SCIEX

Answers for Science.
Knowledge for Life.™

LCMSMS Solutions For Steroid Analysis

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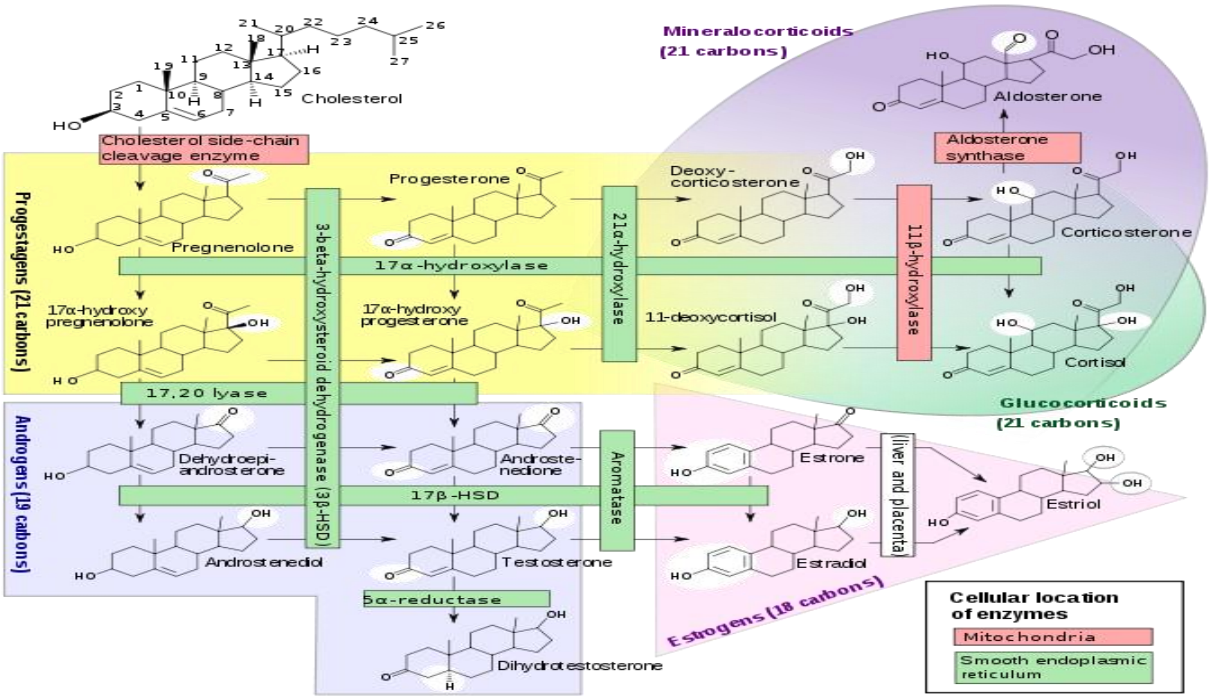
Agenda

- Special considerations for steroid analysis
- Methods of Steroid analysis 1: Simple & Fast
  - Testosterone iMethod™
  - Online Extraction with 2DLC and simple sample processing
  - Low Level Estrogen iMethod™
- Improving Selectivity with Ion Mobility
- Methods of Steroid Analysis 2: Extending the coverage
  - Steroid Panel iMethod™
  - Commercial Kits
- Summary of and conclusions from the different approaches

Special considerations for steroid analysis

- Traditional immunoassays, while convenient, are unreliable for reasons previously discussed
- Varying sample volumes available
  - Paediatric/neonatal samples
- Pressures of sample number and turnaround time demand a fast and easy to setup method
- Again, similar pressures require the use of rapid, automatable extraction procedures
  - Ideally some form of protein precipitation
  - Liquid Liquid extraction is an option but not appropriate for all laboratories
  - SPE is an option but can be costly
  - Derivitisation, for example with dansyl chloride, is an option for challenging steroids but should be considered as a last resort.

- What Steroids to analyse?



- The LC/MS/MS method should give us as much information as possible
- However, increasing the number of analytes could potentially increase the complexity of the assay

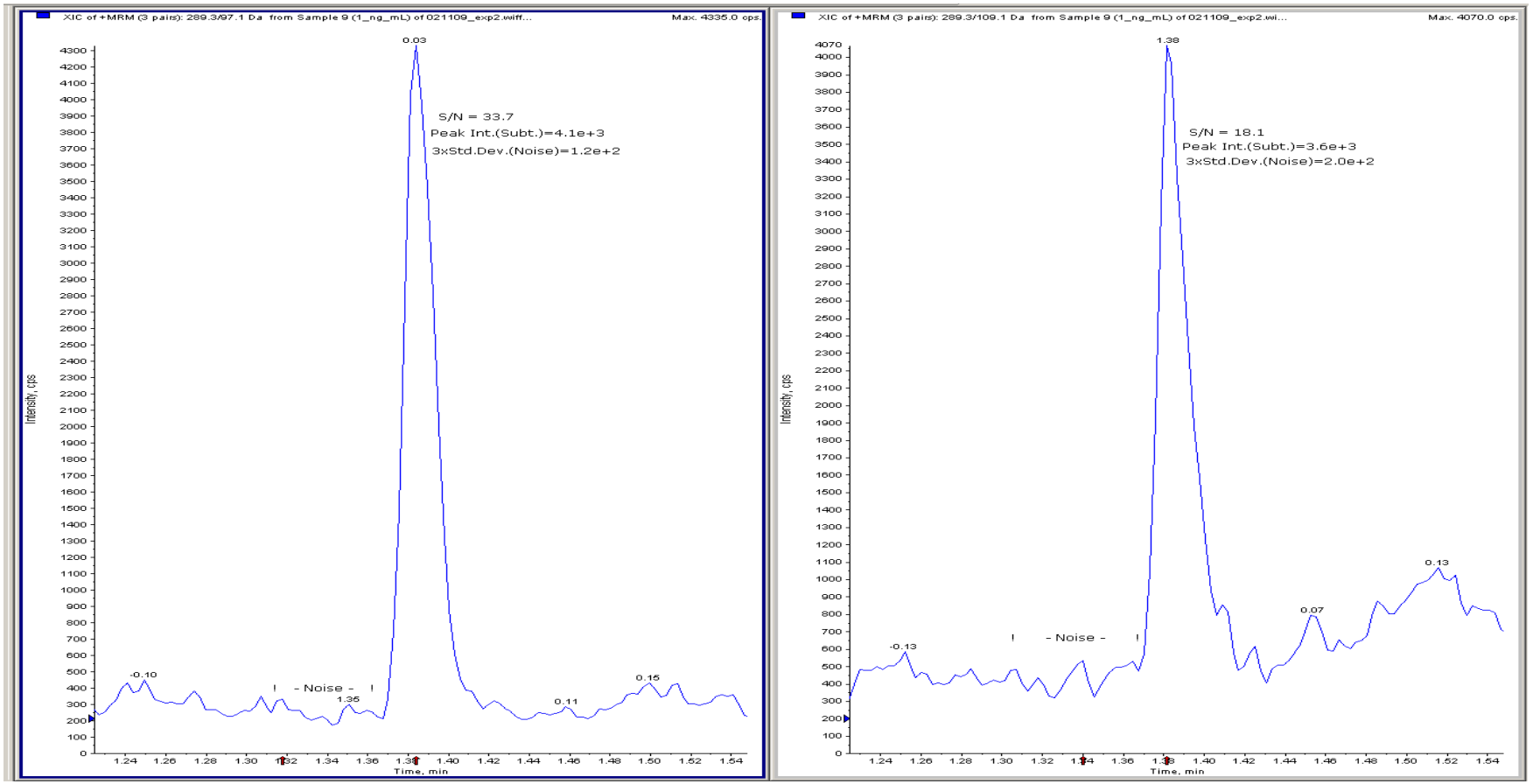
The “ideal” LC/MS/MS Steroid Application

- The convenience of an immunoassay style kit approach
- Sensitive enough to assay the lowest level steroids
  - The “which instrument” part of the question
- Able to cope with sample volume and throughput demands
- The highest control
  - Wide dynamic range standard curve
  - Deuterated internal standards, for each analyte if possible
  - Ability to utilise traceable Quality Control material

Testosterone iMethod™

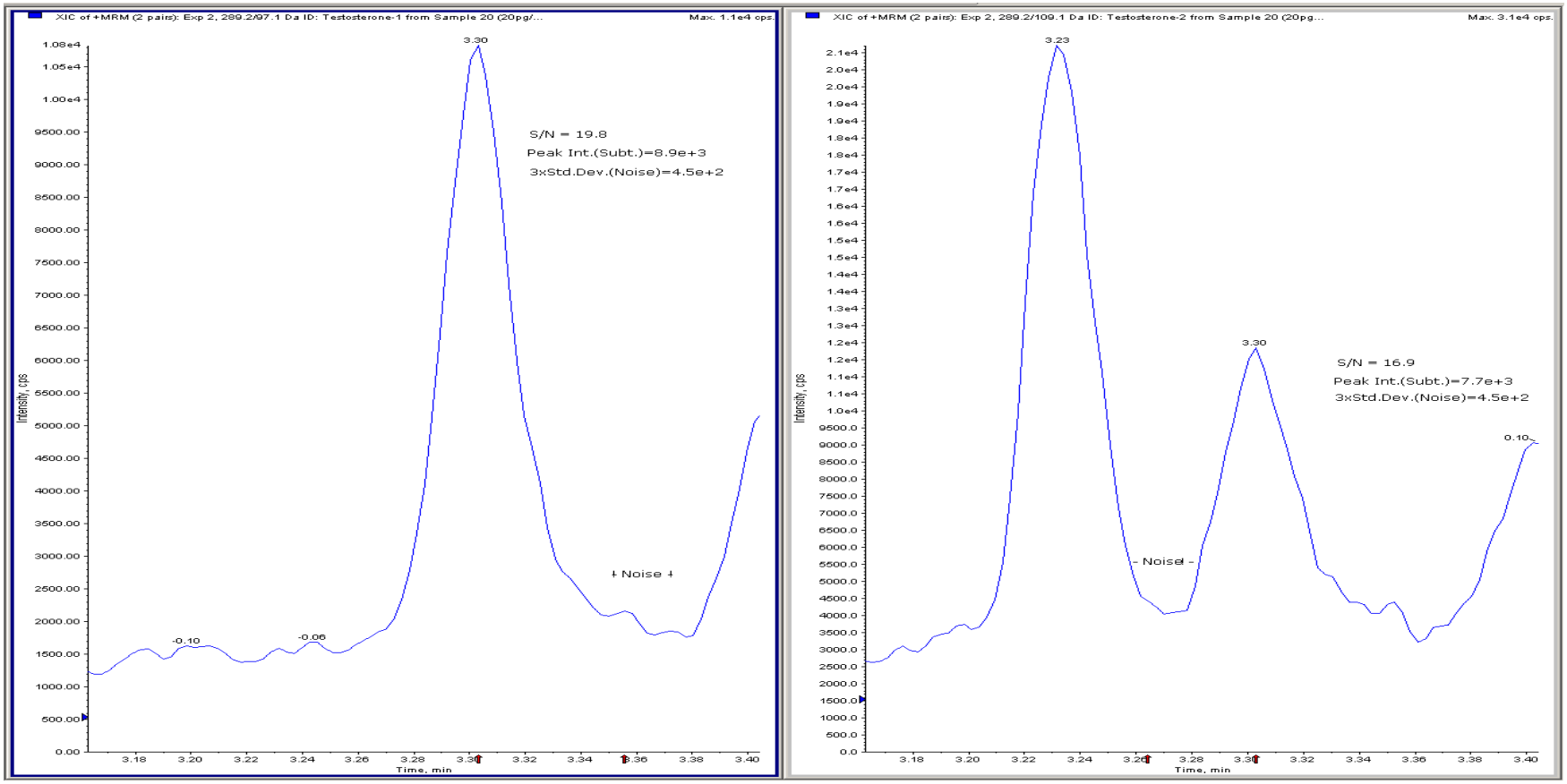
- Testosterone is one of the most widely measured steroids
- Measurement of testosterone is used to research a variety of disorders, such as congenital adrenal hyperplasia and hypogonadism.
- Measurement of the typical levels in men is straightforward; however, measurement of the low levels present in women and children can be especially challenging.
- SCIEX provides solutions for routine analysis for both circumstances.
  - Simple male testosterone assay on the API3200™ platform using protein precipitation
  - Low level (female) assay on the API5000™ or Triple Quad 5500™ using more thorough sample preparation

Testosterone iMethod™, API3200™



Data from a 3.4 nMol/L testosterone sample analyzed using an SCIEX API 3200™ LC/MS/MS System.

