

## Isomer-specific analysis and large-volume sampling of estrogens and their conjugates in water samples

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This work was done in collaboration with

- **Mehran Alaee, Steve Cagampan and associates at National Water Research Institute, Environment Canada**
- **Curtis Campbell, Masayuki Nishimura, Kerry Hill and Masatoshi Takahashi, Shimadzu Scientific Instruments, Inc.**
- **Becky Wittrig and Rick Lake, Restek Corporation**
- **Paul Lynch, Applied Biosystems**



## Introduction

- **At last year's ASMS (Indianapolis, IN, June 2007 ) National Water Research Institute and we jointly presented a poster titled "Determination of Natural and Synthetic Steroids and their Conjugates in Environmental Samples by LC/MS/MS" .**
- **We examined estrone (E1), estradiol (E2), estriol (E3), ethynyl-estradiol (EE), their sulfate (-S) and glucuronide (-G) conjugates present in samples collected from Ontario water treatment plants. These are known endocrine disruptors, and affect aquatic animals and potentially humans.**



## Introduction

- **We used Zorbax XDB-C8 (3  $\mu\text{m}$ , 100 x 3.5 mm) and several Zorbax HT 1.8-  $\mu\text{m}$  (100 x 2.1mm) columns that became available only a few years ago. We used an isocratic condition of 20% water (pH=9.0) + 80% acetonitrile. Although the method worked well for parent molecules, conjugated metabolites were not retained well. There was also no isomer separation of E2-3S and -17S, -3G and -17G.**
- **So, we set 2 goals for this study.**

## Goal 1 of this Study

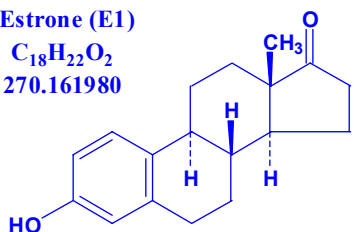
- Since we do not know much about the fate, distribution and biological/environmental effects of positional isomers of conjugated metabolites in the aquatic environment, we felt it important to separate and quantify each isomer so that a detailed toxicological study could be conducted. **Isomer separation of E2-3S, -17S, E2-3G and E2-17G was our first objective.** These isomers are commercially available and act as model compounds for other potential positional isomers (such as estriol conjugates that can have 3 positional isomers each).

## Goal 2 of this Study

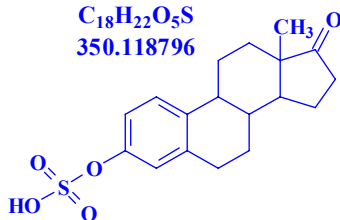
- We felt we could use better detection limits for steroids.
- We have examined several ionization techniques including Heated Nebulizer™, chemical ionization, Photospray® and electron capture after derivatization in addition to our standard TurbolonSpray® technology. We managed a gain of 5 – 10 in response, but some of the methods suffered from interference or other operational issues.
- **The second objective was to see if large-volume sampling (1 L) coupled with Poros® perfusion technology would decrease the current length of time needed to conduct sample preparation by on-line concentration of water samples, since we are not sample-limited (unlike clinical application where volume of blood is limited).**

# Structures of compounds examined in this study

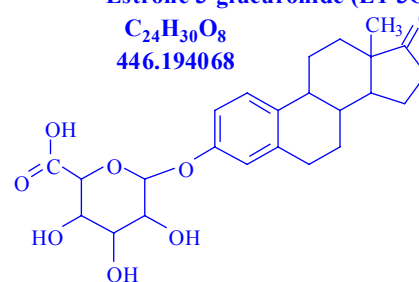
**Estrone (E1)**  
 $C_{18}H_{22}O_2$   
 270.161980



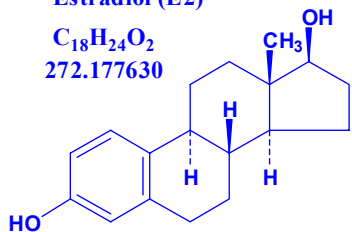
**Estrone 3-sulfate (E1-3S)**  
 $C_{18}H_{22}O_5S$   
 350.118796



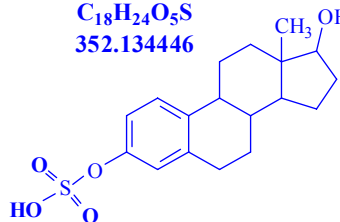
**Estrone 3-glucuronide (E1-3G)**  
 $C_{24}H_{30}O_8$   
 446.194068



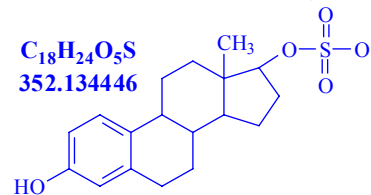
**Estradiol (E2)**  
 $C_{18}H_{24}O_2$   
 272.177630



**Estradiol 3-sulfate (E2-3S)**  
 $C_{18}H_{24}O_5S$   
 352.134446

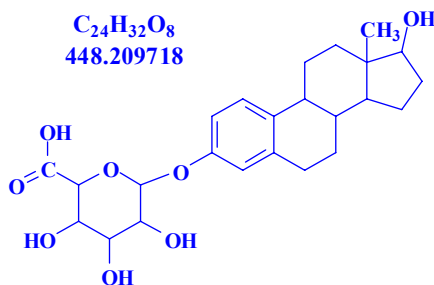


**Estradiol 17-sulfate (E2-17S)**



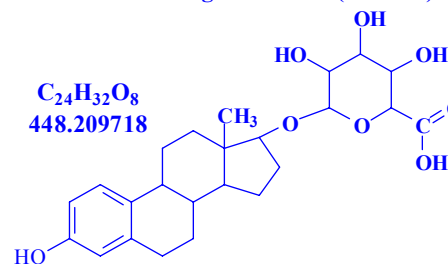
**Estradiol 3-glucuronide (E2-3G)**

$C_{24}H_{32}O_8$   
 448.209718



**Estradiol 17-glucuronide (E2-17G)**

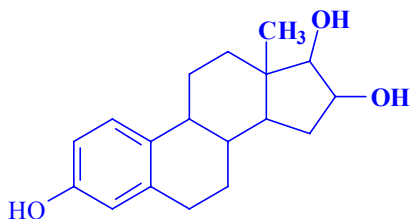
$C_{24}H_{32}O_8$   
 448.209718



# Structures of compounds examined in this study

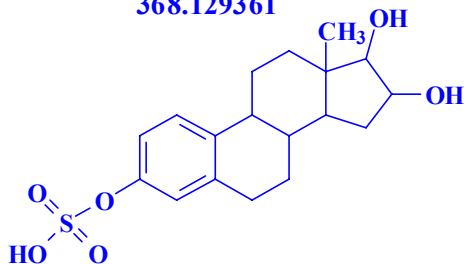
**Estriol (E3)**

$C_{18}H_{24}O_3$   
 288.172545



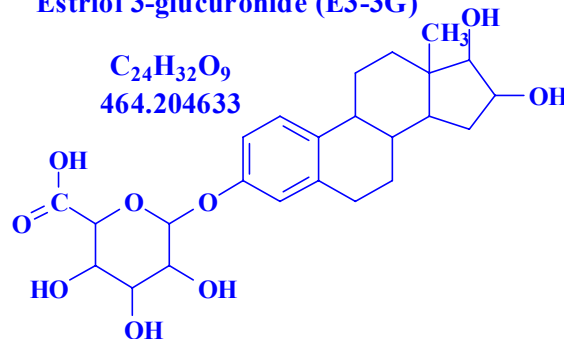
**Estriol 3-sulfate (E3-3S)**

$C_{18}H_{24}O_6S$   
 368.129361



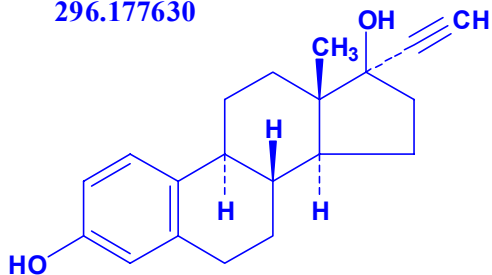
**Estriol 3-glucuronide (E3-3G)**

$C_{24}H_{32}O_9$   
 464.204633



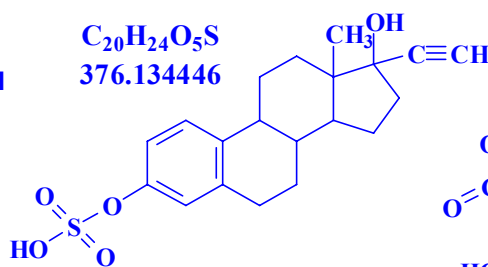
**Ethinylestradiol (EE)**

$C_{20}H_{24}O_2$   
 296.177630



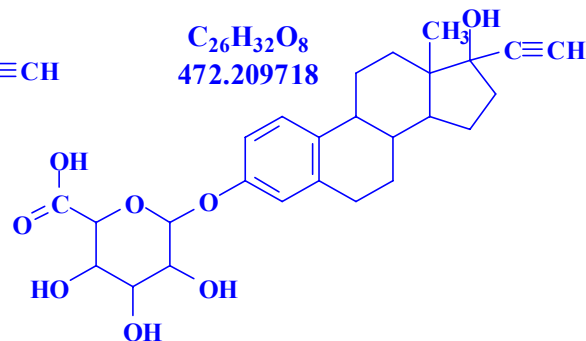
**Ethinylestradiol 3-sulfate (EE-3S)**

$C_{20}H_{24}O_5S$   
 376.134446



**Ethinylestradiol 3-glucuronide (EE-3G)**

$C_{26}H_{32}O_8$   
 472.209718



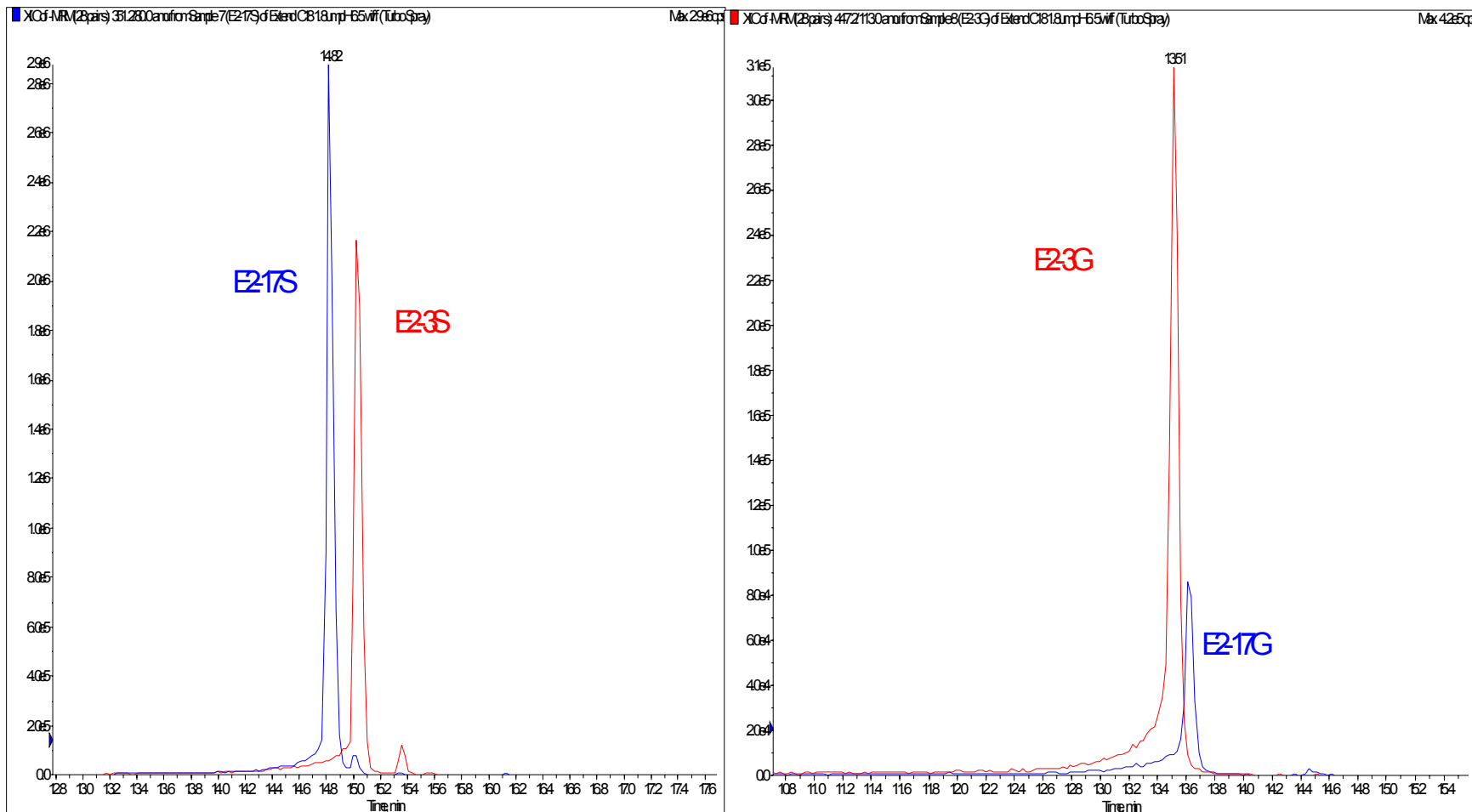
## Analytical Method for column selection

