Endogenous Steroids Analysis in Plasma by LC/MS

Analysis by LC-MS/MS
for Clinical Research

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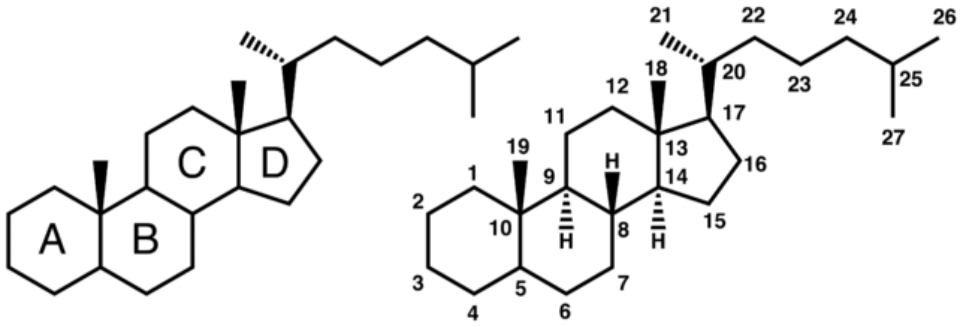
Agilent Technologies

May 1, 2014

Overview

- Introduction
- 13 Steroids Panel Analysis
  - Sample Preparation & Methodology
  - Results
- Conclusion
- Questions

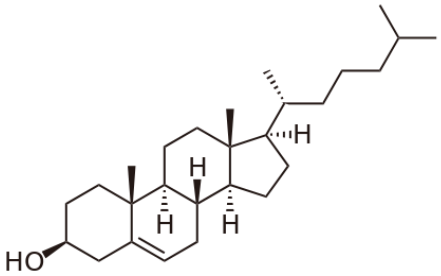


Why Do Clinical Research Laboratories Adopt LC/MS?

- High Selectivity
- High Sensitivity
- Accurate Quantification
- No Sample Derivatization
- Measure Analytes and their Metabolites Simultaneously
- Easier Research Method Development vs. Antibody-based Techniques

Cholesterol Steroidogenesis

Analysis of 13 Steroids for research



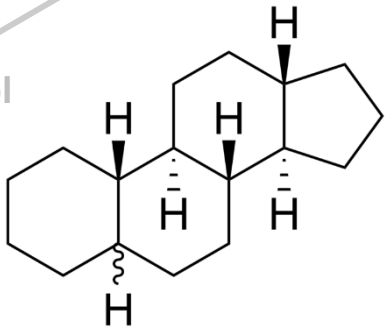
Cholesterol

Pregnenolone → **Progesterone** → Deoxycorticosterone → **Corticosterone** → **Aldosterone**

17α-OH Pregnenolone → **17α-OH Progesterone** → **11-Deoxycortisol** → **Cortisol** → **Cortisone**

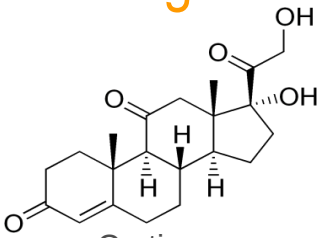
Dehydroepiandrosterone → **Androstenedione** → Estrone → Estradiol → Estriol

Androstenediol → **Testosterone** → **Dihydrotestosterone**

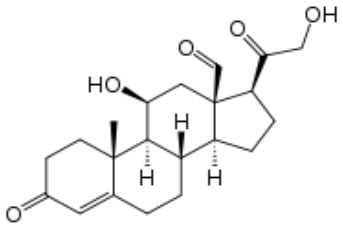


Structures and Molecular Weights

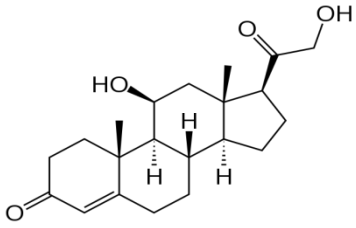
Steroidogenesis



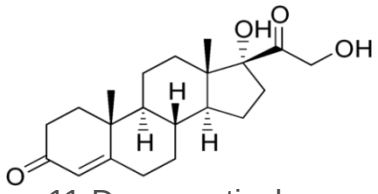
Cortisone
$C_{21}H_{28}O_5$
M.W. 360.2



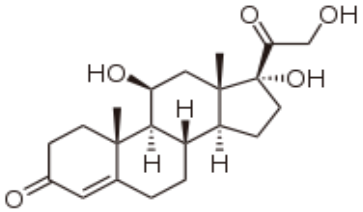
Aldosterone
$C_{21}H_{28}O_5$
M.W 360.2



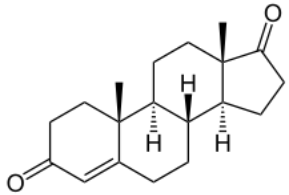
Corticosterone
$C_{21}H_{30}O_4$
M.W 346.2



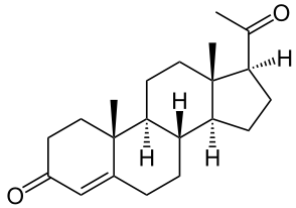
11-Deoxycortisol
$C_{21}H_{30}O_4$
M.W 346.2



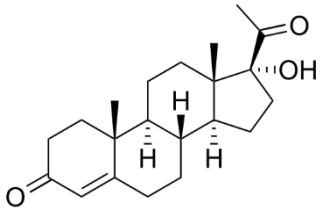
Cortisol
$C_{21}H_{30}O_5$
M.W. 362.46



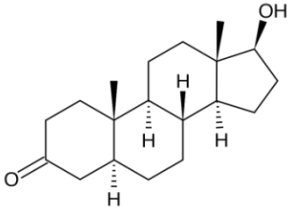
Androstenedione
$C_{19}H_{26}O_2$
M.W. 286.2



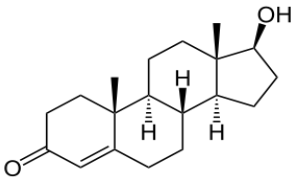
Progesterone
$C_{21}H_{30}O_2$
M.W. 314.2



17-Hydroxyprogesterone
$C_{21}H_{30}O_3$
M.W. 330.2

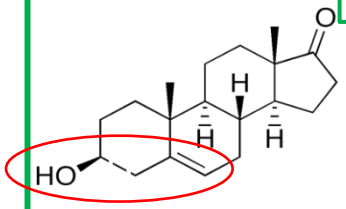


Dihydortestosterone
$C_{19}H_{30}O_2$
M.W. 290.2

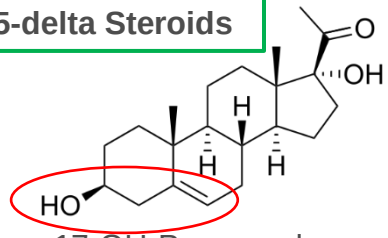


Testosterone
$C_{19}H_{28}O_2$
M.W 288.2

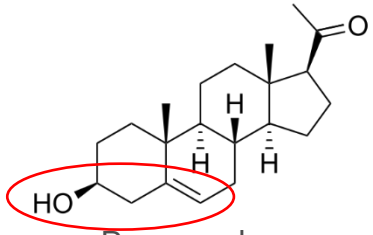
5-delta Steroids



DHEA
$C_{19}H_{28}O_2$
M.W 288.2



17-OH-Pregnenolone
$C_{21}H_{32}O_3$
M.W. 332.2



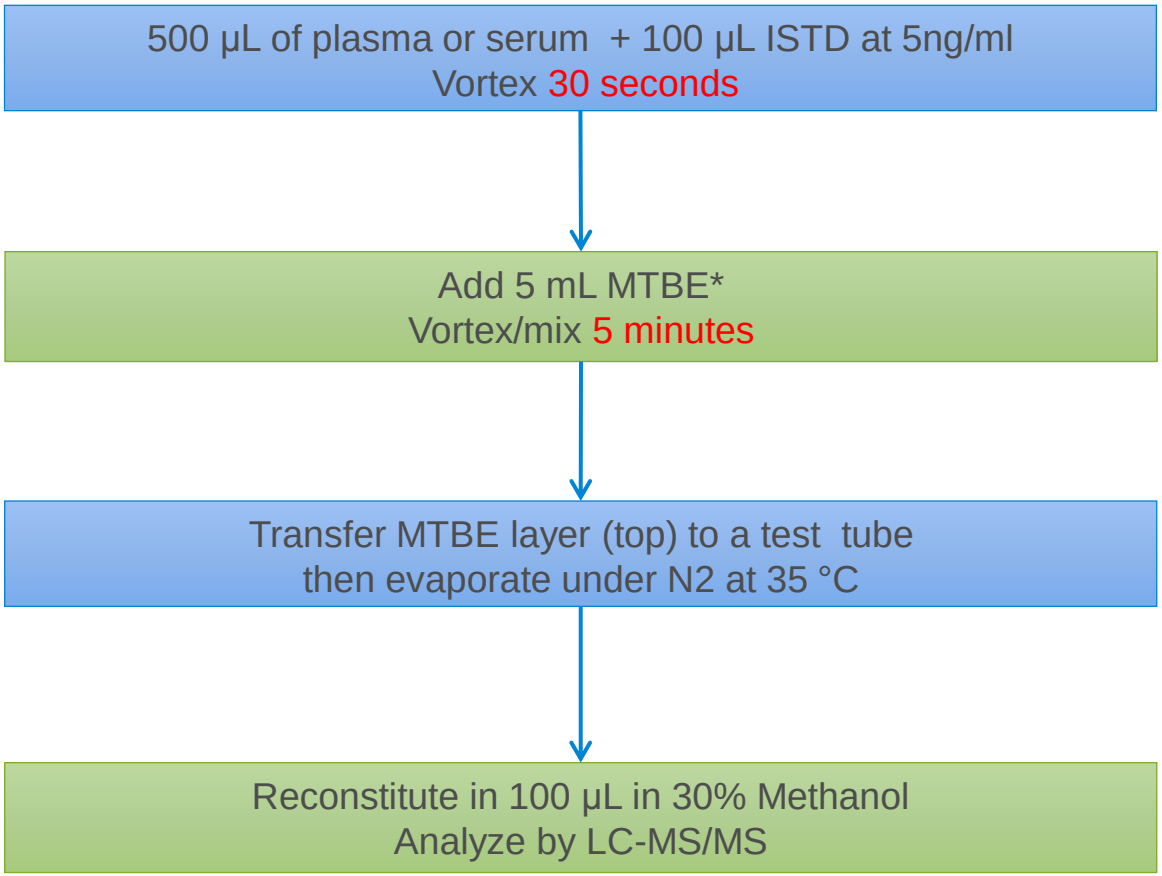
Pregnenolone
$C_{21}H_{32}O_2$
M.W. 316.2

