



Fast separation of hydroxytestosterone isomers using chip-based FAIMS combined with mass spectrometry for high-throughput drug assays

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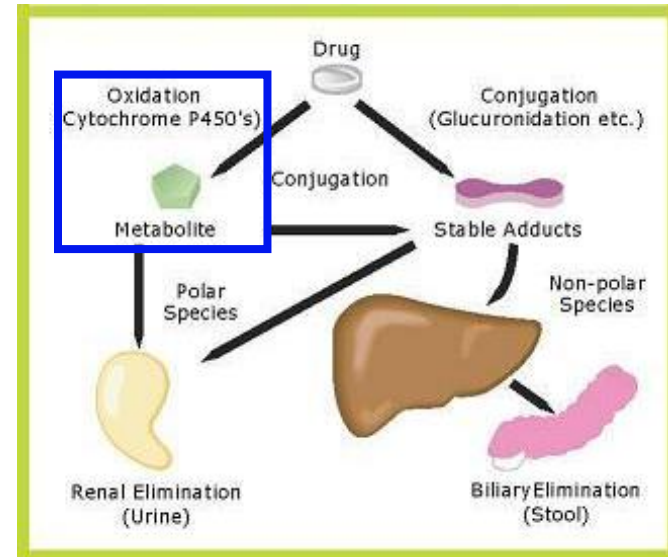
Vaughn Miller



Agilent Technologies

Drug Metabolism

- Elimination of a drug or its metabolites occurs by:
 - metabolism, usually by the liver or gut mucosa
 - excretion, usually by the kidneys and liver
- Hepatic elimination occurs primarily by the cytochrome P450 family (CYP) of enzymes
- The presence of other medications can alter metabolism significantly, which can lead to possible adverse side effects



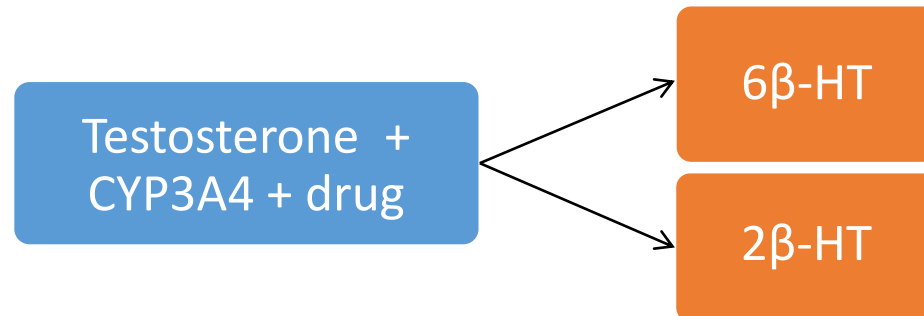
- As a result, investigation of drug-drug interactions is required as part of the approval process for a new drug

P450 inhibition (CYP3A4)

- Testosterone is one of the FDA's preferred substrates for measuring inhibition of the enzyme CYP3A4 (a standard measure of drug-drug interactions)
- 6 β -hydroxytestosterone (6 β -HT) is the major metabolite of testosterone through CYP3A4 action

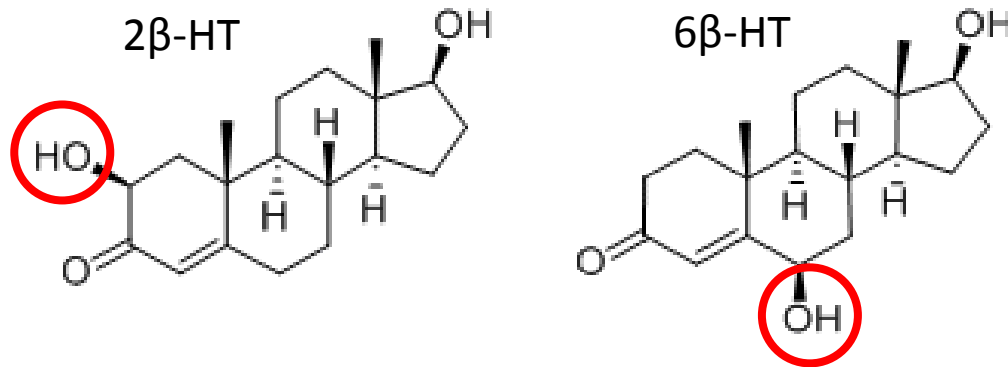


- However some drug candidates promote formation of 2 β -HT while inhibiting 6 β -HT



The Challenge: determination of 6β -HT concentration in the presence of 2β -HT

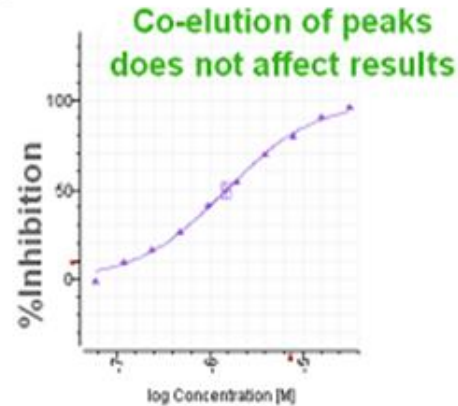
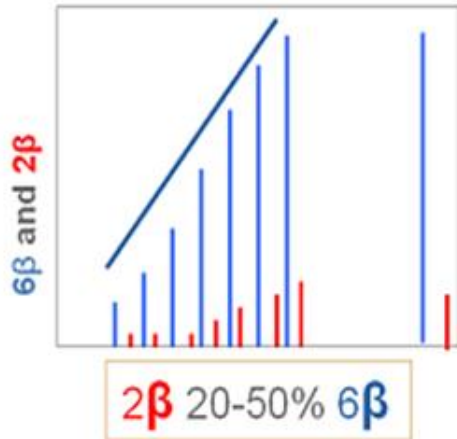
- 2β -HT and 6β -HT are isobaric with no unique MRM transition to distinguish them



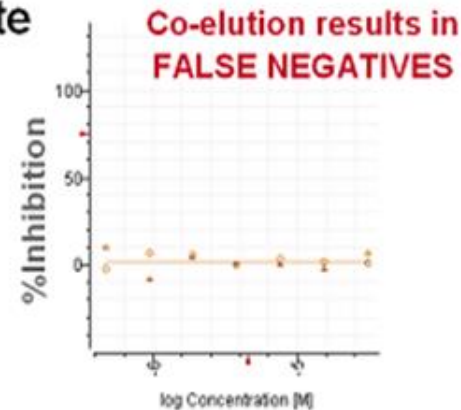
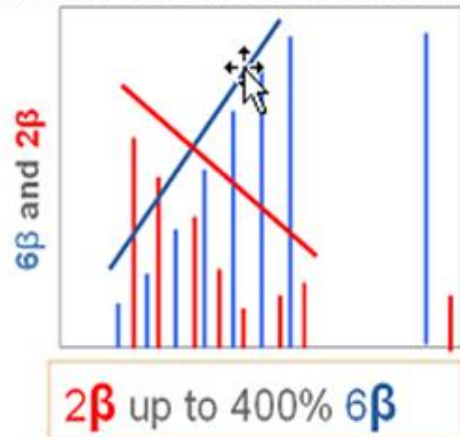
- LC-MS methods are currently used to separate these isomers
- A faster method for testing potential drug candidates is highly desirable
 - Online-SPE methods (e.g. RapidFire)

