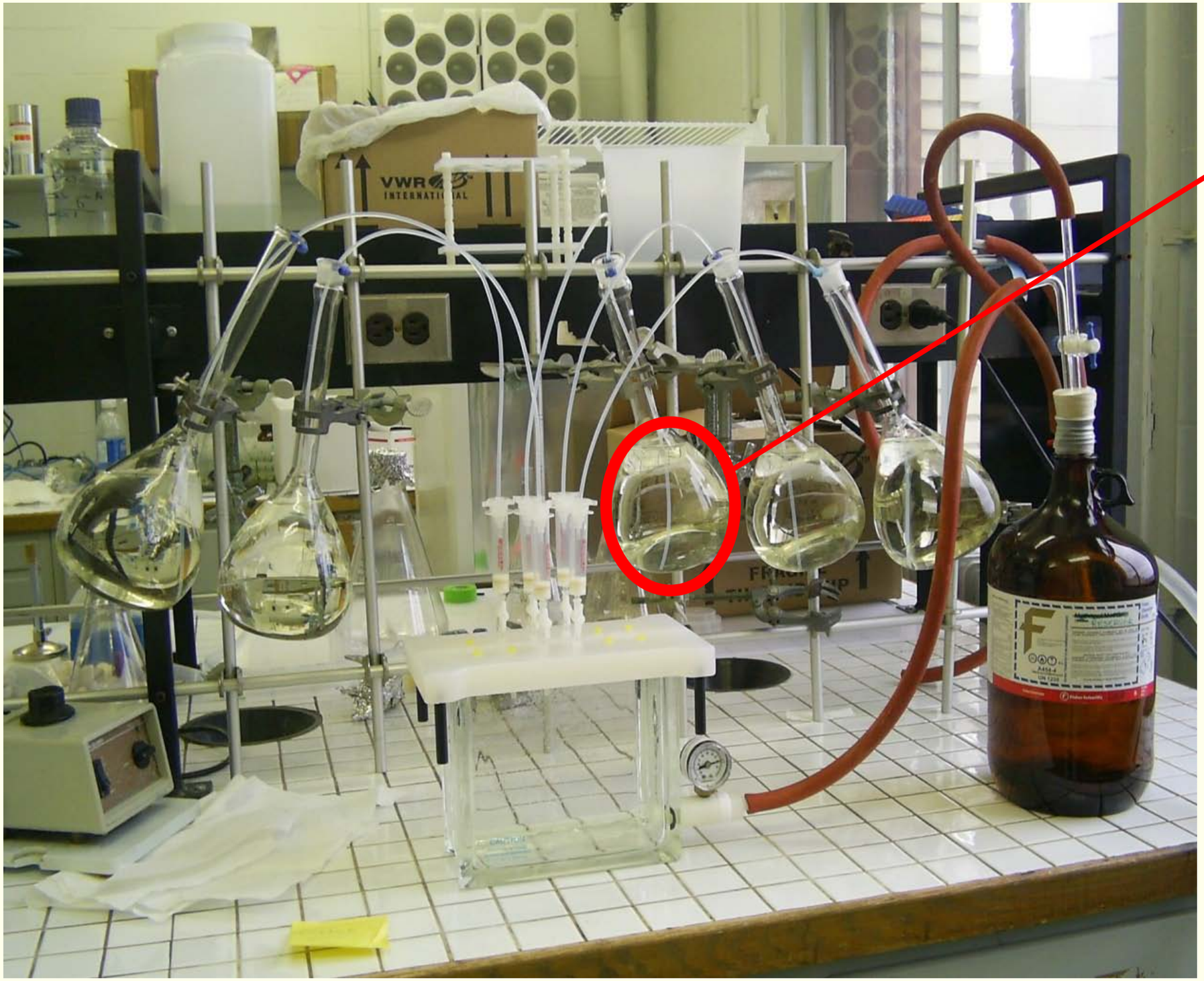




- **Off-line SPE is still more popular and more prevalent than on-line SPE. With very good limits of detection, large volume of sample can be used and is versatile (many stationary phase option)…*<u>but</u>***

is very time consuming, 15 hours for 12 samples in our lab

Off-line SPE



Here,

V_i = 500 mL

V_f = 0.250 mL

→CF = 2000

Off-line SPE

The time consuming off-line SPE procedure coupled to the limited number of samples capable of being analyzed each day (maximum 12 samples a day in our lab) makes this technique very laborious.

Therefore we need to develop a new, more practical pre-concentration technique while having similar performances as off-line SPE methods

Solution: on-line SPE

On-line SPE

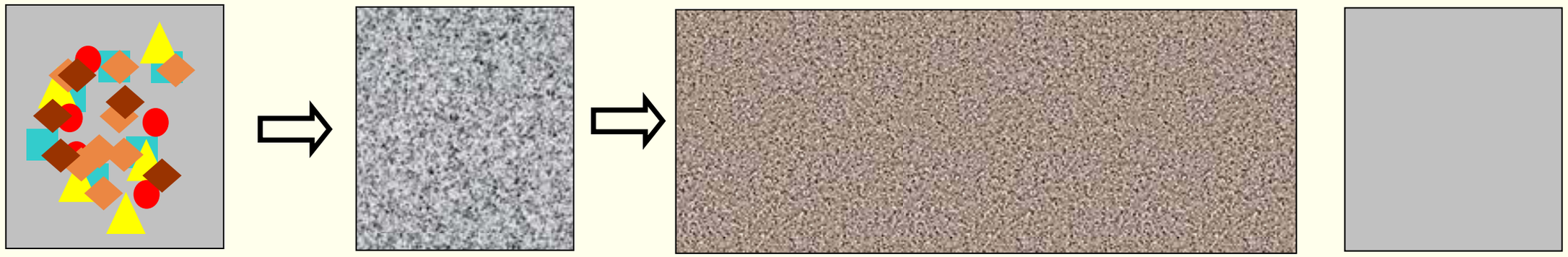
- There are many advantages of using on-line SPE:
  - ✓ **reduced sample handling and preparation** (minutes instead of hours)
  - ✓ **reduction of sampling size and storage volume** (1 to 10 mL versus 250 to 1000 mL)
  - ✓ **improved reproducibility** (because of automation)
  - ✓ **higher sample throughput per day** (between 50 and 100 versus 12 for off-line SPE)
  - ✓ **less waste and solvent consumption** (1 on-line SPE cartridge will be used for up to 200 samples depending on the matrix)

- The same steps (1. conditioning, 2. charging, 3. wash, 4. elution) as for off-line SPE will still be applied to on-line SPE. **The difference is in the automation of the process.**