Research Method Development Objectives

- Comprehensive, quantitative method for the analysis of major cholesterol steroidal metabolites for use in the clinical research laboratories
- Simple and economical sample preparation
- Robust and reliable research workflow
- Easily modifiable to accommodate and adjust changing research requirements

Sample Preparation

Liquid-Liquid Extraction



* Methyl *tert*-butyl ether

LC Research Method Parameters

1290 UHPLC System

Injection volume: 10 µL

Autosampler temp: 5 ºC

Column Temp: 50 ºC

Needle Wash: 50%IPA : 25% MeOH : 25% H_2O

Column: Poroshell 120 EC-C18, 2.1x50 mm, 2.7 µm

Pump Flow: 0.4 mL/min

Mobile Phase: A = Water + 5mM NH_4FA

B = Methanol + 5mM NH_4FA

Gradient:

Time	%B
0.00	10
0.50	10
8.00	40
9.00	60
9.50	60
9.60	95
10.4	95
10.5	10

Stop time – 10.5 min

Post time – 1.00 min

MS Research Method Parameters

6460 QQQ System

Ion Mode:	Jetstream, Positive
ESI conditions	
Drying Gas Temperature:	250 ºC
Drying Gas Flow:	11 L/min
Nebulizer Pressure:	35 psi
Sheath Gas Temperature:	350 ºC
Sheath Gas Flow:	11 L/min
Capillary:	3000 V
Nozzle Voltage:	0 V
EMV:	300 V
Resolution:	MS1 - Unit, MS2 – Unit
Dwell Time	20 ms
Cell Acceleration	3 V

- The selection of the spray chamber conditions for a **multi-component method** always involves a trade-off between the optimum settings of the individual compounds.

MS/MS Research Method Parameters

MRM Transition Parameters for Analytes

Compound Name	Precursor Ion	Product Ion Quant-Qual	Frag (V)	CE (V) Quant-Qual
11-Doxycortisol	347.2	109.1 - 97.1	95	28 - 28
17a-Hydroxypregnenolone	315.2	297.1 - 255.1	93	2 - 7
17a-Hydroxyprogesterone	331.2	109.1 - 97.1	100	27 - 27
Aldosterone	361.2	343.2 - 315.2	100	15 - 16
Androstenedione	287.2	109.1 - 97.1	100	25 - 17
Corticosterone	347.2	329.2 - 121.1	110	12 - 24
Cortisol	363.2	121.1 - 91.1	105	24 - 60
Cortisone	361.2	163.1 - 121.1	120	20 - 32
DHEA	289.2	271.2 - 231.2	95	2 - 20
Dihydrotestosterone	291.2	255.2 - 91.1	120	12 - 52
Pregnenolone	317.2	299.2 - 159.1	102	4 - 16
Progesterone	315.2	109.1 - 97.1	100	25 - 20
Testosterone	289.2	109.1 - 97.1	100	25 - 25

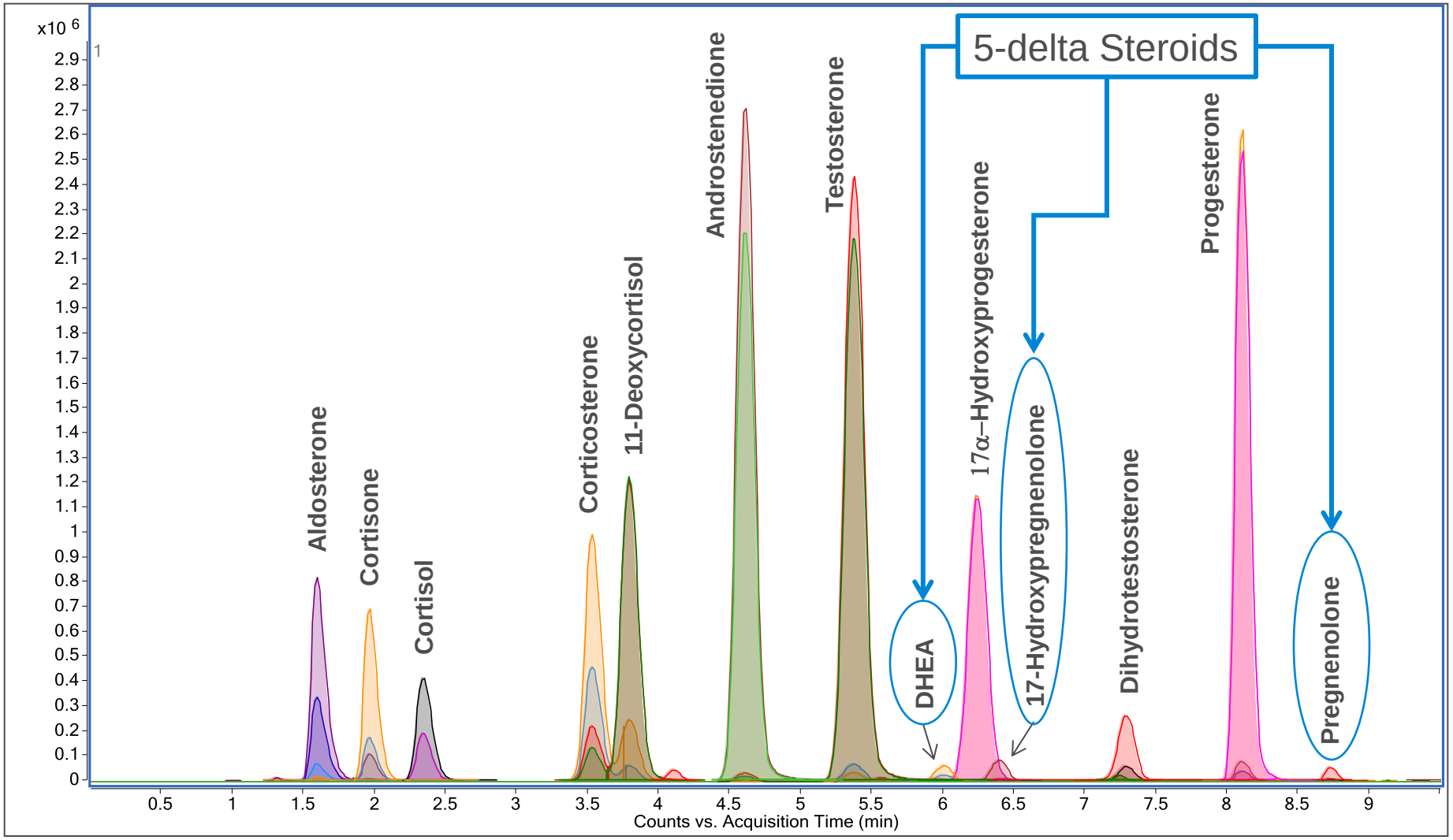
Research Method Parameters

MRM Transition Parameters for Internal Standards

Compound Name	Precursor Ion	Product Ion	Frag (V)	CE (V)
11-Doxycortisol-D5	352.2	100.1	95	28
17a-Hydroxypregnenolone-13C2-D2	319.2	301.1	93	2
17a-Hydroxyprogesterone-D8	339.2	100.1	100	27
Aldosterone-D8	369.2	351.2	100	15
Androstenedione-3C13	290.2	100.1	100	17
Corticosterone-D8	355.2	337.2	110	12
Cortisol-D4	367.2	121.1	105	24
Cortisone-D7	369.2	169.1	120	20
DHEA-D5	294.2	258.2	95	2
Dihydrotestosterone-D3	294.2	258.2	120	12
Pregnenolone-13C2-D2	321.2	303.2	102	4
Progesterone-D9	324.2	100.1	100	20
Testosterone-D3	292.2	97.1	100	25