Effect of 2 β -HT interference on inhibition measurements

When LC-MS and RF-MS correlate well



When LC-MS and RF-MS don't correlate

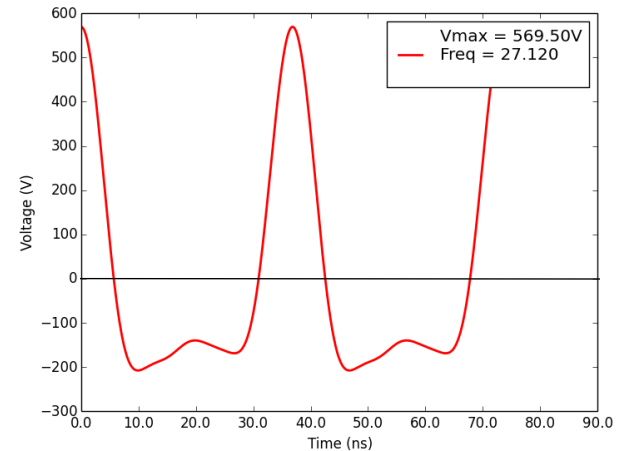
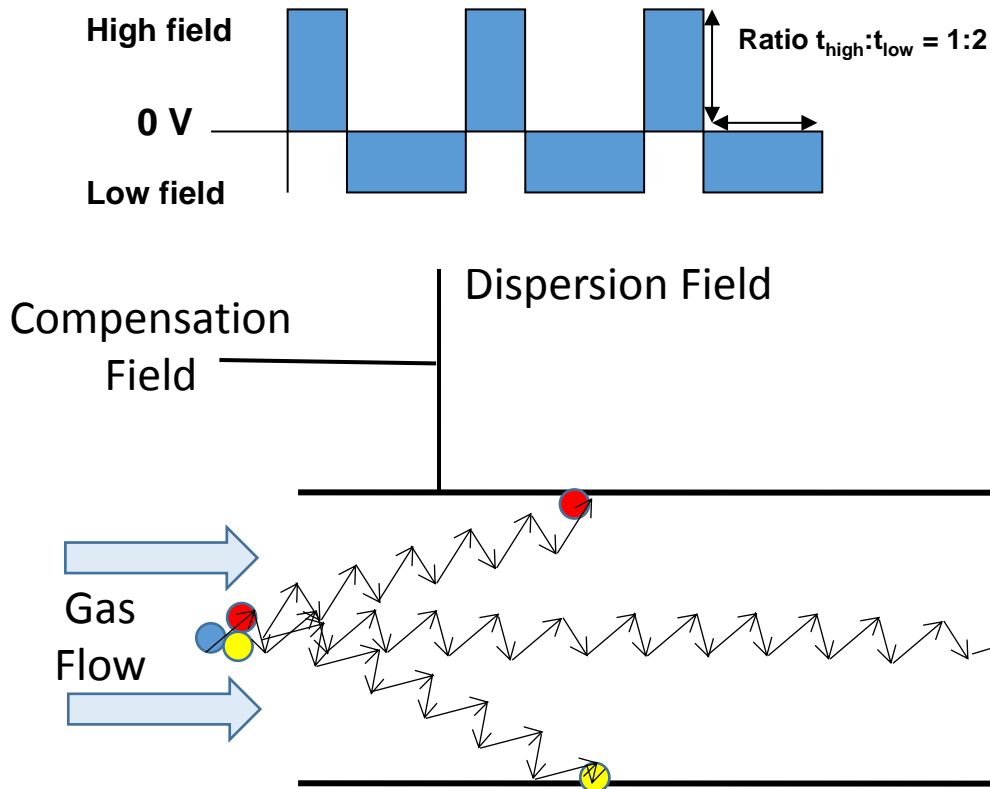


Decreasing [Inh] \rightarrow

P450 inhibition determined using change in 6 β -HT response, but hidden by 2 β -HT interference.

FAIMS basics

- FAIMS subjects ions to an alternating asymmetric waveform of low and high electric fields of opposite polarities, known as the dispersion field (DF)
- Ion mobility (K) has a non-linear behaviour in high electric fields

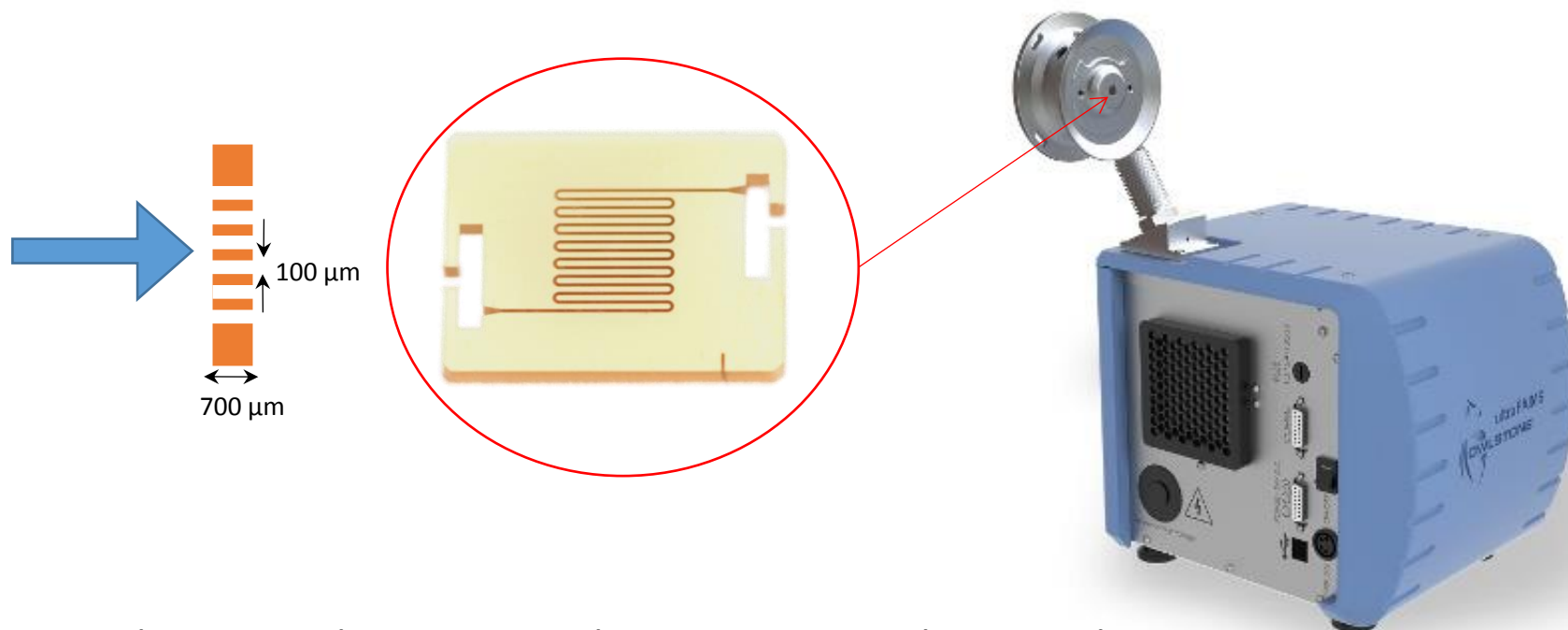


Separation depends on:

- Dispersion field
- Temperature
- Pressure
- Carrier gas composition

Experimental Approach - Instrumentation

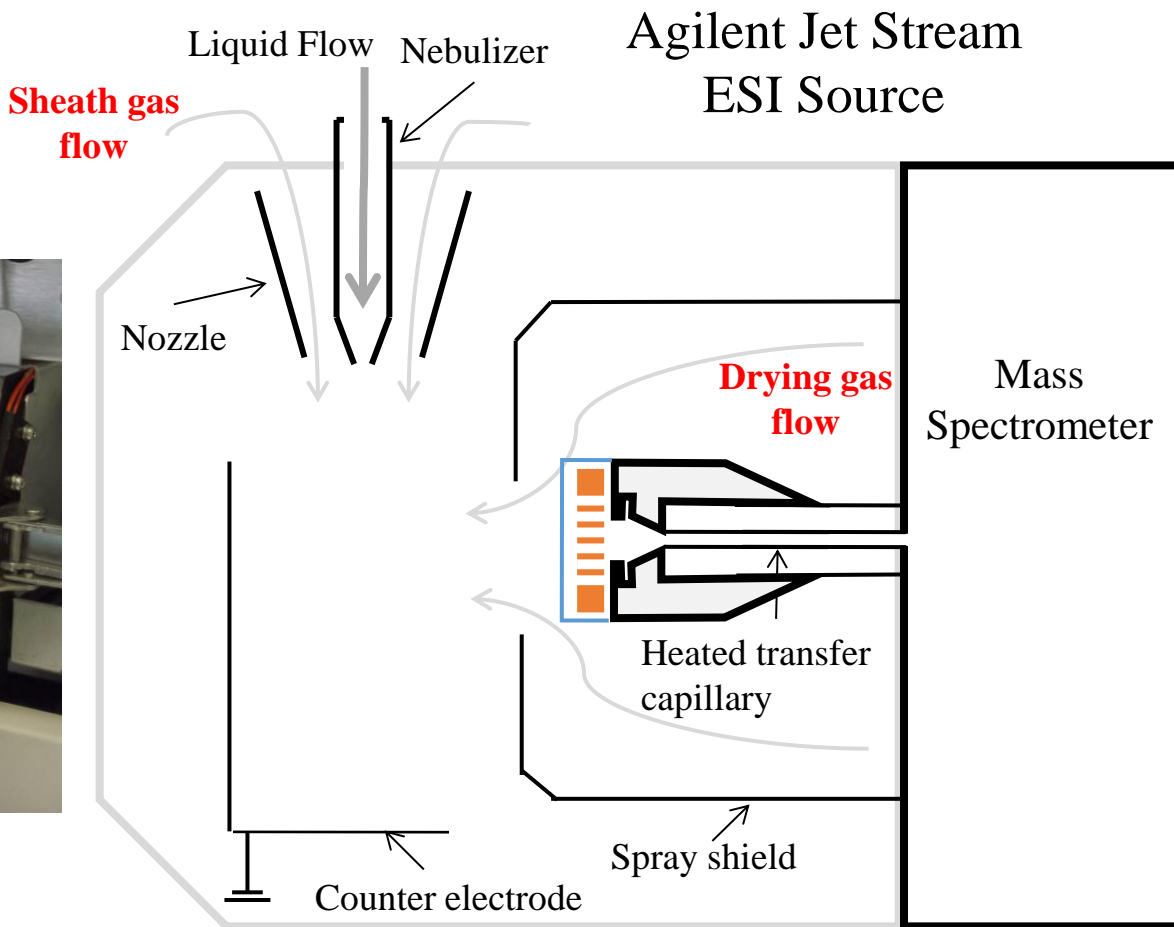
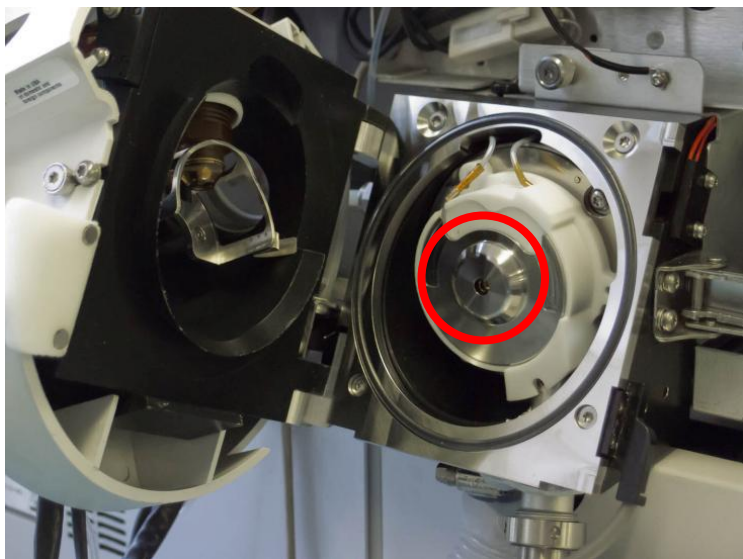
- Owlstone UltraFAIMS system