- Shimadzu Prominence system consisting of 4 x LC-20AD, 1 x CBM-20A, 1 x SIL-20AC, 1 x CTO-20AC with 2 FCV-12AH valves, 1 x DGU-20A5.
- Mobile phase A = 95% water + 5% acetonitrile + 2 mM ammonium acetate, B = 5% water + 95% acetonitrile + 2 mM ammonium acetate, eventual flow rate = 0.3 mL/min for 2.0 – 2.1 mm i. d. columns. For evaluation, a linear gradient of 0 – 100%B/30 min was used.
- MS : API 5000<sup>TM</sup> LC/MS/MS system (most sensitive MRM machine) under Analyst<sup>®</sup> 1.4.2 control

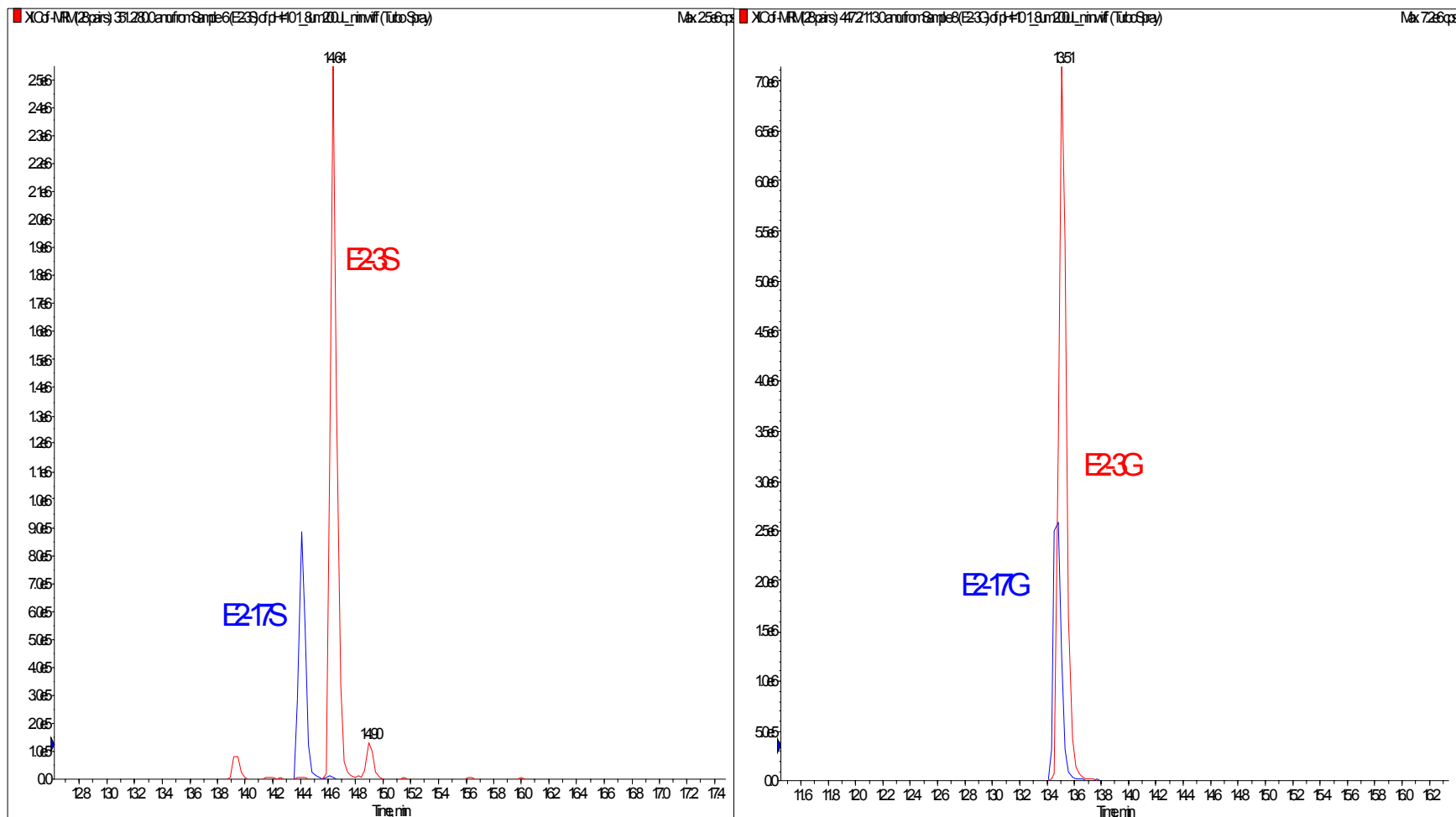
## List of LC columns tested

Name	ID(mm)	Length(mm)	Particle size (mm)
Zorbax HT Extend C18*	2.1	100	1.8
Zorbax HT Eclipse XDB C18	2.1	50	1.8
Inertsil ODS-3	3	250	3
Luna PFP(2)	2	150	3
Gemini C18*	2	150	3
Synergi Polar RP	2	150	4
Pinnacle DB Biphenyl	2.1	50	1.9
Pinnacle DB PFP Propyl	2.1	50	1.9
Pinnacle DB cyano	2.1	50	1.9
Pinnacle DB C18	2.1	50	1.9
Pinnacle DB AqC18	2.1	50	1.9
Allure AK	2.1	150	5
<b>*pH range 2 - 10</b>			

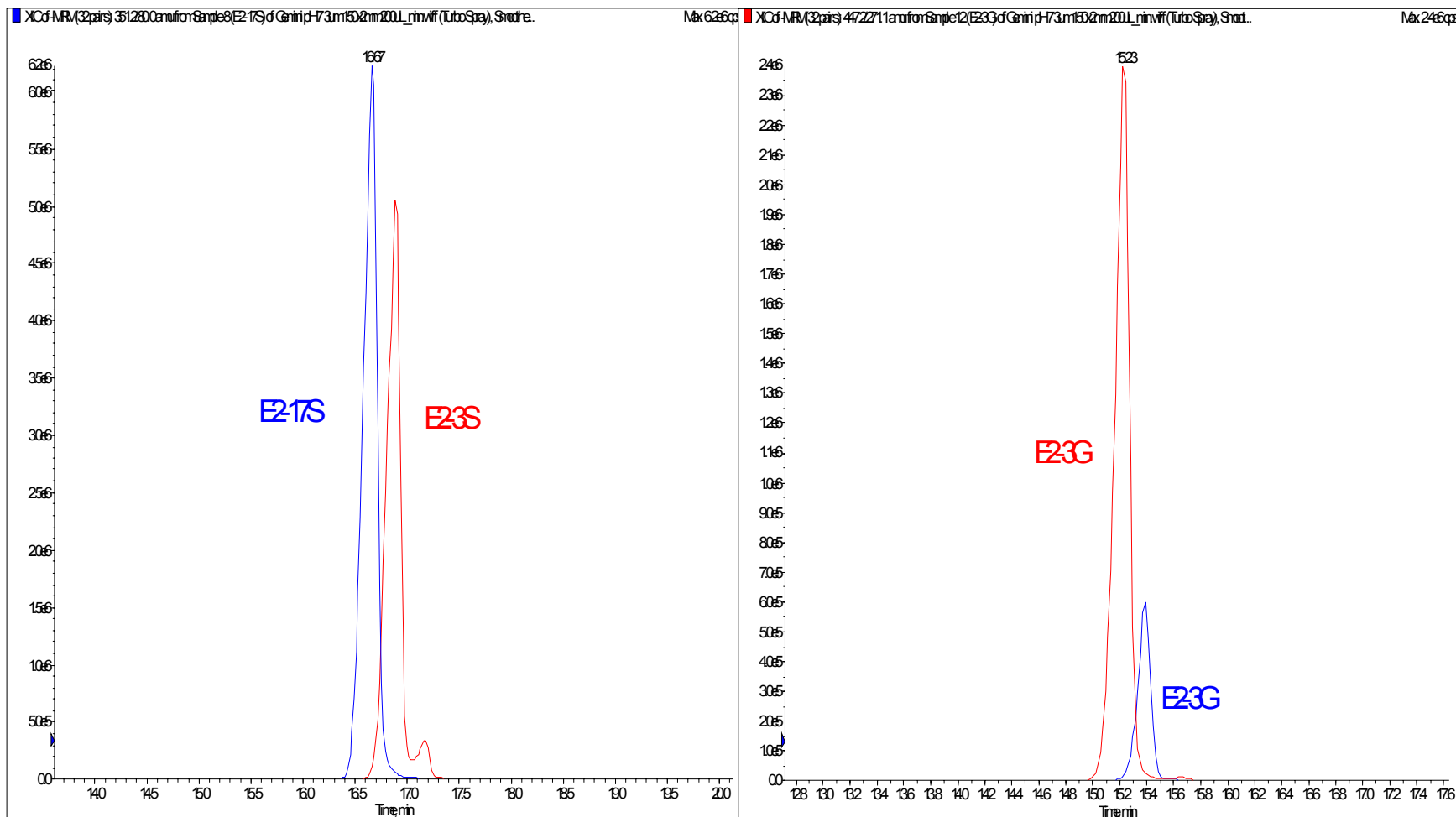
# 1.8- $\mu$ m 2.1 x 100mm Agilent Extend C18 0.2 mL/min pH=6.5 partial separation of E2-3G and -17G, leading edges