Testosterone iMethod™, API5000™

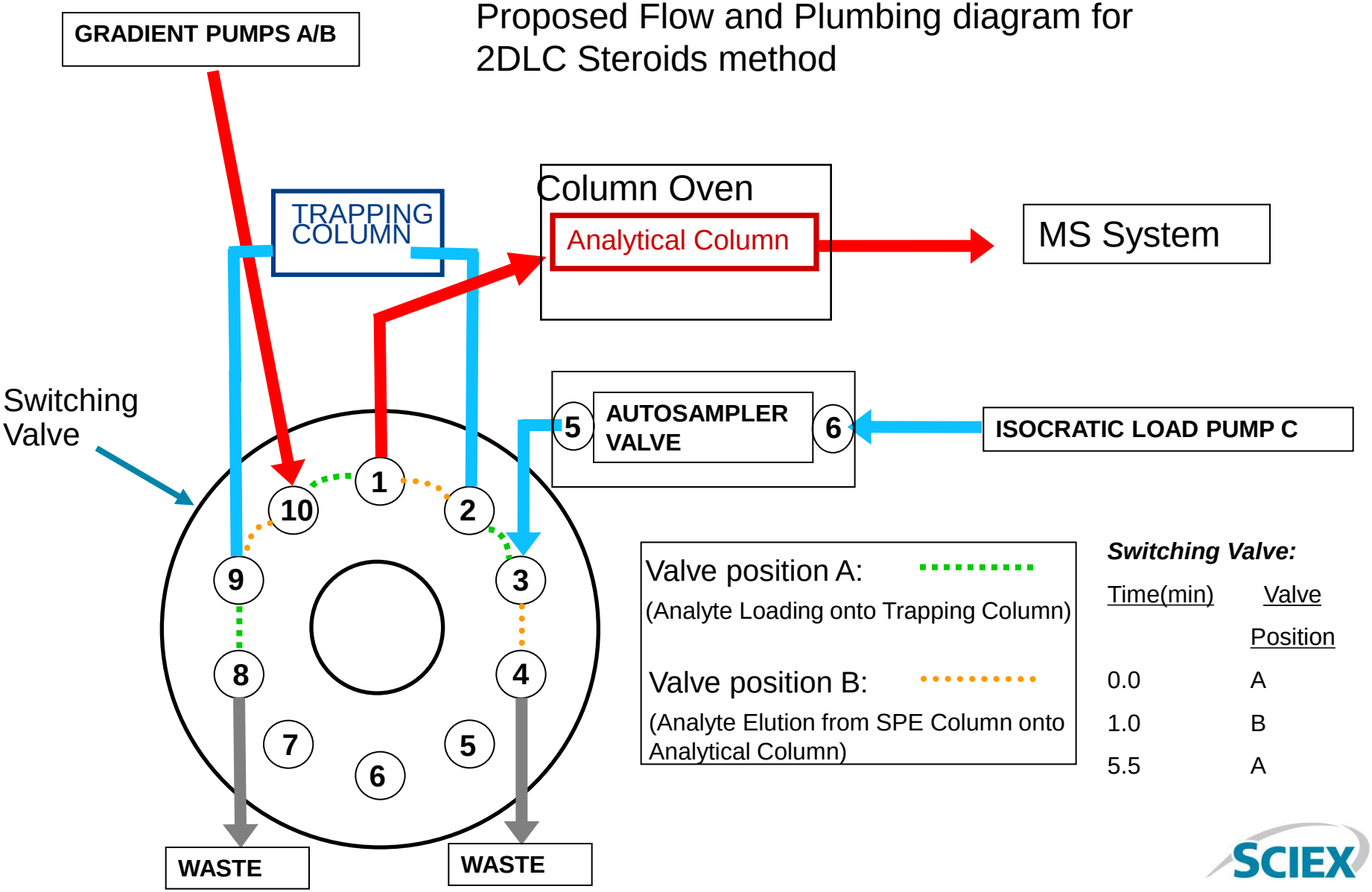


Data from a 0.069 nMol/L testosterone sample analyzed using an SCIEX API 5000™ LC/MS/MS System.

Testosterone iMethod™

- A simple method for analysis of a small number of routinely measured steroids has been proposed
- The method employs a simple protein precipitation with direct injection onto a 2DLC system (no need for offline sample concentration)
- The remainder of the sample cleanup process is performed on a trapping column, followed by a reversed flow gradient onto a common C18 HPLC column, with a total runtime of 5 minutes
- As a consequence, sample preparation time is kept to a minimum and sample throughput is maximised
- The trap column is reusable, with a lifetime in excess of 1000 injections

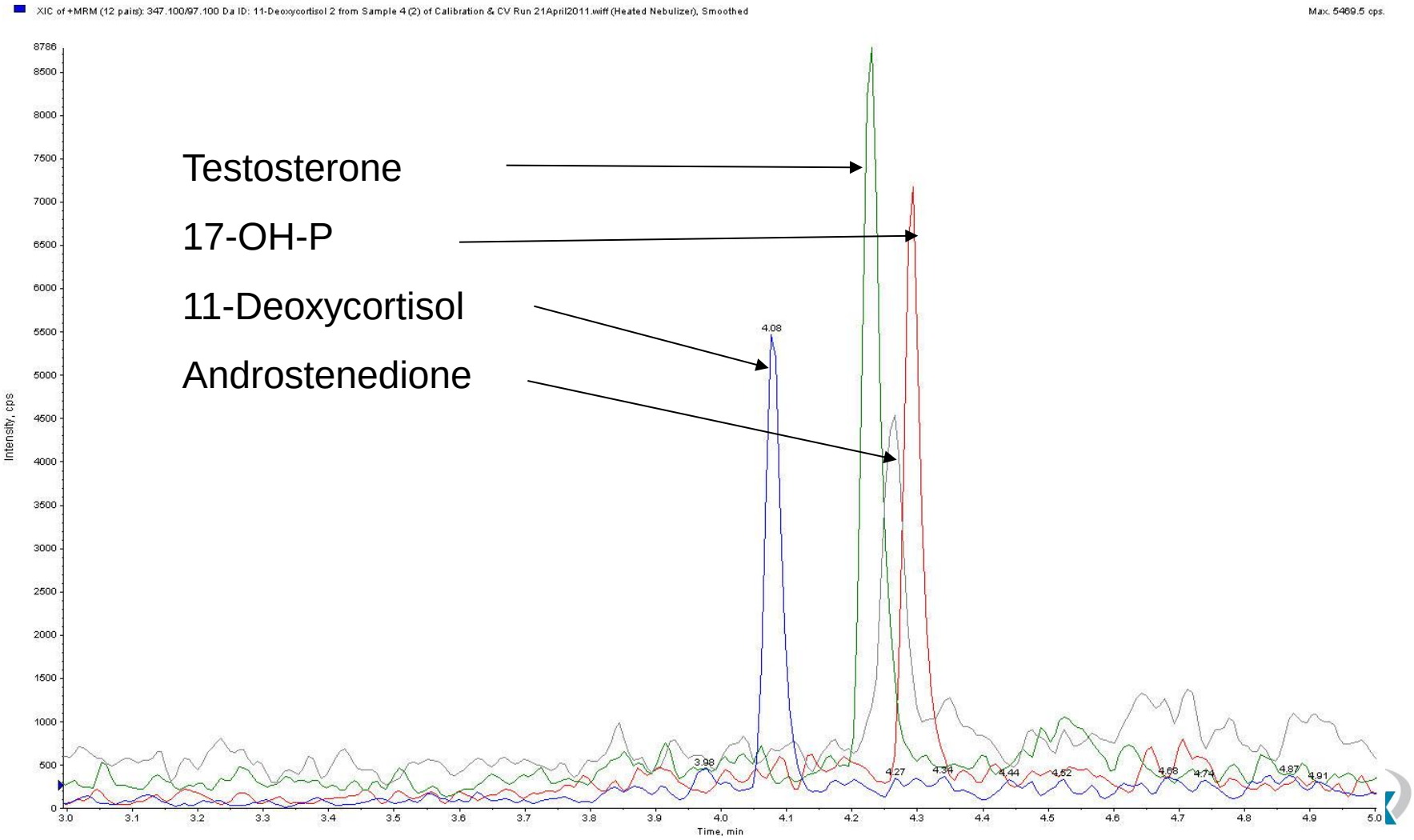
Proposed Flow and Plumbing diagram for 2DLC Steroids method



Switching Valve:

Time(min)	Valve Position
0.0	A
1.0	B
5.5	A

Extracted Steroid sample, approx 0.15nmol/L, Analysed by 2DLC approach



Low Level Estrogen iMethod™

The Importance of Estrogens

- Estrogen is an entire class of related female sex hormones: estrone (E1) estradiol (E2), and estriol (E3)

- Estrone (E1) is widespread throughout the body. It is the only one of estrogens that's present in any amount in women after menopause.

- Estradiol (E2) is the primary sex hormone of childbearing women. It is formed from developing ovarian follicles. Estradiol is responsible for female characteristics and sexual functioning. Also, estradiol is important to women's bone health. Estradiol contributes to most gynecologic problems and even female cancers.

- Estriol (E3) is made from the placenta. It's important only during pregnancy.