- In UltraFAIMS, the separation device is a microscale FAIMS chip
- The small scale enables very fast separation, due to
 - short ion residence times $<200\mu\text{s}$ (MS dependent)
 - low-voltage drive electronics
- Separation speed is compatible either with LC timescales or with online SPE sample introduction

Experimental Approach - Instrumentation

- For this study, an Owlstone UltraFAIMS system was used, interfaced with an Agilent 6230 TOF and an Agilent 6460 triple quad MS



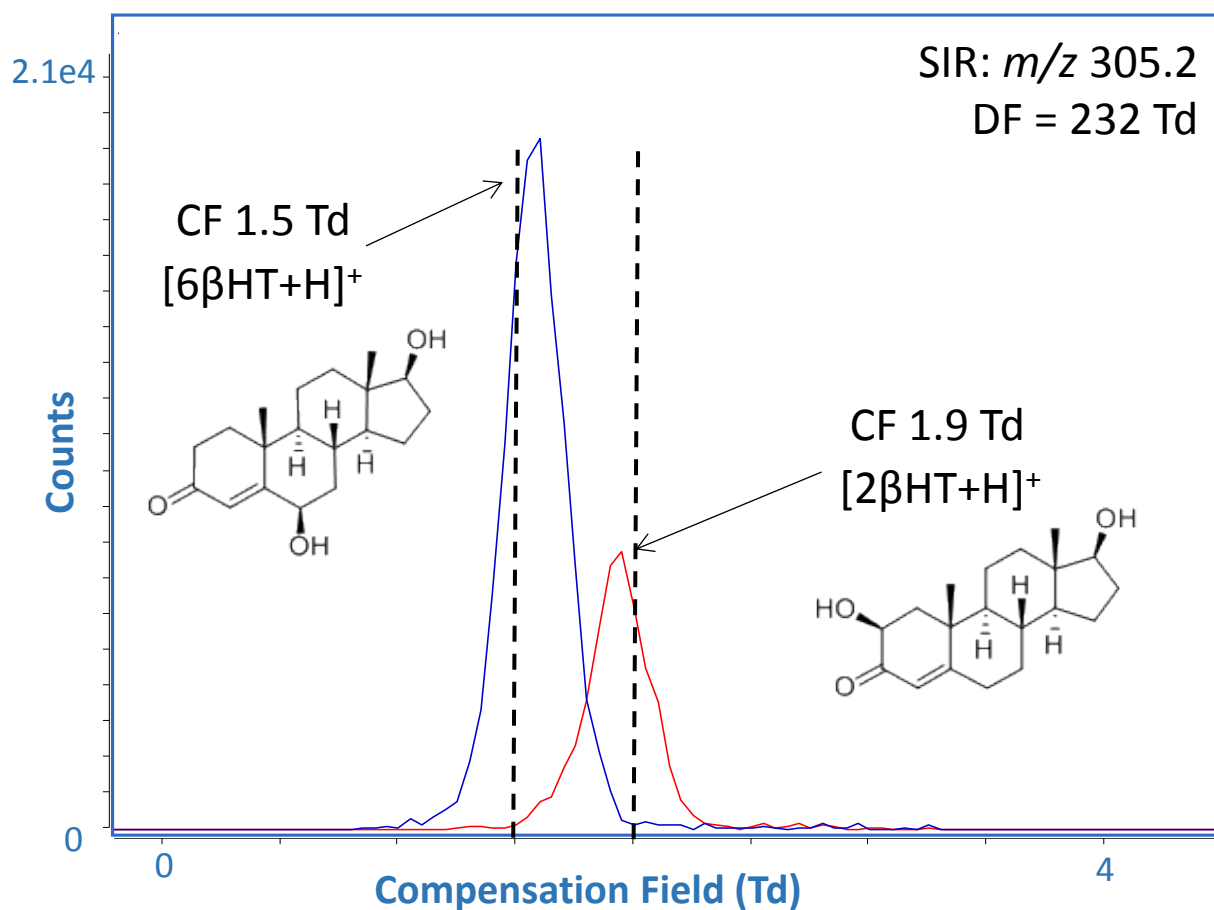
- UltraFAIMS CF can be scanned to produce a spectrum or held in static mode for better quantitation

Aim of study

- Can FAIMS be used transmit only 6β -HT and enable use of high-throughput techniques?
- To test the feasibility of using FAIMS with high-throughput techniques
 - Establish a separation
 - Evaluate compatibility
 - Assess quantitative performance

Initial optimization experiments

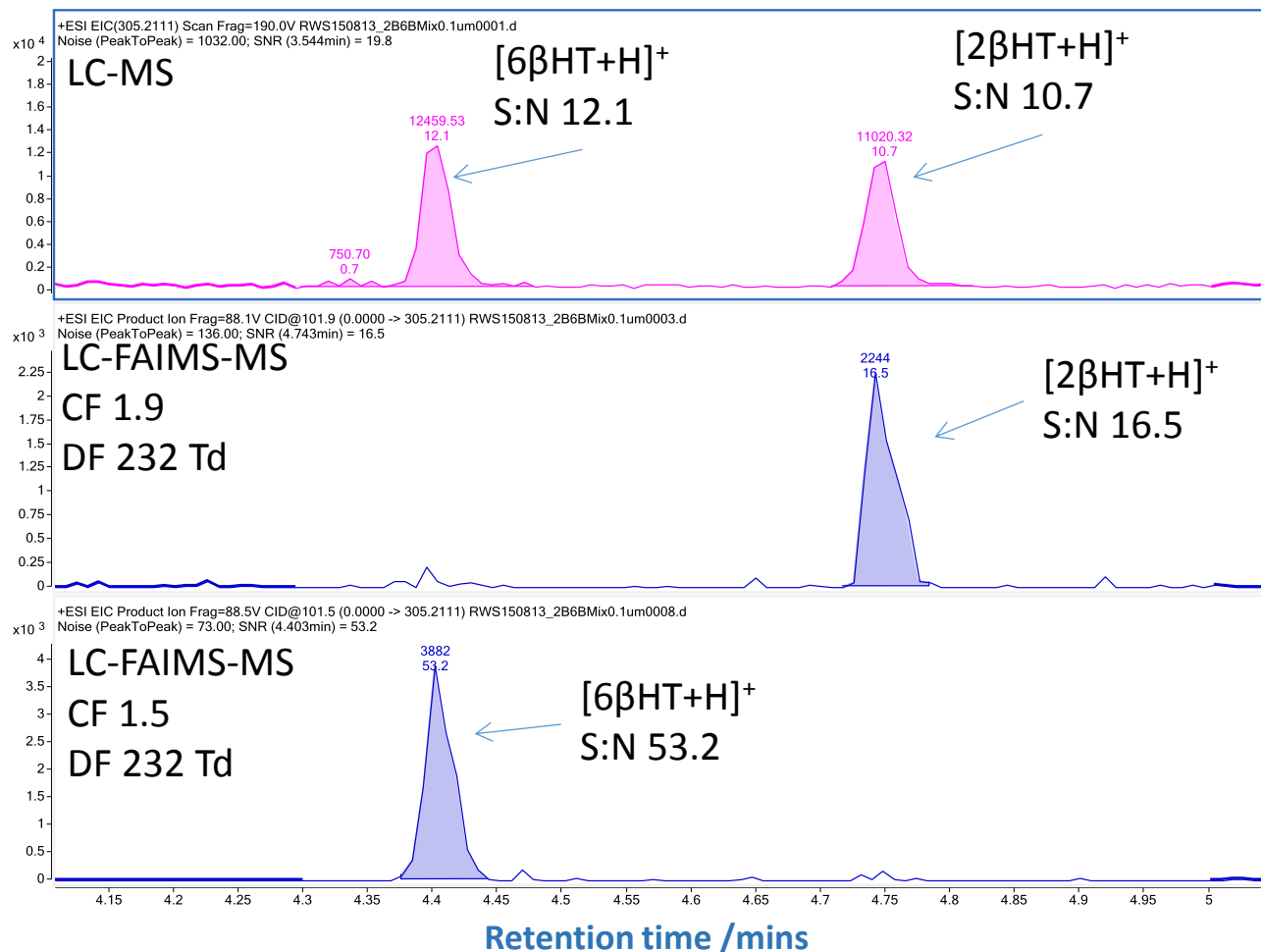
Direct infusion (individual standards of 2 β -HT and 6 β -HT, 5 μ M) on ESI-FAIMS-TOF MS