# 1.8- $\mu\text{m}$ 2.1 x 100 mm Agilent Extend C18 0.2 mL/min pH=10 a higher pH did not help.

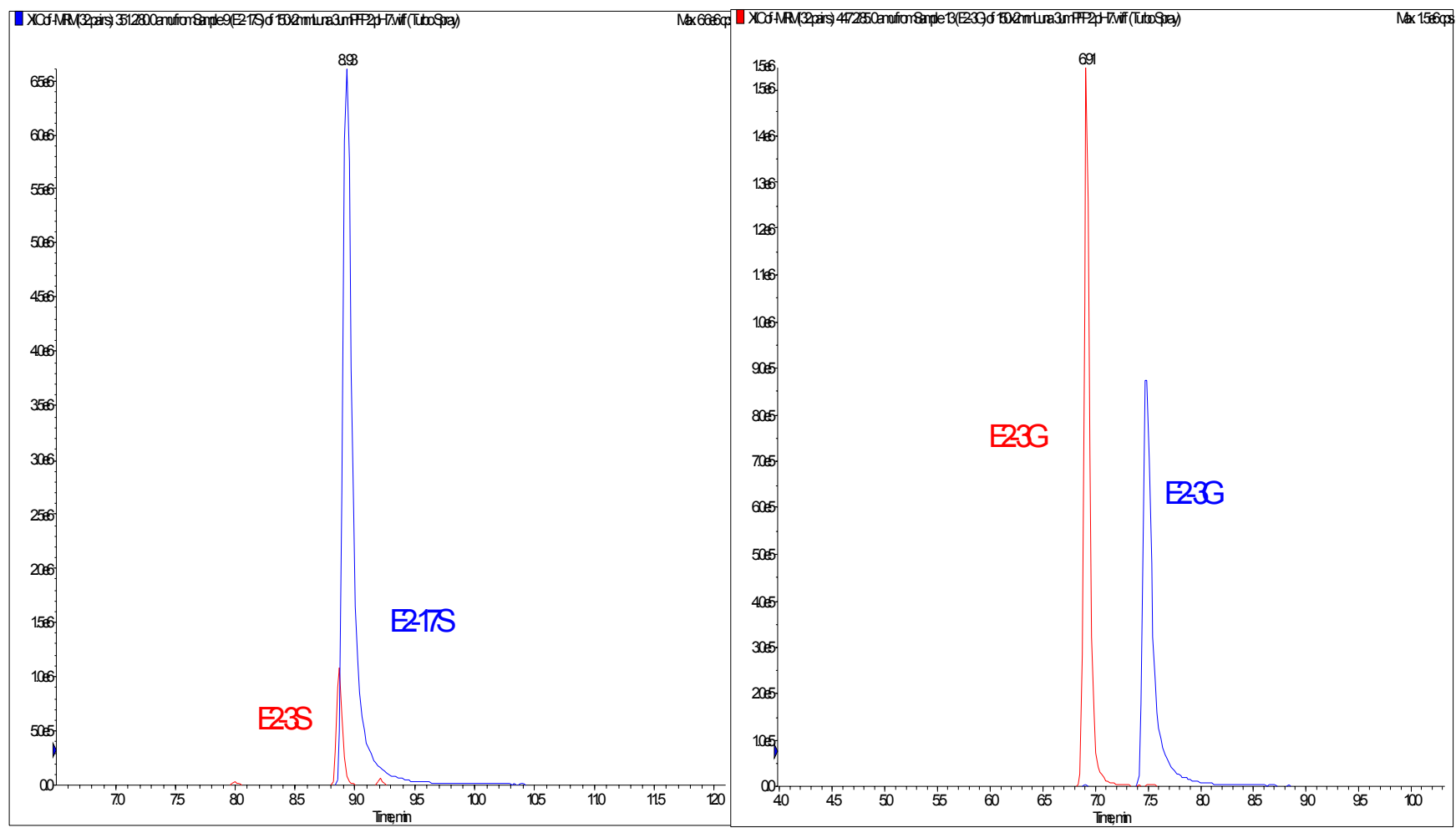


## 2.1 x 150 mm Gemini-C18 0.2 mL/min pH=6.5 – 7.0 partial separation

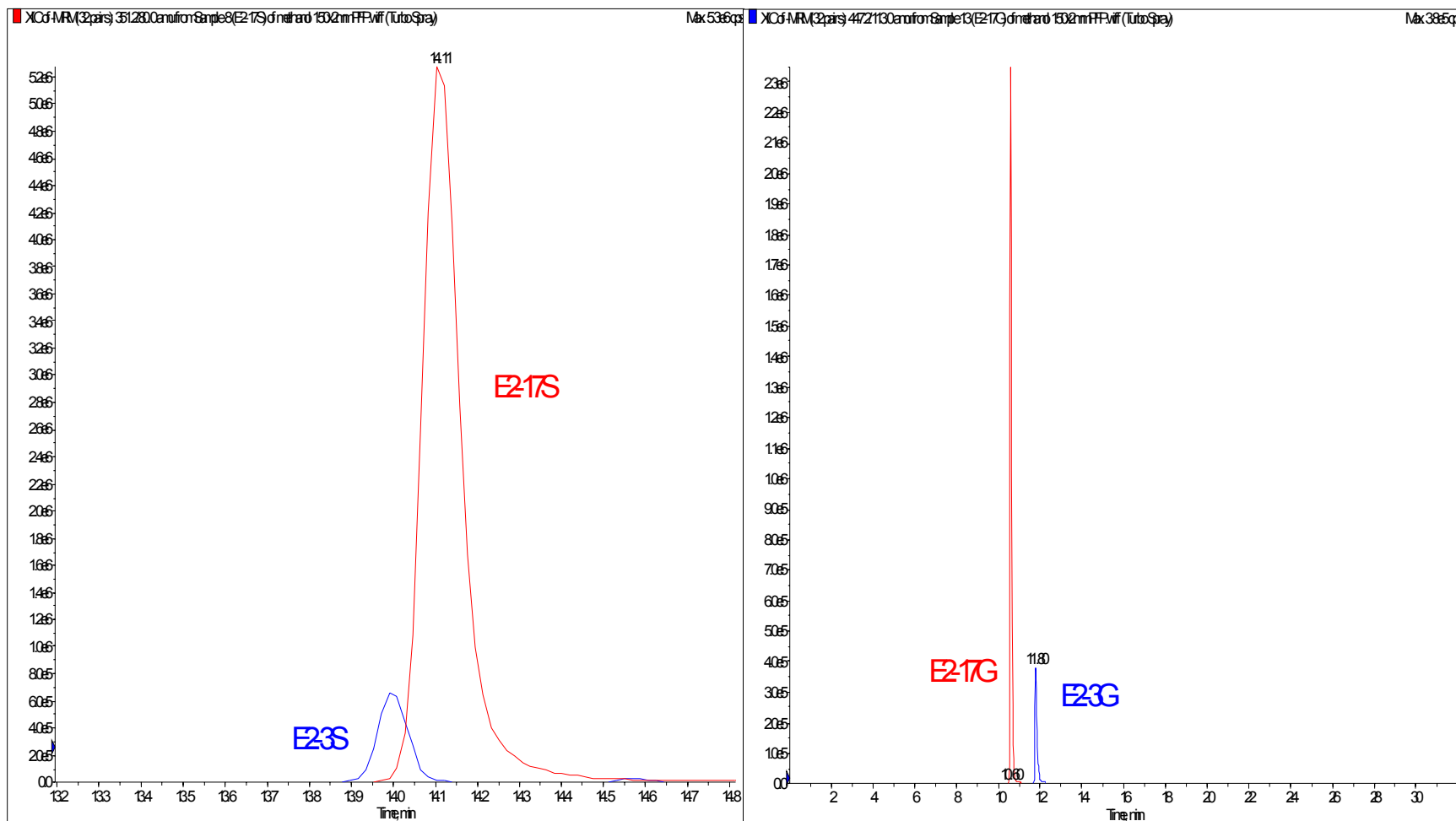




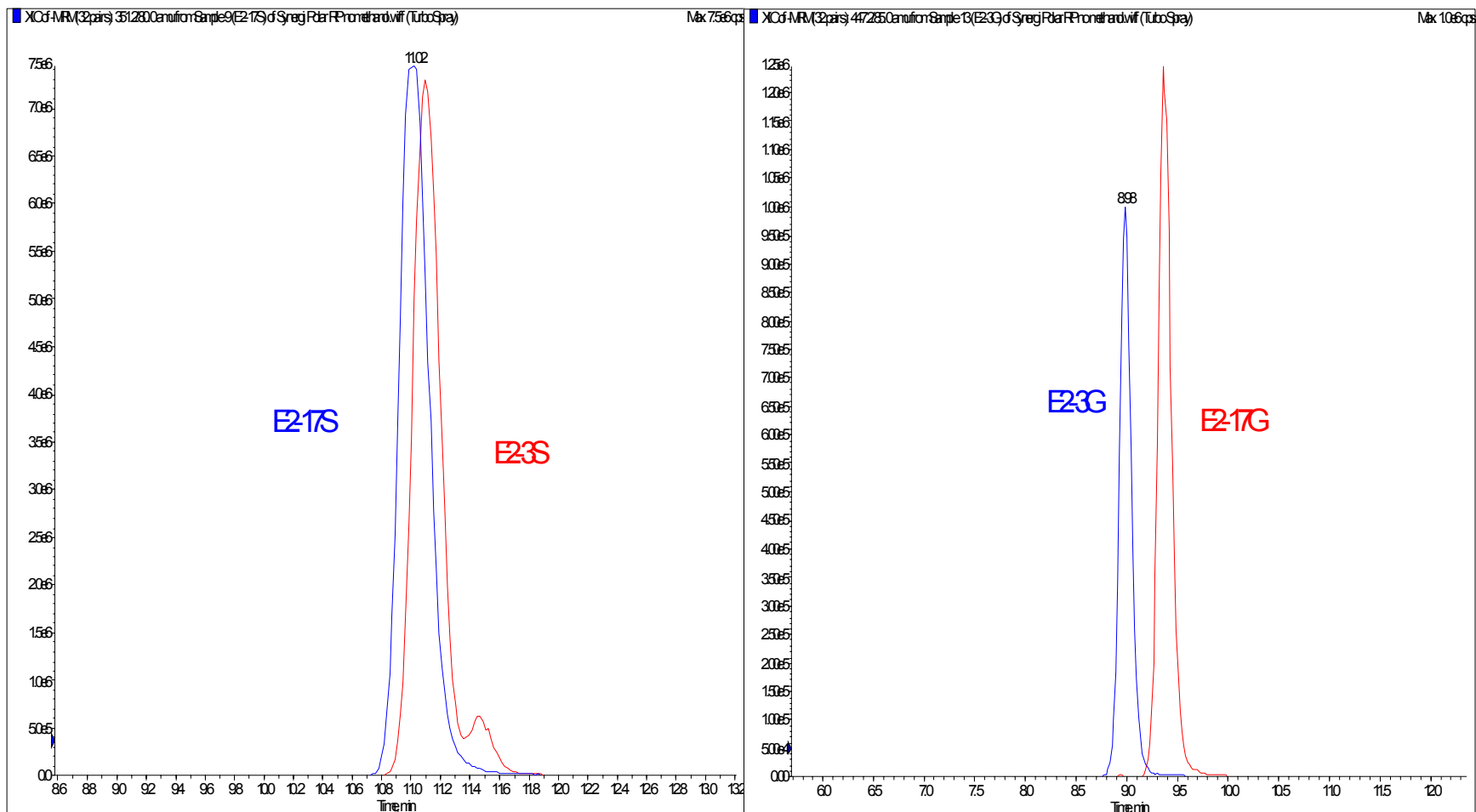
# 150 x 2 mm 3- $\mu$ m Luna PFP column pH=6.5, separated glucuronides, but not sulfates



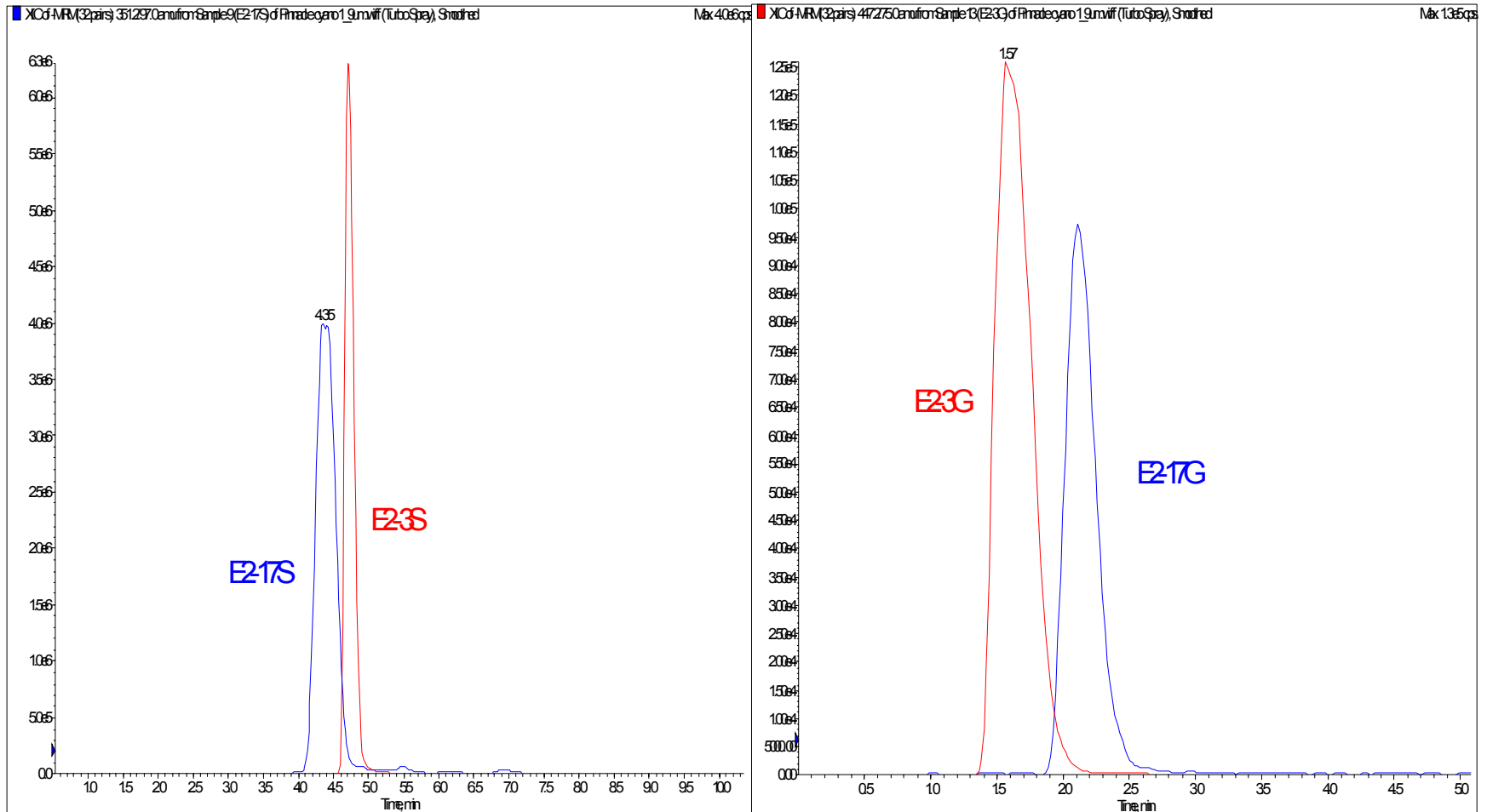
# 150 x 2 mm Luna PFP(2) 3- $\mu$ m with 30% methanol in mobile phase B improved separation of sulfates slightly