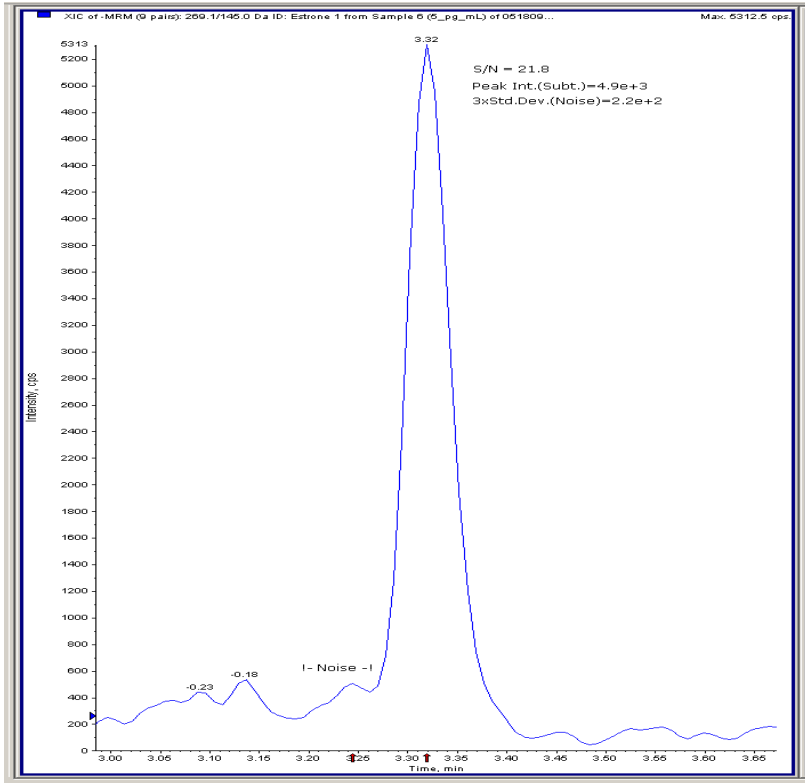
Estrone and Estradiol:

Conditions:

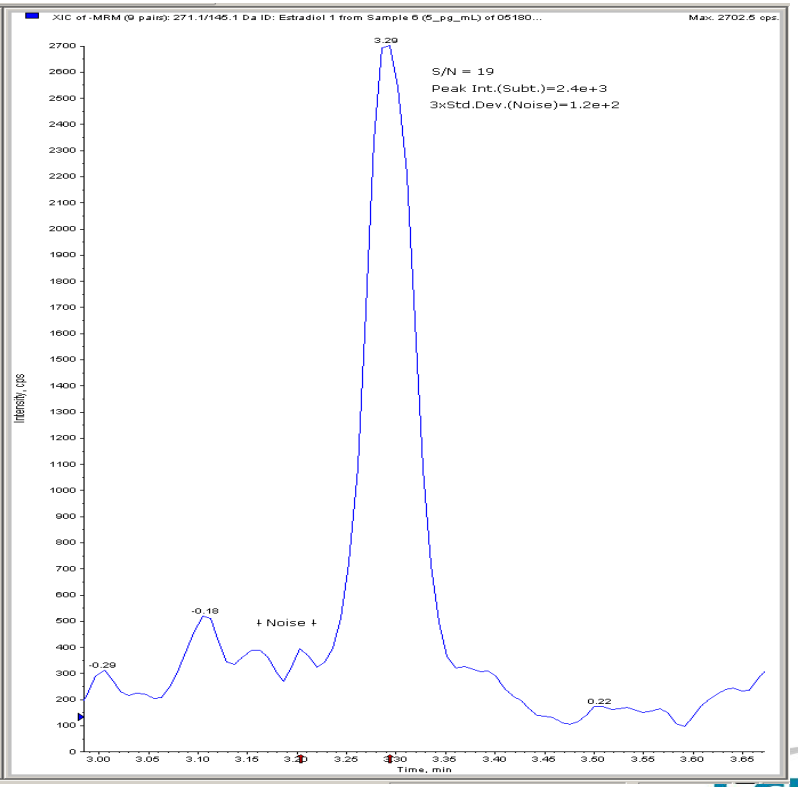
- Instrumentation
  - API 5000™ and SCIEX Triple Quad™ 5500 Mass Spectrometers
  - Shimadzu Prominence 20A and Agilent 1200 integrated systems
- Method Information
  - 500 µL sample volume required
  - Liquid-liquid extraction **without derivatization**
  - Clinically relevant dynamic range of 5-500 pg/mL
  - 6 minute total run time

Estrone and Estradiol: Lower Limit of Quantitation

Estrone 5 pg/mL LLOQ, S/N=22



Estradiol 5 pg/mL LLOQ, S/N=19



Estrone and Estradiol: Precision and Accuracy

Estrone (E1)

Sample (pg/mL)	%CV	% Accuracy
	Day 1-3	Day 1-3
LLOQ (5)	9	104
Low QC (15)	3	104
High QC (100)	3	101
ULOQ (500)	3	100

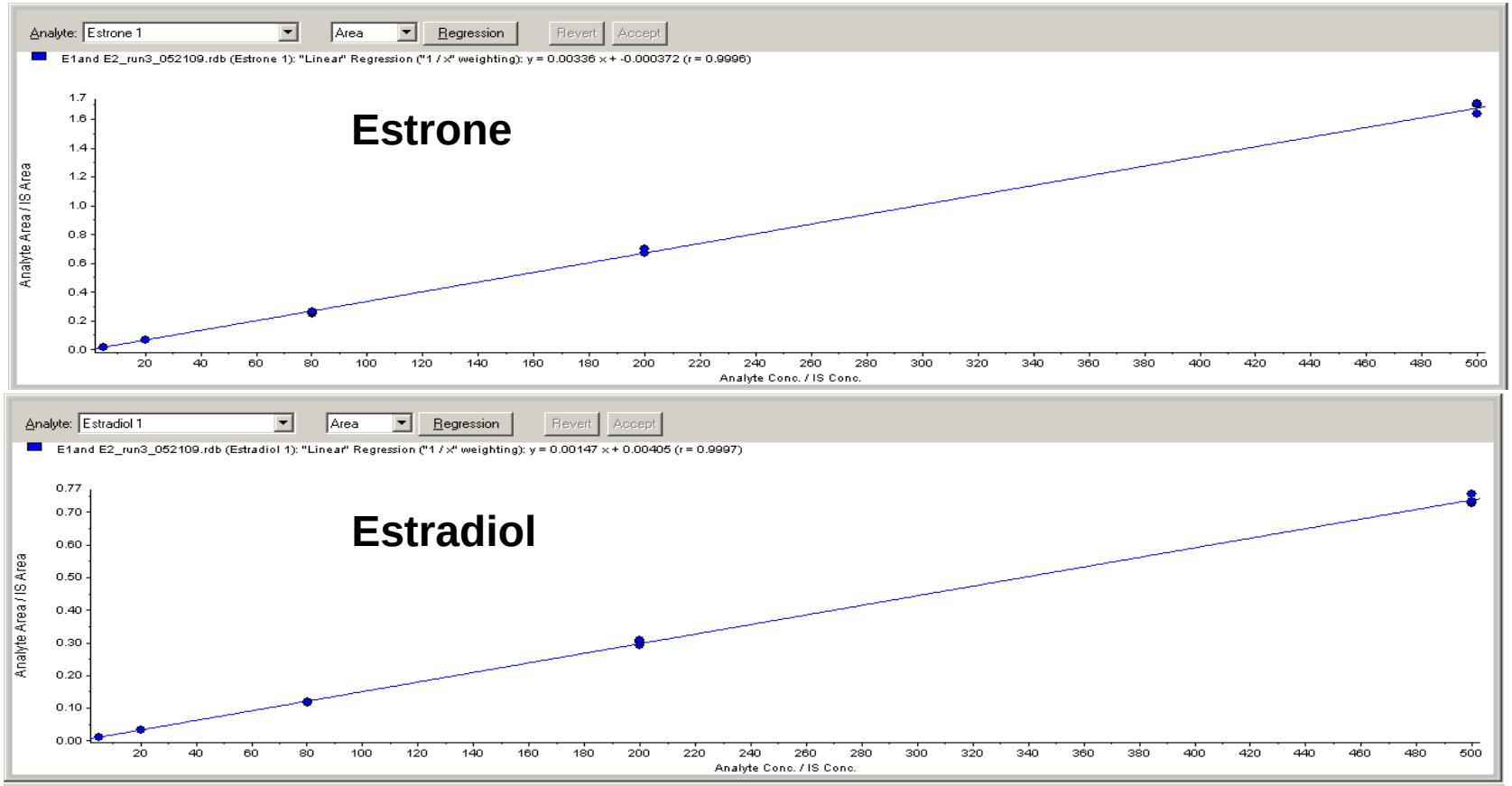
- Estrone (inter-day)
- LLOQ CV's ≤ 9% ,QC's CV's ≤ 5%
- Accuracy deviation ≤ 4%

Estradiol (E2)

Sample (pg/mL)	%CV	% Accuracy
	Day 1-3	Day 1-3
LLOQ (5)	10	101
Low QC (15)	4	96
High QC (100)	5	102
ULOQ (500)	4	101

- Estradiol (inter-day)
- LLOQ CV's ≤ 10% ,QC's CV's ≤ 5%
- Accuracy deviation ≤ 4%