- Best separation achieved in dry N₂ with 50°C drying gas and 250°C sheath gas

Validation of Separation with LC-FAIMS-MS/MS

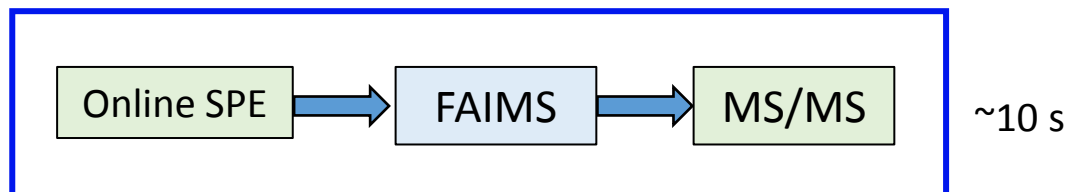
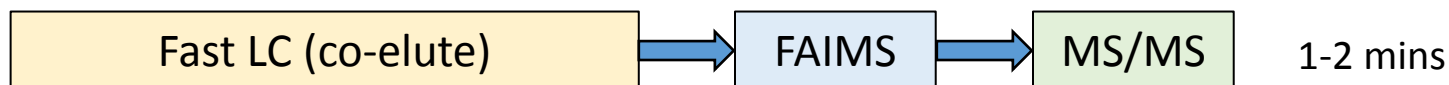
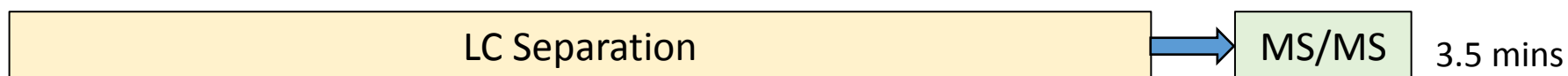
Mixture of 2 β -HT and 6 β -HT (0.1 μ M) injected onto C18 column



- Able to use FAIMS to select which of the metabolites are detected

Is this separation useful?

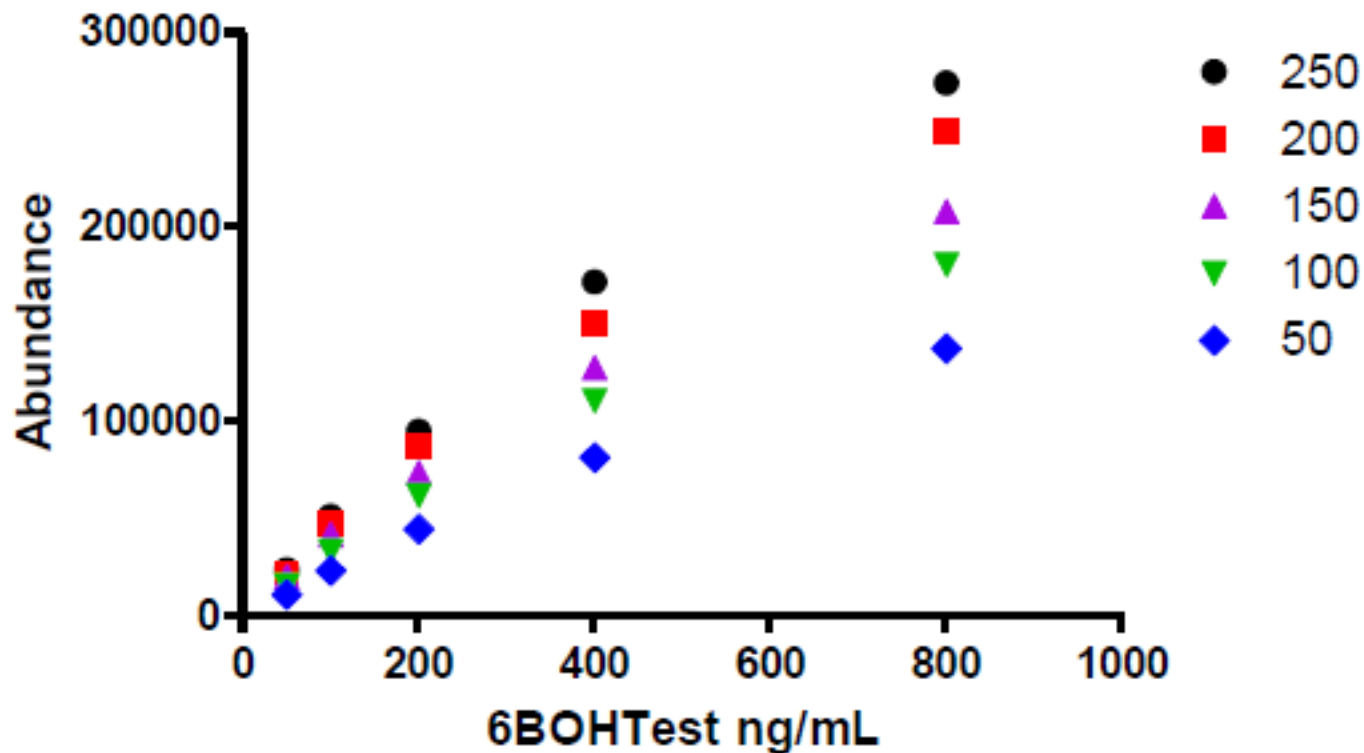
- Goal was to look at high-throughput workflows
- Given the separation capabilities, the options were to:
 1. Shorten LC separation using a quick gradient to remove salts prior to mass spectral detection and use FAIMS to separate the co-eluting isomers
 2. Lose LC completely, remove salts via SPE and use FAIMS to separate isomers prior to mass spectral analysis



- Our co-authors were interested in the latter option so pursued this area of study

Effect of temperature on RapidFire methods

The standard RapidFire method uses much higher source temperatures (300-350°C)