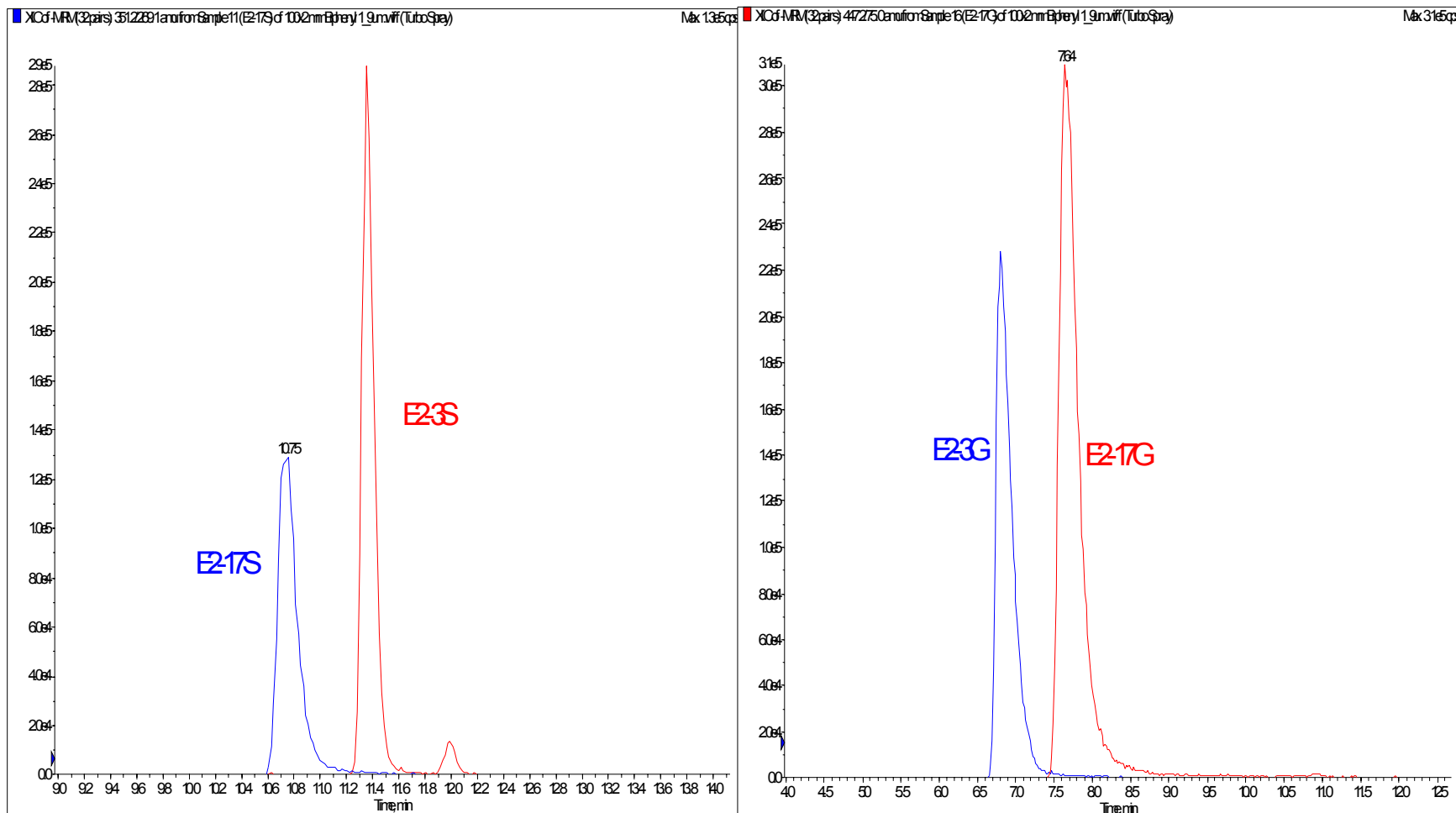
# Synergi Polar RP 4- $\mu$ m 150 x 2 mm, separated glucuronides but not sulfates



## 50 x 2 mm 1.9- $\mu$ m Pinnacle DB Cyano: E1 and EE tailed

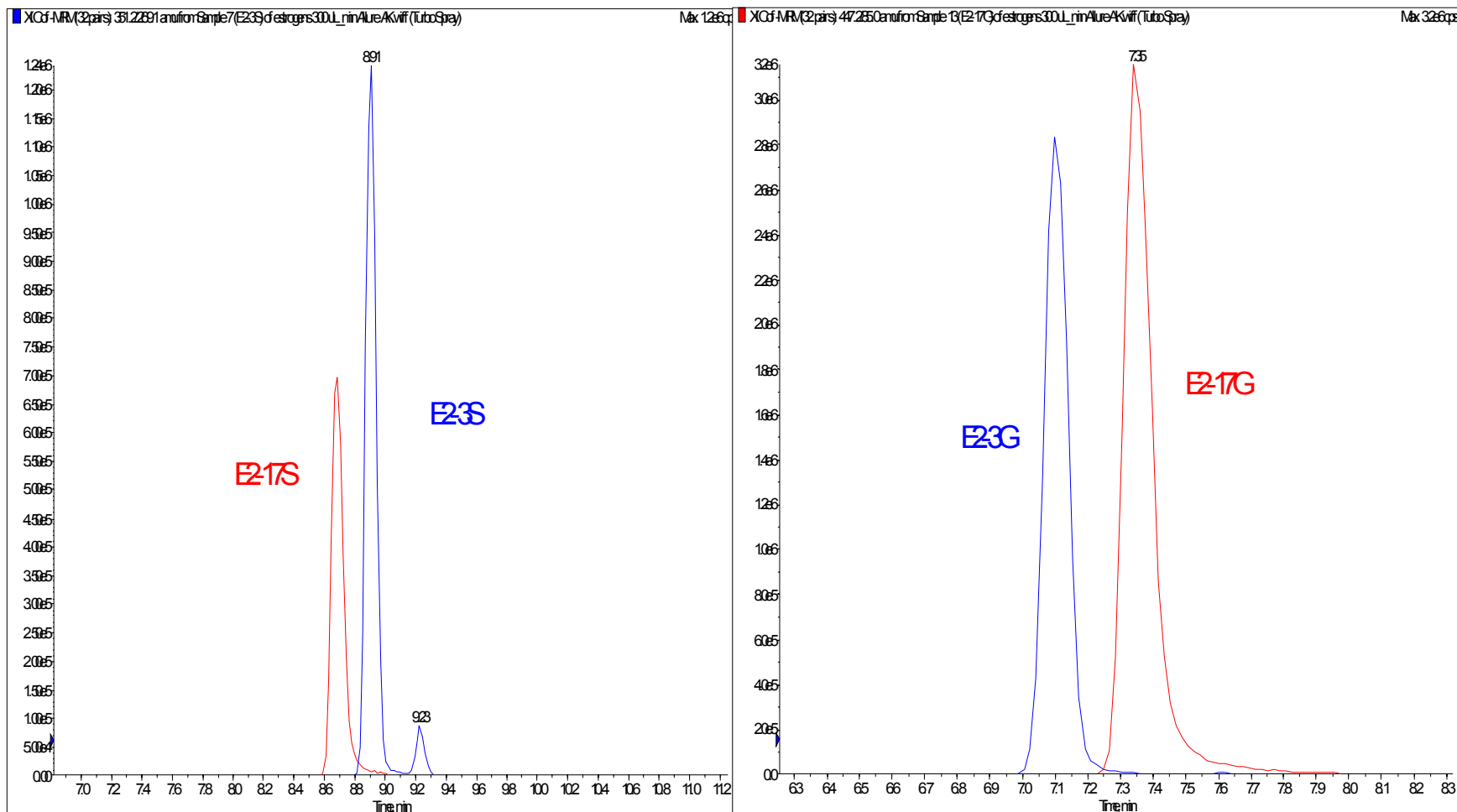


## 100 x 2.1 mm 1.9- $\mu$ m Pinnacle DB Biphenyl separated E2-3S, -17S, -3G and -17G, but E3-3S and E3-3G eluted too soon





## Allure AK 150 x 2.1 mm, 5- $\mu$ m, separates E2-S and E2-G's





## MRM table with retention time for estrogen analysis

Abbreviation	Chemical	Q1 (m/z)	Q3 (m/z)	Dwell (msec)	DP (V)	CE (V)	CXP (V)	Allure AK ret. time
E1	Estrone	269.2	143.1	50	-148	-53.0	-14.5	19.26
		269.2	145.1	50	-148	-53.0	-14.5	
E2	Estradiol	271.2	145.1	50	-159	-54.0	-9.9	17.36
		271.2	183.1	50	-159	-53.0	-12.4	
E3	Estriol	287.2	145.0	50	-163	-58.0	-10.0	12.27
		287.2	255.1	50	-163	-54.0	-16.0	
EE	Ethinylestradiol	295.2	147.1	50	-140	-58.0	-14.8	18.40
		295.2	159.1	50	-140	-47.0	-15.5	
E1-3S	Estrone 3-sulfate	349.2	80.0	50	-100	-42.0	-9.8	10.31
		349.2	269.2	50	-100	-44.0	-15.2	
E2-3S	Estradiol 3 or 17-sulfate	351.2	80.0	50	-150	-106.0	-12.0	9.34
		351.2	97.0	50	-150	-50.0	-8.0	
E2-17S	Estradiol 3-sulfate	351.2	269.1	50	-150	-60.0	-10.0	9.08
		351.2	271.1	50	-150	-49.0	-9.5	
d4-E1-3S	d4-Estrone 3-sulfate	353.2	80.0	50	-100	-42.0	-9.8	
		353.2	273.2	50	-100	-44.0	-15.2	
d4-E2-3S	d4-Estradiol 3-sulfate	355.2	80.0	50	-150	-106.0	-12.0	
		355.2	275.1	50	-150	-49.0	-9.5	
E3-3S	Estriol 3-sulfate	367.1	80.0	50	-106	-45.0	-9.9	6.34
		367.1	287.1	50	-106	-50.0	-10.2	
EE-3S	Ethinylestradiol 3-sulfate	375.2	80.0	50	-105	-45.0	-9.9	10.01
		375.2	295.2	50	-105	-46.5	-9.9	
E1-3G:	Estrone 3-glucuronide	445.2	113.0	50	-115	-30.0	-14.0	8.22
		445.2	269.1	50	-115	-52.5	-9.2	
E2-3G	Estradiol 3-glucuronide	447.2	113.0	50	-115	-31.0	-14.0	7.38
		447.2	271.1	50	-115	-57.0	-9.6	
E2-17G	Estradiol-17-glucuronide	447.2	75.0	50	-115	-36.0	-15.1	7.67
		447.2	85.0	50	-115	-43.0	-5.8	
E3-3G	Estriol 3-glucuronide	463.2	113.0	50	-150	-31.0	-14.0	4.85
		463.2	287.1	50	-150	-30.0	-14.6	
EE-3G	Ethinylestradiol 3-glucuronide	471.2	113.0	50	-115	-31.0	-14.0	7.94
		471.2	295.1	50	-115	-52.0	-10.0	

2 ion pairs were used for each species, one for quantification, and the second one for confirmation by comparing ion intensities. If an intensity ratio does not fall into a pre-set value, it means we have a matrix interference problem. The following time program was used for direct injection at 0.3 mL/min:

Time (min)	% B
0.1	0
20.0	66
22.0	100
23.0	100
23.1	0
27	stop



## Detection limits: 2007 ASMS vs this study (ng/ $\mu$ L)

Compound	API2000™system	API5000™ system	API5000™system
E1	4.75	0.08	0.001
E2	20.38	0.16	0.001
EE	17.50	0.14	0.001
E1-3S	0.78	0.01	0.0001
E2-3S	1.38	0.02	0.0001
E2-17S	0.66	0.03	0.0001
EE-3S	0.62	0.10	0.0001
E1-3G	29.57	0.47	0.0001
E2-3G	4.10	0.07	0.0001
E2-17G	20.16	0.32	Not tested
EE-3G	29.22	0.47	Not tested