Estrone and Estradiol: Linearity



Clinically relevant dynamic range 5-500 pg/ml, R≤ 0.999

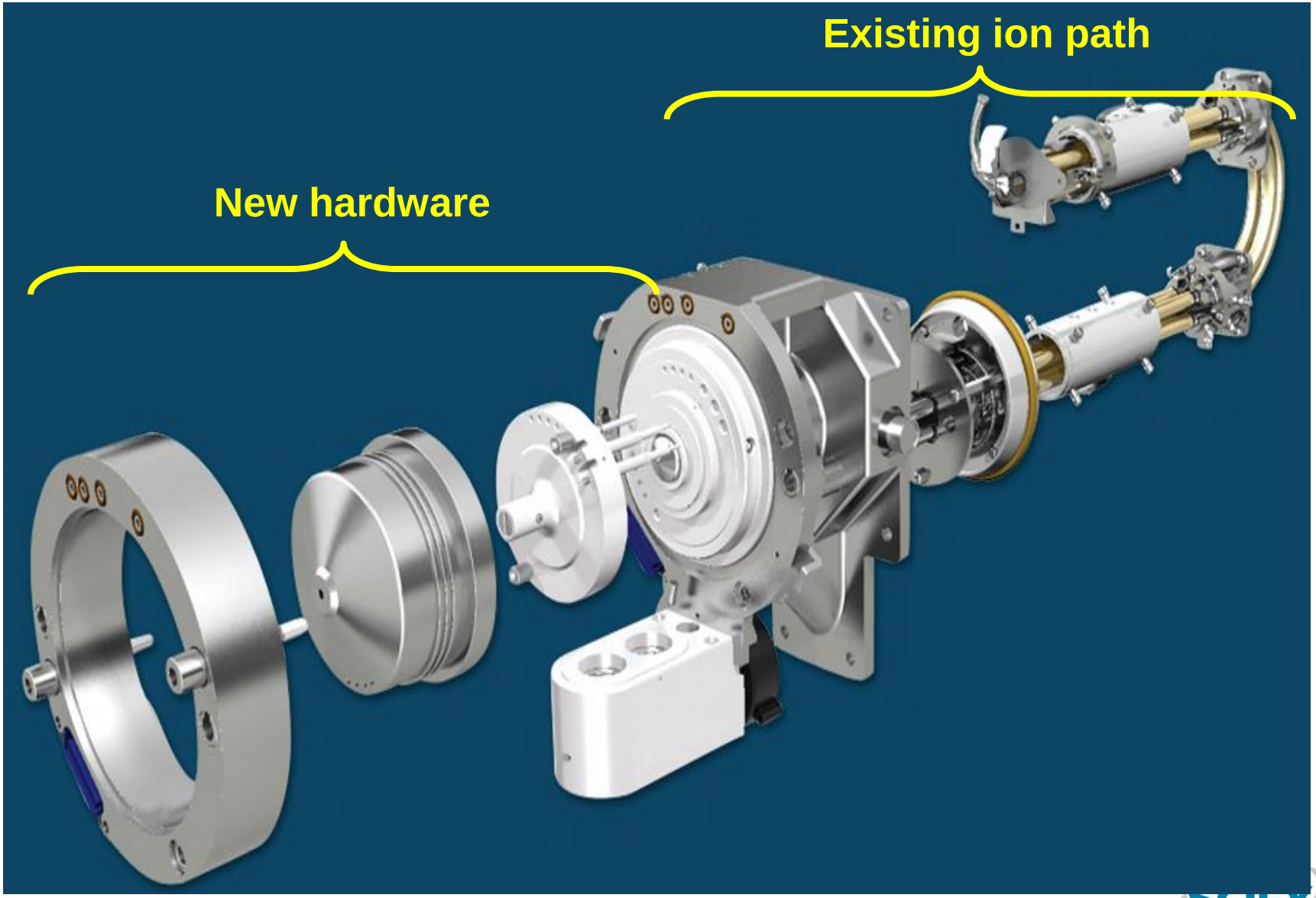
Estrone and Estradiol: Summary

- The method was verified internally across laboratories for analytical analysis
- LLOQ with suitable signal to noise (20)
- Inter-day Precision and Accuracy
  - LLOQ CVs ≤ 10% ,QCs CV's ≤ 5%
  - Accuracy Deviation ±4%
- Control samples at clinically relevant low and high concentrations (5-500 pg/mL) with linear response r ≤ 0.999
- Liquid-Liquid extraction without derivatization
- 6 minutes run time

Improving Selectivity with Ion Mobility

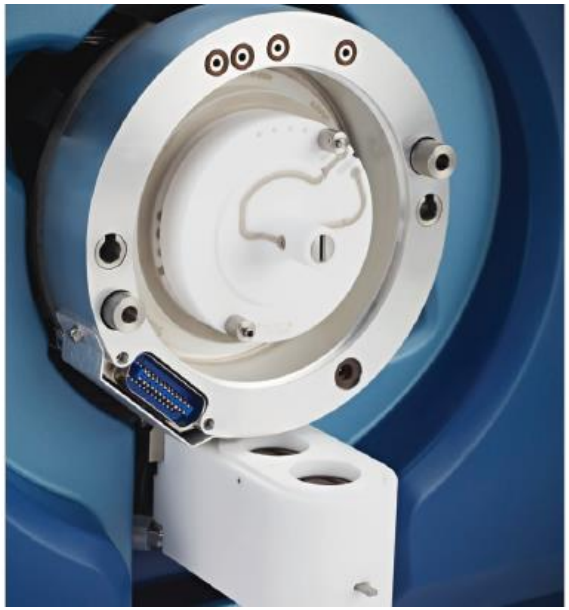
Ion Mobility to improve selectivity

- There are many different ways to achieve improved selectivity in applications of LC-MS/MS, including:
  - Improved chromatography
  - Tandem mass spectrometry (MS/MS, or even higher MS^3)
  - High-resolution mass spectrometry
  - More up-front sample cleanup
- In many applications of LC-MS/MS, mass measurements alone (even high resolution) cannot provide sufficient selectivity
  - Isobaric interferences may have the same “exact mass” as analyte ions
  - Interferences may have common fragment ions
  - Chemical background may pose a challenge
- The SelexION™ ion mobility technology provides an orthogonal means of separating / filtering ions prior to, and independent of, MS/MS analysis.

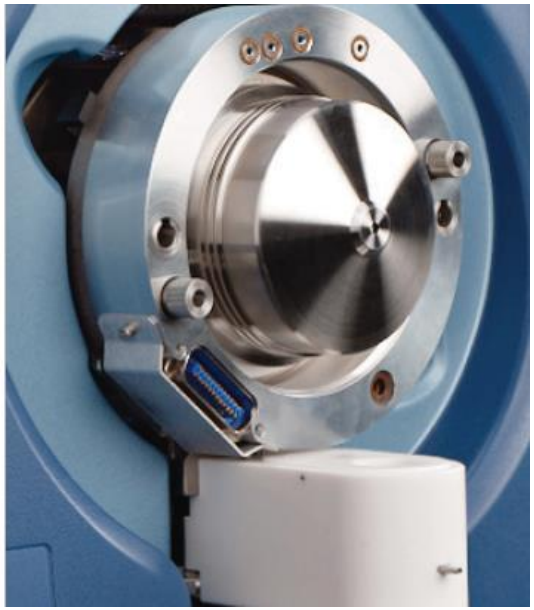


1. Orifice Plate

2. Ion Mobility Cell

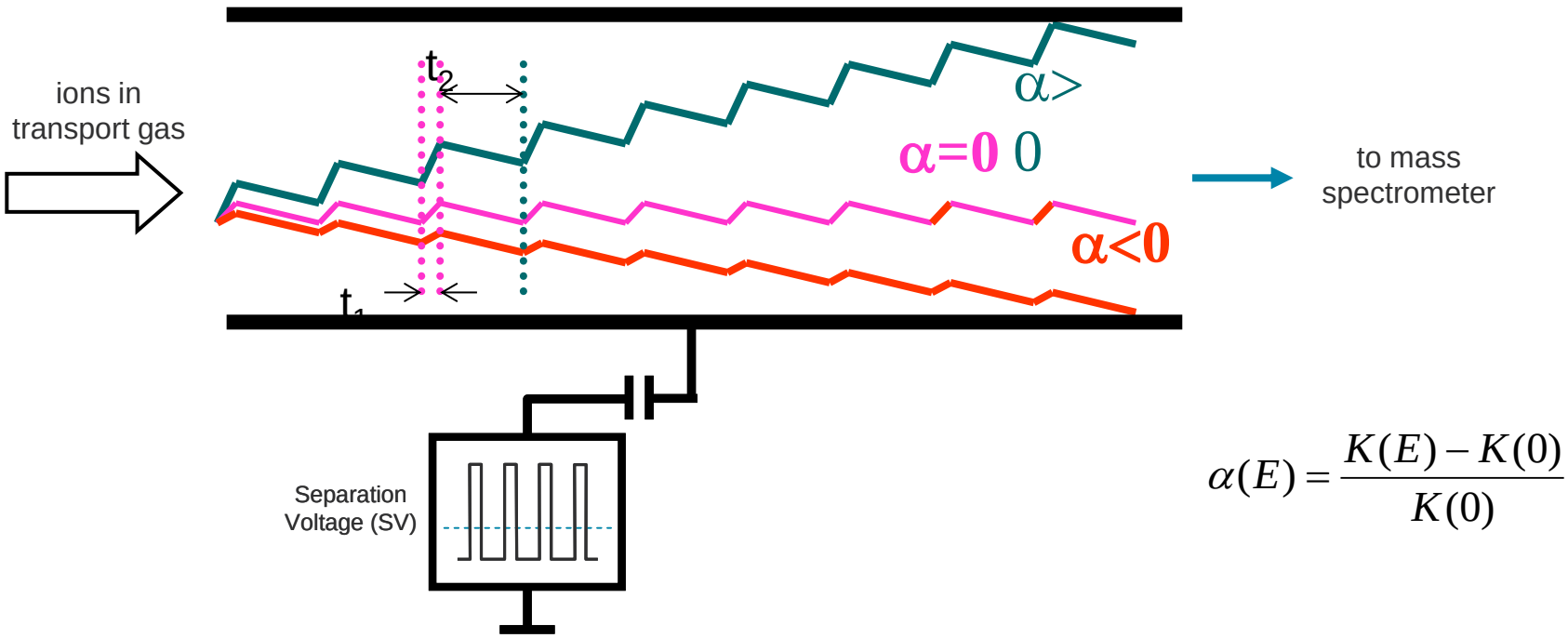


3. Curtain Plate



Robust, easy-to-install, hardware components:

- No tools required
- No cables
- No need to break vacuum
- Installation in about 2 minutes

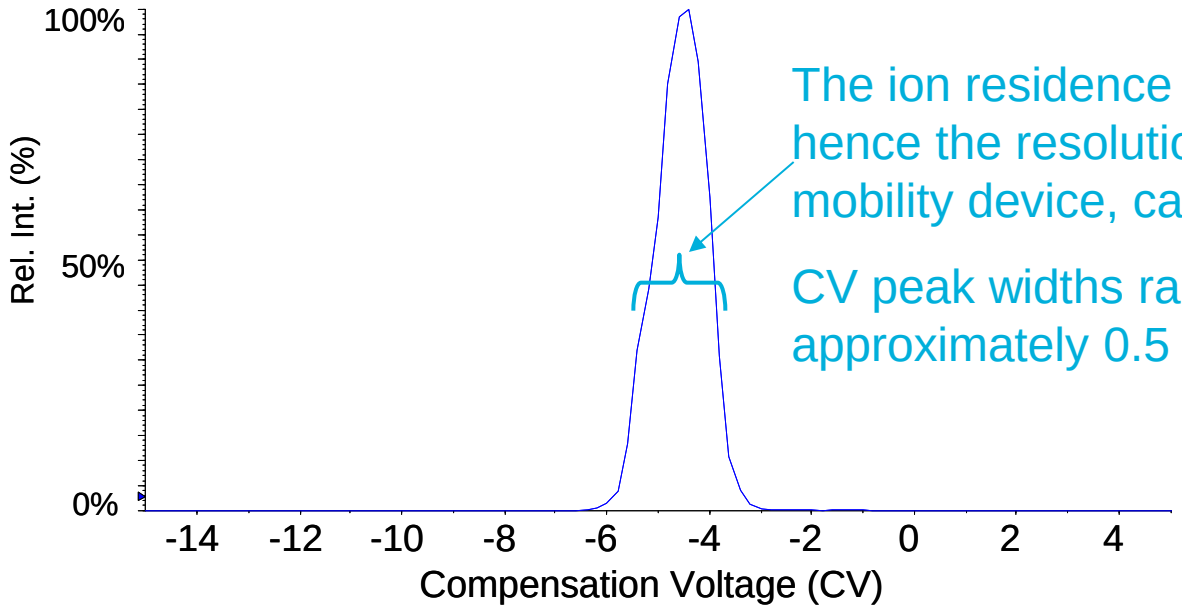


$$\alpha(E) = \frac{K(E) - K(0)}{K(0)}$$

- Ions migrate towards one of the planar electrodes
  - If an ion's high-field mobility K(E) is larger than its low-field mobility K(0), $\alpha>0$
  - If an ion's low-field mobility K(0) is larger than it high-field mobility K(E), $\alpha<0$

- Any ion can be steered back onto the center-line, by application of a compound-specific DC compensation voltage (CV).