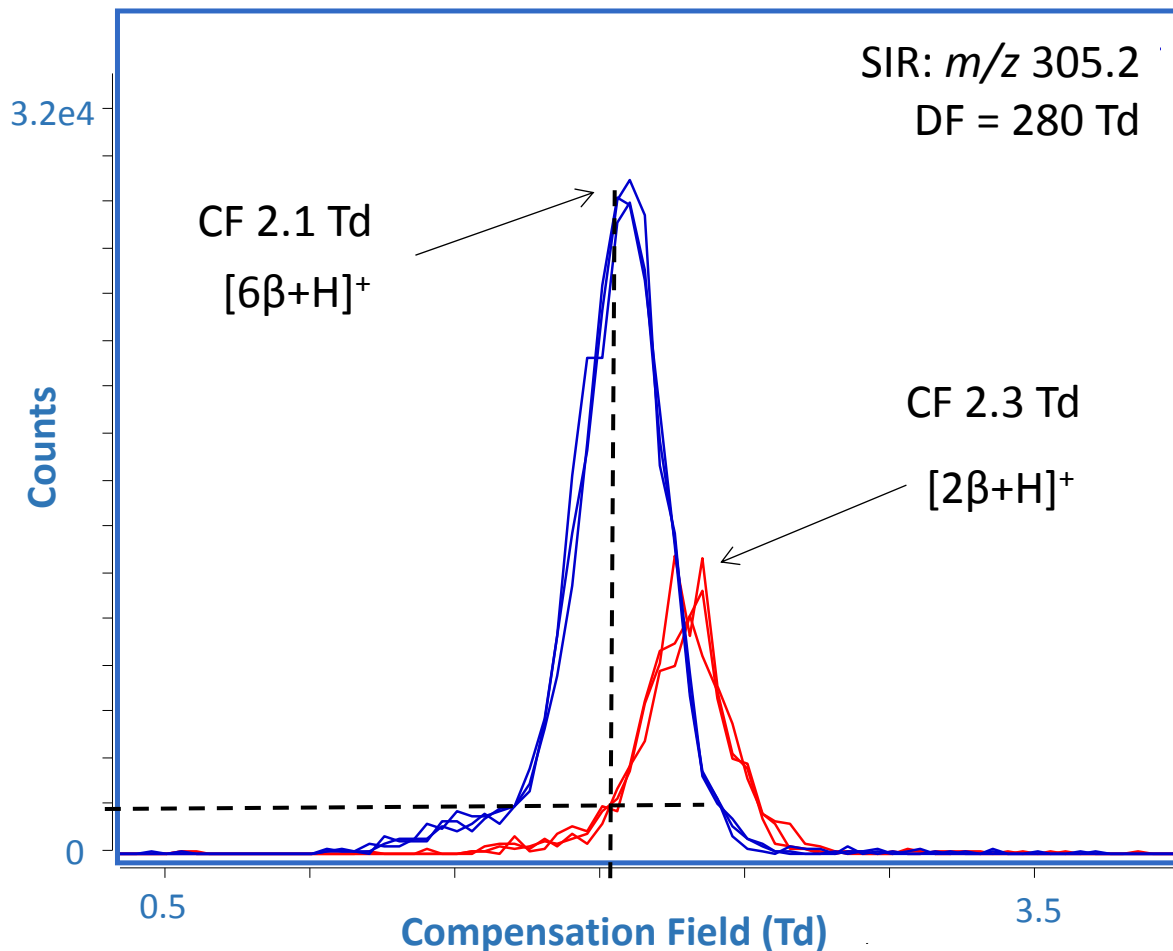


Source temperatures > 150°C advised for

- Sensitivity
- Robustness of method for routine use

Investigation of Increased Source Temperatures

Nitrogen drying gas temperature was increased to 150°C and sheath gas to 250°C on ESI-FAIMS-TOF MS

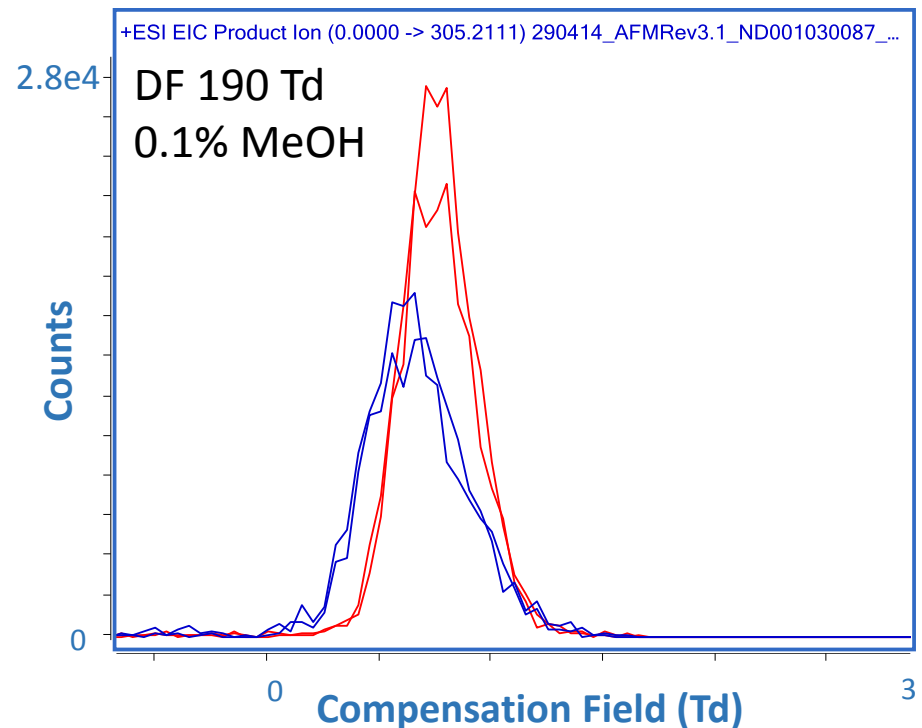
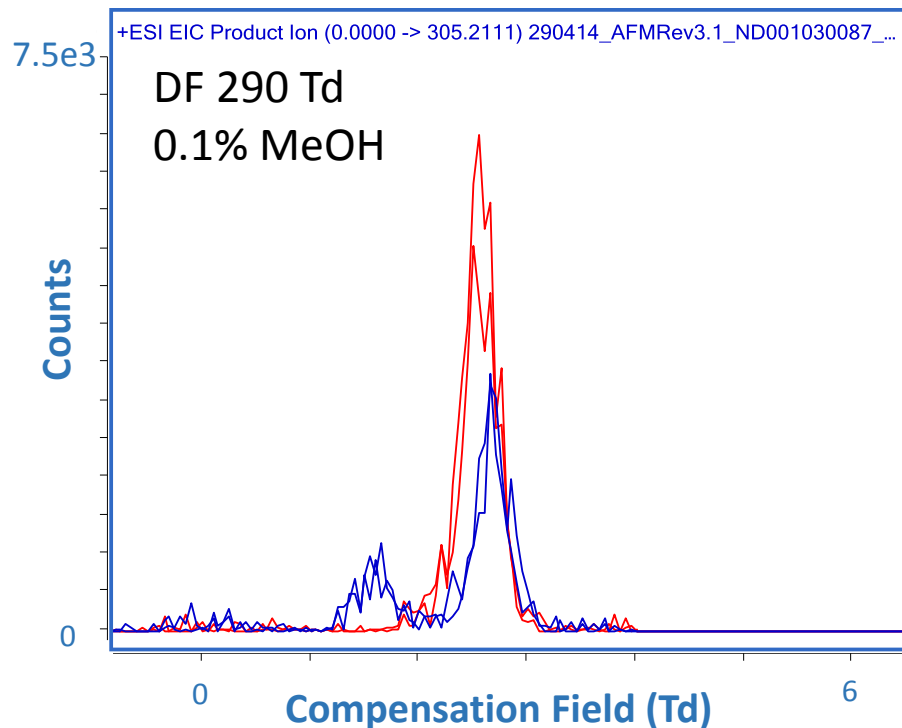


- Interference from other isomer is too high (> 5%)

FAIMS with Solvent Modifiers

The addition of solvent modifiers to affect separation is a common strategy with FAIMS

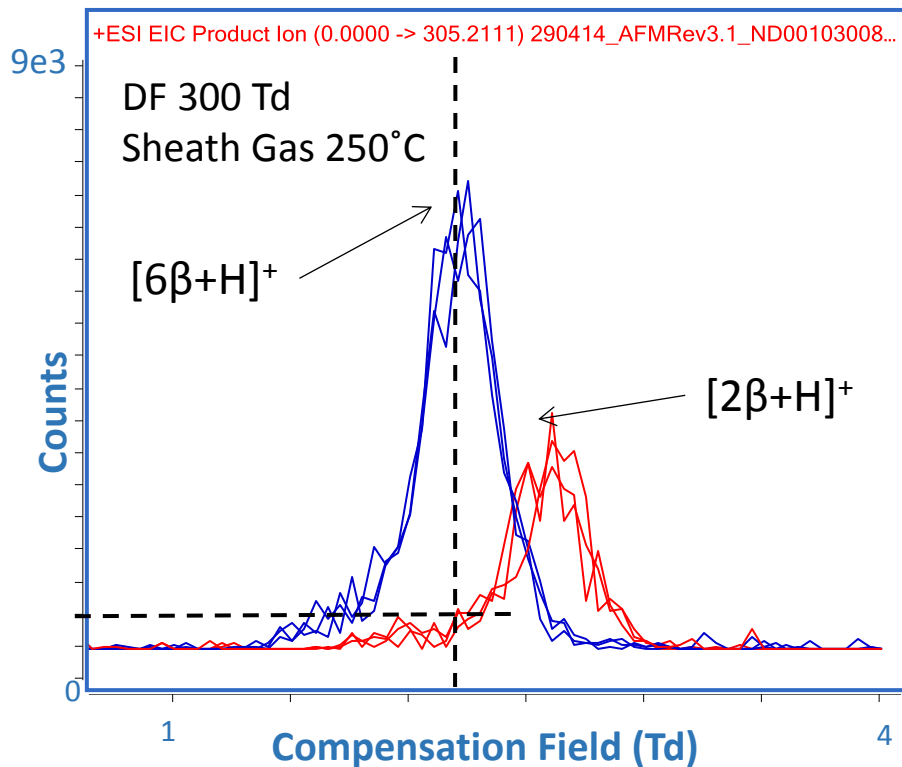
- A controlled evaporator system (Bronkhorst, UK) was used to deliver precise and accurate vapour concentrations into the drying gas