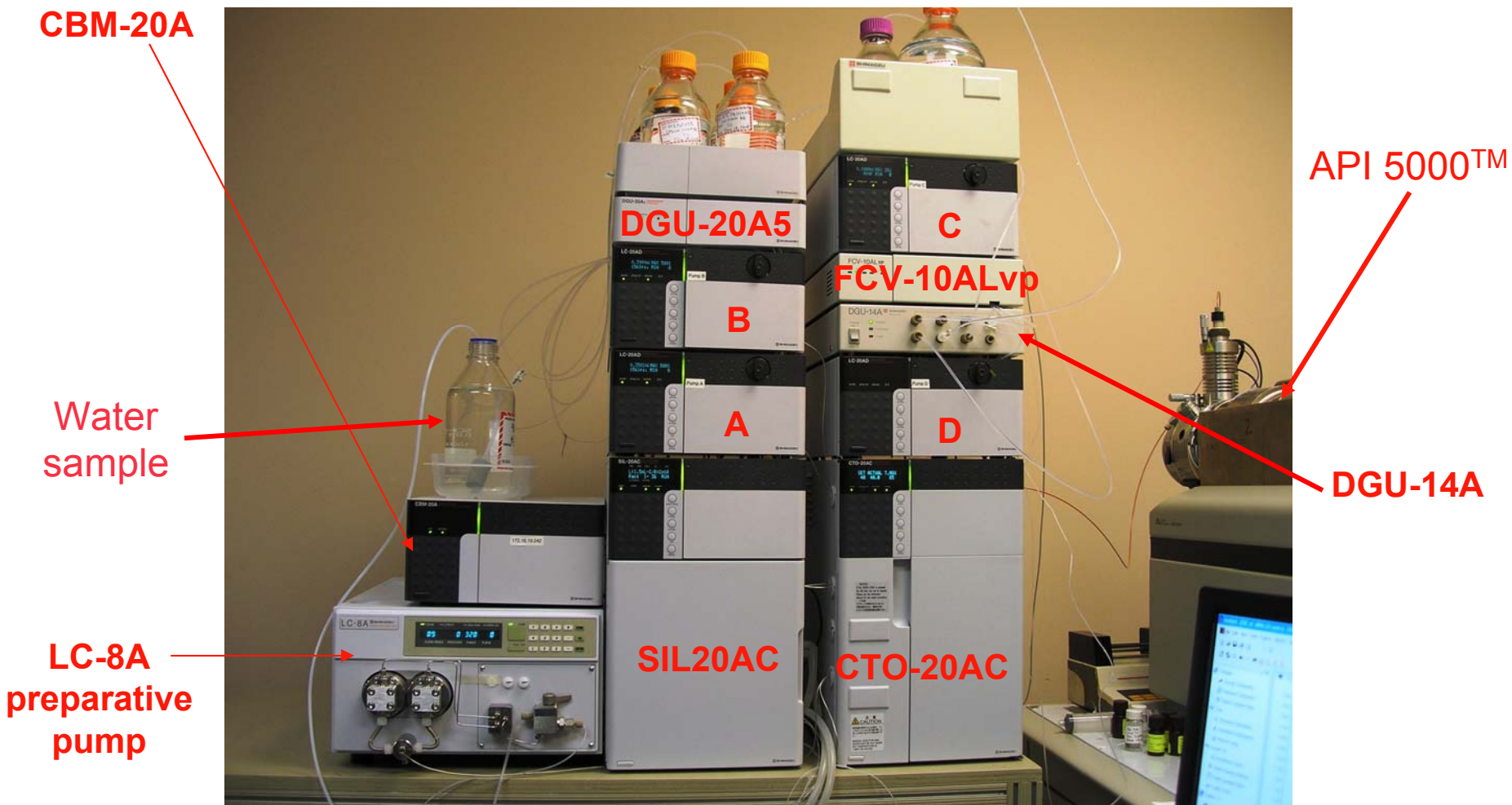
Improvement due to better chromatography and 100- $\mu$ L injection

## Large-volume sampling by Poros<sup>®</sup> perfusion column

- Pump D (LC-20AD) was replaced with an LC-8A preparative pump rated at 150 mL/min.
- We have been using several Poros<sup>®</sup> (2.1 x 100 mm, 4.6 x 100 mm) and Shimpack MAYI columns for concentrating medicinal chemicals and metabolites in whole blood, serum, plasma, bile, inner tissue fluids, etc.
- We decided to try a large trap (16 mm i.d. x 100 mm long) that is rated up to 150 mL/min of loading flow rate.
- A larger Allure AK 150 x 3.2 mm, 5- $\mu$ m, was specially packed and used at 1 mL/min to elute from the 16 mm x 100 mm Poros<sup>®</sup> trap.