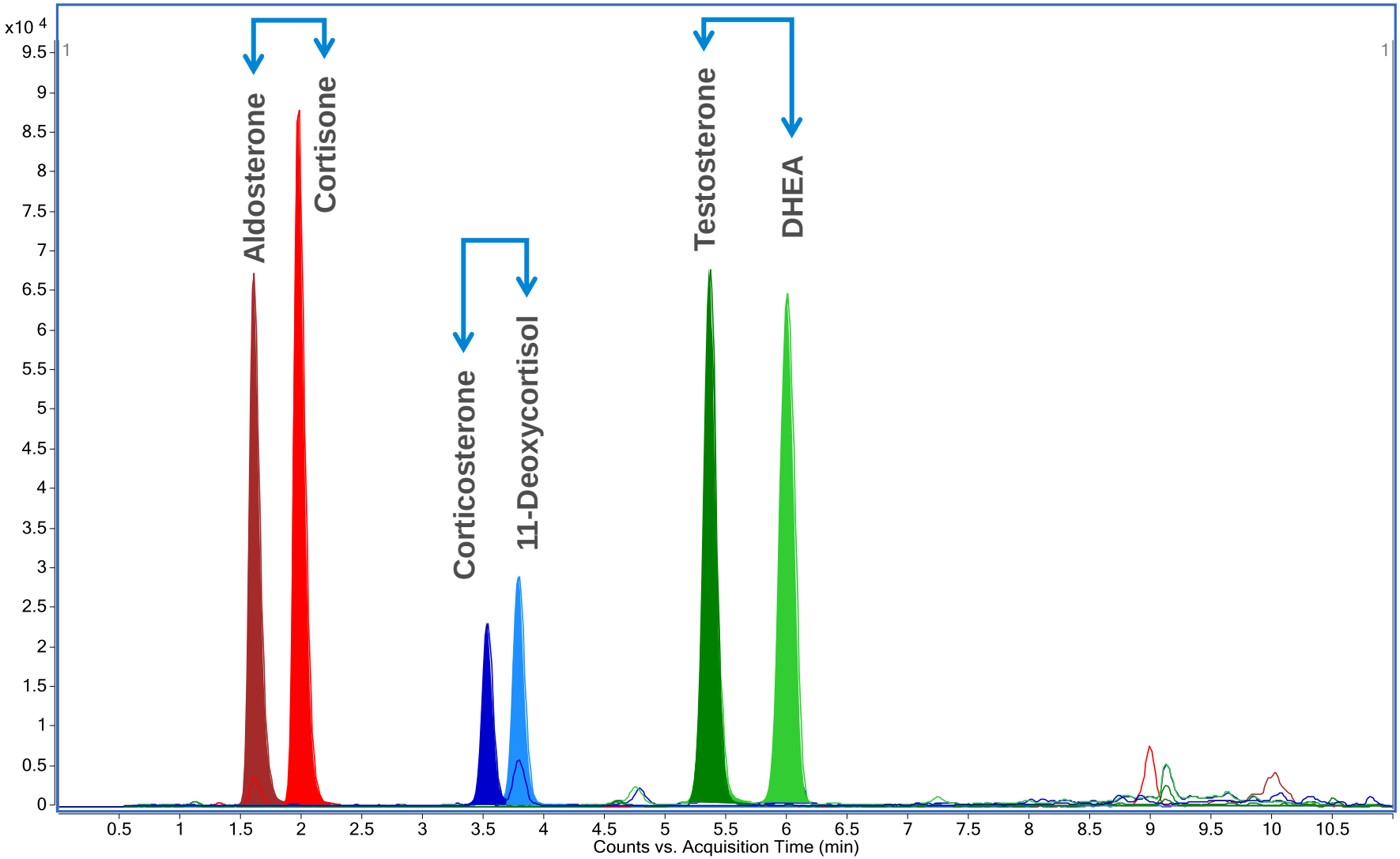
Chromatography

13 Steroid Panel for Research



Chromatography

Isobaric Pairs Need for Baseline Separation



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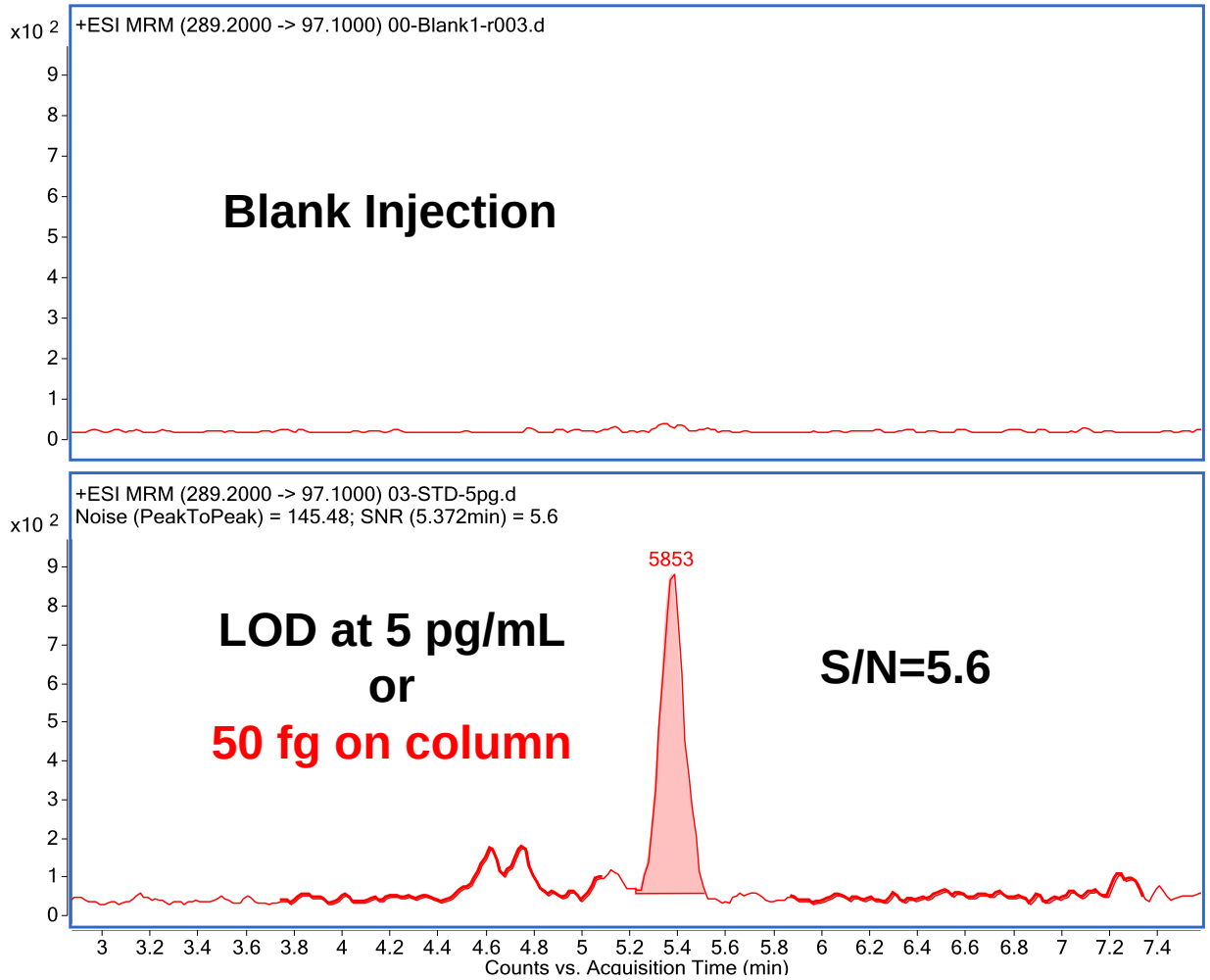
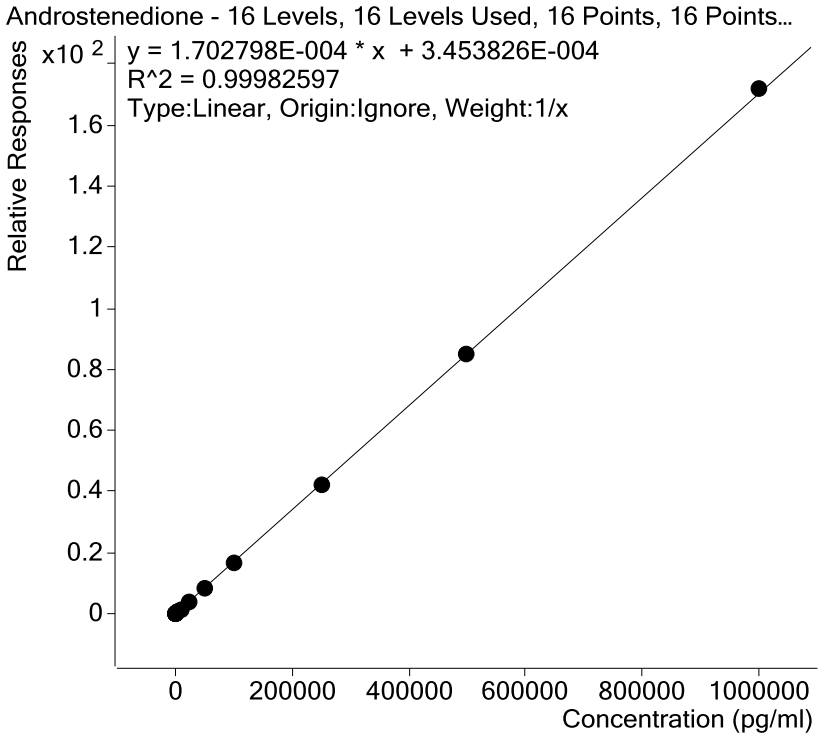
Limit Of Detection Example: Testosterone at 5 pg/mL