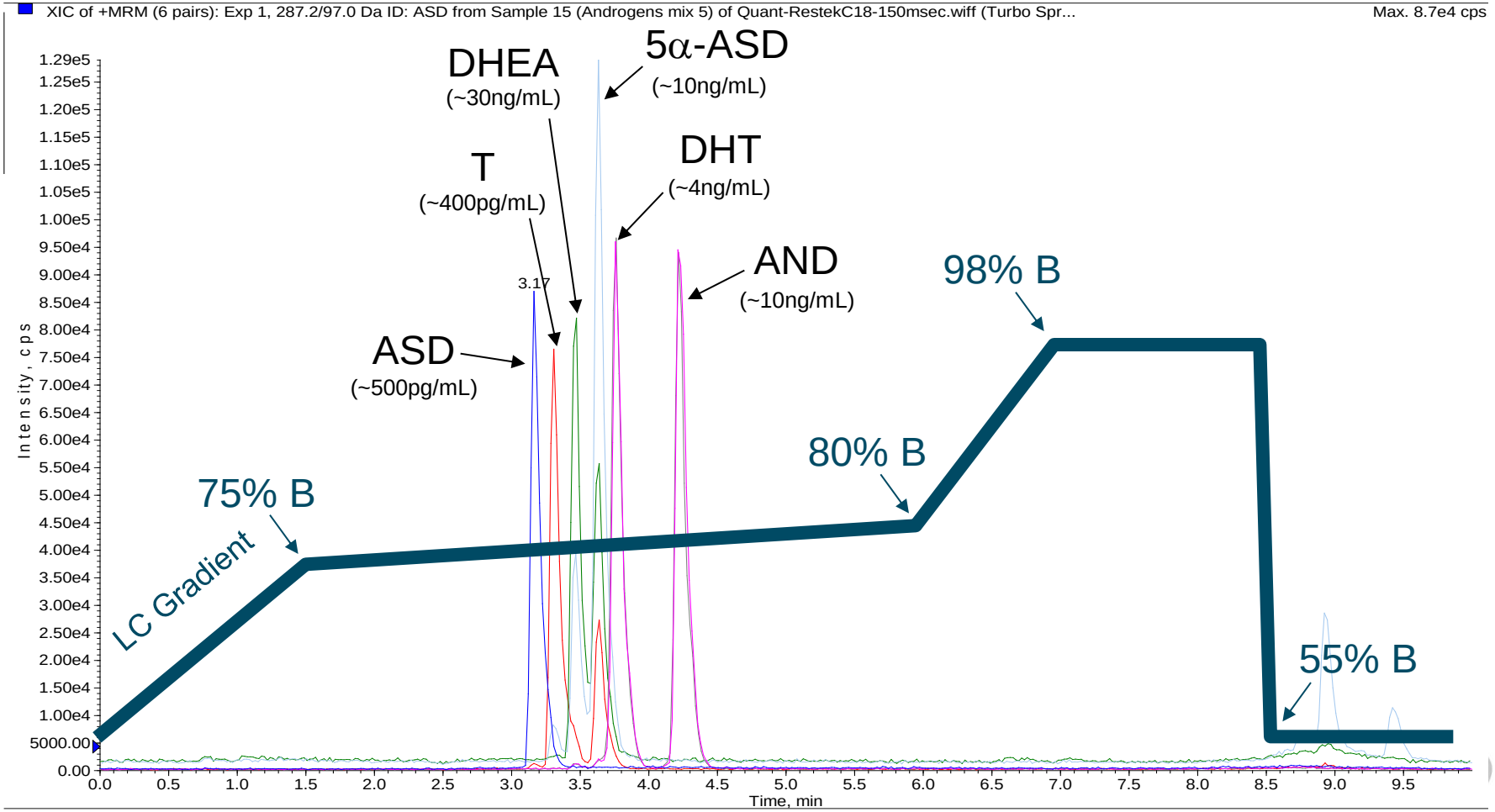
Tuning the SelexION™ Ion Mobility Device



- In infusion mode, the compensation voltage (CV) parameter is ramped in order to determine the optimized value.
- Note that this is analogous to tuning any other compound-specific parameter, e.g. the collision energy (CE)

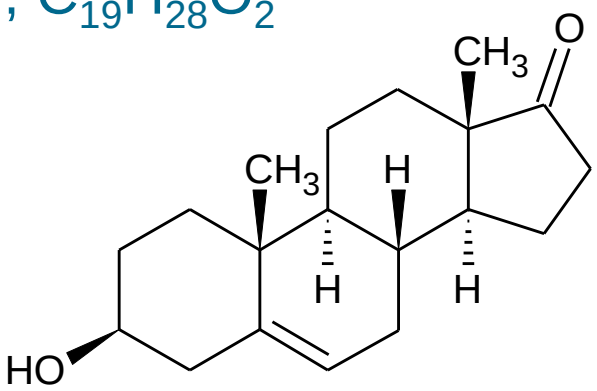
Mixture of 6 androgens in 60:40 Methanol:Water

- Sample chromatogram from QTRAP® 5500 LC/MS/MS system



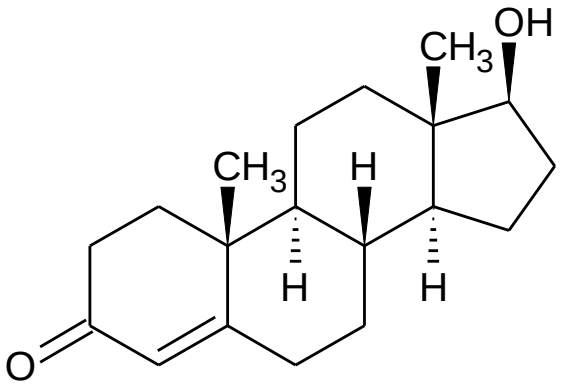
Structures of two androgens, DHEA and T

- Testosterone, $C_{19}H_{28}O_2$



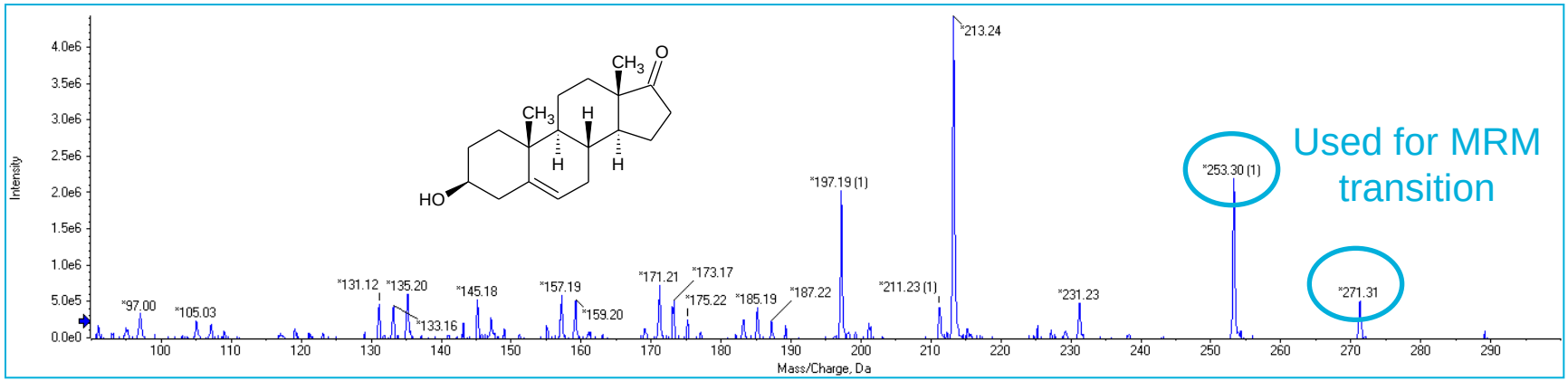
- **Low ionization efficiencies** ✔
- **Common precursor ion masses (m/z 289)** ✔
- **Similar fragmentation patterns** ✔
- **Similar chromatographic retention properties** ✔

- Dehydroepiandrosterone (DHEA), $C_{19}H_{28}O_2$

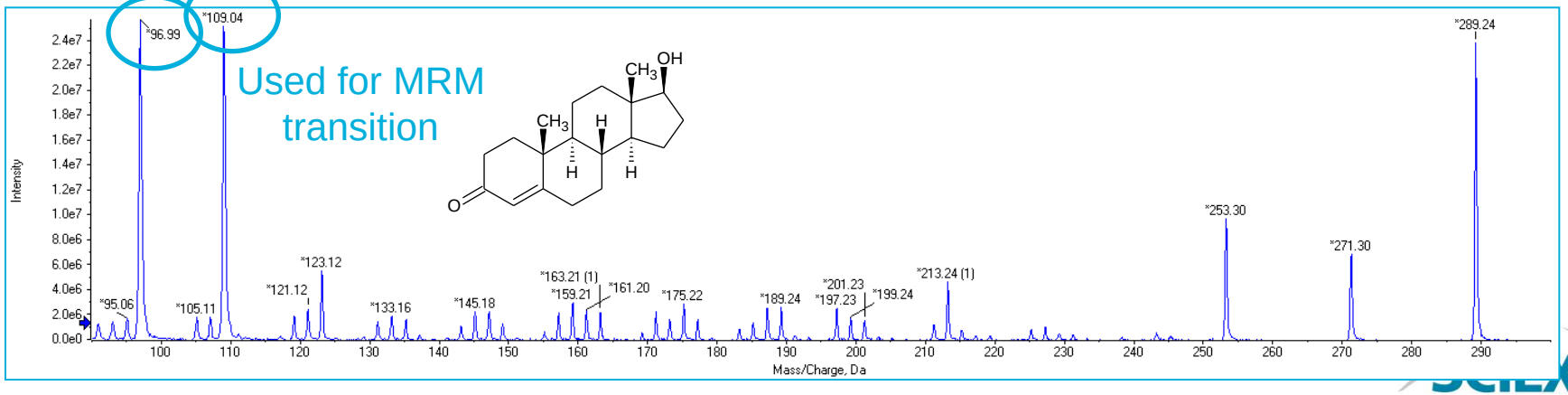


MS/MS of DHEA and Testosterone (m/z 289)