On-line SPE

1. Pre-concentration, wash

2. Elution, separation, quantification



1 - 10 ml

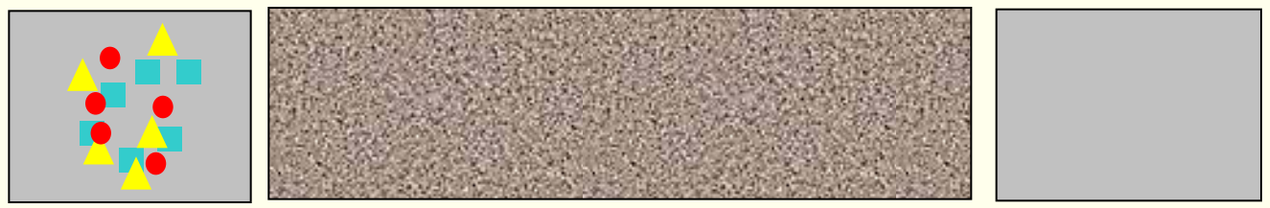
SPE

Chromatography

MS/MS

1. Off-line SPE

2. Separation and quantification



Waste

10 µl of
0.250 ml

Chromatography

MS/MS

▲ ■ ● Analytes

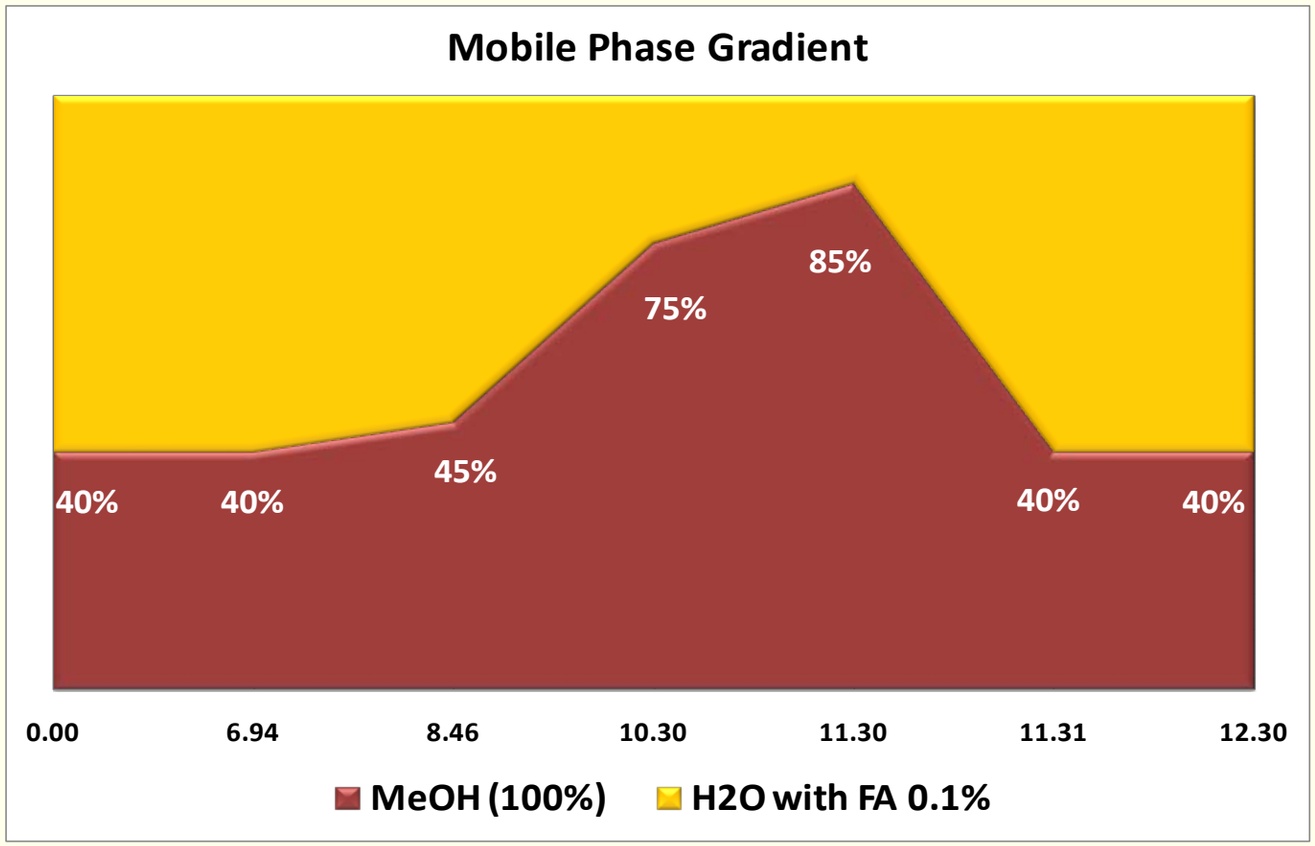
◆ ◆ Interferences

⇨ Permutation (valve)

On-line SPE

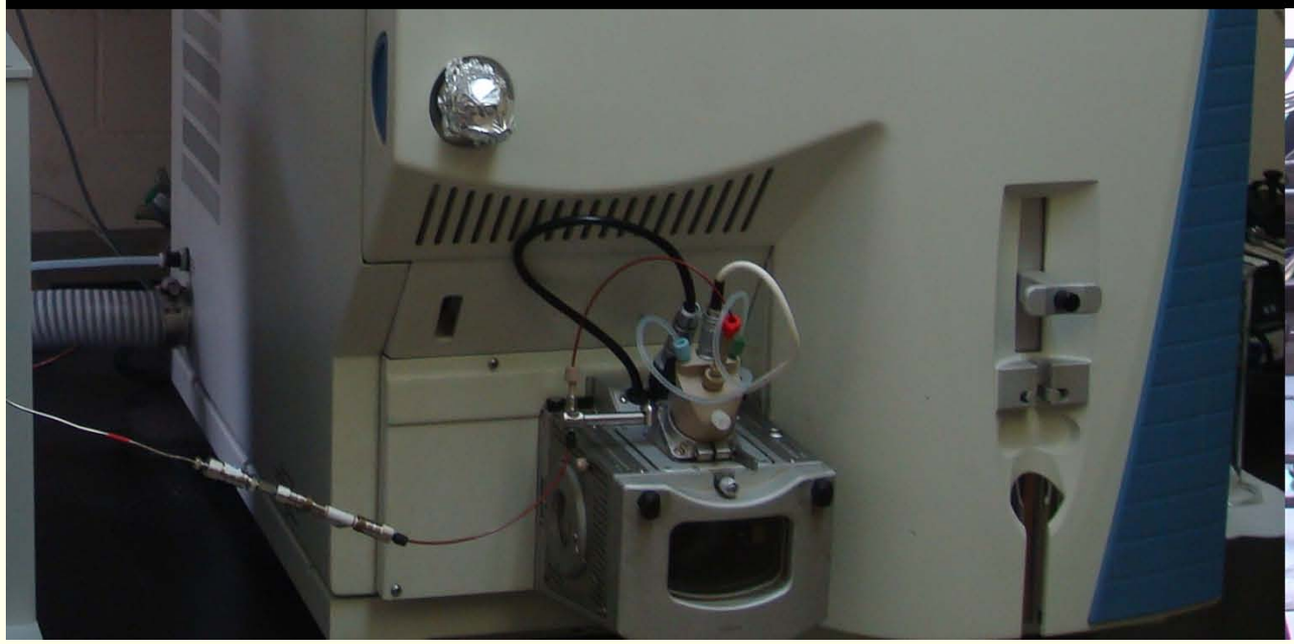
- Analytical column:

  - ✓ **Hypersil GOLD™ (1.9 µm, 100 × 2.1 mm)**
- On-line SPE column:
  - ✓ **Hypersil GOLD™ aQ (12 µm, 20 × 2.1 mm)**
- Injection volume:
  - ✓ **1 to 10 mL (final volume used was 5 mL)**
- Mobile phases:
  - ✓ **A: Water FA 0.1 %**
    B: MeOH
- Ionization source:
  - ✓ **APCI**
- Temparature:
  - ✓ **60°C**