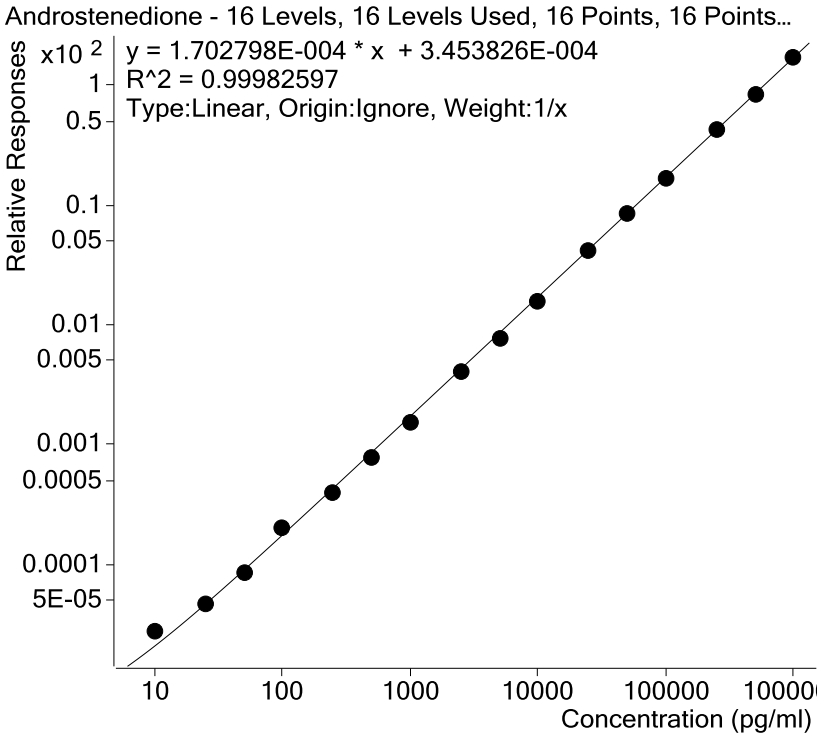
50 femtograms injected on Column



Androstenedione Calibration: 10pg/mL-100 ng/mL

Regular Scale vs. Log Scale





1 2 3 4 5

Linearity of 5 Orders of Magnitude

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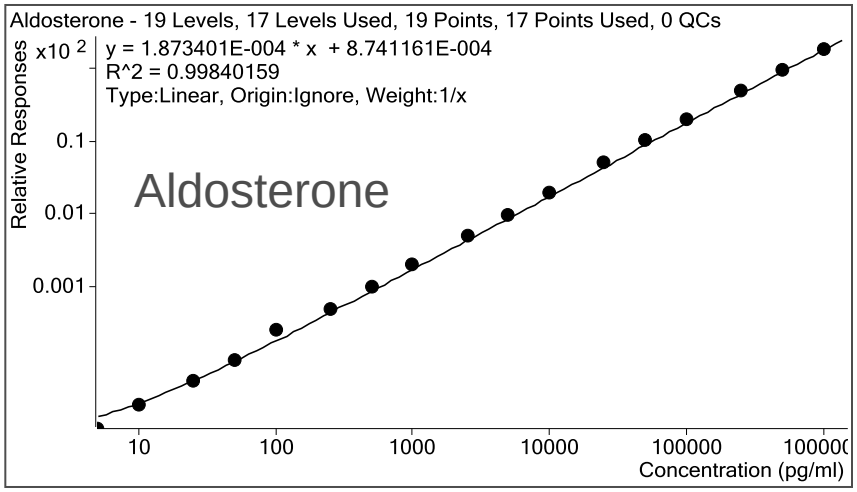
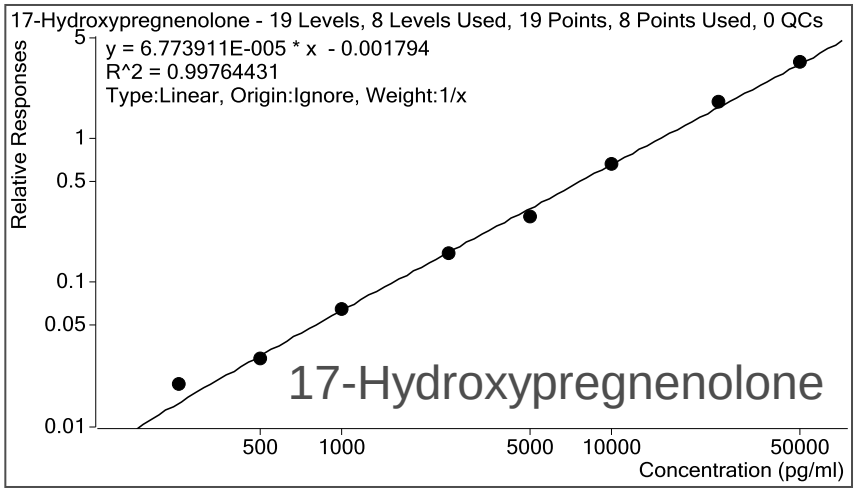
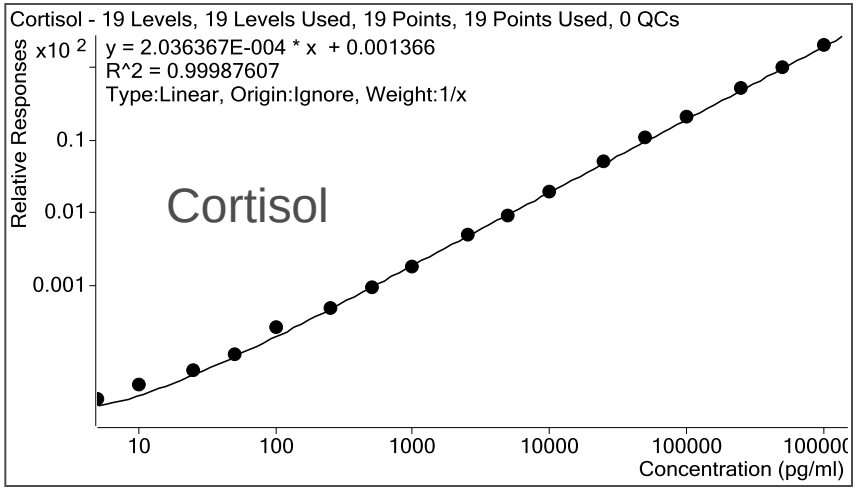
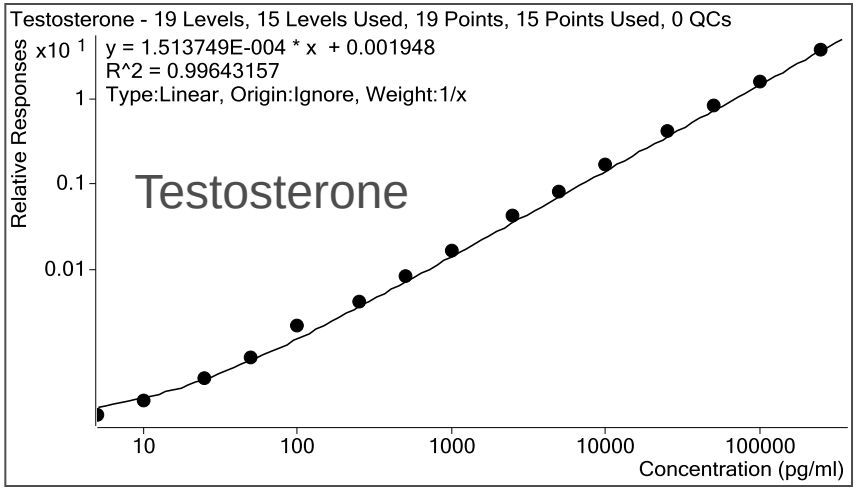
Calibration Curves

Representative Steroids









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Results

Summary of Analytical Performance

Compound	R^2	Level	Conc. (pg/mL)
Aldosterone	0.998	LOD LLOQ ULOQ	5 10 500000
Cortisone	0.998	LOD LLOQ ULOQ	5 10 1000000
Cortisol	0.999	LOD LLOQ ULOQ	1 5 1000000
Corticosterone	0.996	LOD LLOQ ULOQ	5 10 100000
11-Deoxycortisol	0.995	LOD LLOQ ULOQ	2.5 5 500000
Androstenedione	0.999	LOD LLOQ ULOQ	2.5 5 1000000
Testosterone	0.996	LOD LLOQ ULOQ	5 10 500000

Compound	R^2	Level	Conc. (pg/mL)
DHEA	0.995	LOD LLOQ ULOQ	250 500 100000
17α-Hydroxyprogesterone	0.996	LOD LLOQ ULOQ	10 25 100000
17-Hydroxypregnenolone	0.995	LOD LLOQ ULOQ	250 500 250000
Dihydrotestosterone	0.997	LOD LLOQ ULOQ	5 10 500000
Progesterone	0.995	LOD LLOQ ULOQ	25 50 500000
Pregnenolone	0.999	LOD LLOQ ULOQ	1000 2500 500000

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Conclusions

- Comprehensive analytical research method for steroids profile and a highly sensitive and robust research method for low-level quantitation of 13 steroids panel has been developed
- Sample preparation consisted of a straight forward LLE
- Steroid Panel LLOQs were in the low pg/ml range while 5-Δ steroids were in high pg/mL. Dynamic range of up to 5 orders of magnitude with $R^2 \geq 0.995$
- Number of steroids and analysis time can be reduced according to research needs.