(A) MS/MS fragmentation pattern (Collision Energy ramp) for DHEA

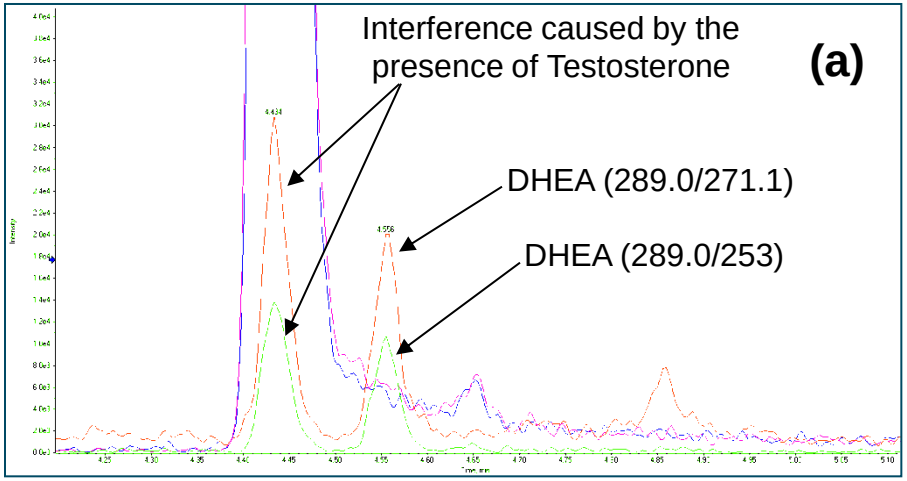


(B) MS/MS fragmentation pattern (Collision Energy ramp) for Testosterone

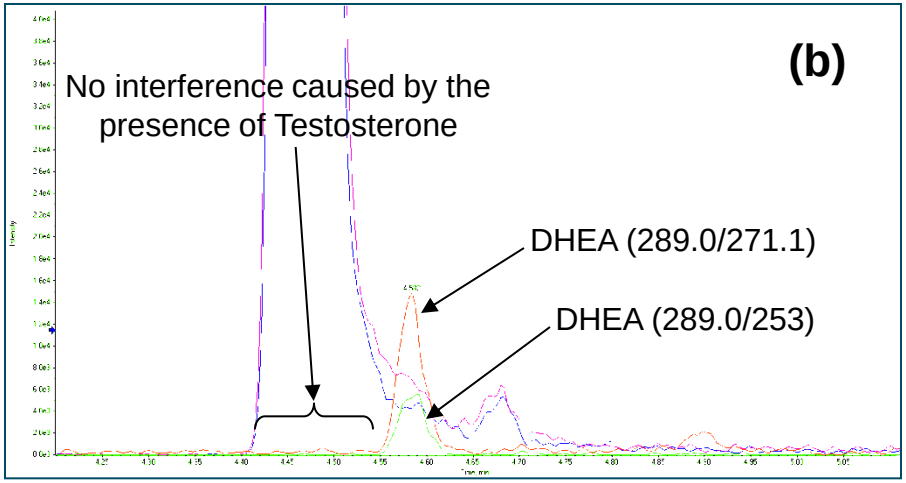


Resolving DHEA and Testosterone using the SelexION™ Ion Mobility Device

SelexION™ device “off”



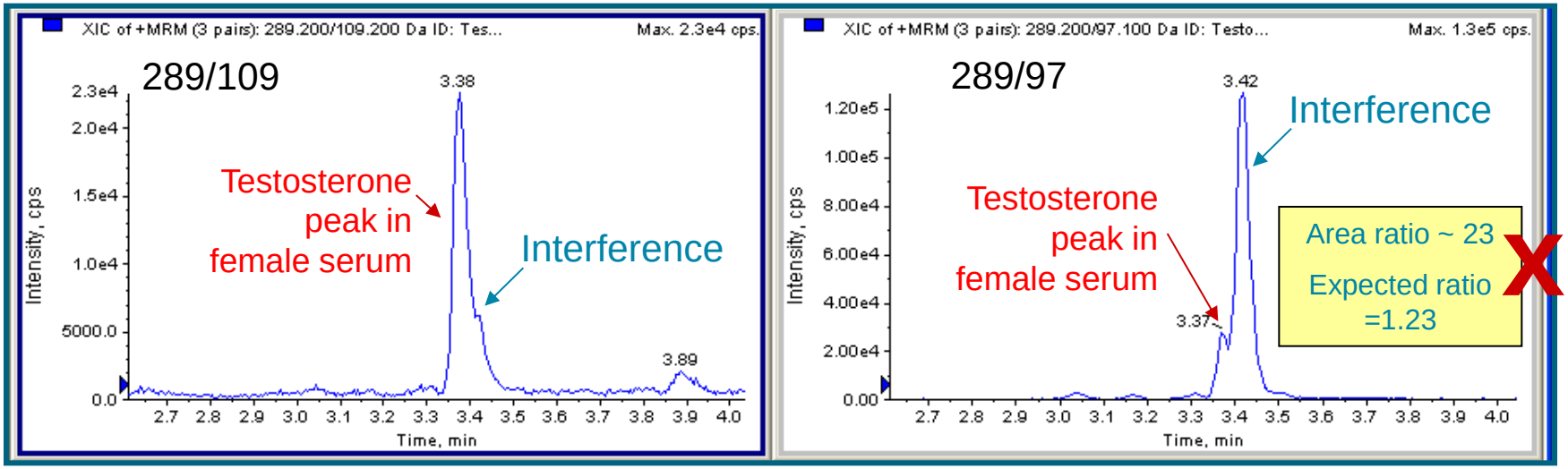
SelexION™ device “on”



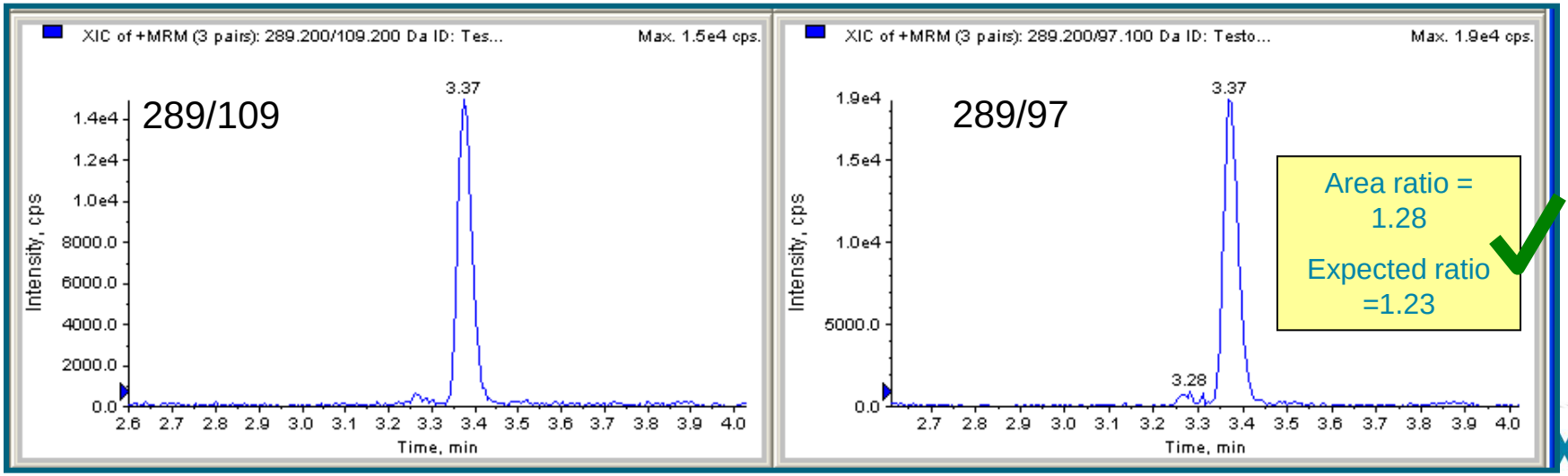
a) There is a significant interference in the MRM channel for DHEA, caused by the presence of testosterone

b) When the SelexION™ ion mobility device is employed the interference is completely removed. Thus there is no longer a need for chromatographic separation, and run-times can be reduced dramatically.

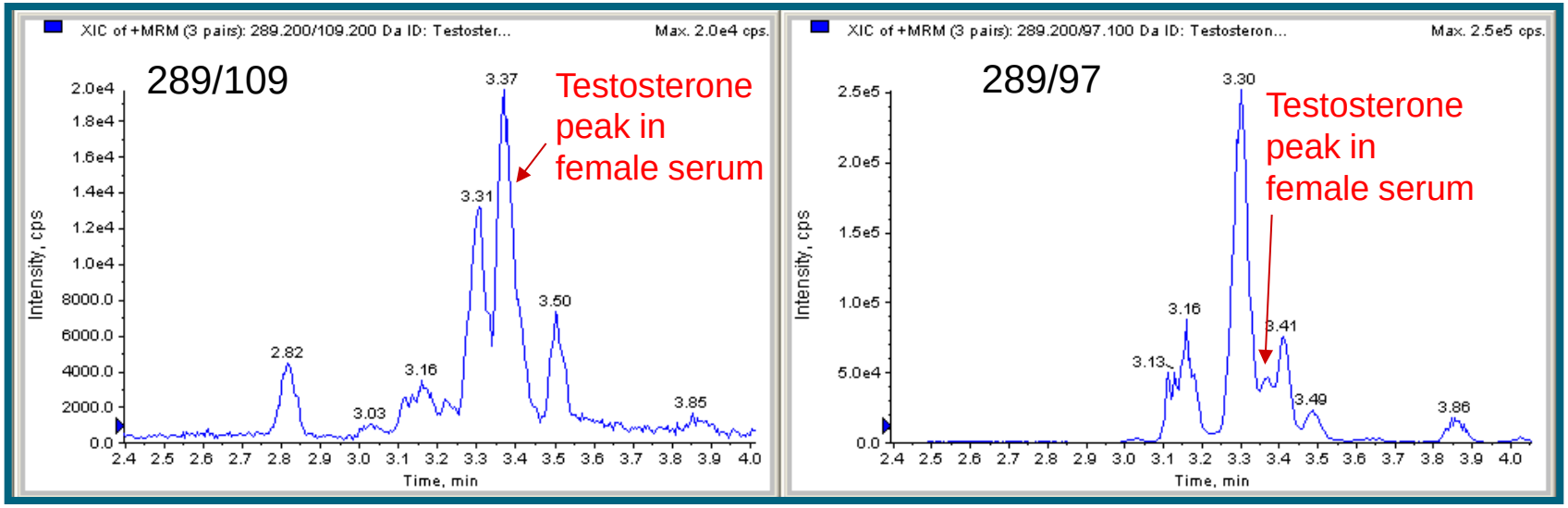
Liquid-Liquid Extraction (LLE) – LC-MS/MS



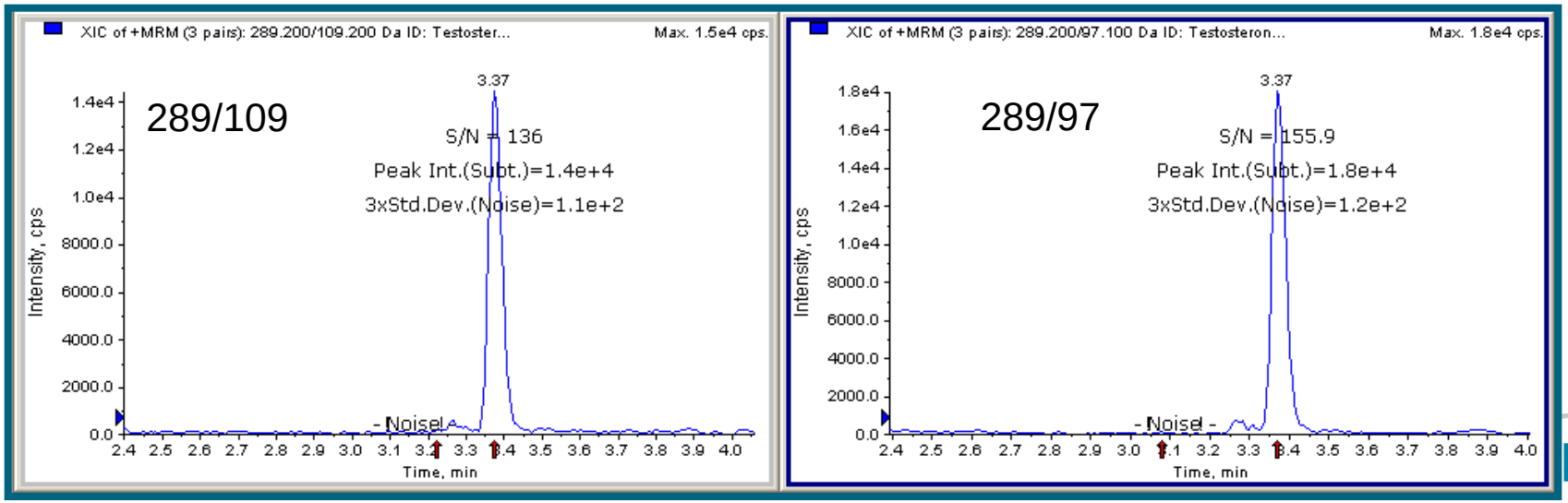
Liquid-Liquid Extraction (LLE) – LC-MS/MS with SelexION™ Technology