On-line SPE


Thermo
Thermo
FINNIGAN
TSQ Quantum
ULTRA
AM
Thermo




Thermo
MS Pump Analytical #2
ACCELA
LC Pump Loading #1
Thermo
ACCELA

On-line SPE

STEP 1 =

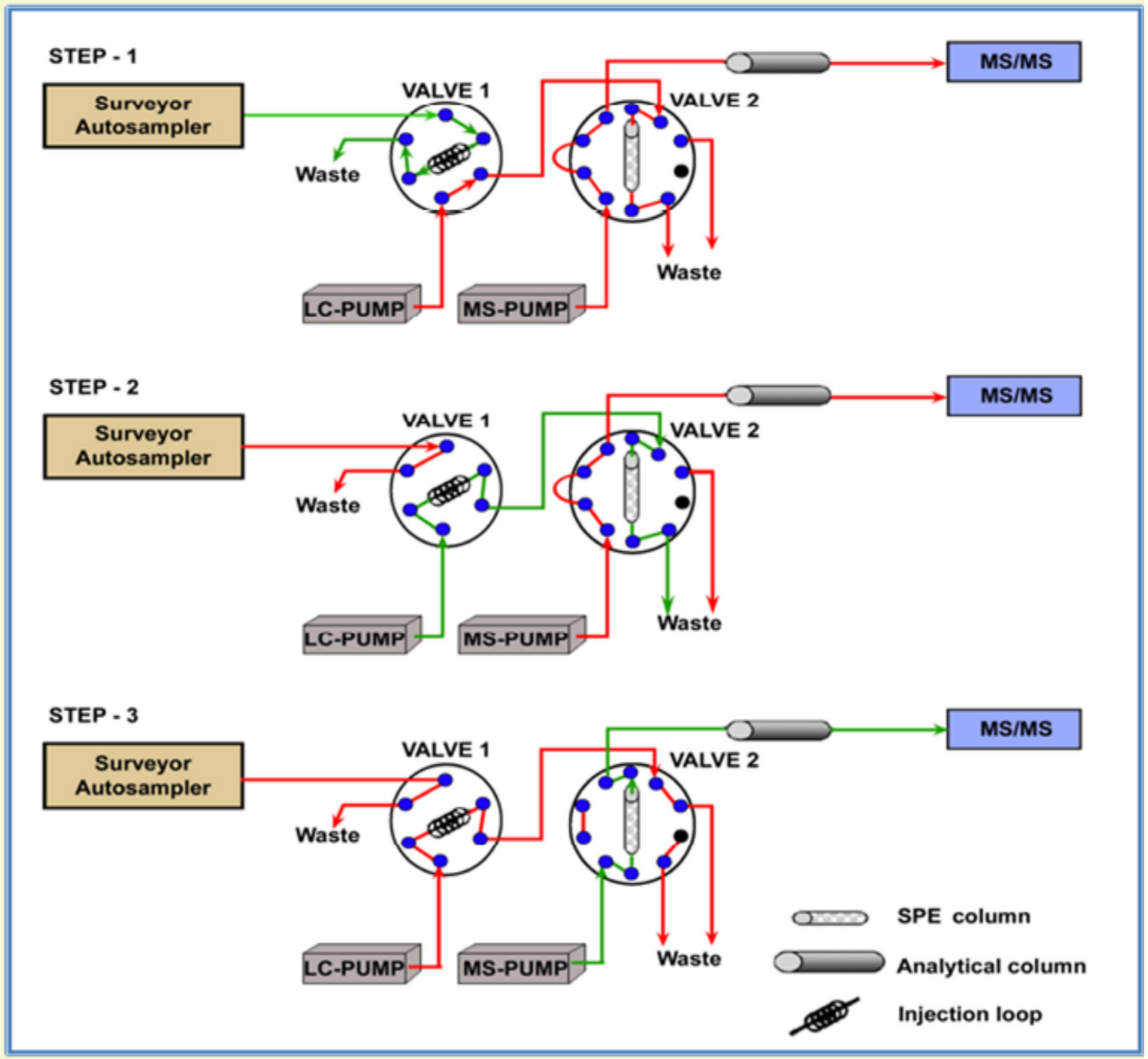
Injection loop loading

STEP 2 =

SPE column loading

STEP 3 =

Injection mode

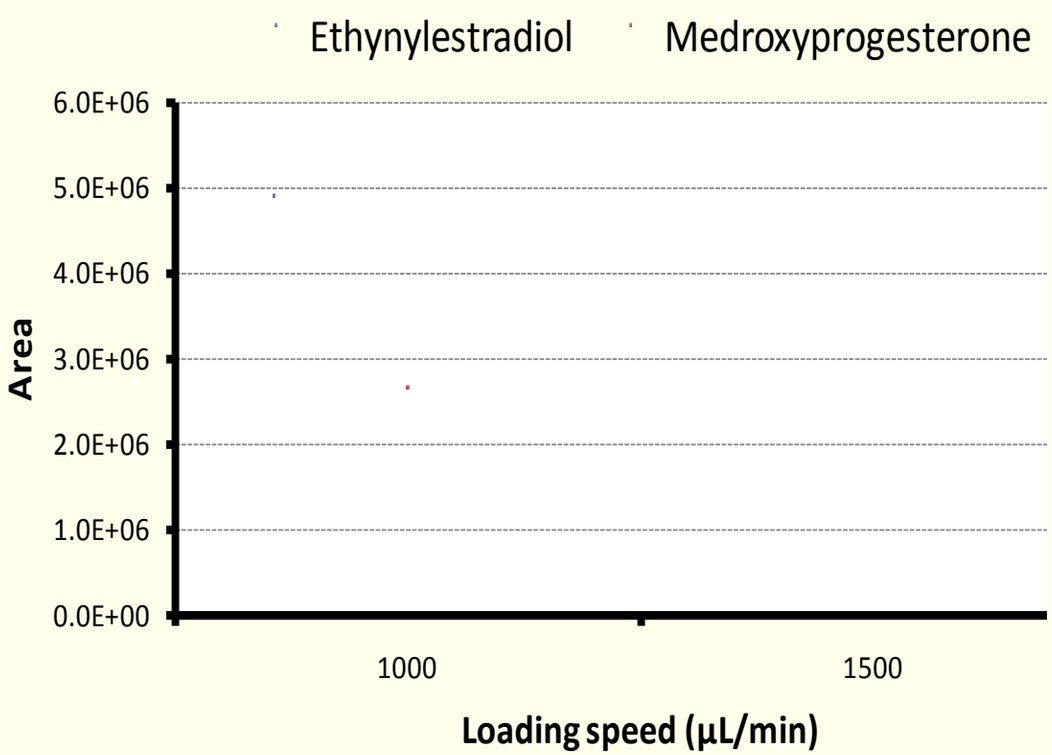


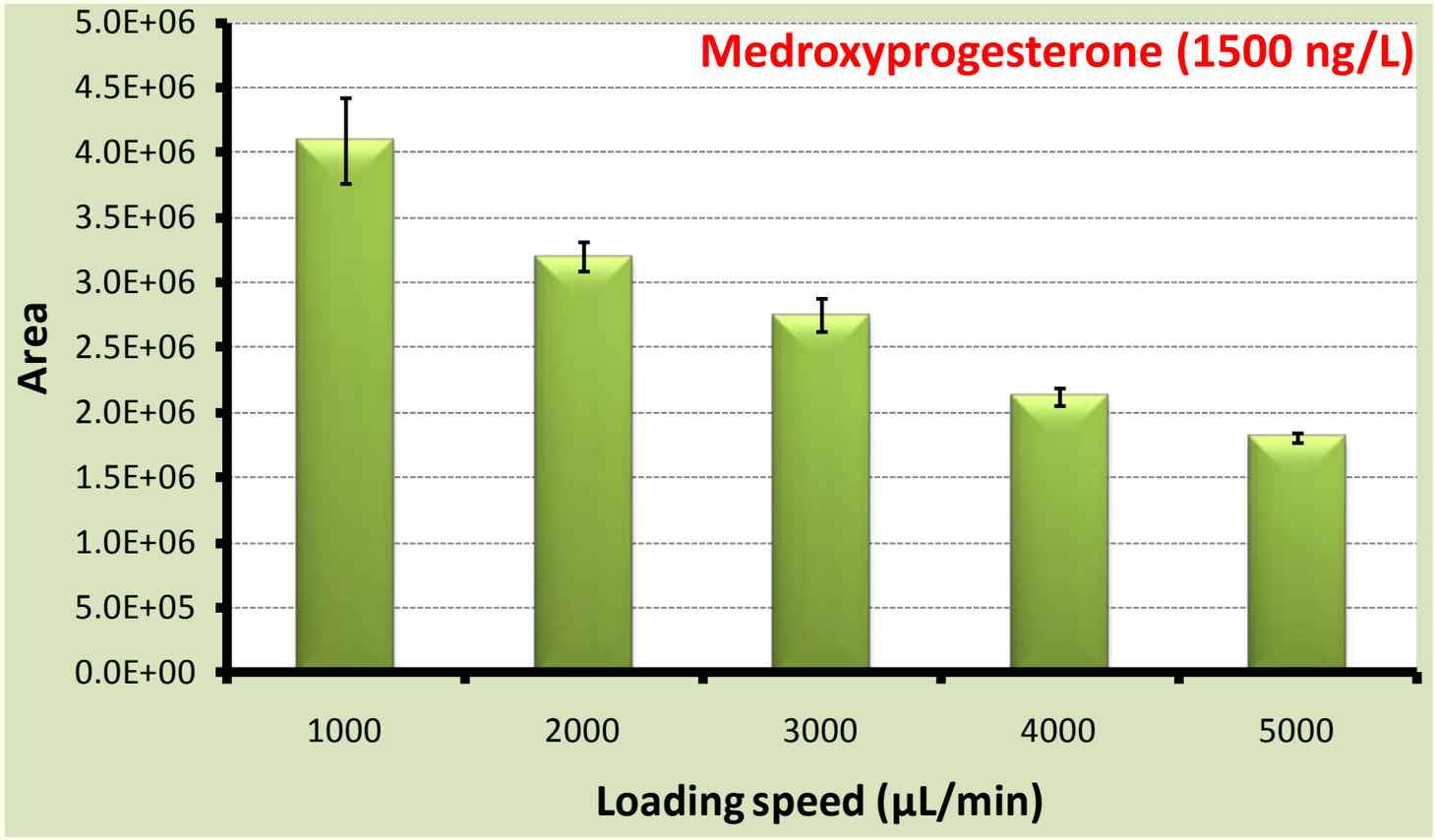
On-line SPE-LC-MS/MS method optimization

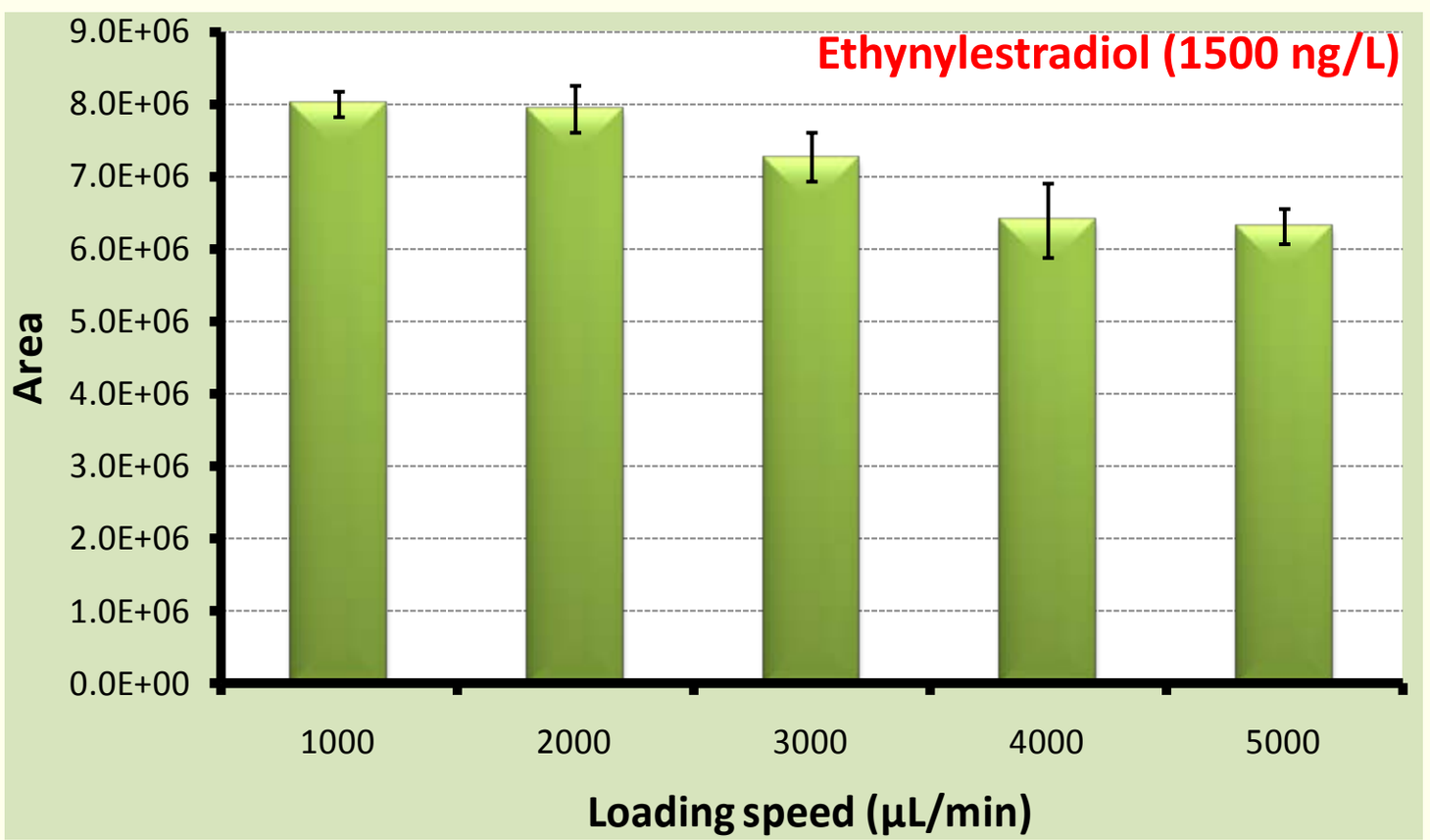
Loading speed

- The sample transfer time (or loading speed) from the injection loop to the SPE column will be important in diminishing total analysis time.