Questions

Thank You!

Acknowledgements

Kevin McCann
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Applications Specialist

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Vitamin D Metabolite Analysis in Serum by LC/MS

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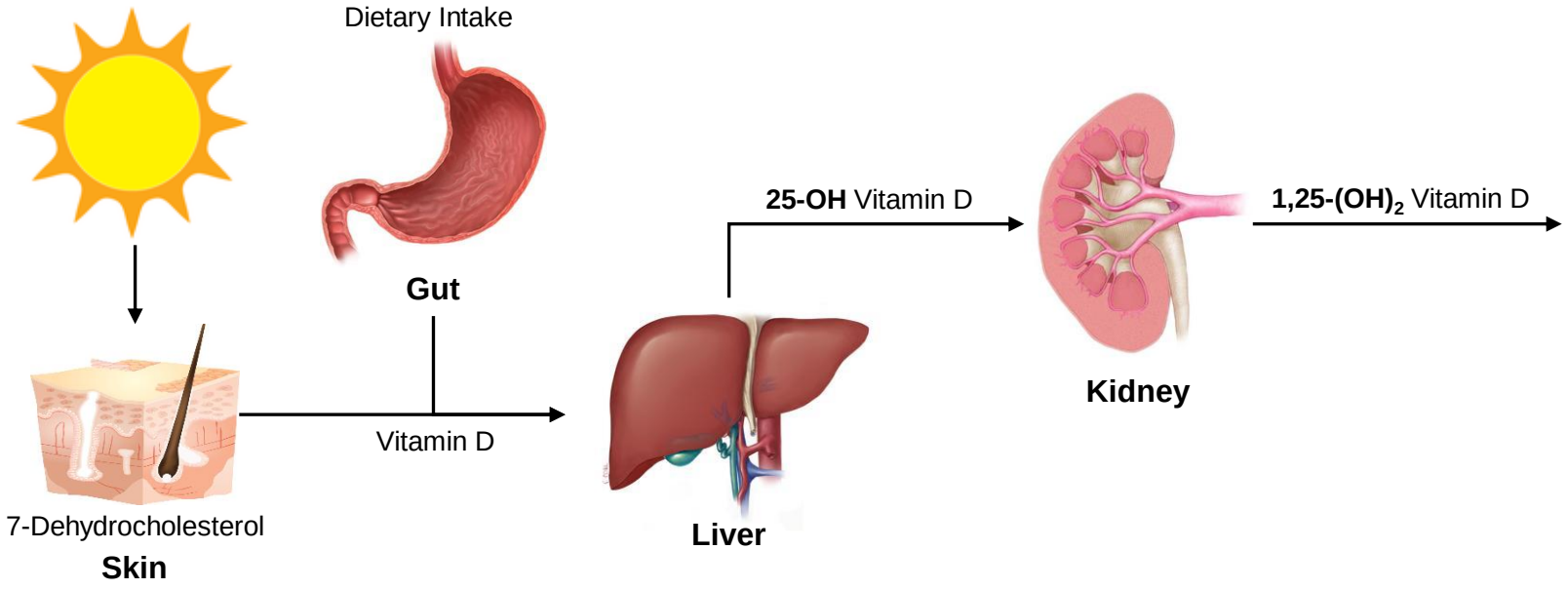
Agenda

- Researching Vitamin D metabolites
- LCMSMS reserach methods for the accurate analysis of Vitamin D metabolites and isoforms
- How automated, online sample clean-up can be used to reduce offline sample preparation
- Technological advances enabling the analysis of low-level Vitamin D metabolites
- Quantitative data analysis

Rapid Analysis of Vitamin D Metabolites for Research

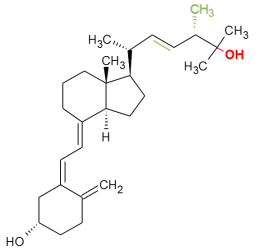
- 25-OH, epimers, 1,25-$(OH)_2$, and more
- Chromatic separation of isoforms for each metabolite
- Internal standard corrected quantification
- Multi-point calibration curve covering a wide dynamic range
- Secondary qualifier ion for each analyte