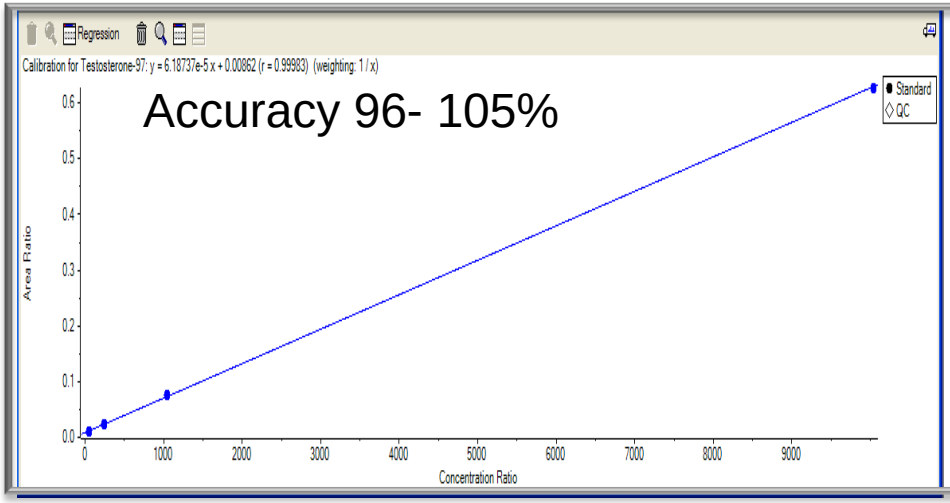
Protein Precipitation (PPT) – LC-MS/MS

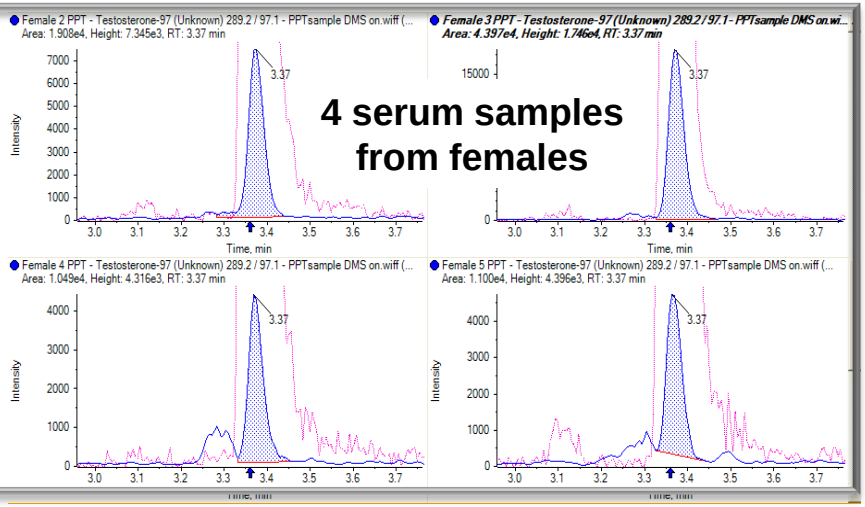


Protein Precipitation (PPT) – LC-MS/MS with SelexION™ Technology



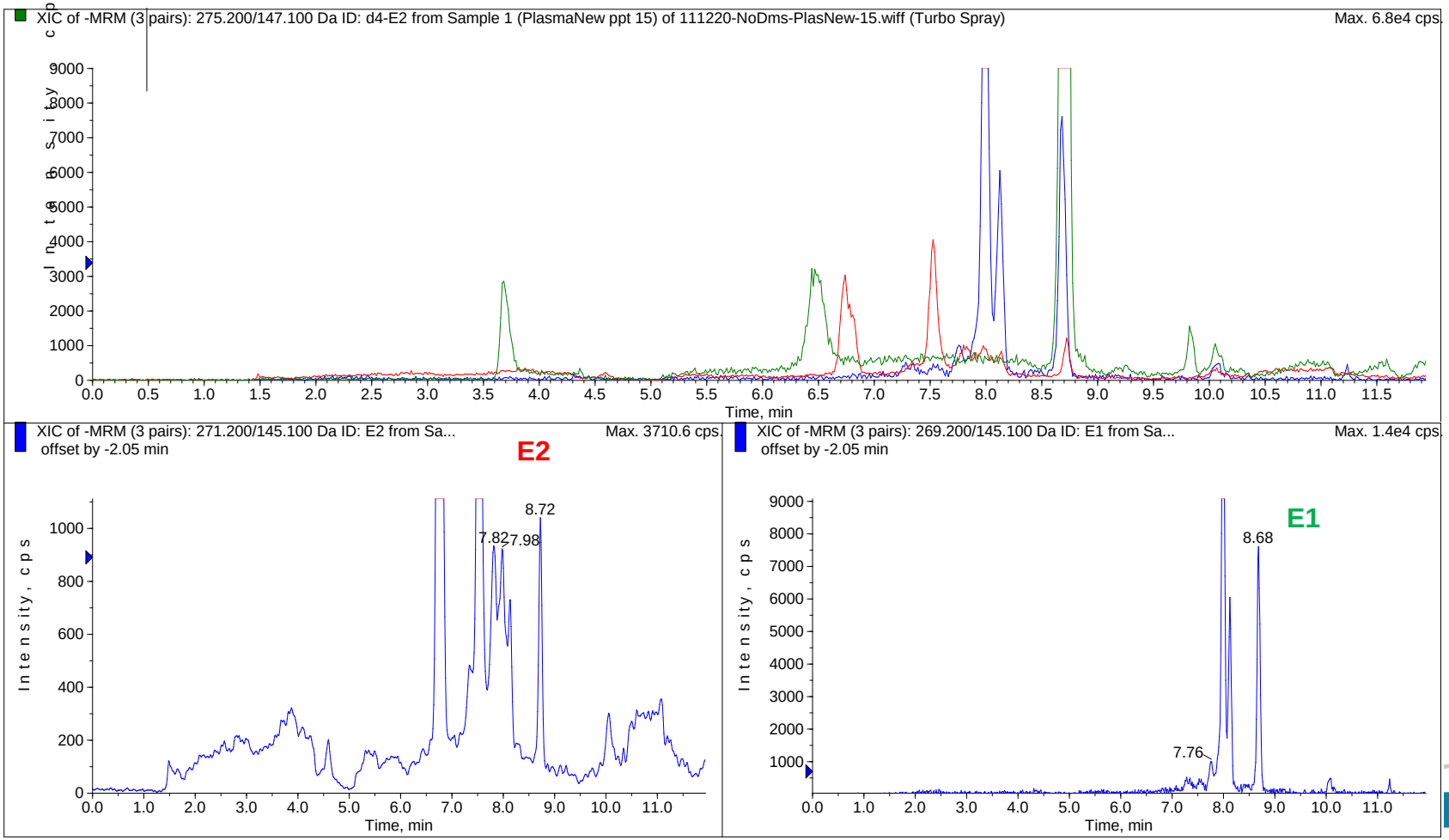
STD Curve in Serum Sample using the SelexION™ Ion Mobility Device (sample preparation = PPT)



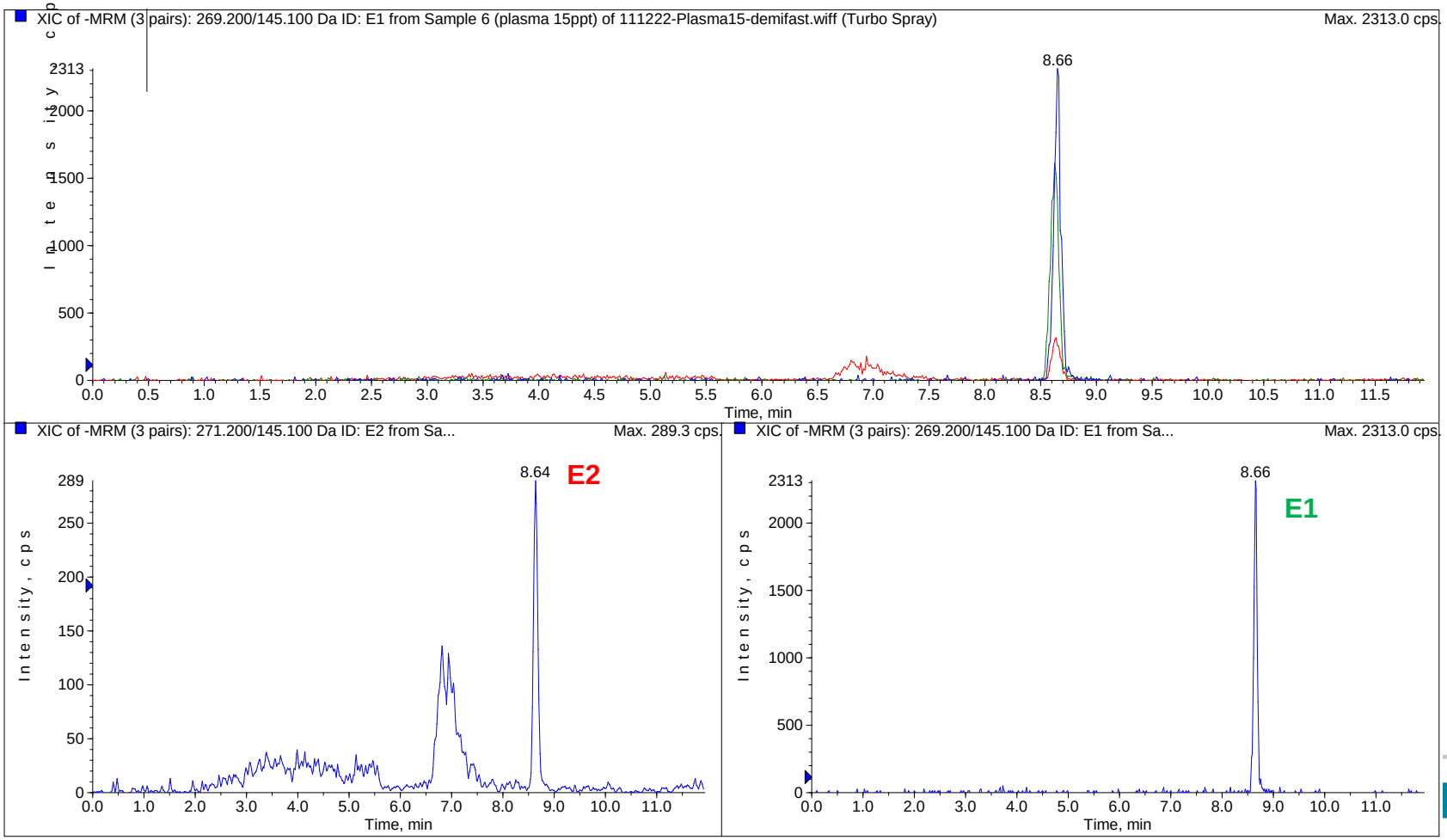


Sample	Neat (N=28)	Serum PPT (N=25)	Serum LLE (N=25)
Average Ion Ratio (97/109)	1.23	1.28	1.24
STDEV	0.04	0.07	0.04
%CV	3.44	5.47	3.12