Optimum speed is 1500 µL/min (tested at 850 ng/L in neat solution)

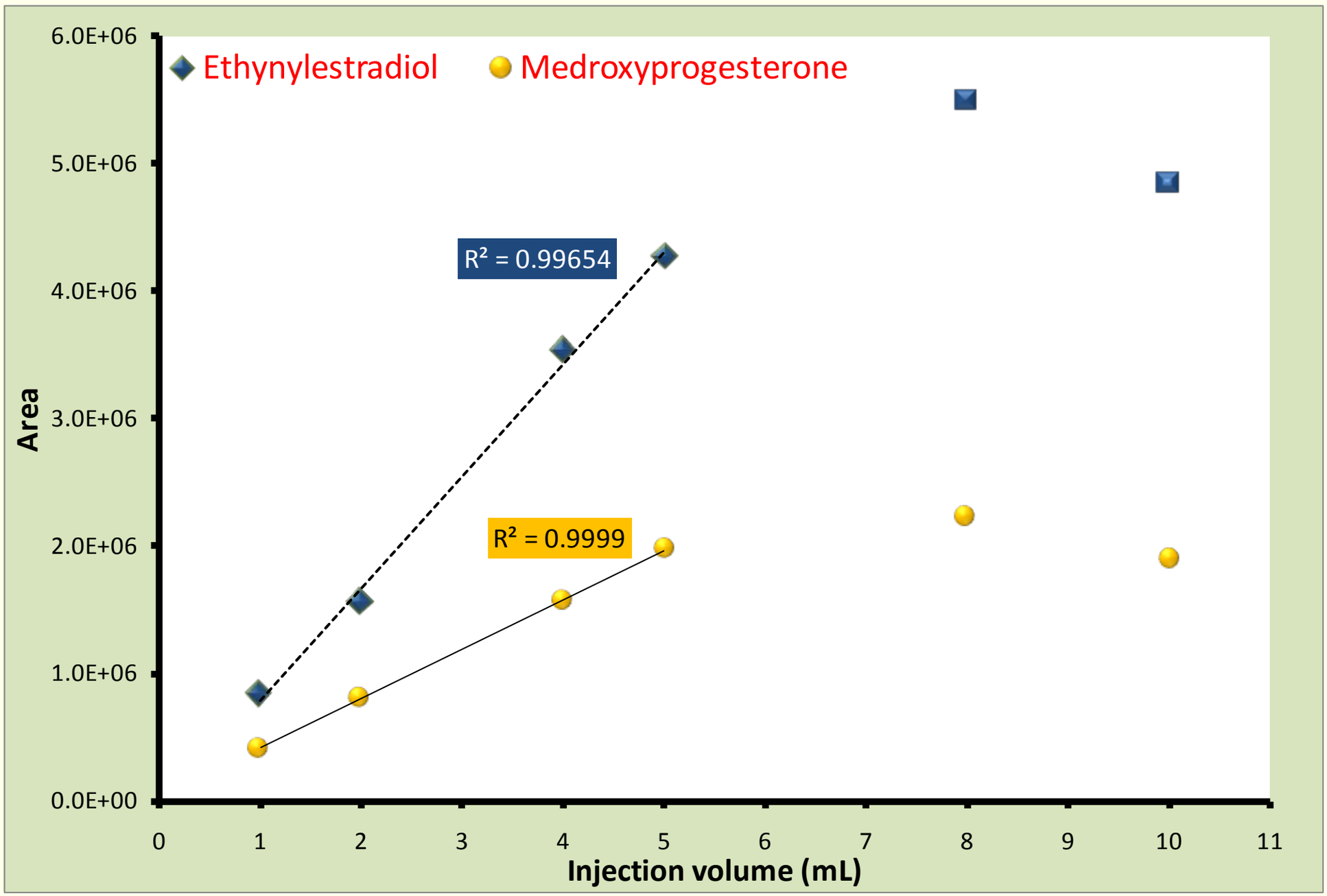






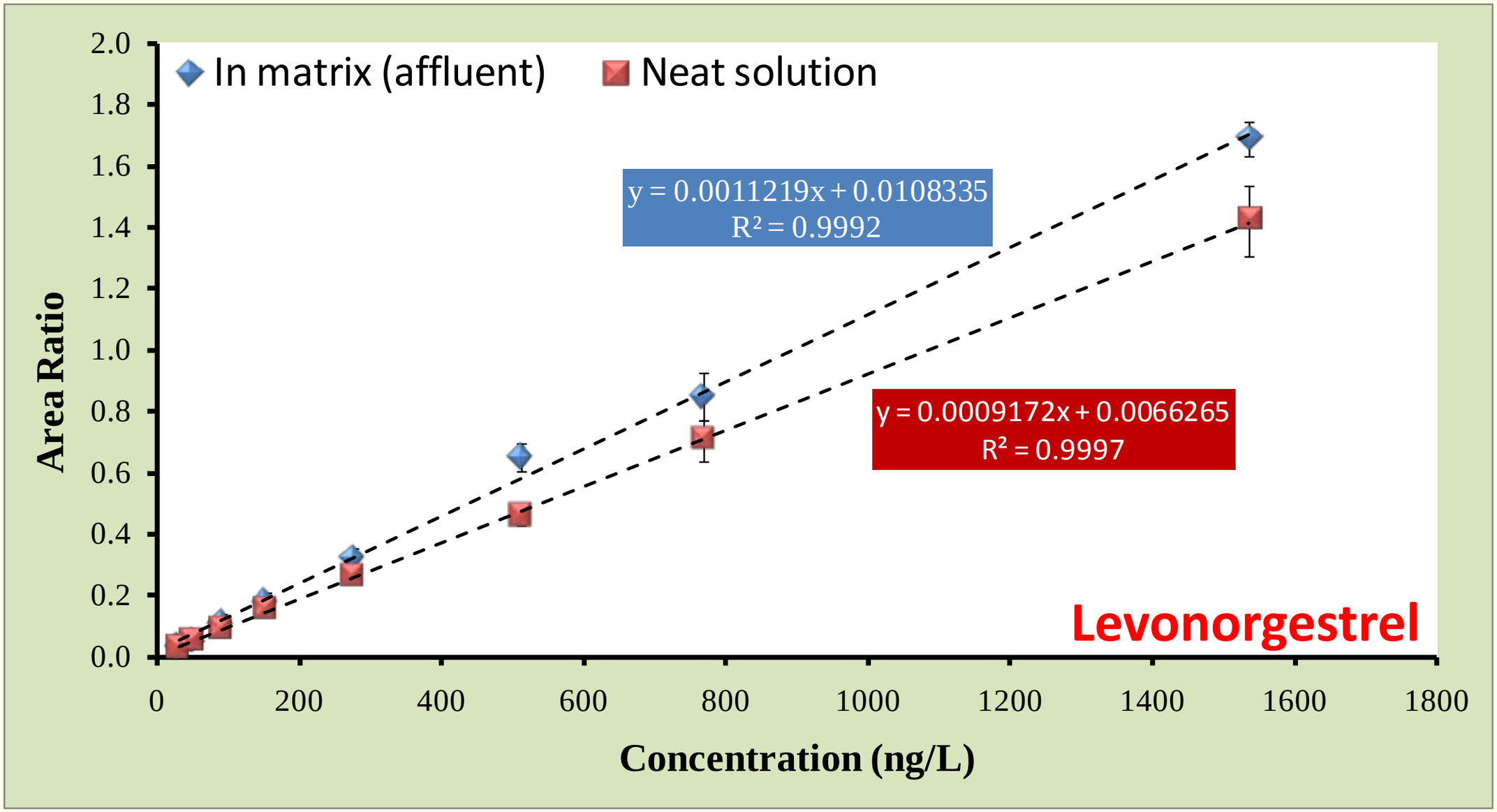
Breakthrough volume

- In order to improve signal intensities and also limits of detection we tested multiple injection volume using a 10mL injection loop and established the maximum injectable volume without loss of analyte (**tested at 200 ng/L in affluent wastewater**).