A Few Metabolites of Vitamin D

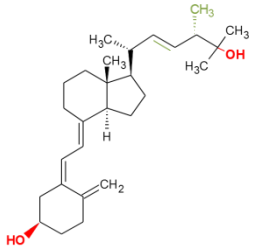
25-OH Vitamin D_3

25-OH Vitamin D_2



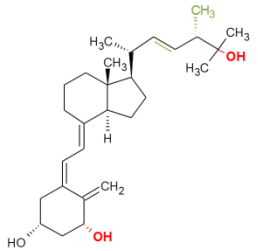
3-epi-25-OH Vitamin D_3

3-epi-25-OH Vitamin D_2

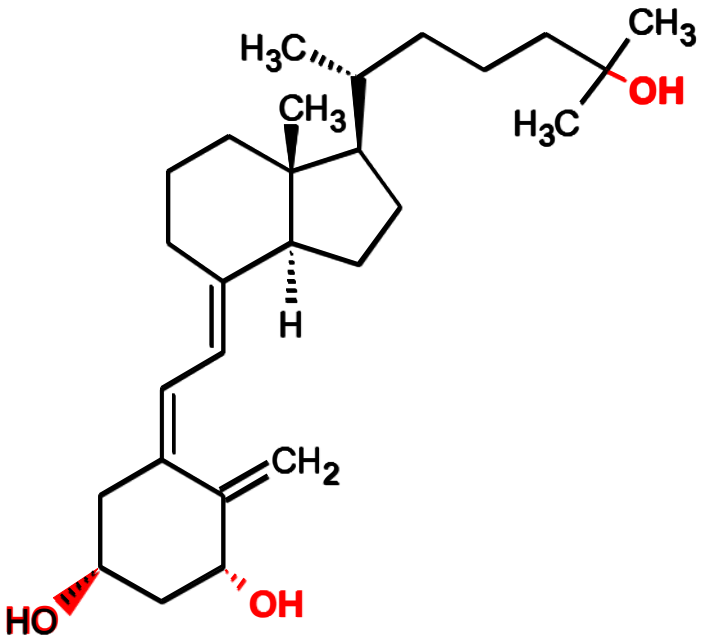


1,25-$(OH)_2$ Vitamin D_3

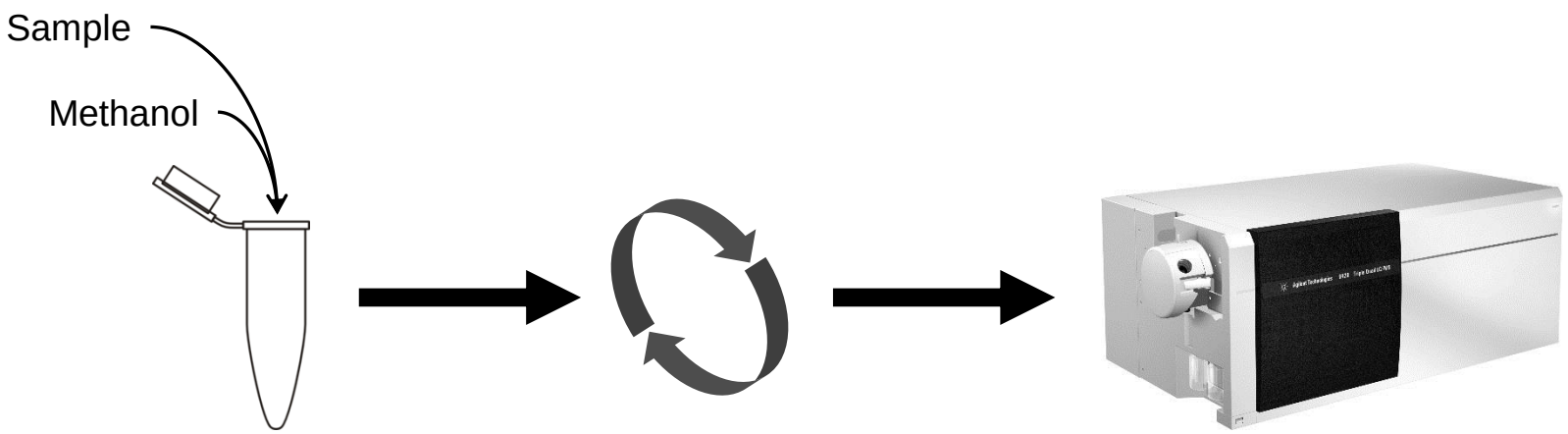
1,25-$(OH)_2$ Vitamin D_2



Vitamin D_3

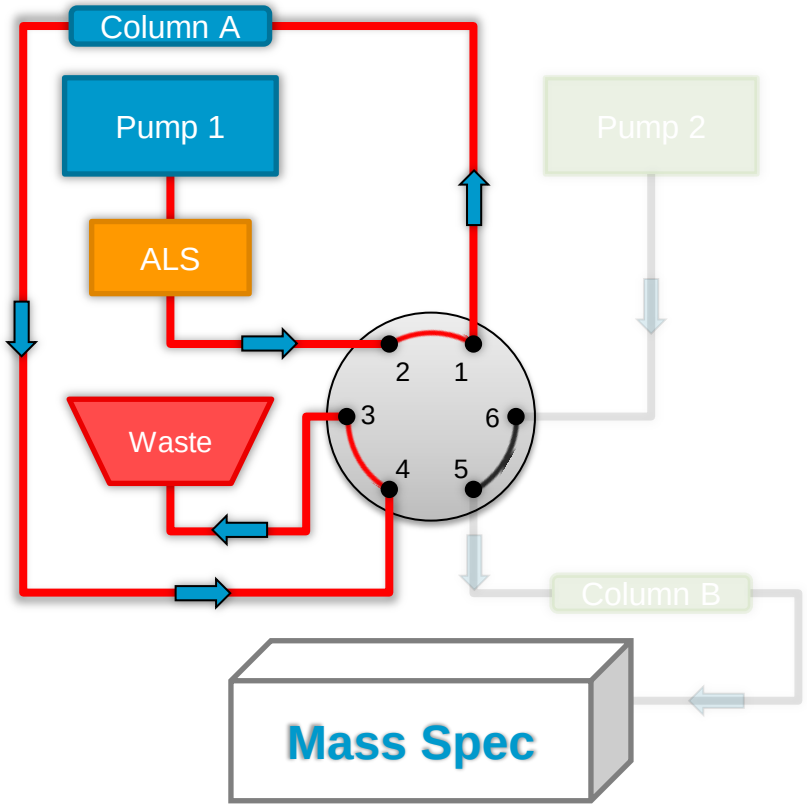


Protein Precipitation for 25-OH Metabolite Research Analysis

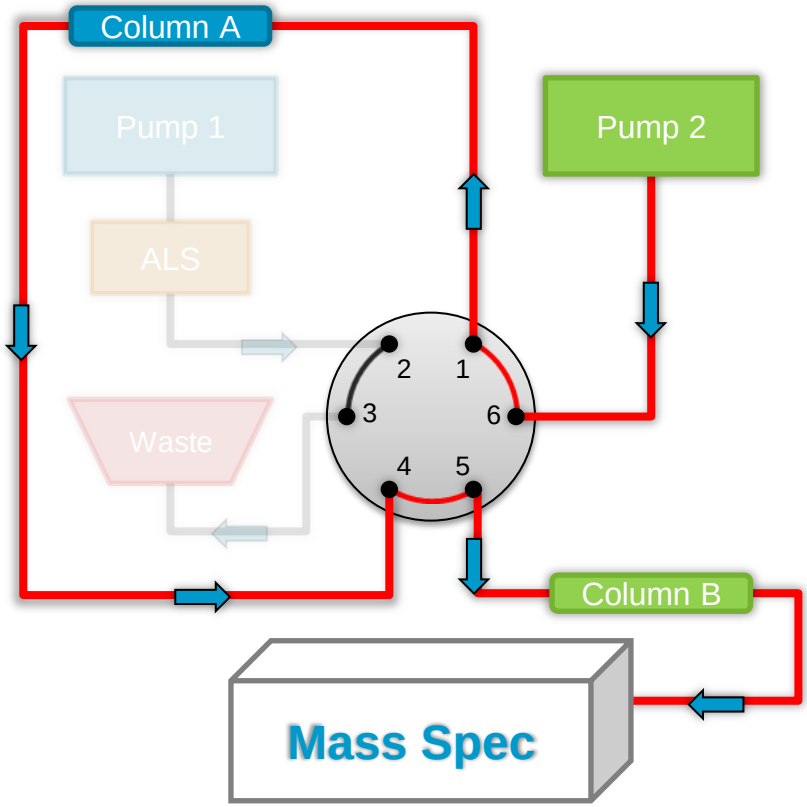


Cleaner Samples with Online Sample Cleanup

Position 1 (Port 1 > 2)



Position 2 (Port 1 > 6)



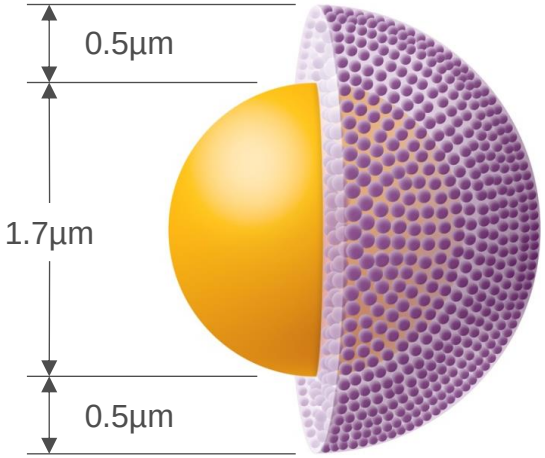
Poroshell 120 Columns for HPLC and UHPLC

Poroshell 120 is a high efficiency, high resolution column choice for enhancing productivity in LC and LC/MS

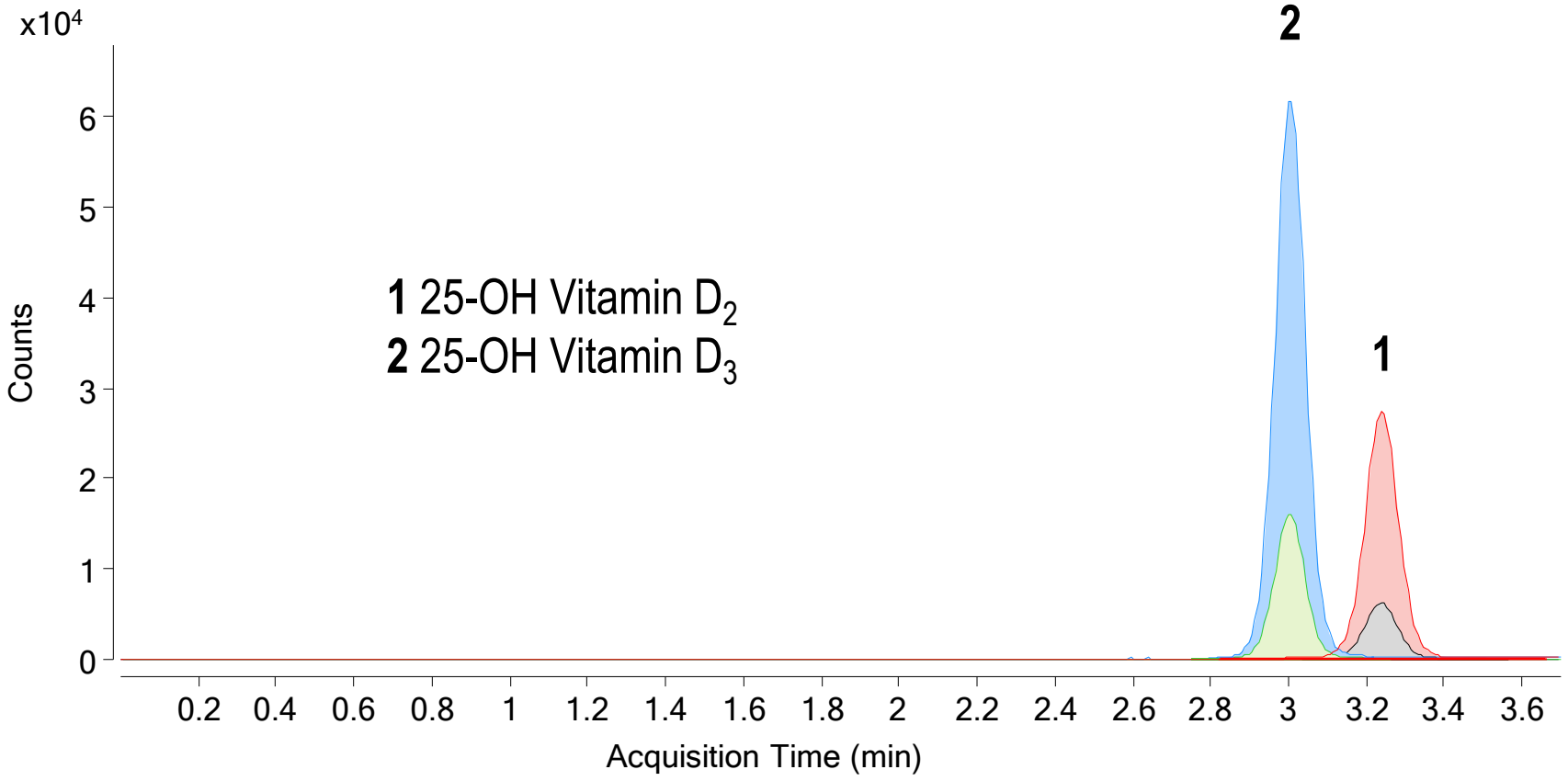
Poroshell 120 Columns have:

- 80-90% efficiency of sub-2µm columns
- ~40-50% lower pressure
- 2x efficiency of 3.5µm (totally porous)
- A 2µm frit to reduce clogging
- A 600 bar pressure limit for HPLC or UHPLC

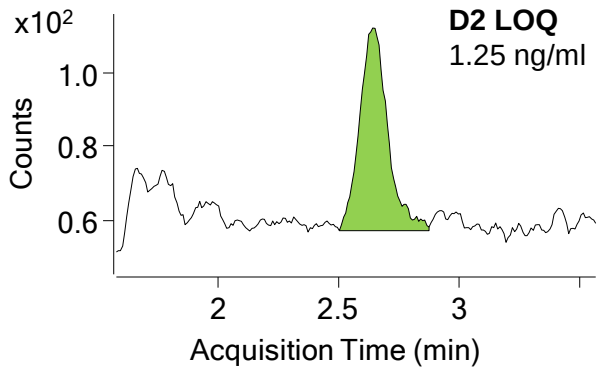
- The superficially porous particle is 2.7um with a solid core (1.7um) and porous outer layer with a 0.5um diffusion path

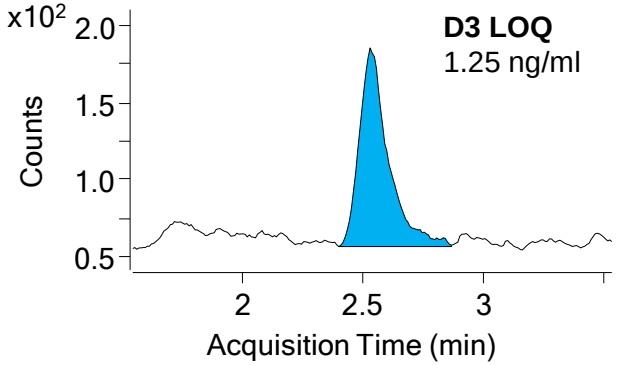


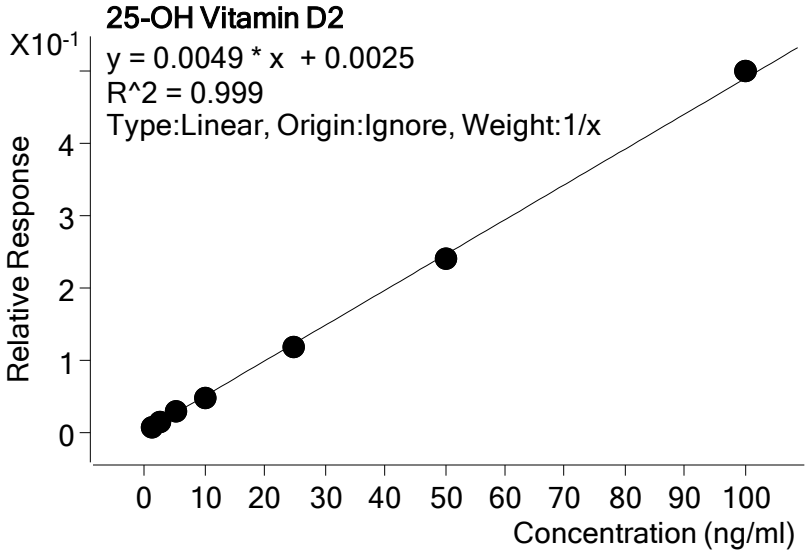
Chromatographic 2D Separation of Metabolites