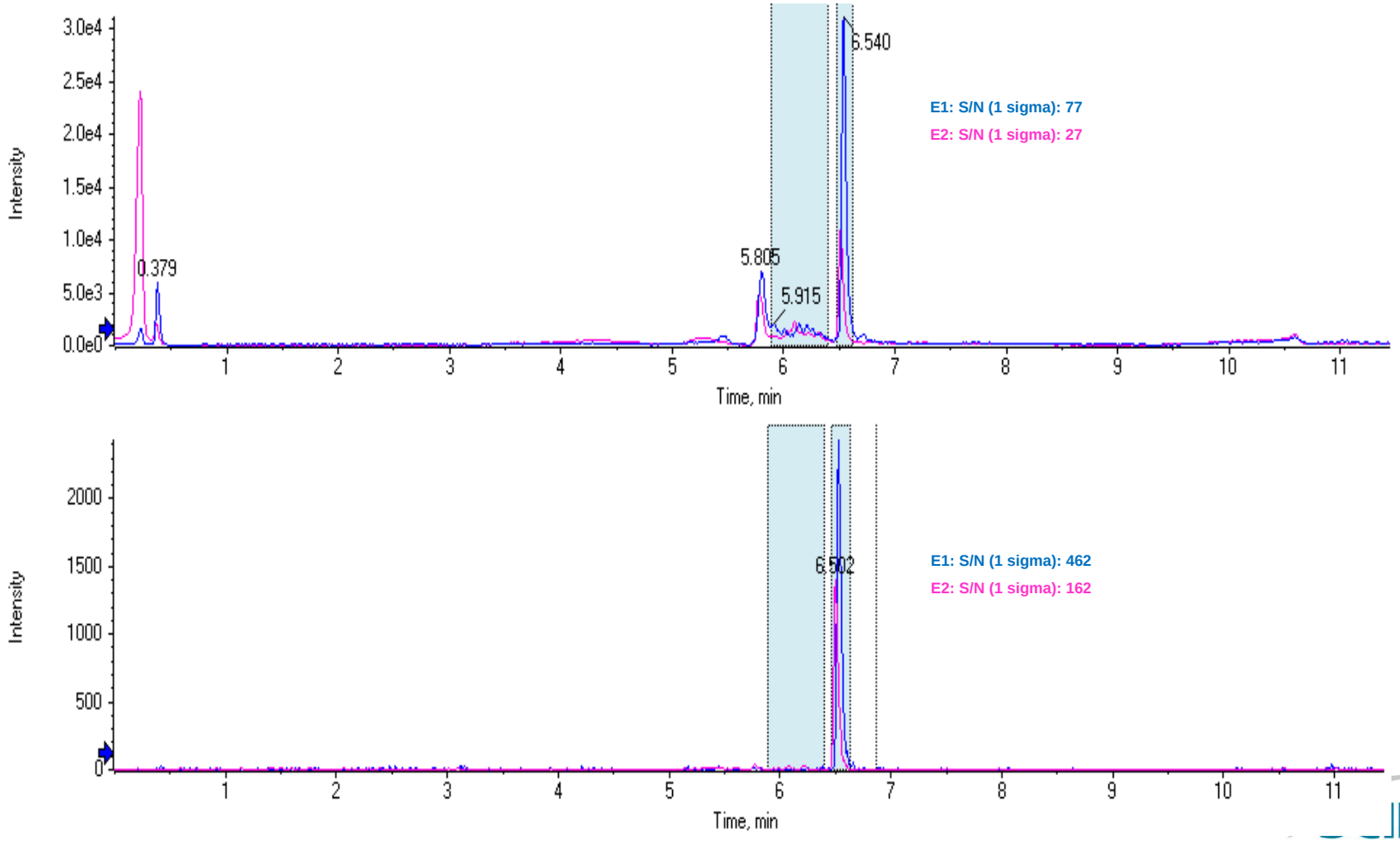
SelexIon performance for Estrogen Analysis
Serum (40 uL) by PPT and no SelexIon™



SelexIon performance for Estrogen Analysis
Serum (40 uL) by PPT with SelexIon™



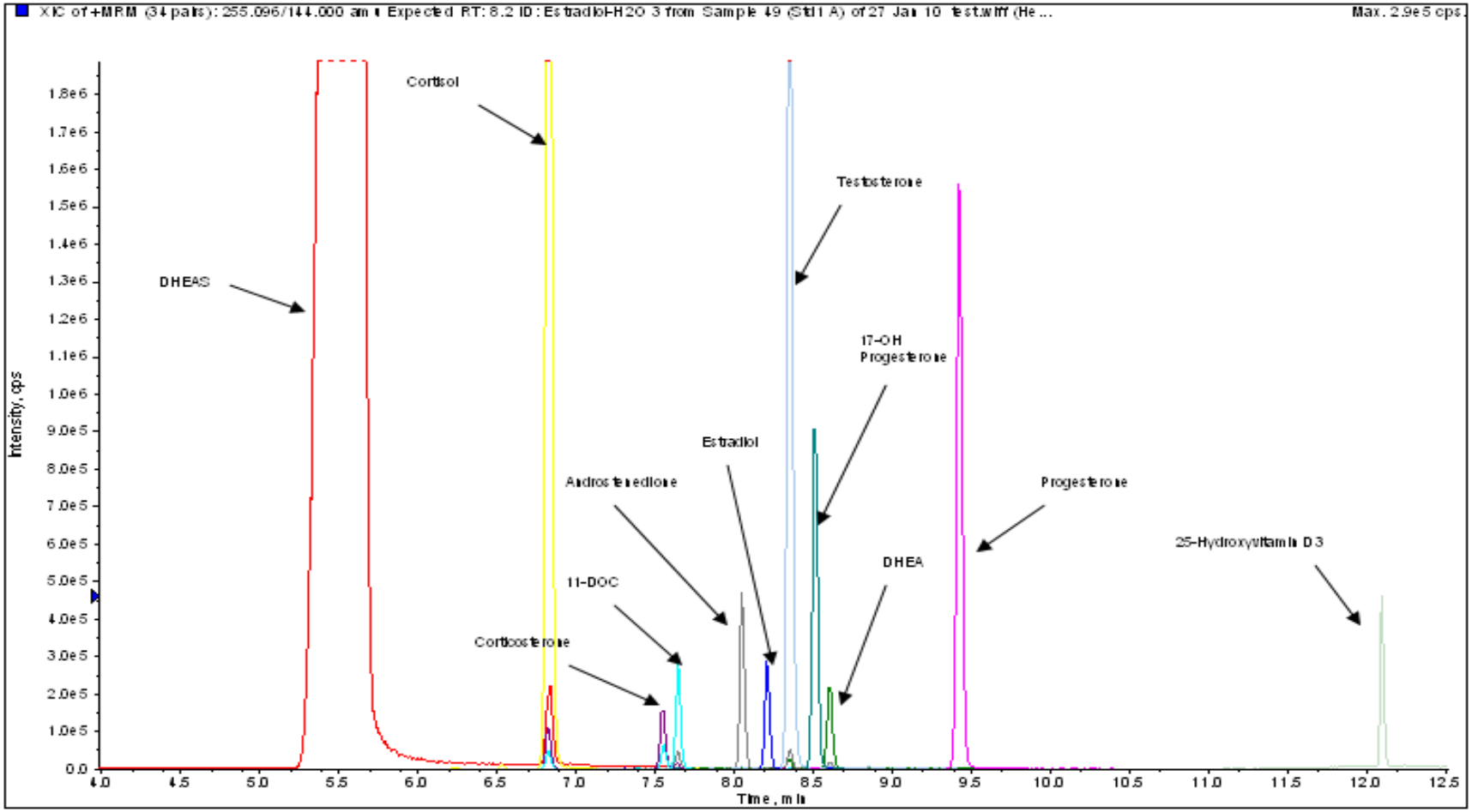
Spiked plasma sample analyzed without SelexION™ (A) and with SelexION™ Technology (B). The light blue marked regions defined where signal and noise were collected.



Steroid Panel iMethod™

- Increasing the number of compounds analysed in an assay is a perfect way of maximising potential return on investment
- LC-MS/MS is ideally suited to this approach
- The SCIEX Steroid Panel iMethod™ allows simultaneous analysis of 10 commonly measured steroids and also 25-Hydroxyvitamin D3
- Sample preparation is simple protein precipitation with dilution
- LC separation is required in order to keep the sample preparation to a minimum, run time is 12.5 minutes
- In order to achieve the required sensitivity for all compounds assayed, this method is designed for use on the API5000™ and Triple Quad 5500™ instruments

Steroid Panel iMethod™, Extracted sample at ULOQ



Performance Data, Steroid Panel iMethod™

Compound	Q1 (amu)	Q3 (amu) Quantifier	Q3 (amu) Qualifier	Limit of Quantitation (ng/mL)	%CV at LOQ (n=4)	Accuracy at LOQ
DHEAS	271.1	213.1	253.2	25	4.58	100
Cortisol	363.1	115.1	121.0	5	3.11	102
11-Deoxycortisol	347.1	97.0	109.0	0.025	13.0	98.7
Androstenedione	287.1	109.0	97.1	0.025	3.09	102
Estradiol (water loss)	255.1	144.0	159.1	0.1	8.63	101
Testosterone	289.1	109.0	97.0	0.1	7.56	96.4
17-OH-Progesterone	331.0	97.0	109.0	0.1	4.40	101
DHEA	271.2	213.1	253.2	0.1	4.40	101
Progesterone	315.1	109.0	97.0	0.1	8.45	98.6
25-Hydroxyvitamin D3	383.3	91.0	365.3	6.0*	7.80	98.2
Corticosterone	347.1	121.1	91.0	0.05	8.92	94.9

* Corrected for endogenous levels in serum.

- SCIEX offers several solutions for steroid analysis
- Various forces will determine which approach is needed
  - Analysis of less challenging steroids
  - Requirements for analysis of multiple compounds
  - Sensitivity requirements on challenging steroids
  - Desire for the convenience of a kit-based assay
  - Multiplexing with additional applications
- From this, the appropriate method and instrument can be matched to the analytical requirements

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