Method validation

- Calibration curves in matrix (affluent) and in neat solution were built in order to asses linearity range as well as matrix effect and recovery values. Injection volume was 5 mL (optimal volume without breakthrough) in a 10 mL loop. (n=3 for each calibration point)

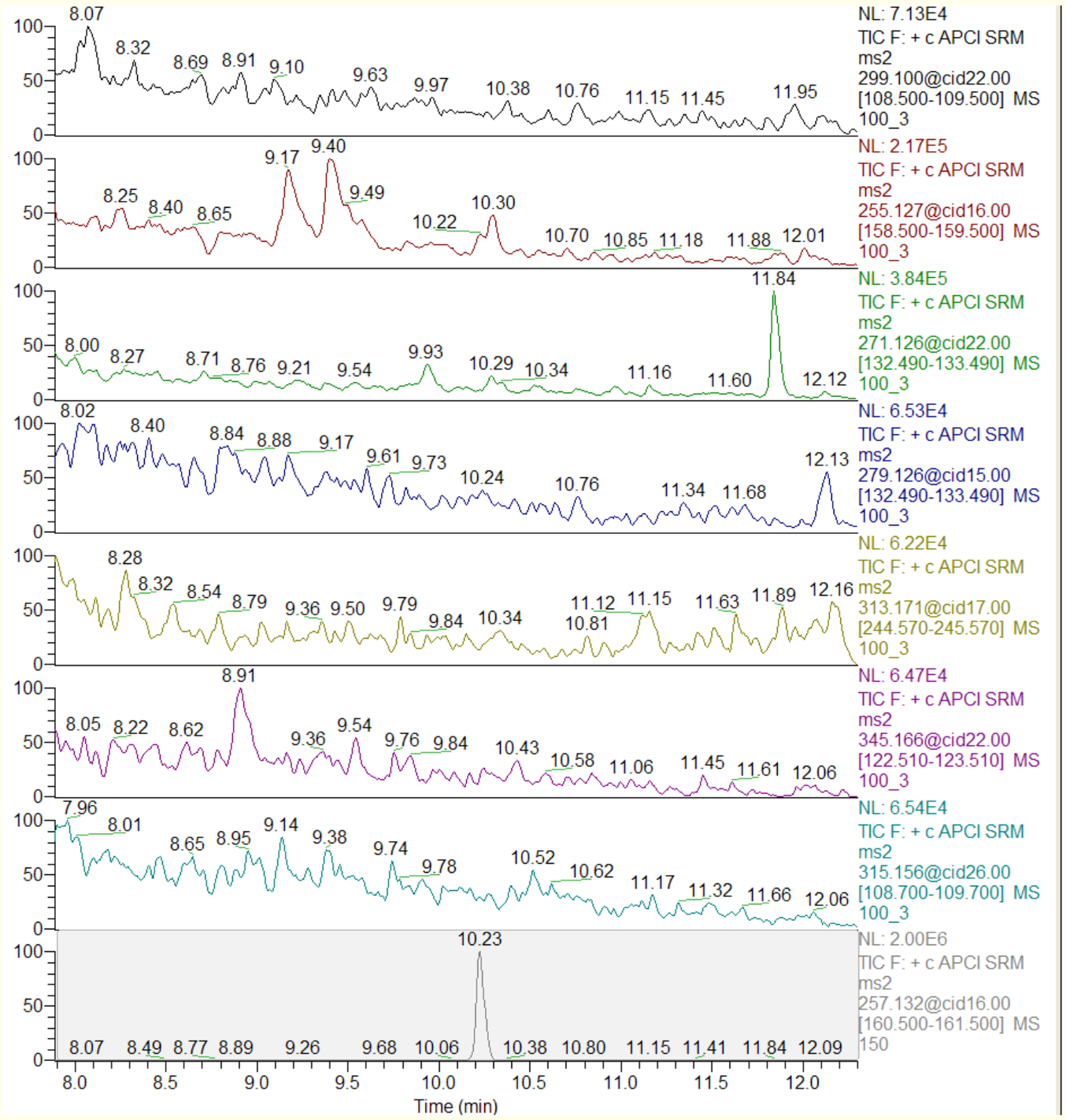


We observed signal enhancement in matrix (affluent)

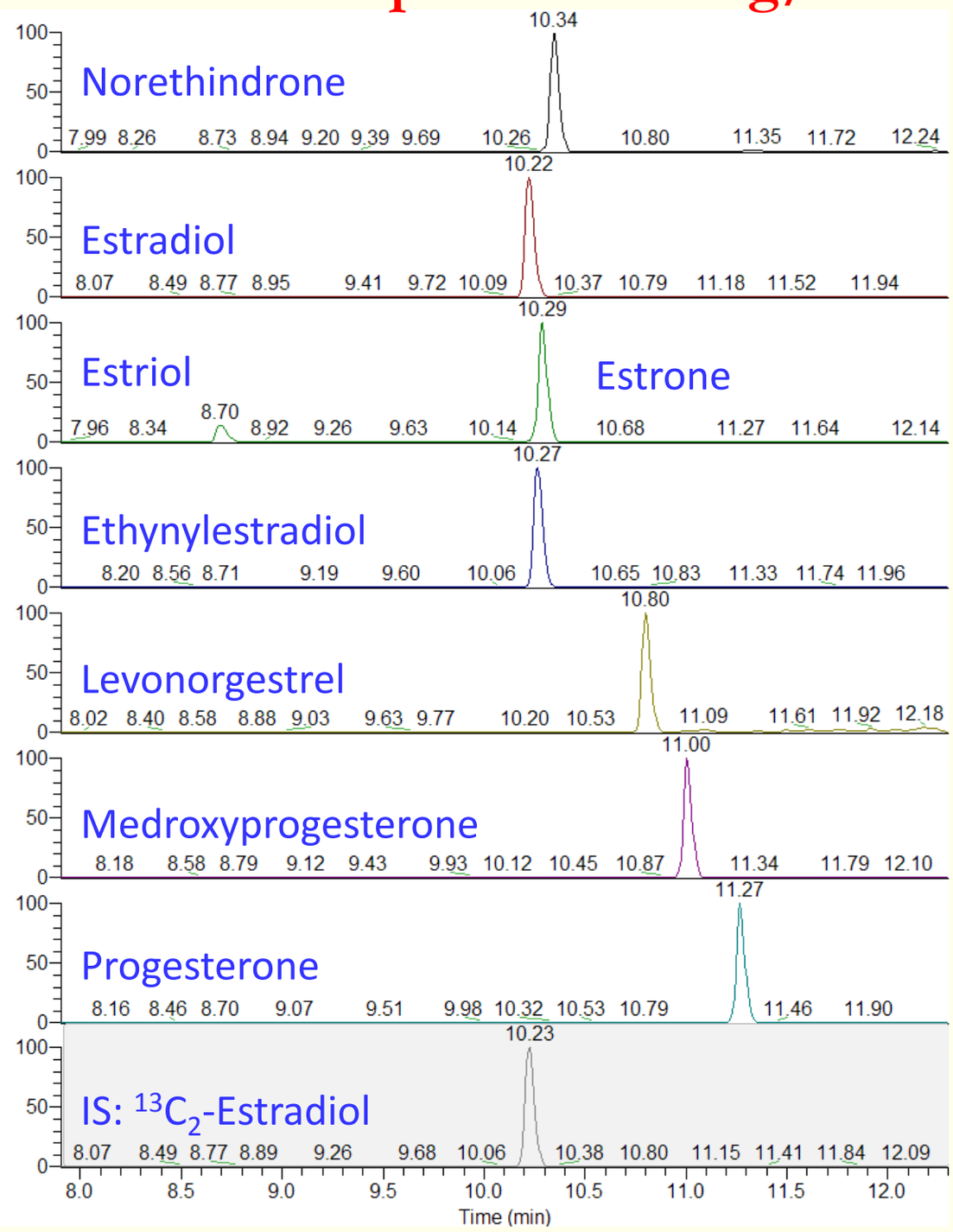
Method validation

- Blanks were evaluated to establish that signal enhancement was not caused by the presence of the analyte of interest in matrix or interfering compounds.