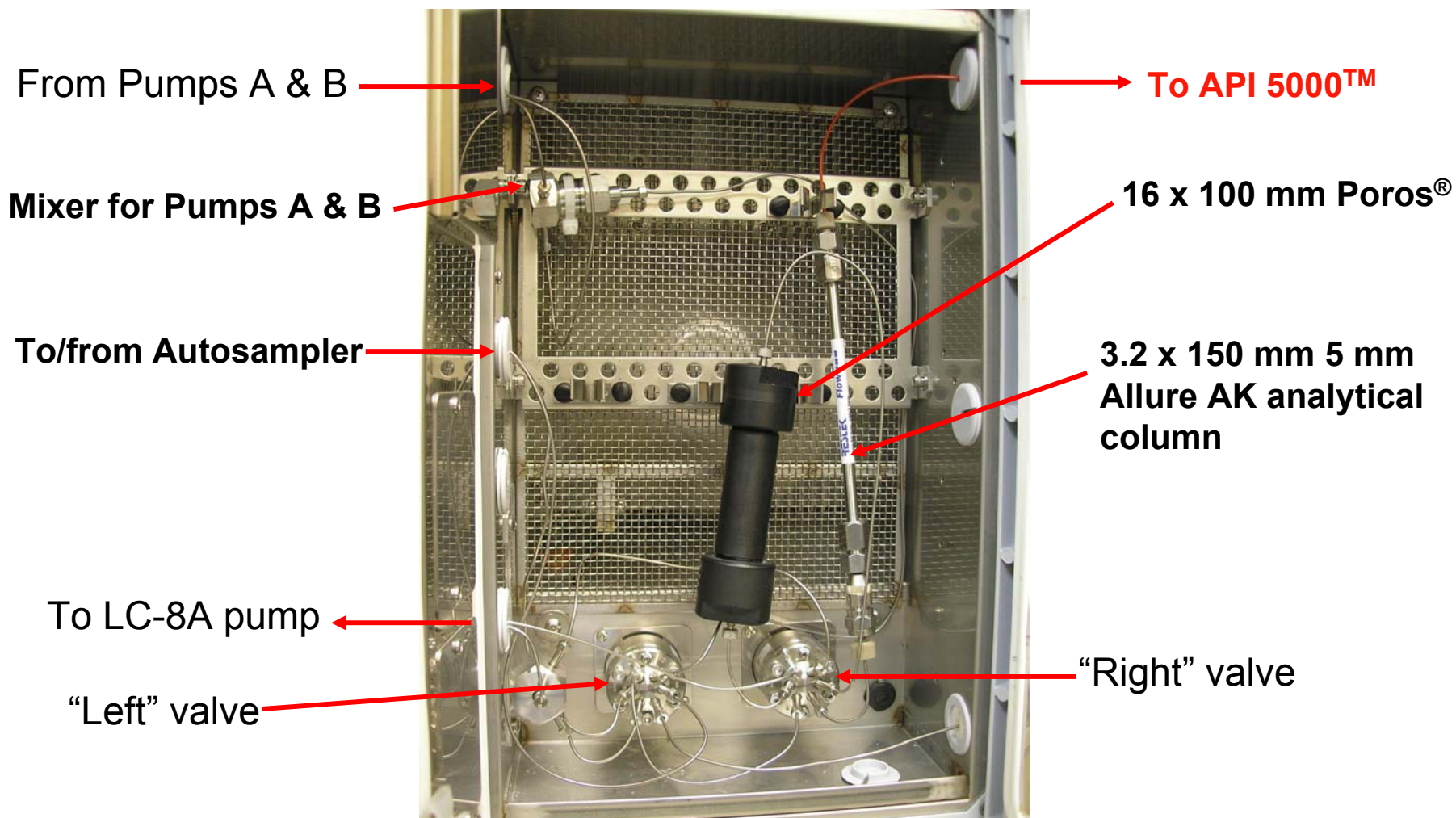


# Experimental setup for large-volume sampling



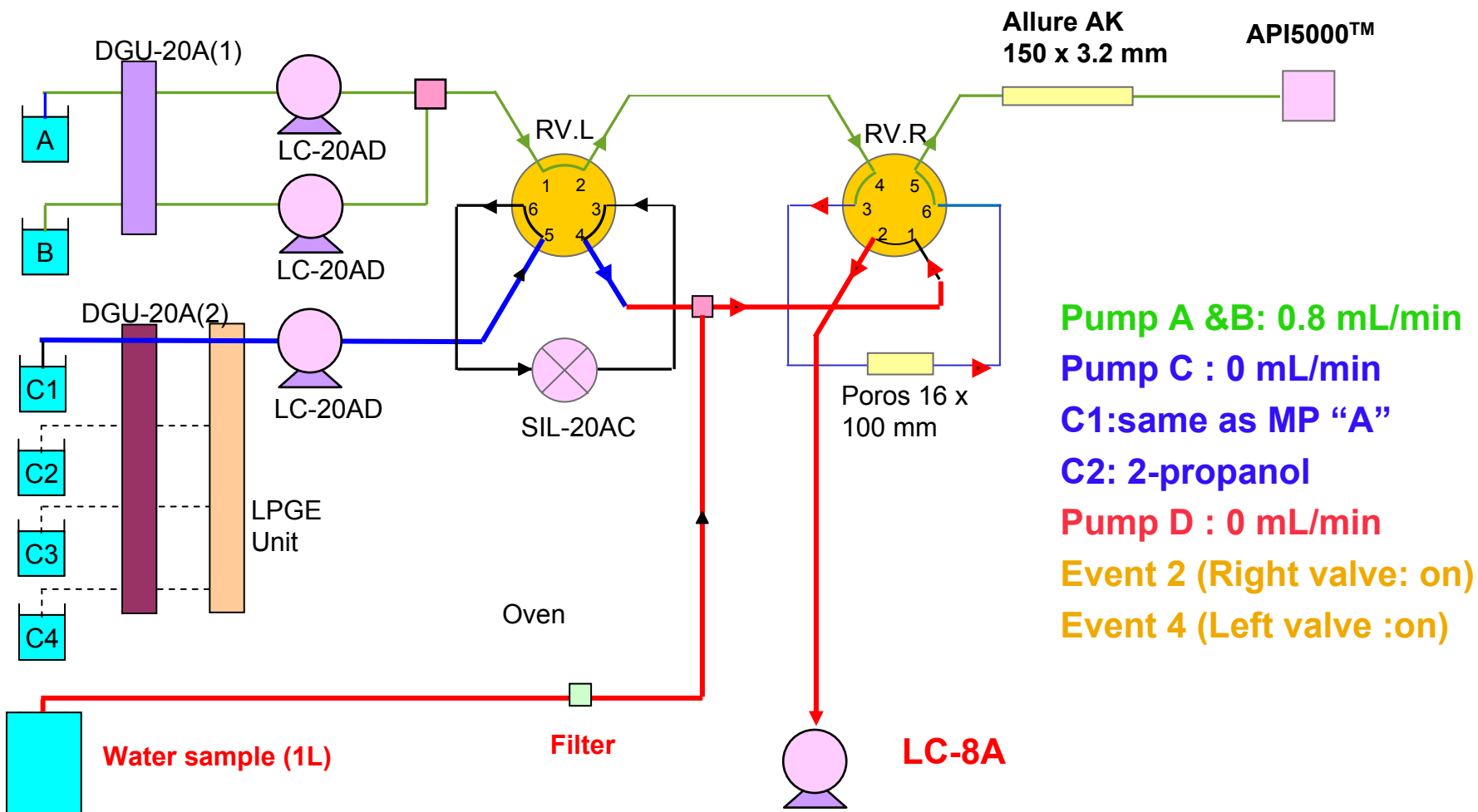


## Layout inside Column Oven (CTO-20AC)



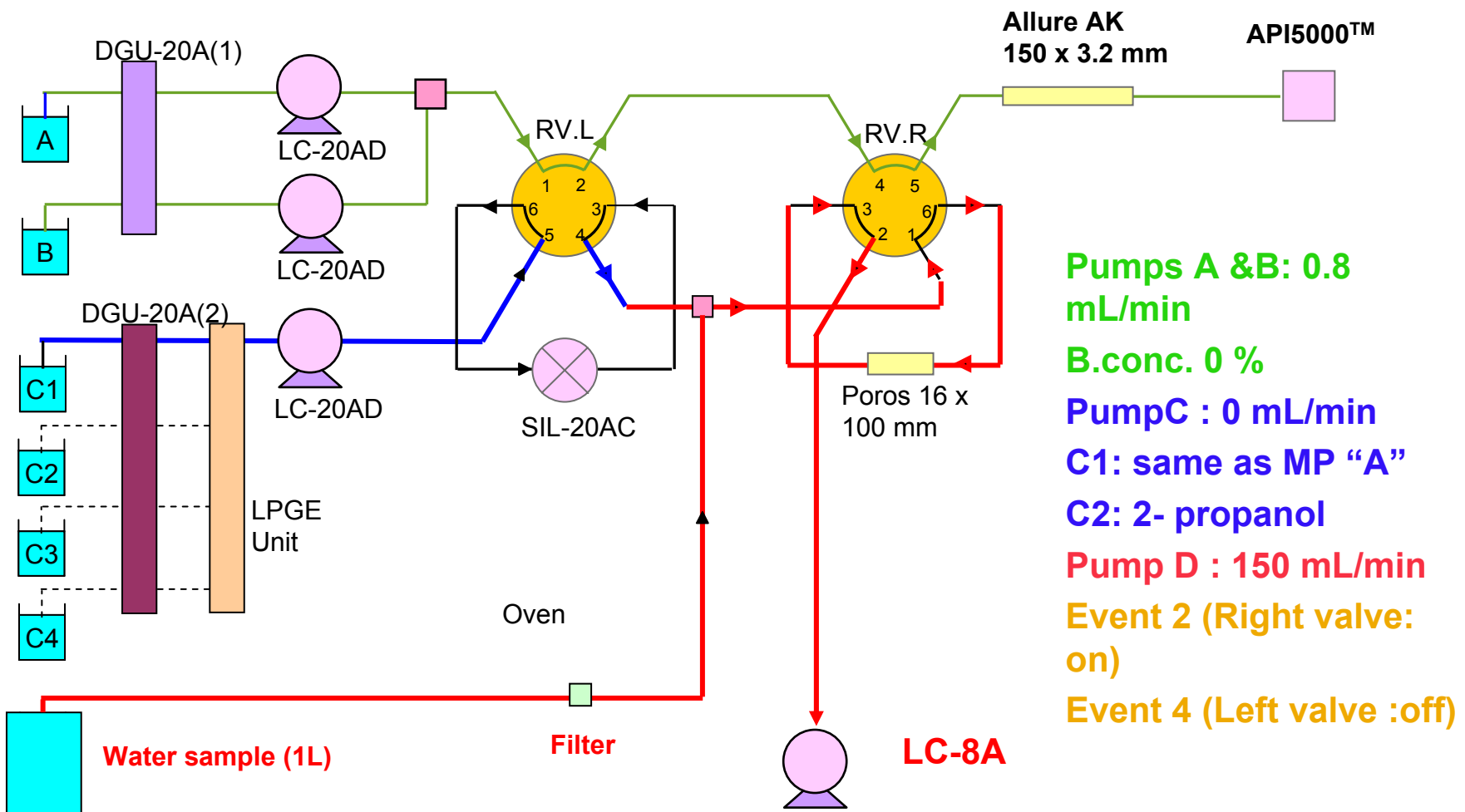


# Large-Volume Sampler Flow diagram “Start” conditions

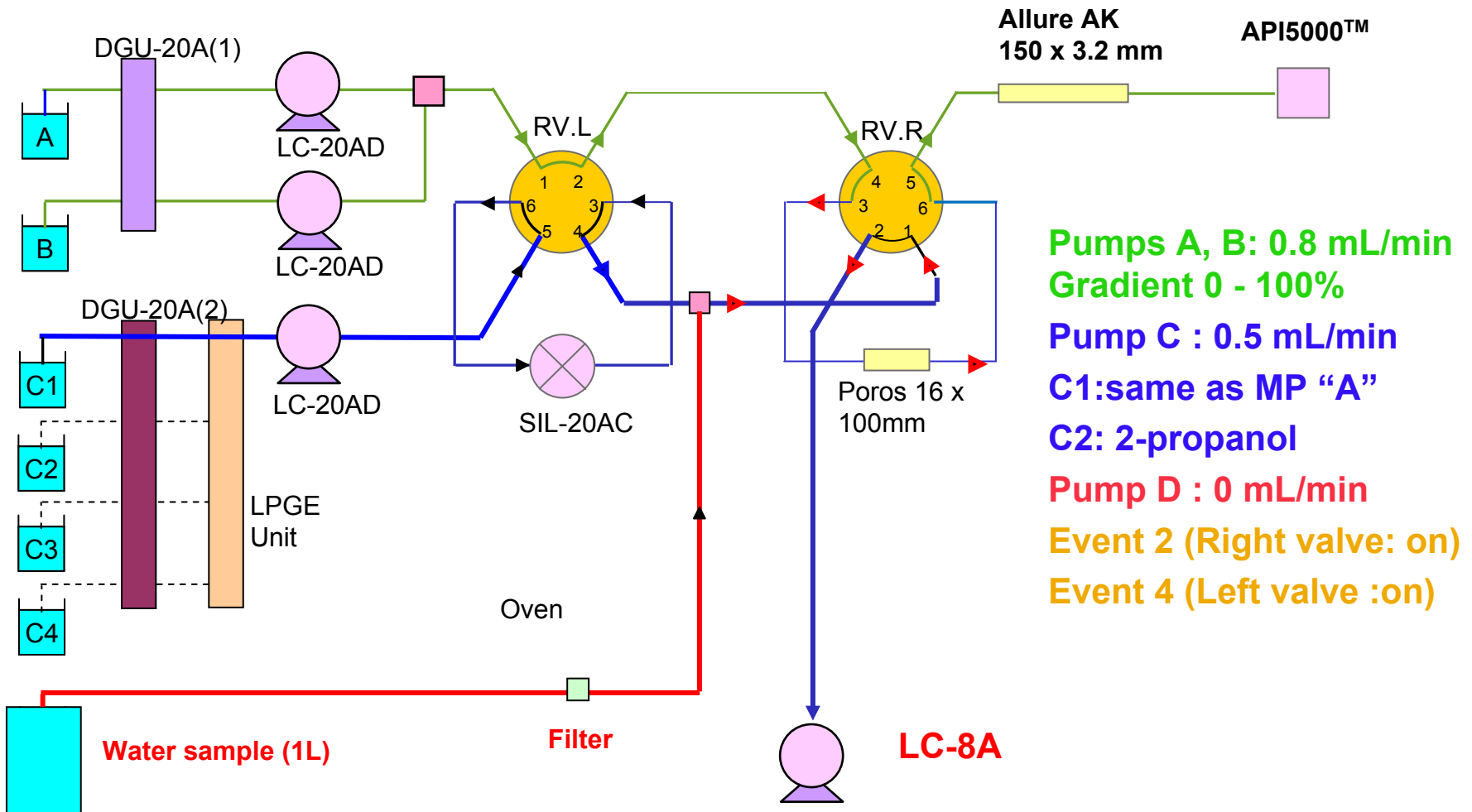




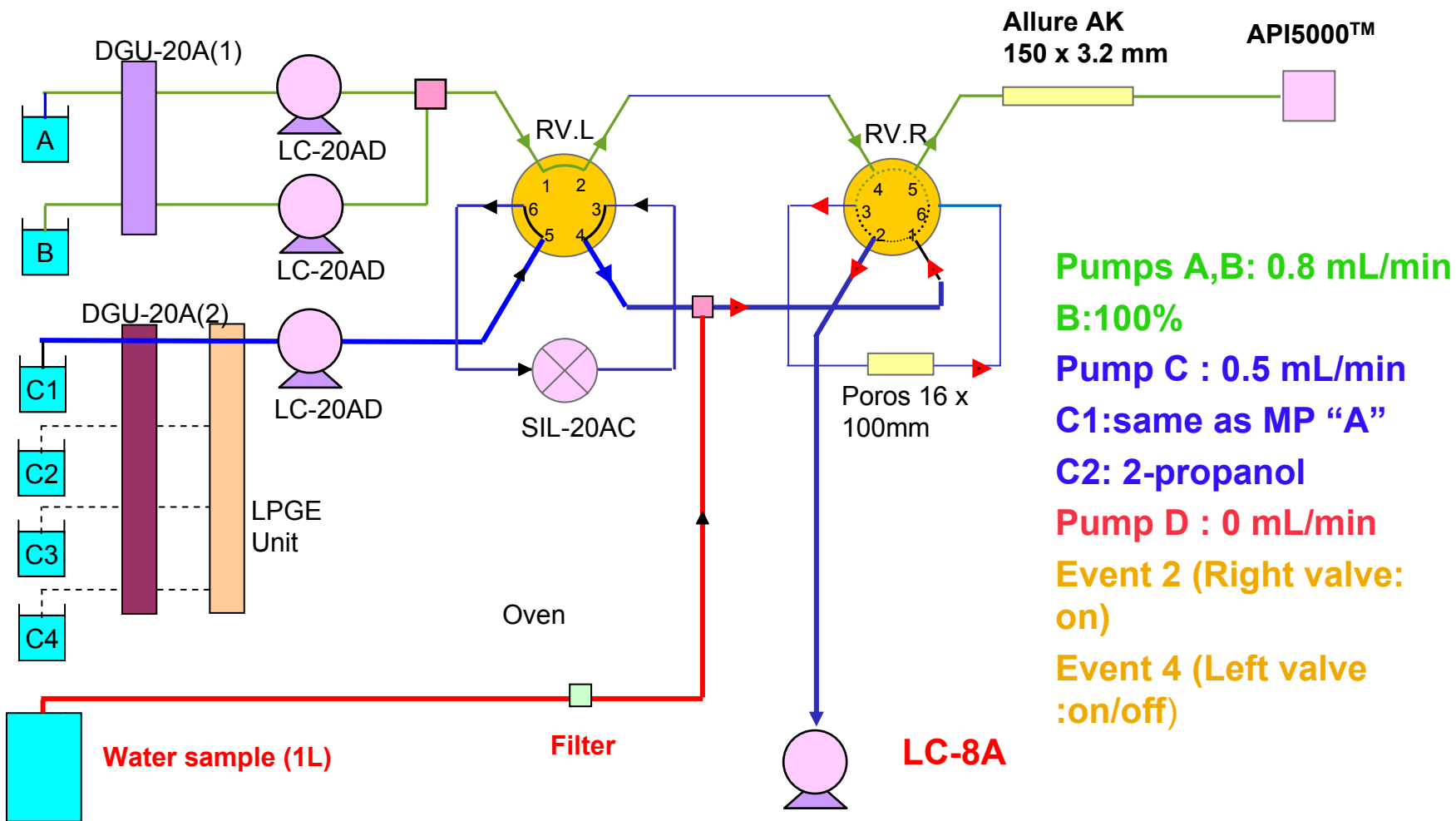
## Large-Volume Sampler Flow diagram: sample loading period 0.01 min-6.66 min



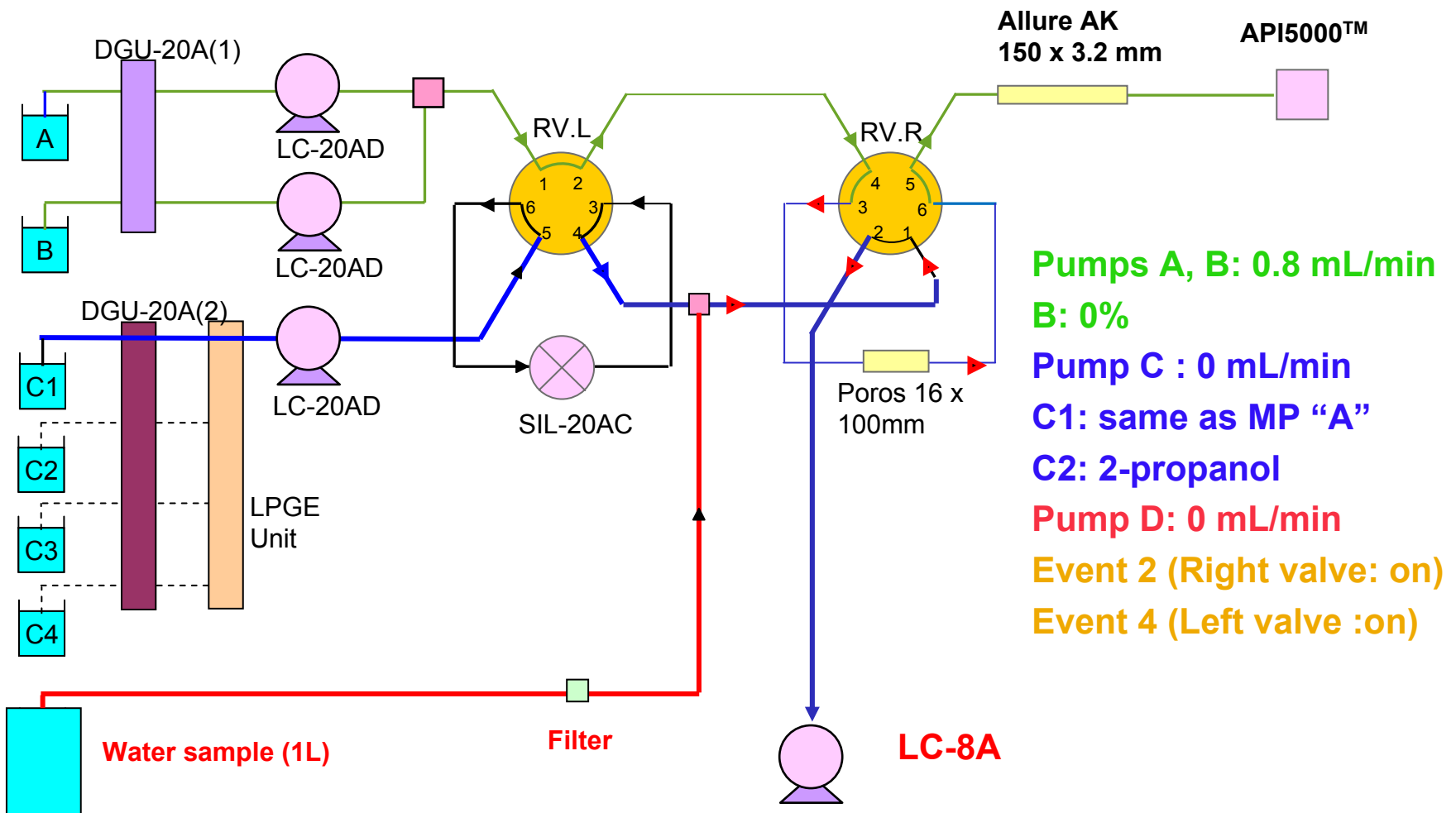
## Large-Volume Sampler Flow diagram : Analysis 6.73-12 .00min