- With methanol at 0.1% and high DFs, we observed suppression and no improvement in separation
- Better response at lower DFs but metabolites not separated

FAIMS Optimization with Solvent Modifiers

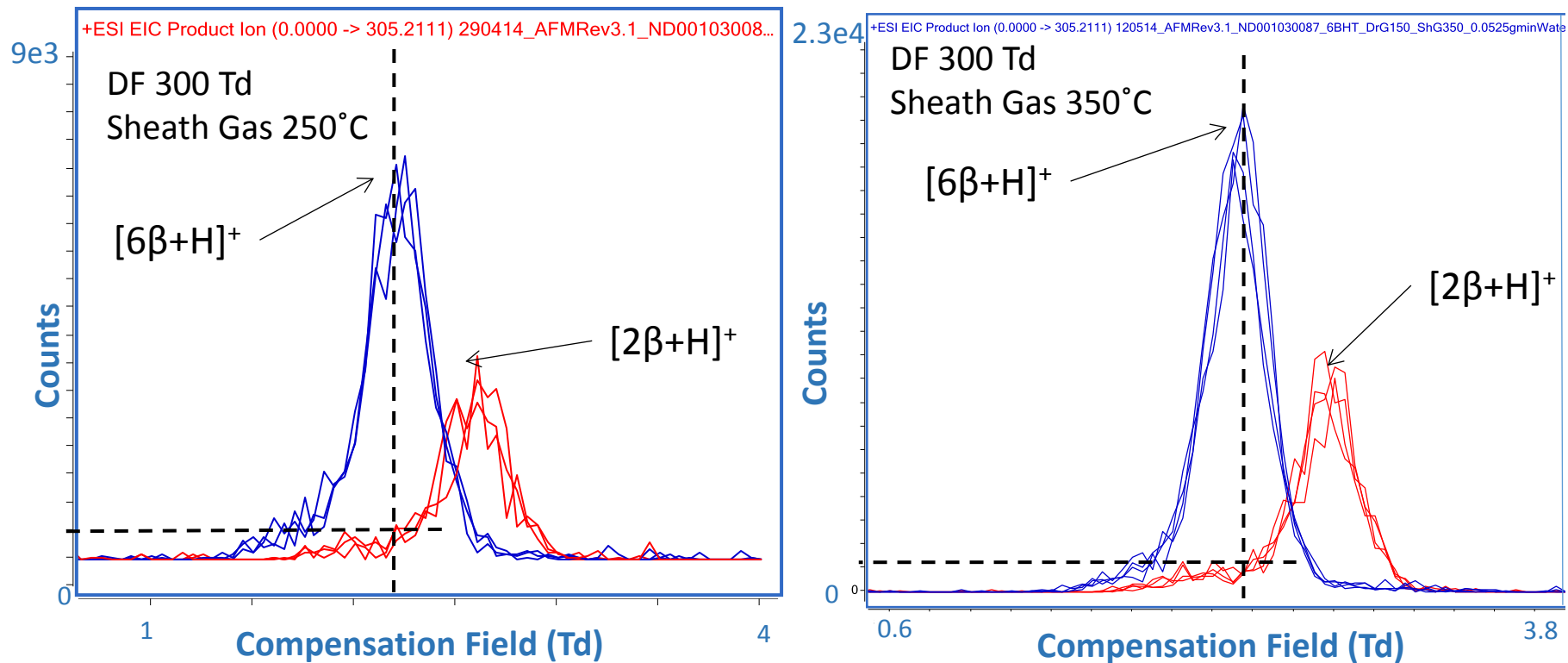
To reduce suppression, modifiers with lower proton affinity were investigated using water on ESI-FAIMS-TOF MS



- Water at 1% gave sufficient separation

FAIMS Optimization with Solvent Modifiers

To reduce suppression, modifiers with lower proton affinity were investigated using water on ESI-FAIMS-TOF MS



- Water at 1% gave sufficient separation
- Increasing sheath gas to 350°C gave 3-fold increase in 6β -HT signal (~7000 counts to ~20000 counts) (right)

FAIMS-QQQ Validation

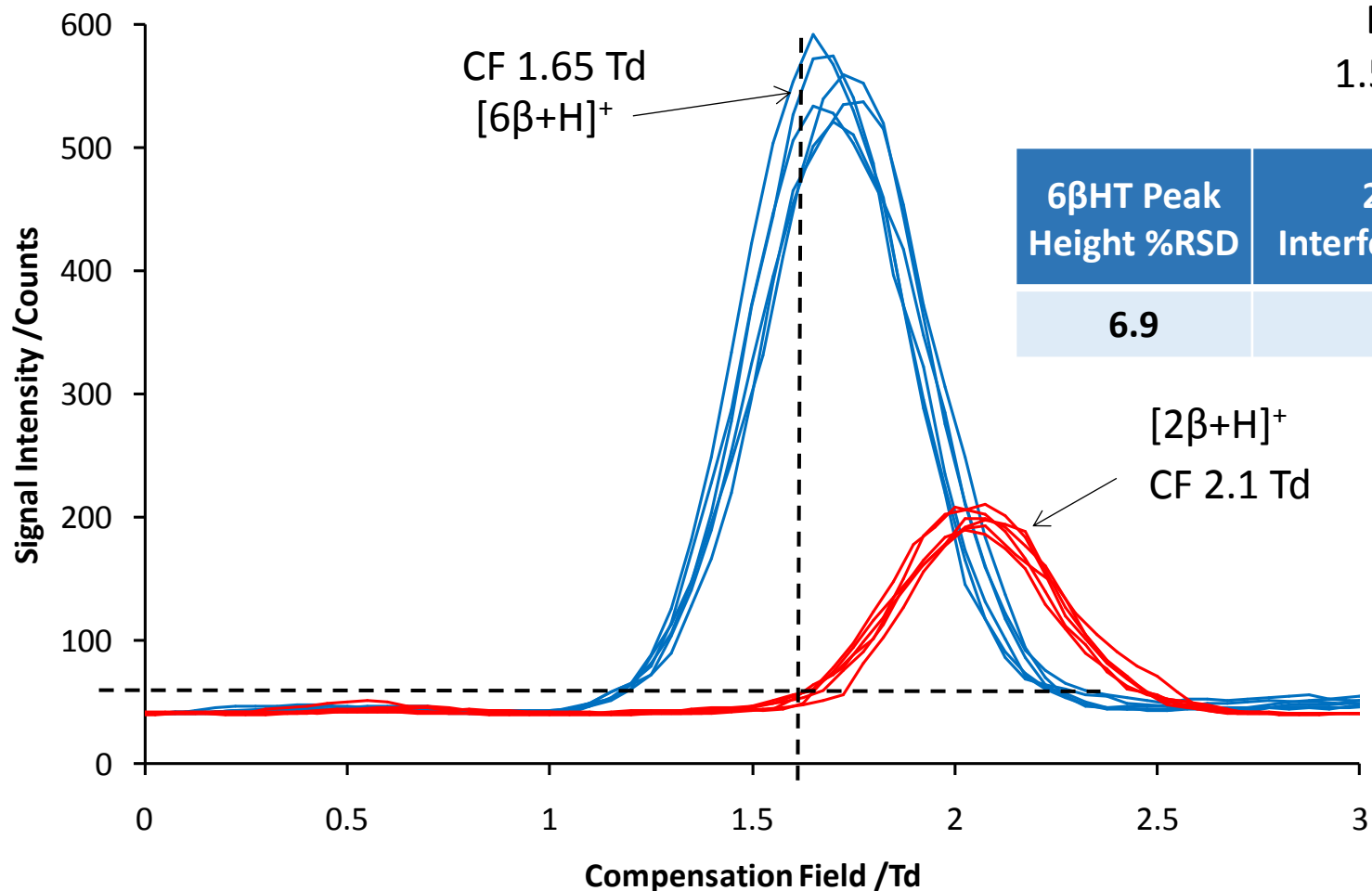
Once ultraFAIMS was installed on a triple quad, the separation conditions were checked and improved