Affluent blank



Affluent spiked at 150 ng/L

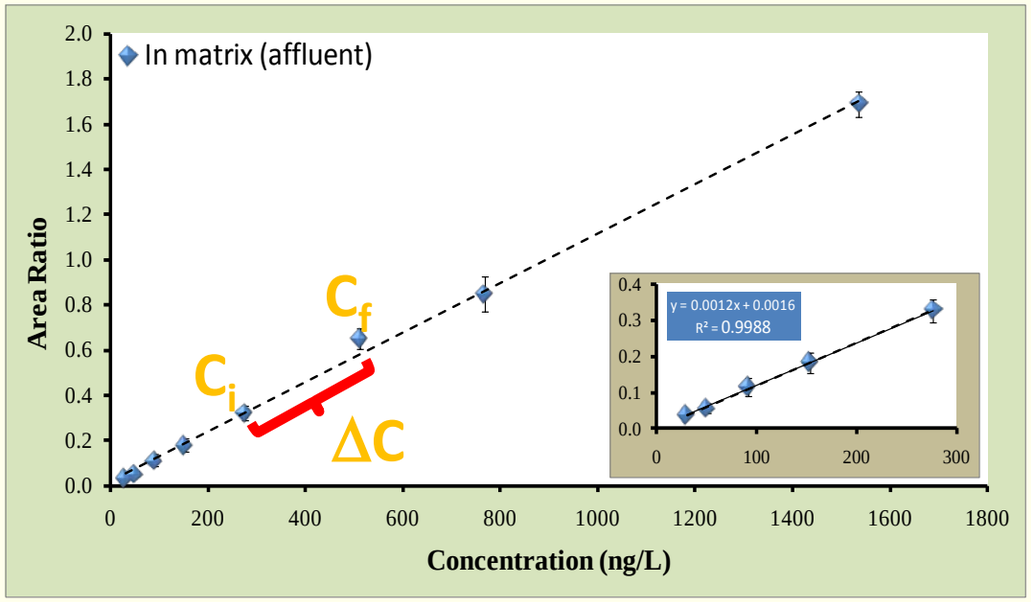


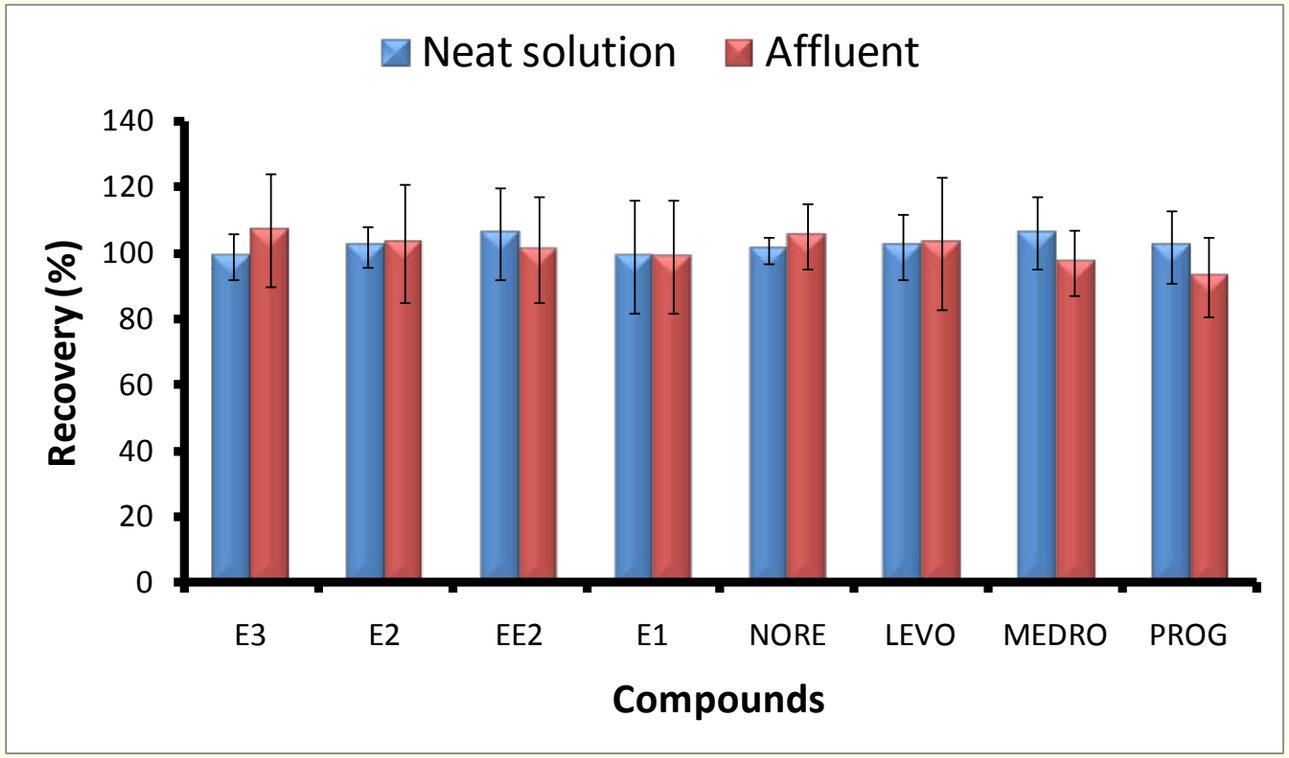
Method validation

- Recoveries were calculated using the calibration curves.

$$\text{Recovery (\%)} = \frac{C_f - C_i}{\Delta C} \times 100$$

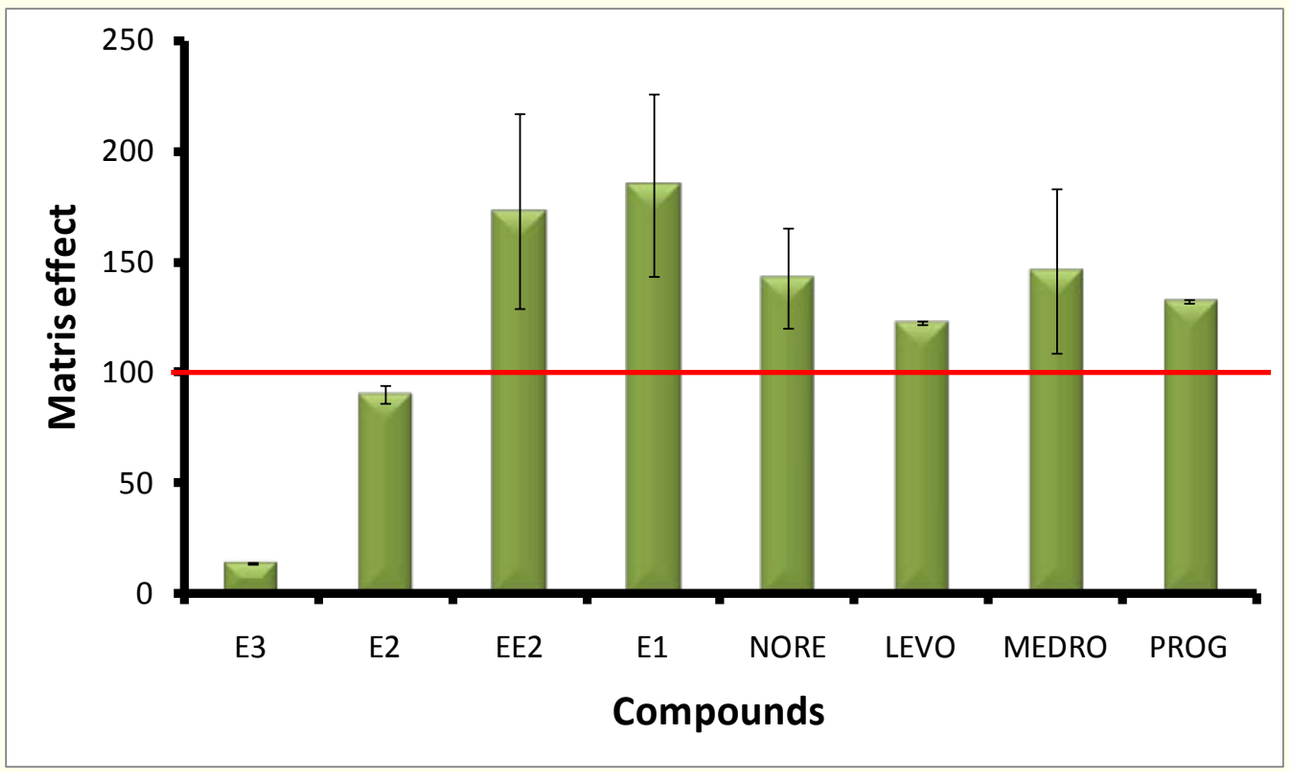
C_i = initial measured concentration of analyte (ng/L)
C_f = final measured concentration of analyte (ng/L)
ΔC = added concentration (ng/L)





- Matrix effect was calculated by dividing the slopes of the calibration curves in affluent solution (B) by those in neat solutions (A).

$$\text{ME (\%)} = \frac{B}{A} \times 100$$



Method validation

- Precision (inter-day, n=4) and accuracy (% Bias, n=4) were evaluated in neat solution as well as in affluent wastewater at two different levels: QC # 1 at 90 ng/L and QC #2 at 500 ng/L.

- Limits of detection (LODs) were evaluated using the calibration curves in both neat and affluent standard solutions (n=3, minimum of 6 calibration points) with the following equation.

Internal Calibration → LOD → $3.3 \times SD_{y\text{-intercept}}/slope$

Compound	RT min	LOD ng/L		QC #1 (90 ng/L) ng/L amount		QC #2 (500 ng/L) ng/L amount		Bias % QC #1		Bias % QC #2	
		neat	affluent	neat solution	affluent	neat solution	affluent	neat solution	affluent	neat solution	affluent
E3	8.70 (0.2)	27	82	87 (8)	56 (7)	525 (9)	440 (17)	8	37	5	12
E2	10.19 (0.2)	22	36	100 (7)	79 (20)	527 (7)	522 (2)	11	12	5	4
E1	10.26 (0.2)	38	46	76 (10)	91 (13)	520 (8)	415 (8)	16	1	3	17
EE2	10.23 (0.2)	21	39	91 (4)	83 (10)	515 (8)	448 (4)	1	7	3	10
NORE	10.31 (0.2)	12	76	100 (8)	63 (7)	536 (4)	440 (5)	11	30	7	12
LEVO	10.76 (0.1)	20	32	91 (8)	75 (10)	532 (6)	446 (2)	1	16	7	10
MEDRO	10.97 (0.1)	35	65	80 (8)	90 (4)	520 (11)	438 (6)	11	1	3	12
PROG	11.23 (0.1)	27	82	93 (7)	67 (2)	526 (12)	517 (3)	3	26	5	3