## Large-Volume Sampler Flow diagram: Washing 12:00 – 12:45 min



## Large-Volume Sampler Flow diagram: re-equilibration 13.00 – 15:00 min



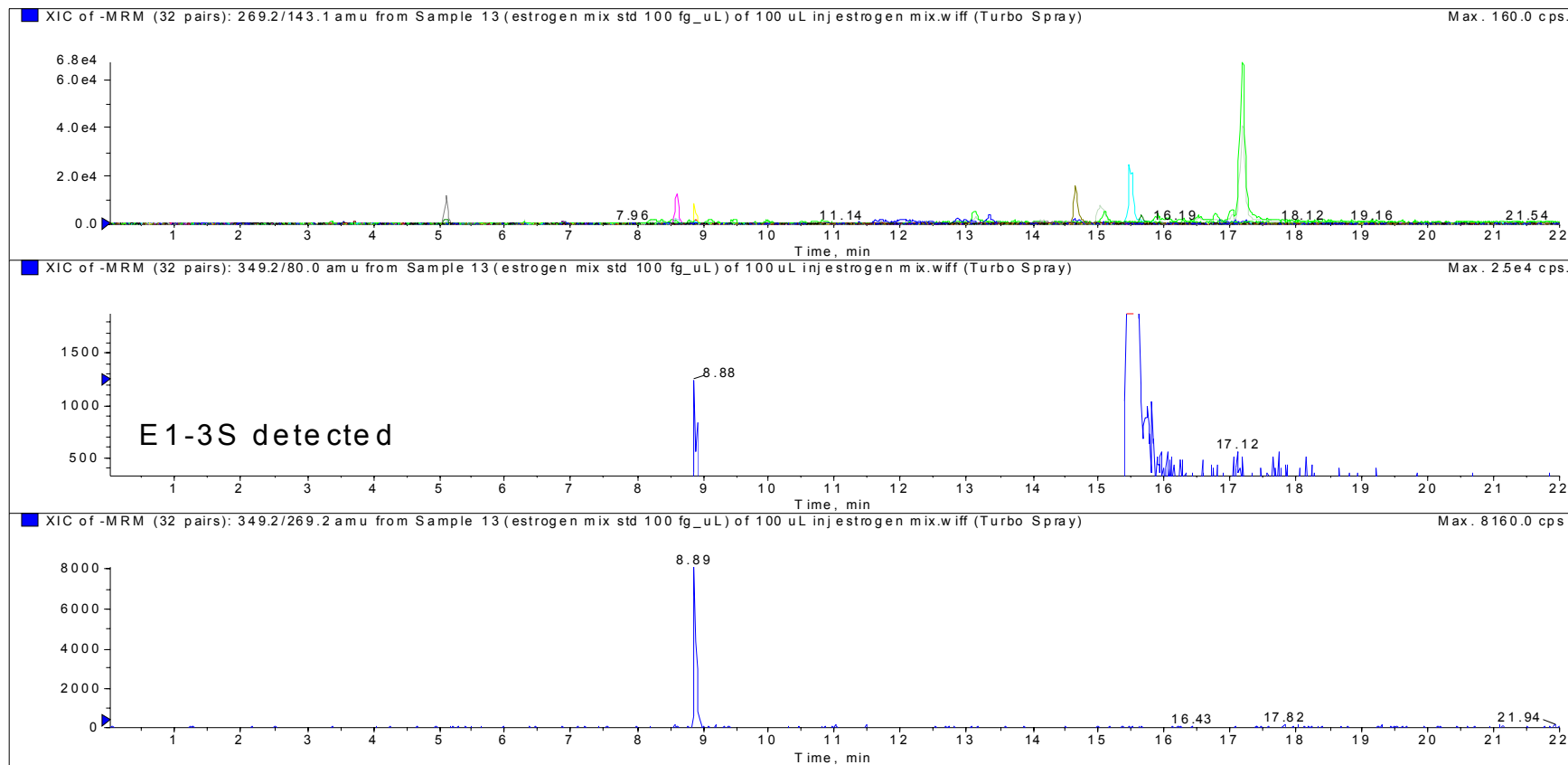


# E1-3S detected at 100 fg/ $\mu$ L in solution

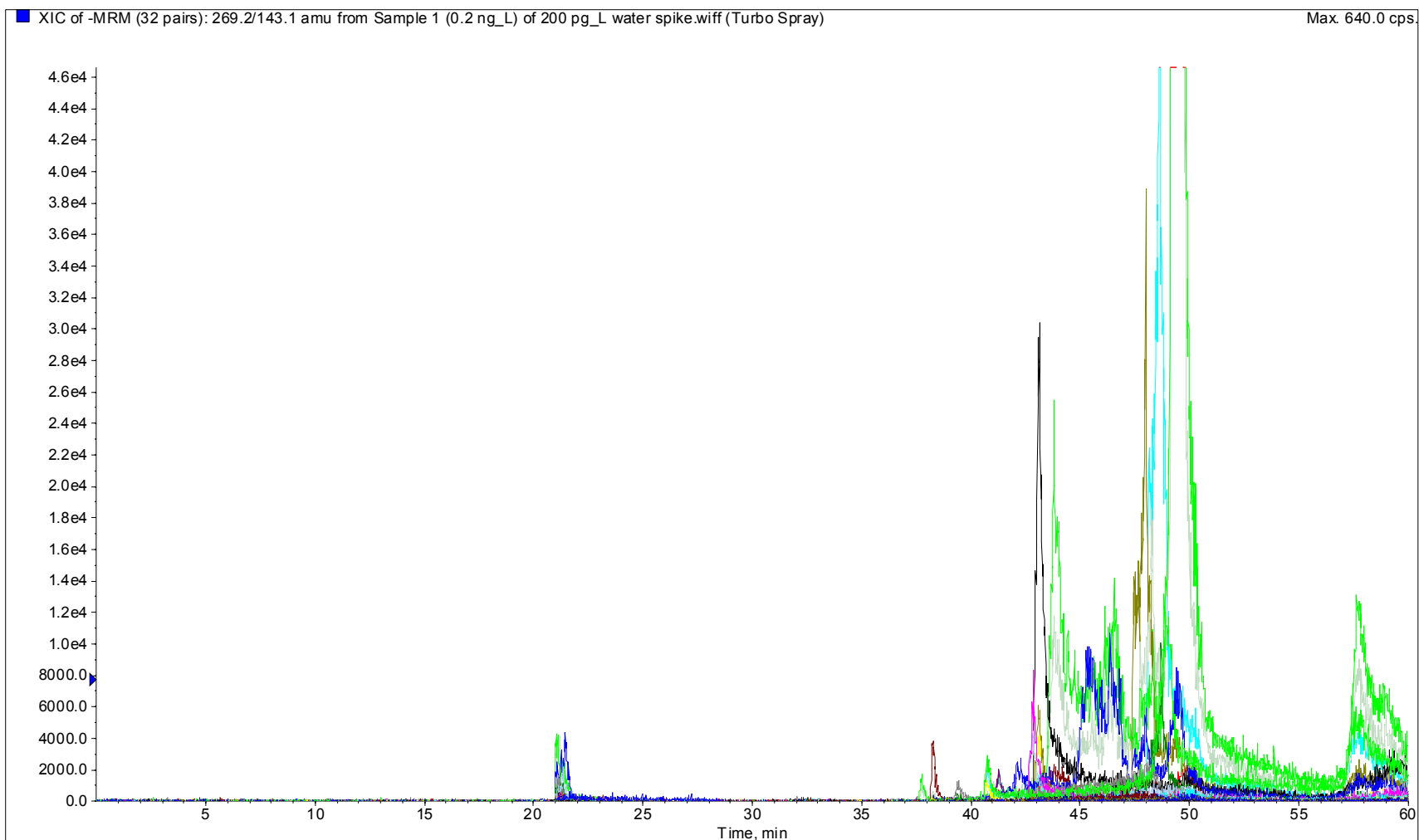
Operator: Demo User  
Analyst Version: 1.4.2

Acq. File: 100 uL inj estrogen mix.wiff  
Sample Name: estrogen mix std 100 fg\_uL

Acq. Date: Monday, June 23, 2008  
Acq. Time: 23:15



# Extracted ion chromatogram of 0.2 ng/L (0.2ppt) estrogens in water – Poros trapped



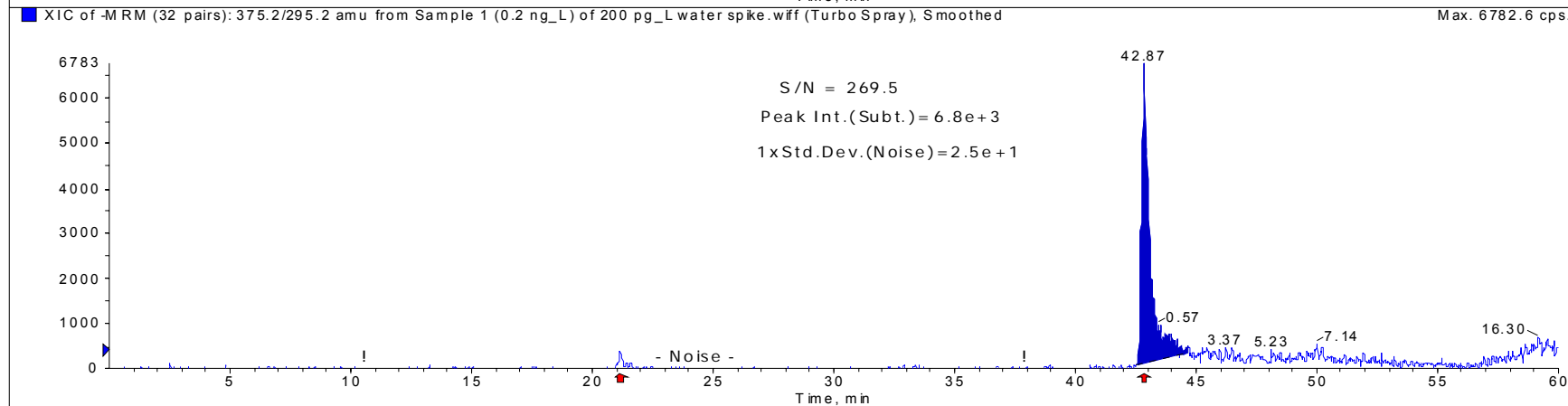
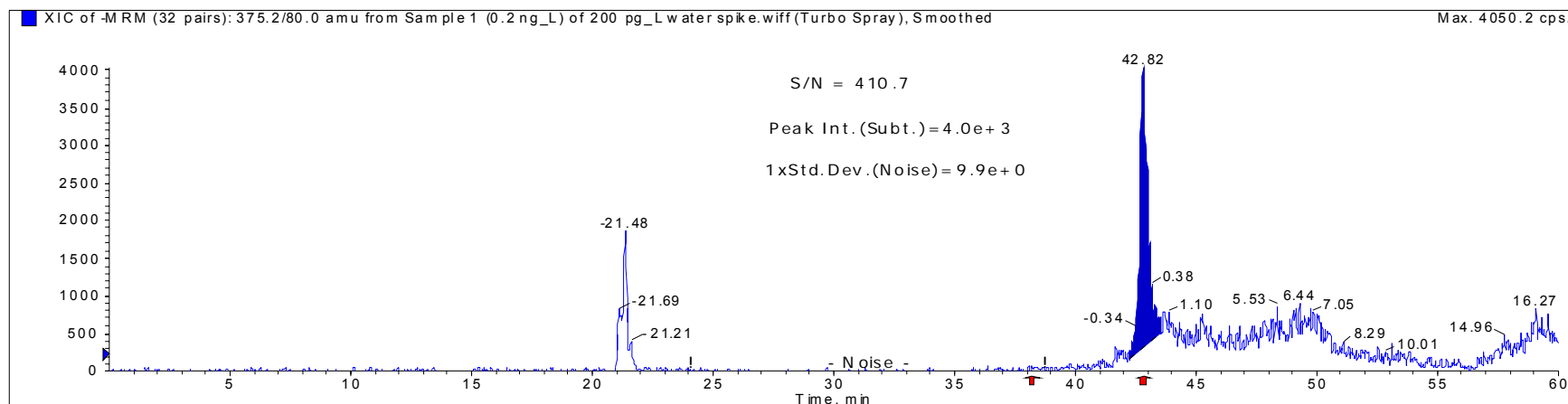