MRM: m/z 305.2>269.4

DF 305 Td

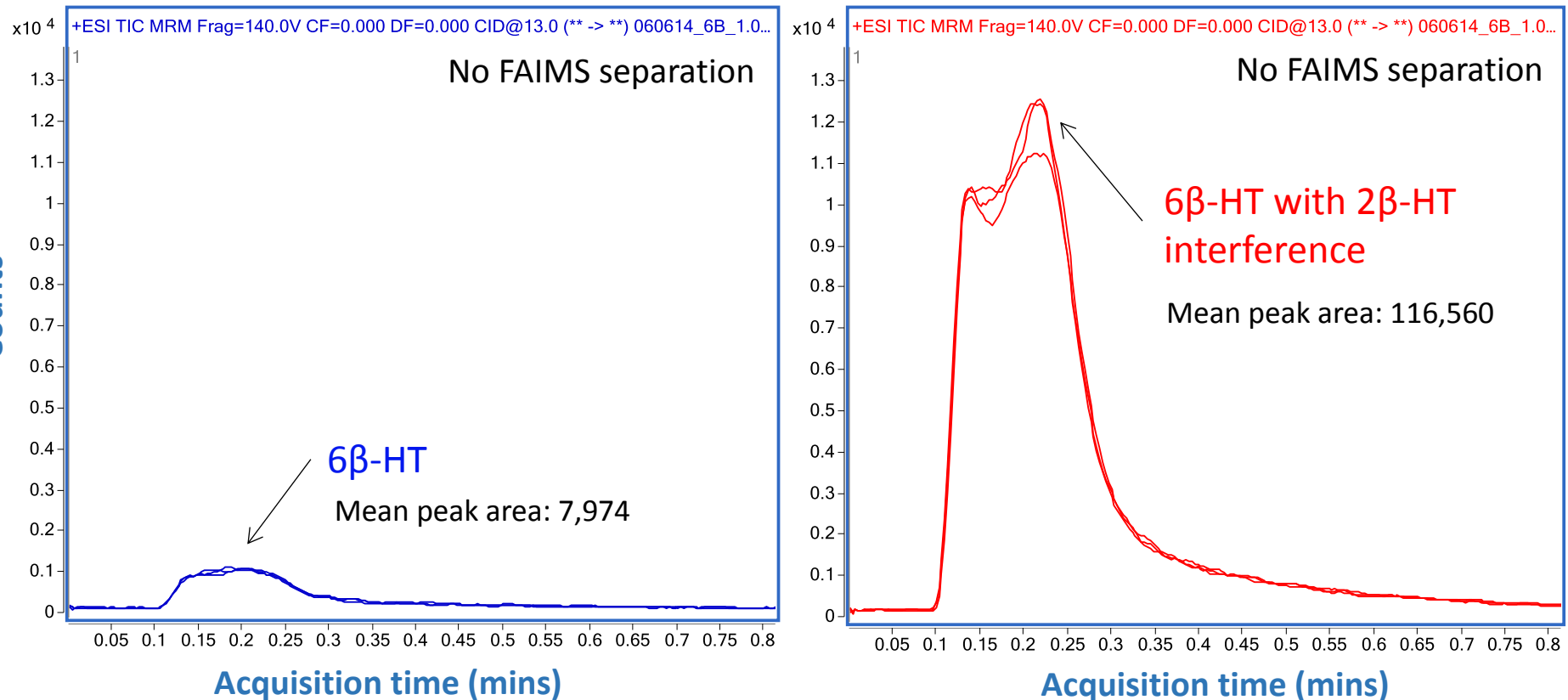
1.5% water.

6 β HT Peak Height %RSD	2 β HT Interference (%)
6.9	3.6



2 β -HT interference over 6 β -HT response

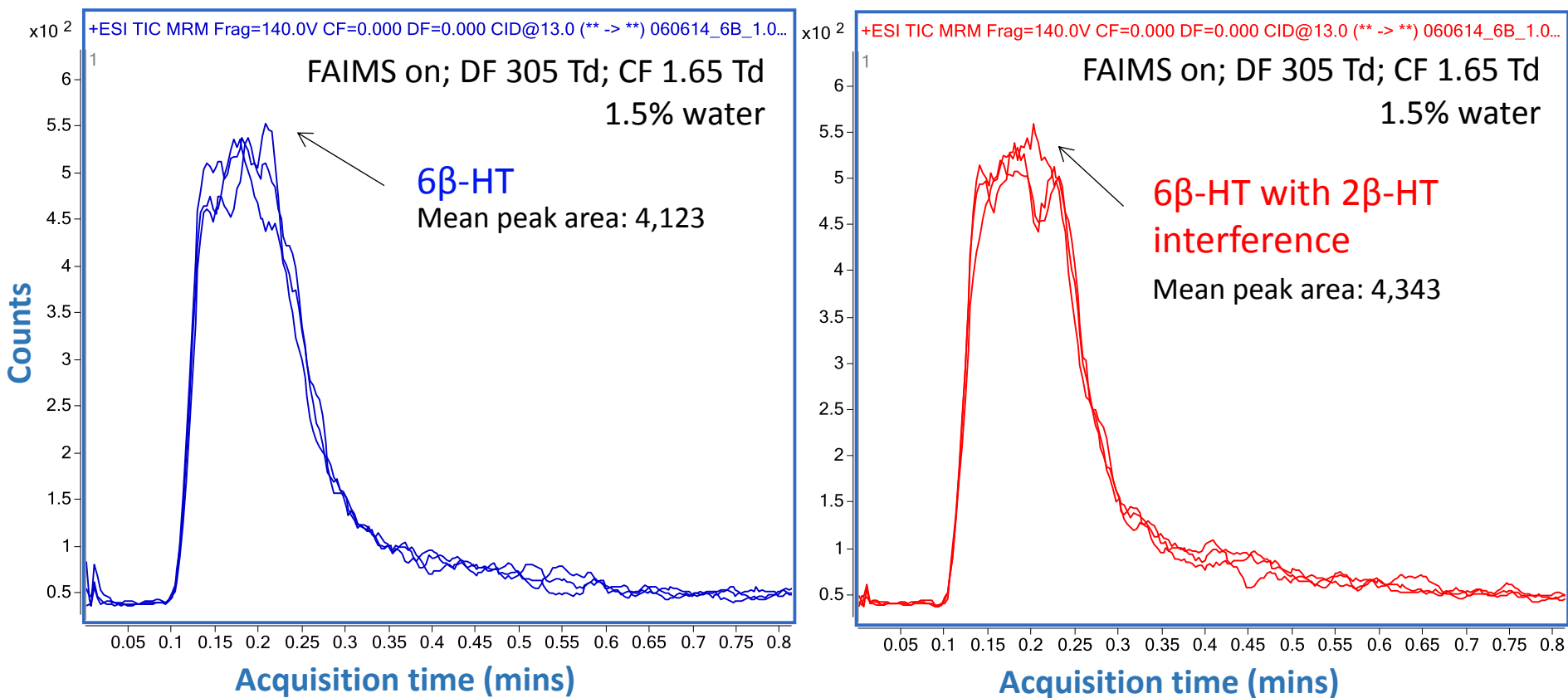
Loop injections of 6 β -HT (blue) and equimolar mixture of 6 β -HT and 2 β -HT (red) at 1 μ M, 0.2ml/min without FAIMS separation.



Large amount of interference from 2 β -HT compared to the response when only 6 β -HT present in sample.

Removal of 2 β -HT interference from 6 β -HT response

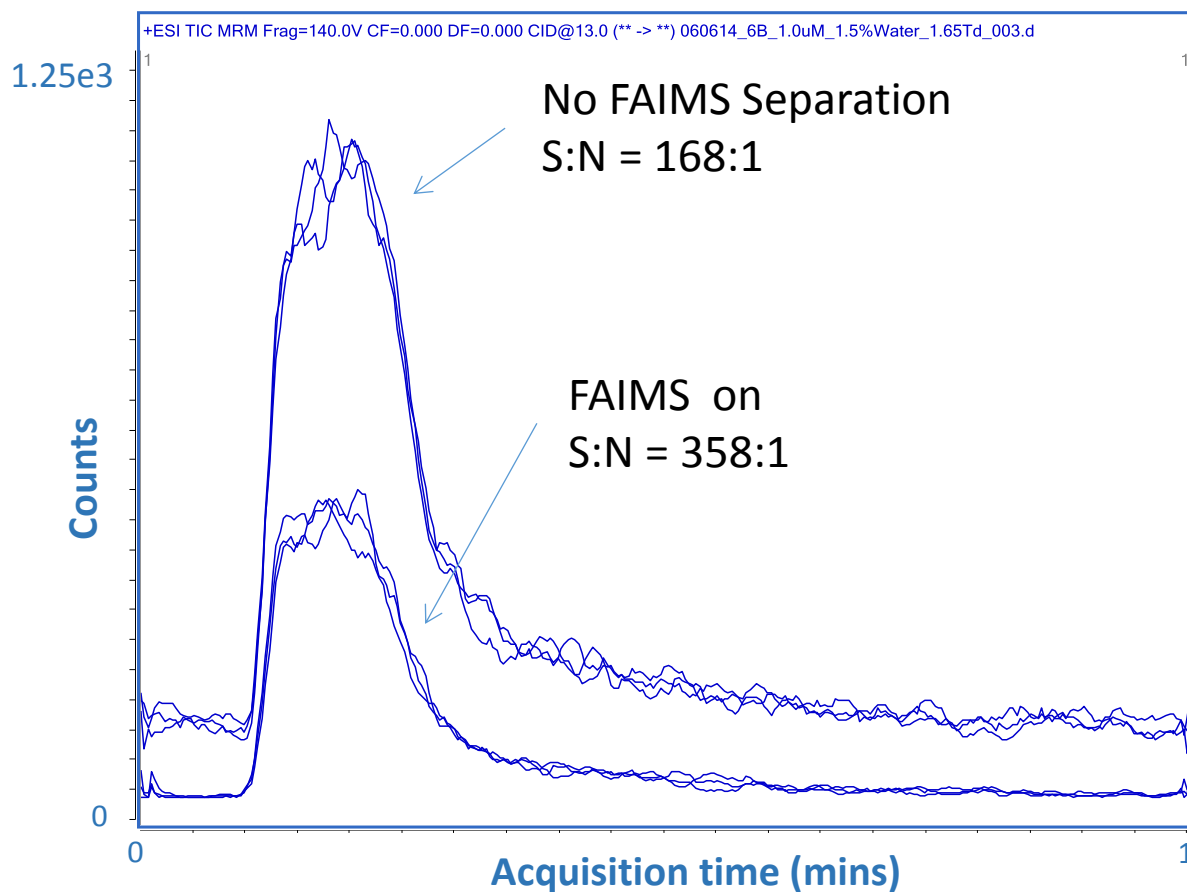
Loop injections of 6 β -HT (blue) and equimolar mixture of 6 β -HT and 2 β -HT (red) at 1 μ M, 0.2ml/min with FAIMS set to transmit 6 β -HT.