*numbers in parentheses represent RSD

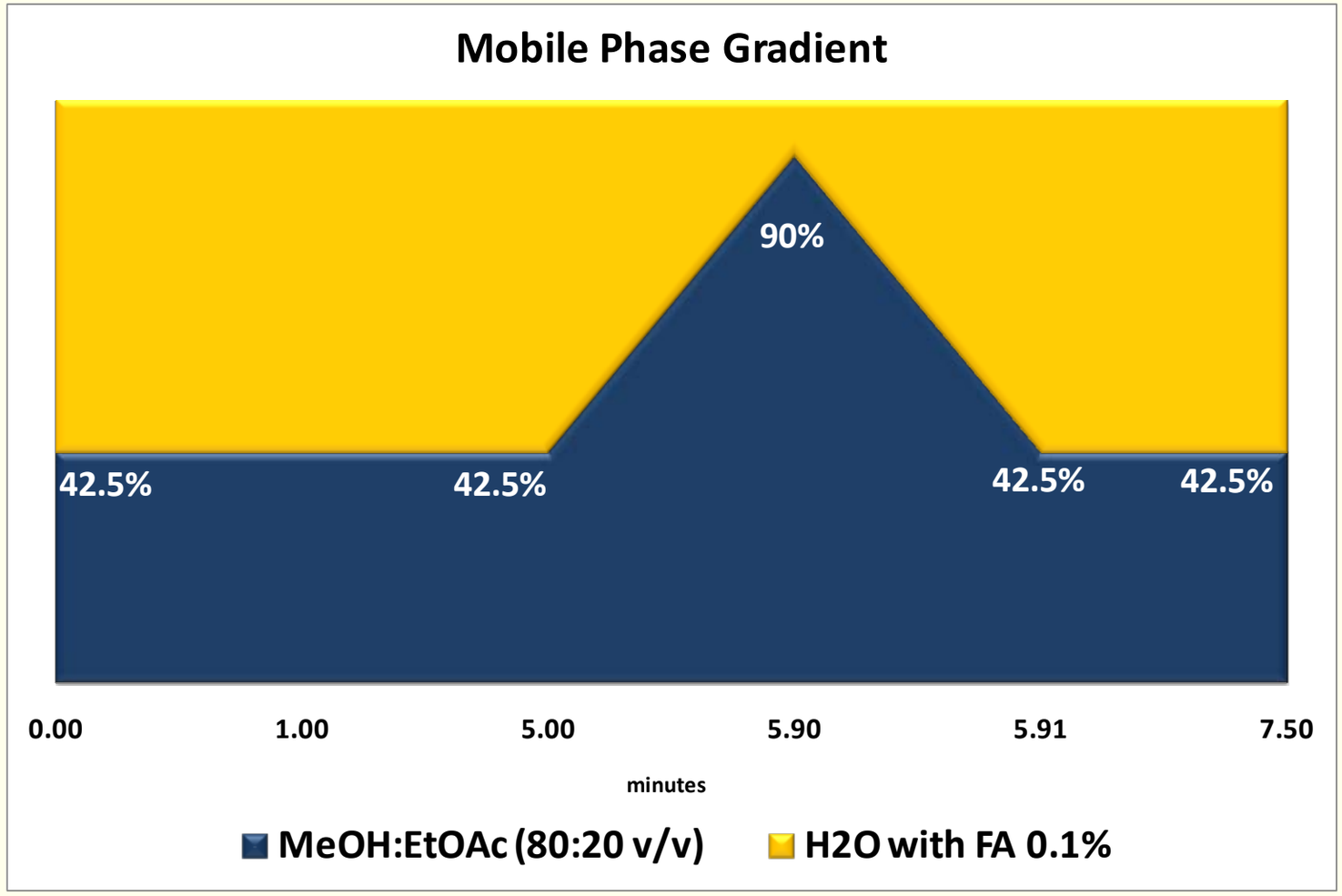
A step further: Chromatographic separation

Separation

- An alternative separation approach employed in order to achieve the chromatographic separation of the four co-eluting compounds of the eight selected steroid hormones with the use of ternary gradient mobile phase composition consisting of water, methanol (MeOH) and ethyl acetate (EA).

- The initial binary mixture of water and MeOH for all the different solvent composition conditions did not allow for peak differentiation.

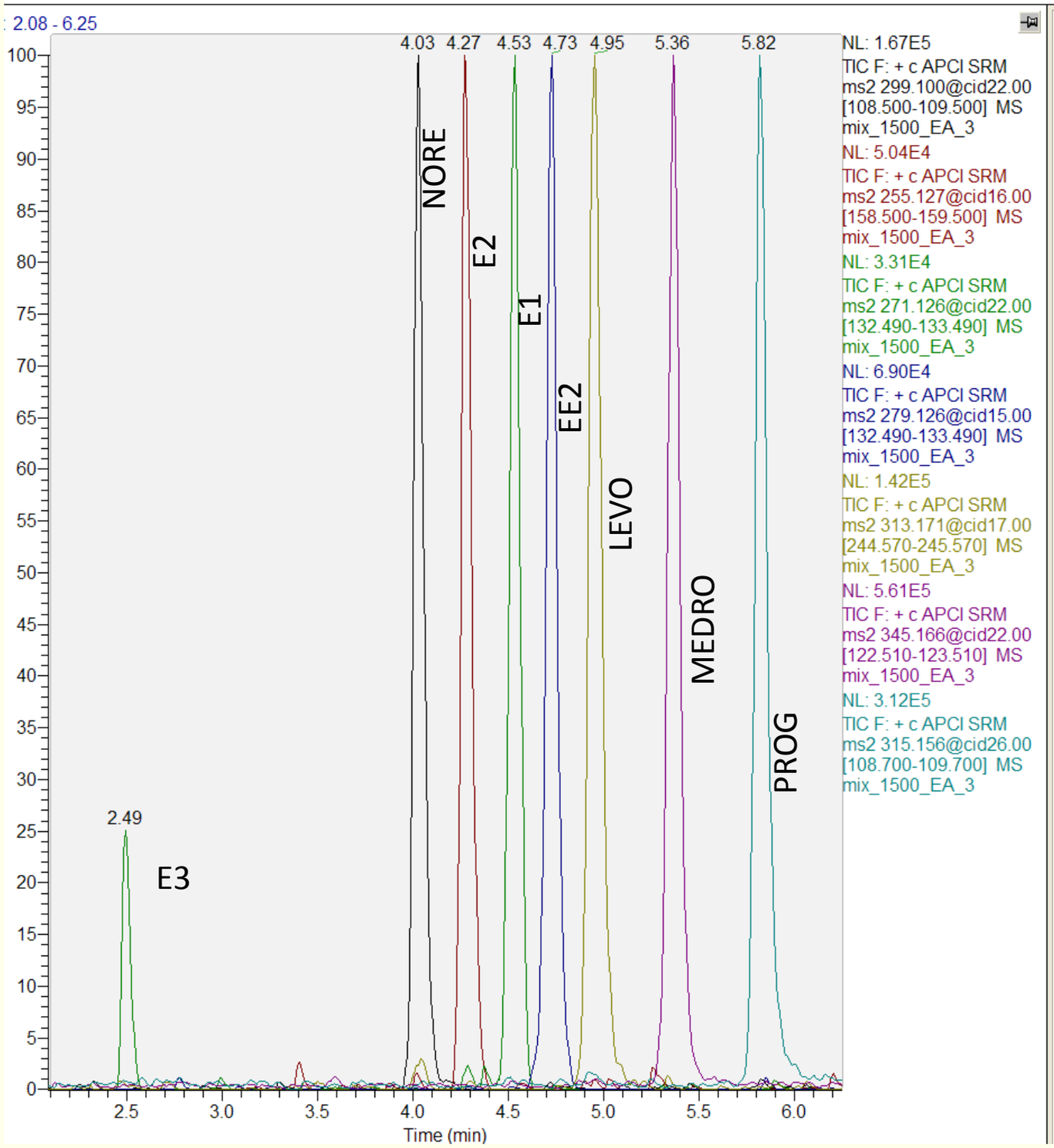
We tested our new gradient for 1 mL volume injections because of lengthy analysis time for our 5 mL validated injection volume method.



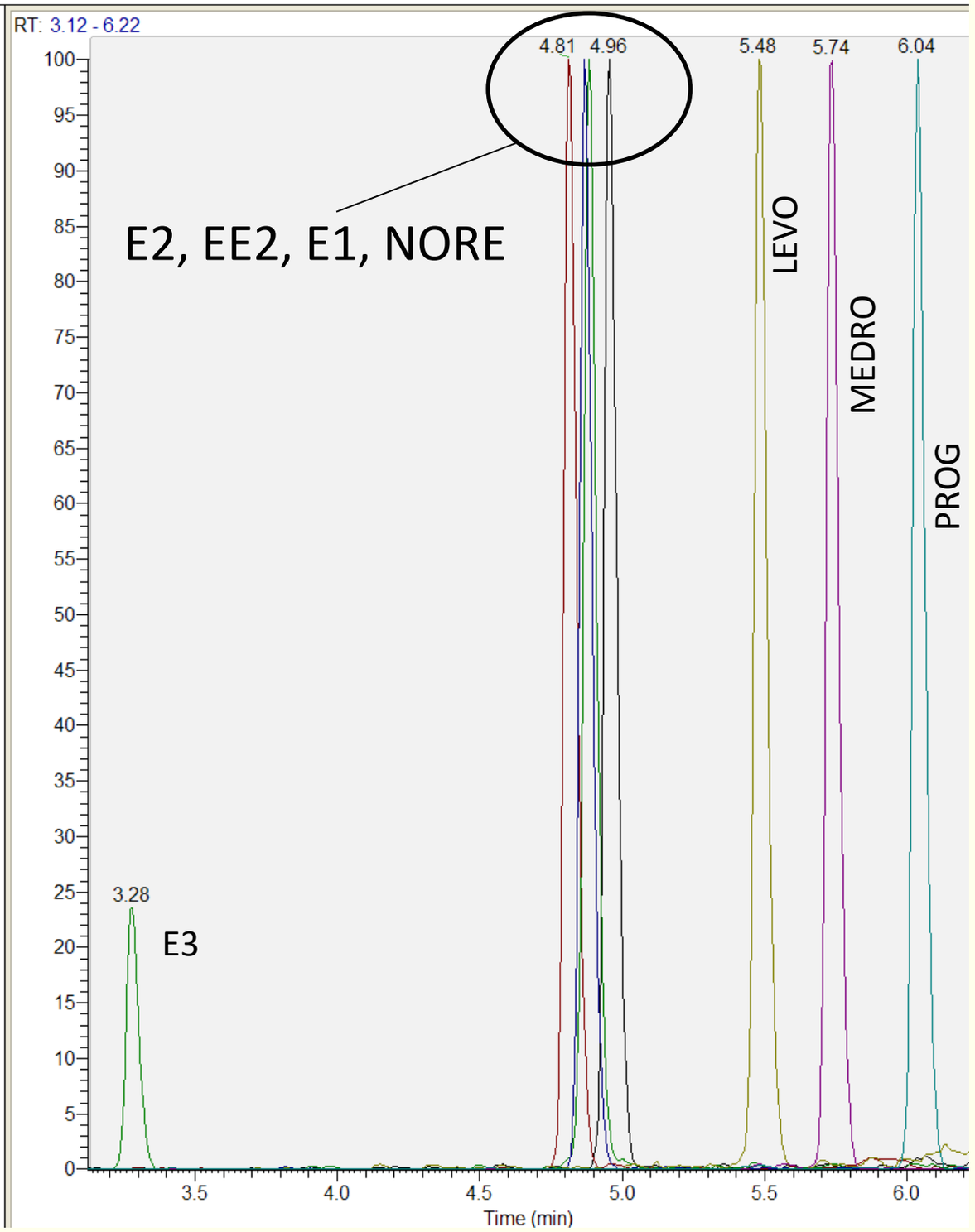
Separation

- Test done in matrix (effluent) at 1500 ng/L.