# EE-3S detected from 0.2 ng/L(0.2ppt) spike, Poros<sup>®</sup>

Operator: Demo User  
Analyst Version: 1.4.2

Acq. File: 200 pg\_L water spike.wiff  
Sample Name: 0.2 ng\_L

Acq. Date: Tuesday, June 24, 2008  
Acq. Time: 11:43



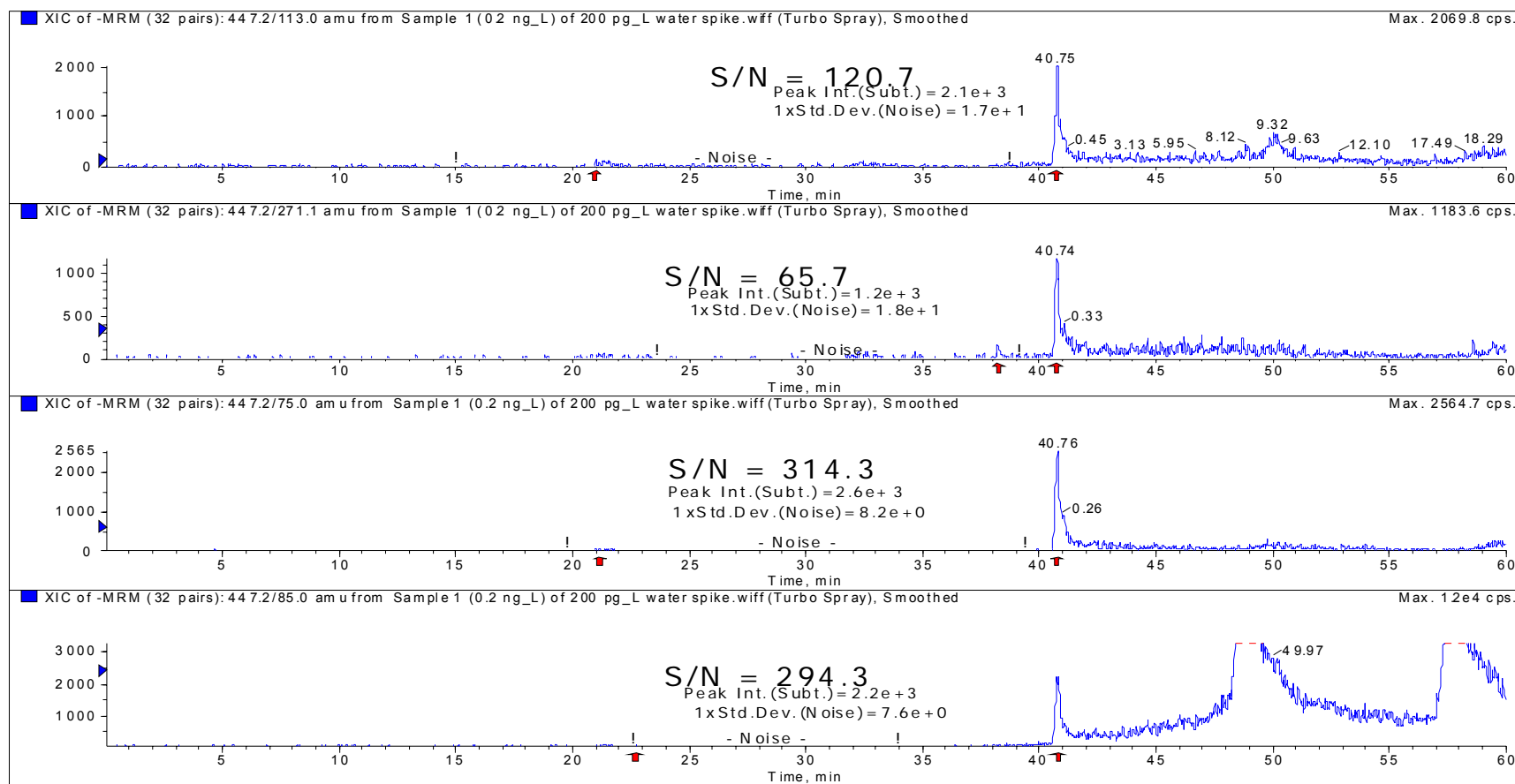


# E2-3G detected at 0.2 ng/L (0.2 ppt) spike level, Poros®

Operator: Demo User  
Analyst Version: 1.4.2

Acq. File: 200 pg\_L water spike.wiff  
Sample Name: 0.2 ng\_L

Acq. Date: Tuesday, June 24, 2008  
Acq. Time: 11:43



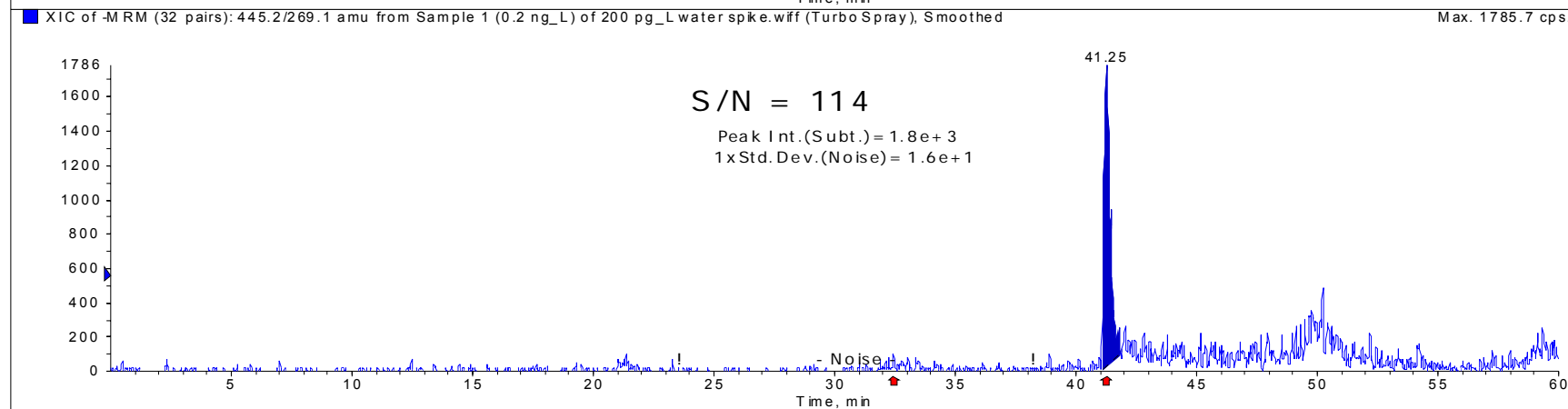
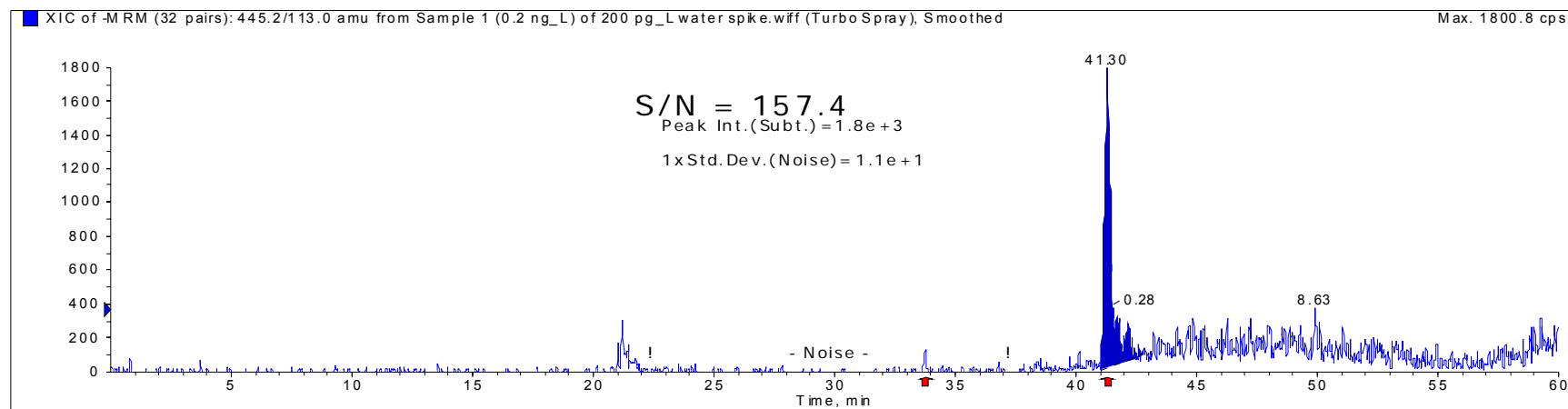


# E1-3G detected from 0.2 ppt spike, Poros®

Operator: Demo User  
Analyst Version: 1.4.2

Acq. File: 200 pg\_L water spike.wiff  
Sample Name: 0.2 ng\_L

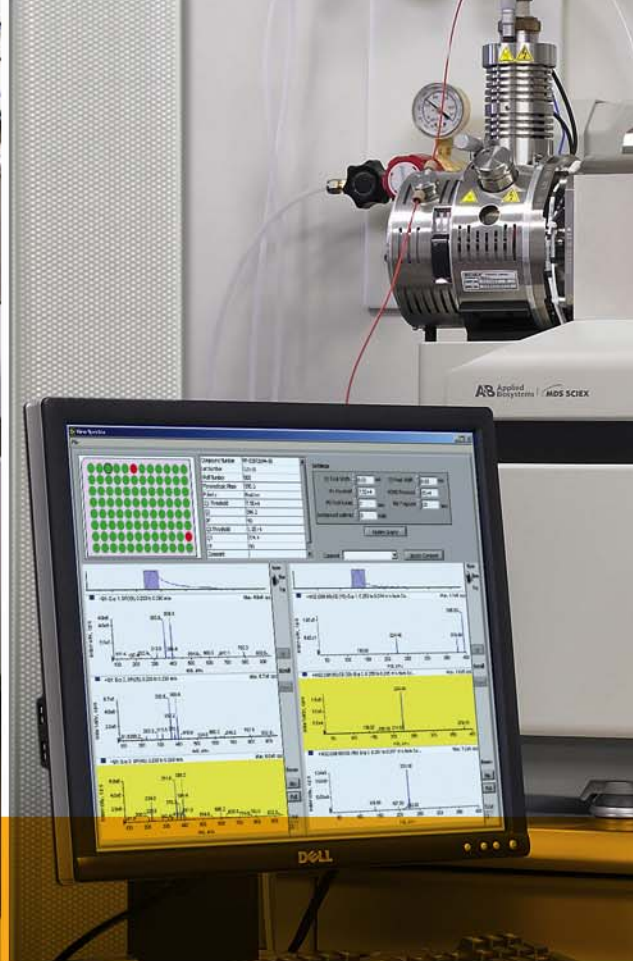
Acq. Date: Tuesday, June 24, 2008  
Acq. Time: 11:43





## Conclusions and future work

- The Allure AK allowed the separation of E2-3S from -17S, and -3G from -17G, and improved detection limits.
- For large-volume sampling, work was just started. It is as sensitive as the conventional OASIS extraction method. We can hopefully improve in terms of time and extraction efficiency for all species.
- Some optimization is needed in the area of trapping flow rate, desorption flow rate and gradient rate, loading volume, column temperature, etc., and then automation.



# Thank you for your attention!

Please contact Takeo Sakuma for any question.

Telephone (905) 660 – 9006 Ext. 2261

E-mail: [takeo.sakuma@sciex.com](mailto:takeo.sakuma@sciex.com)