With FAIMS selection of 6 β -HT, the isobaric 2 β -HT interference has been almost entirely removed and is now comparable with the sample without 2 β -HT present.

Signal to noise improvements

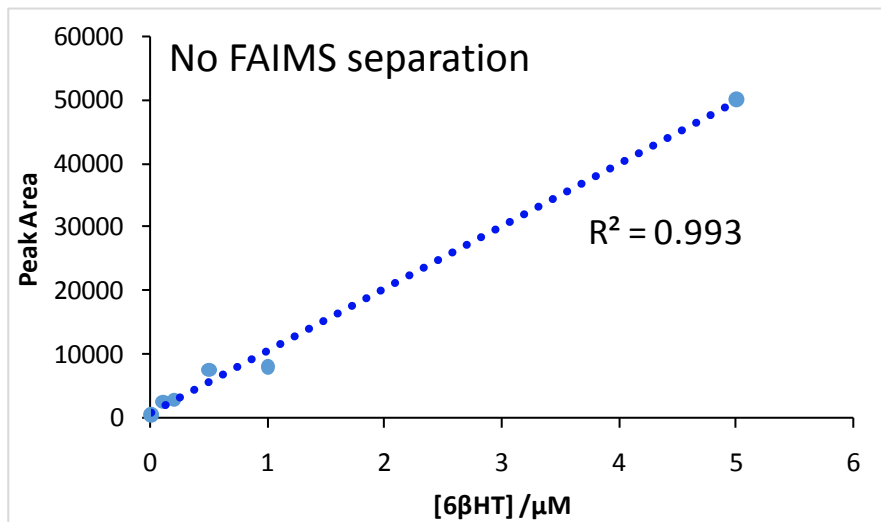
Loop injections of 6 β -HT (no 2 β -HT in sample) at 1 μ M, with and without FAIMS separation.



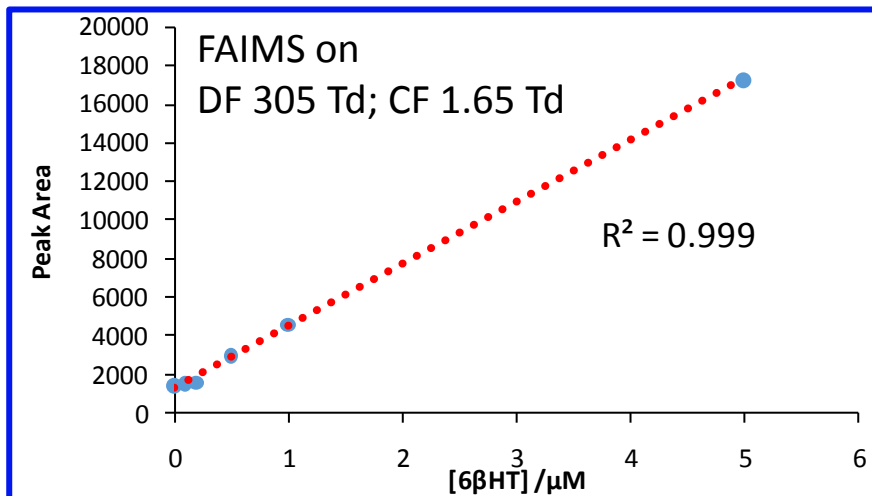
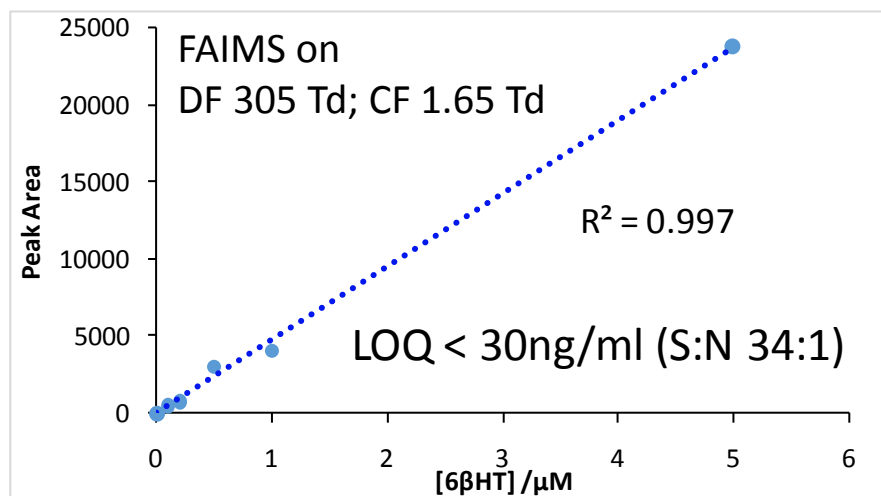
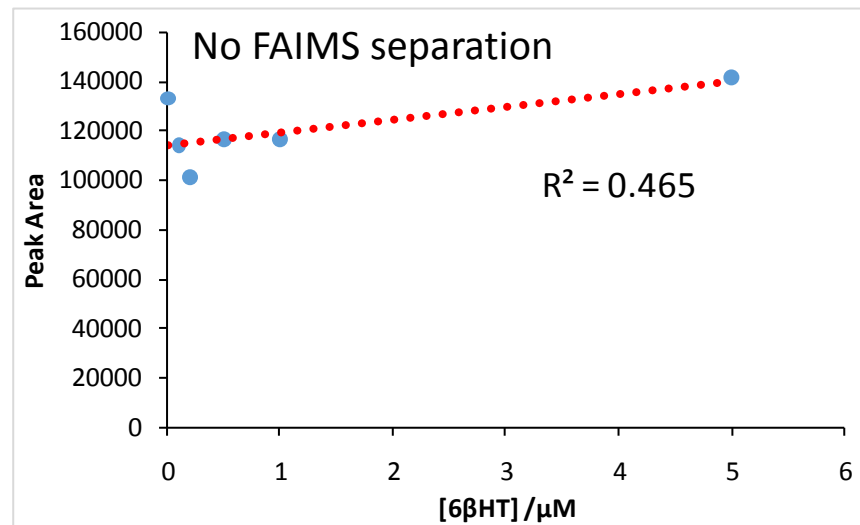
As well as removing interference from the isomer, FAIMS is also improving signal to noise.

Quantitative Performance - 6β-HT Calibration curve

6β-HT only



6β-HT with 2β-HT interference (1μM)



Conclusions and Further Work

- Future work will explore what sensitivity can be achieved with on-line SPE
- FAIMS can be used to separate 6 β -HT from 2 β -HT with 95% selectivity for 6 β -HT
 - Addition of FAIMS would allow a faster LC method to be used
 - Operating conditions are compatible with high-throughput online SPE methods
- Good quantitative performance
 - LOQ <30ng/ml for 6 β -HT
 - 6 β -HT signal-to-noise is doubled with FAIMS
 - Good linearity $R^2 = 0.999$
- UltraFAIMS shows promise for use with high-throughput systems even when isomeric interference would otherwise prevent accurate determination of P450 inhibition



THANK YOU
FOR YOUR ATTENTION!

Any questions?