Ternary mobile phase (MeOH, EA and water)

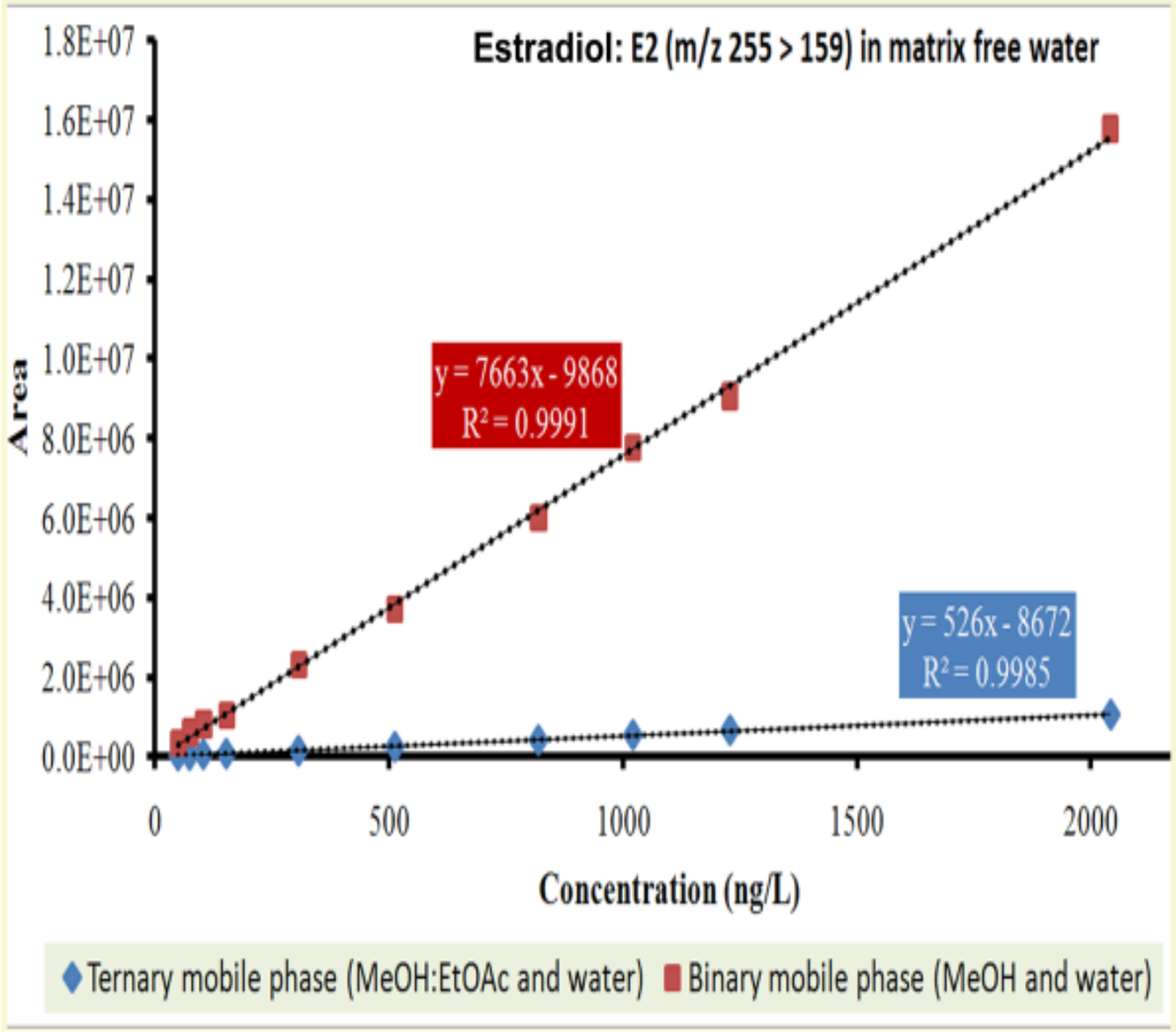


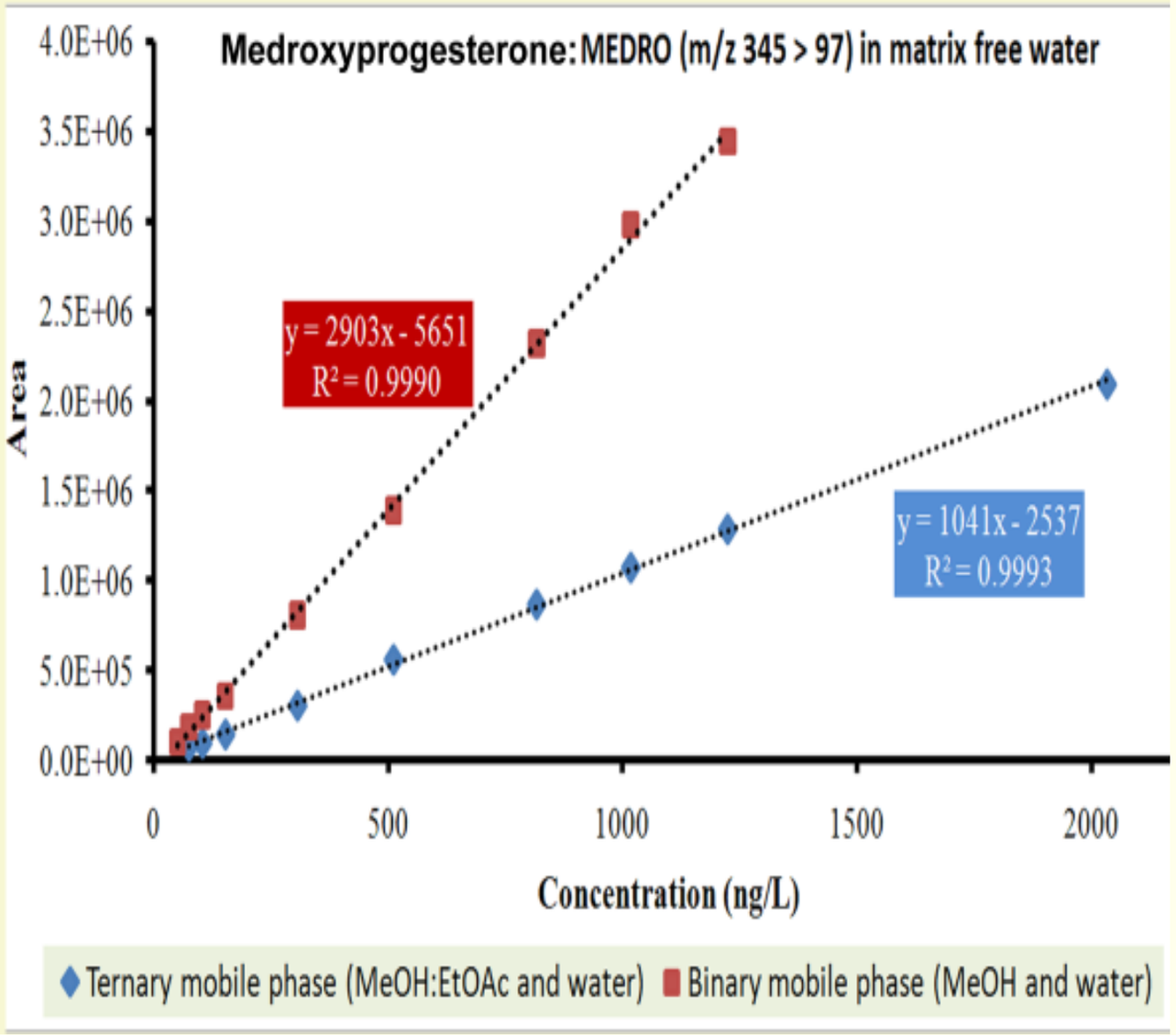
Binary mobile phase (MeOH and water)



Separation

- Although we achieved proper separation, signal intensities and method sensitivity are affected when using EA in the mobile phase because of ionization efficiency affected in APCI.





We still have some work to do !!!

Conlusion

- We developed, optimized and validated a rapid, sensitive and selective method for eight selected steroid hormones with LODs between 12 and 38 ng/L in neat solution and 32 and 82 ng/L in affluent. The method relies on on-line SPE-LC-APCI-MS/MS. These values are similar to off-line SPE methods that are time consuming and need very large sample volume.

- With these values we can analyze wastewater samples (affluent and effluent) when considering their levels in these matrices (between 50 and 250 ng/L). Our goal, ultimately, was to detect these compounds in surface water destined to be used in drinking water facilities. To achieve this we need to lower our LODs by a factor of at least 10.

- Future challenge: lowering the LODs
  - ✓ **use of different SPE column with higher affinity to inject higher volumes (Hypercarb or Phenyl type columns)**
  - ✓ **adopt a wash method into the method to improve our S/N**
  - ✓ **try using tandem SPE on-line method to reduce breakthrough at higher injection volumes**

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