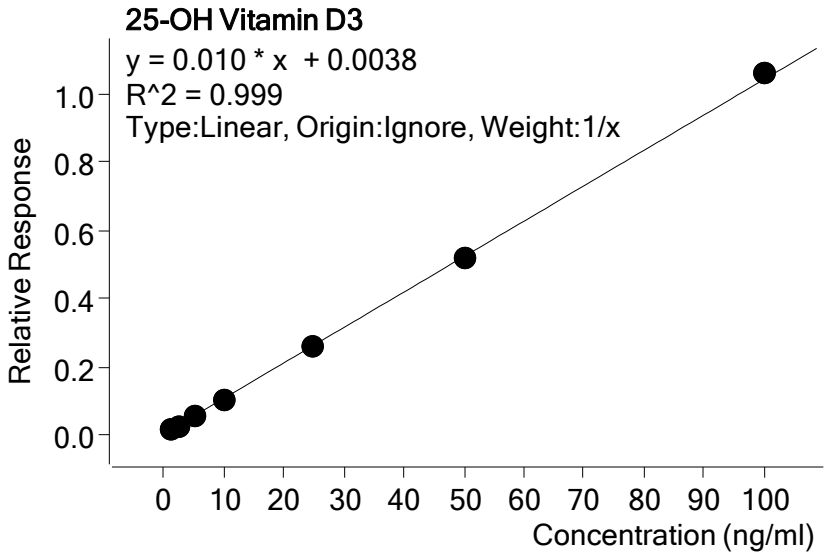


Accurate 1D Quantification of 25-OH Vitamin D for Research

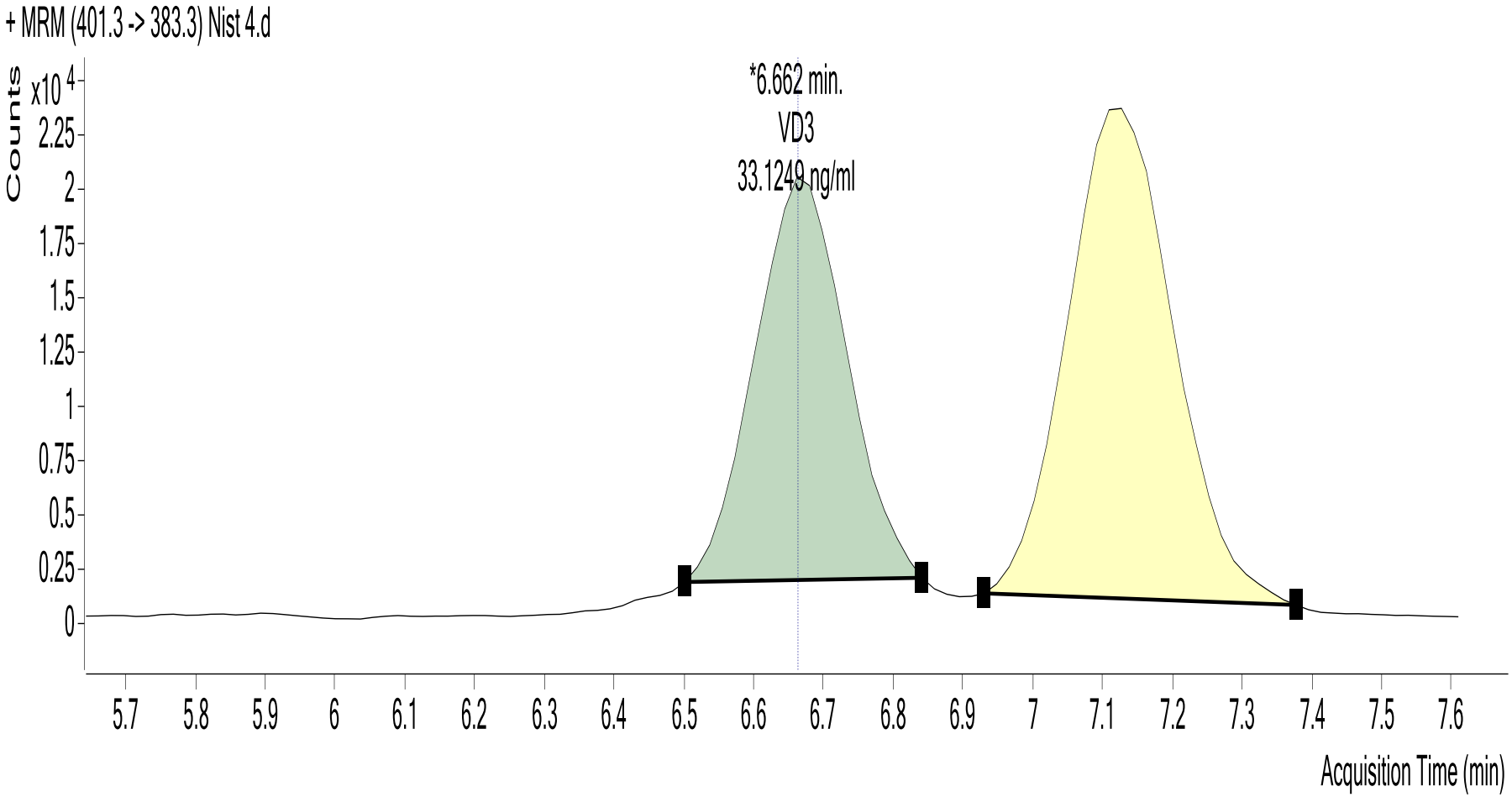




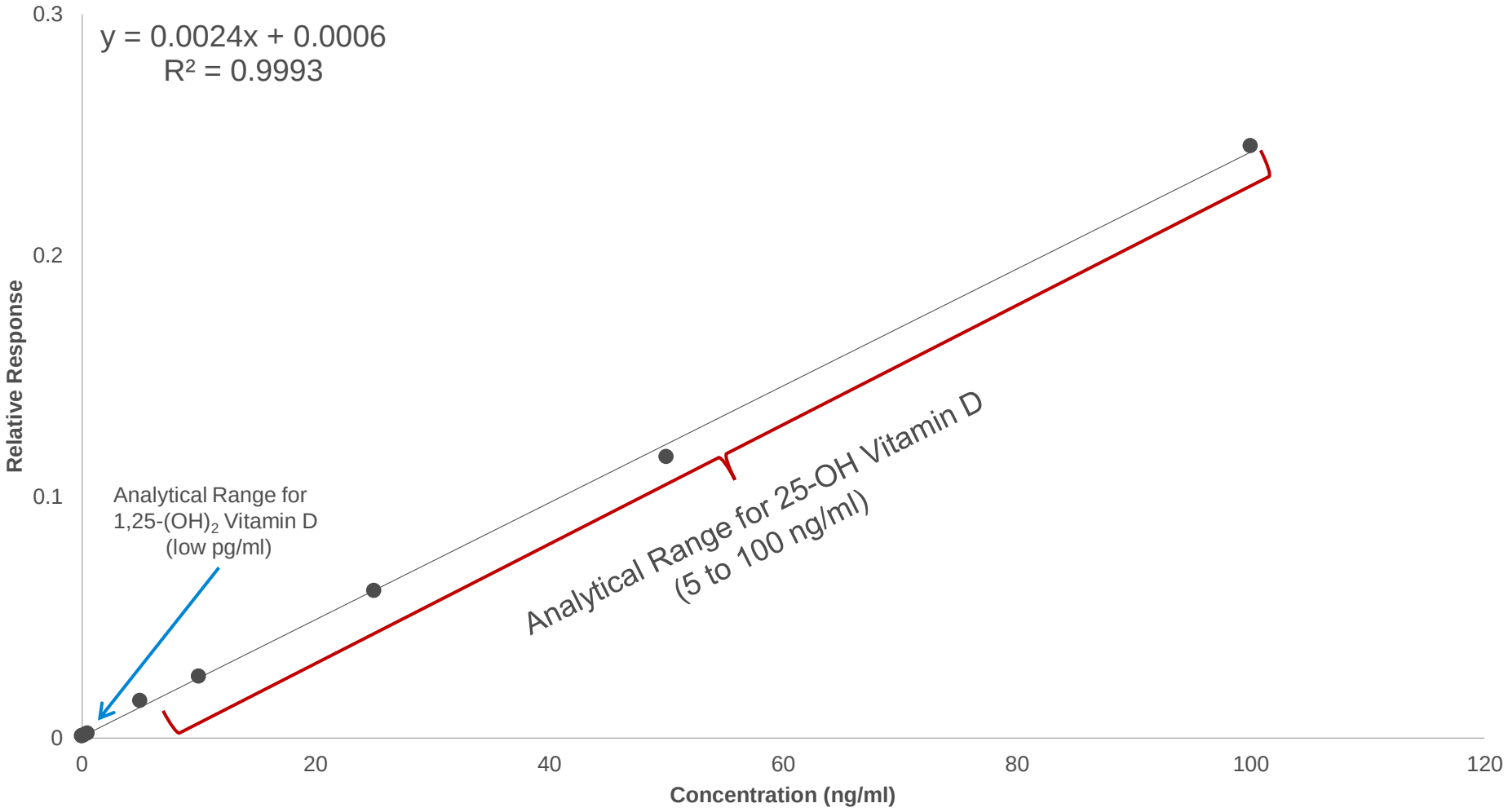




Chromatographic 1D Separation of 3-Epi-25-Hydroxy-Vitamin D using a PFP column



Analytical Range for Vitamin D Metabolites in Research



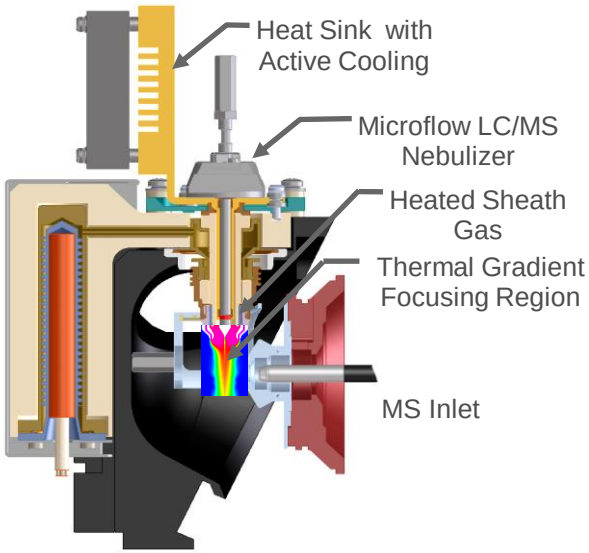
Quantification of 1,25-$(OH)_2$ Vitamin D for Research
Dealing with a Difficult Analysis

Accurate and reliable quantification for research of low level analytes such as 1,25-$(OH)_2$ Vitamin D can be challenging, costly and time-consuming.
Through the implementation of novel and cutting-edge technologies, Agilent makes these research analyses a reality.

iFunnel Technology Revolutionizes Ion Sampling

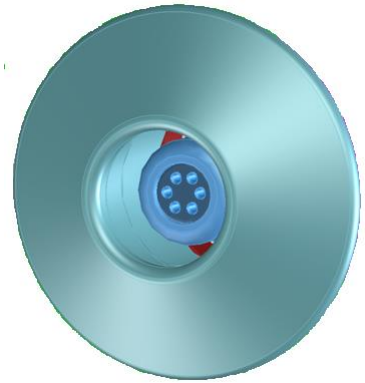
Agilent Jet Stream

- Thermal confinement of ESI plume
- Efficient desolvation to create gas phase ions
- Creates an ion rich zone



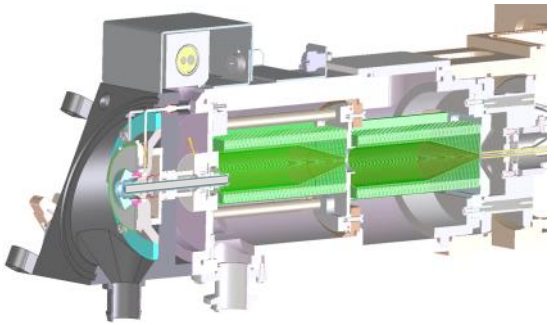
Hexabore Capillary

- 6 capillary inlets
- Samples ~10 times more ion rich gas from the source
- Captures the majority of the gas from the source region



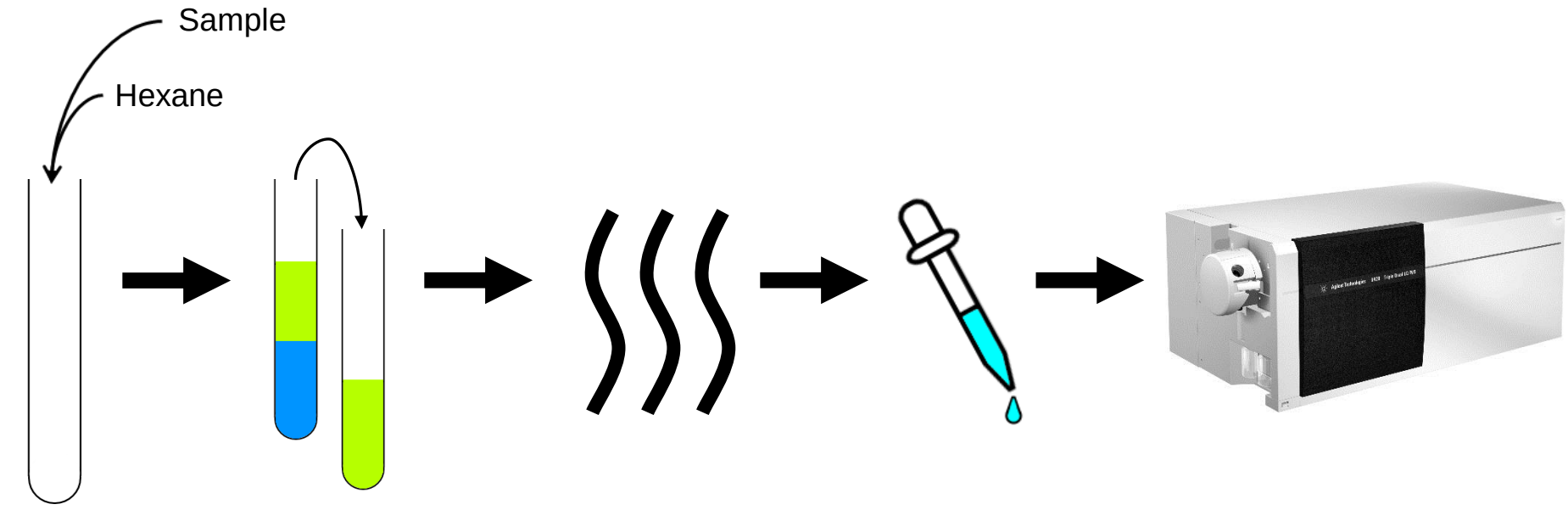
Dual Ion Funnel

- Removes the gas but captures the ions
- Removes neutral noise
- Extends turbo pump life



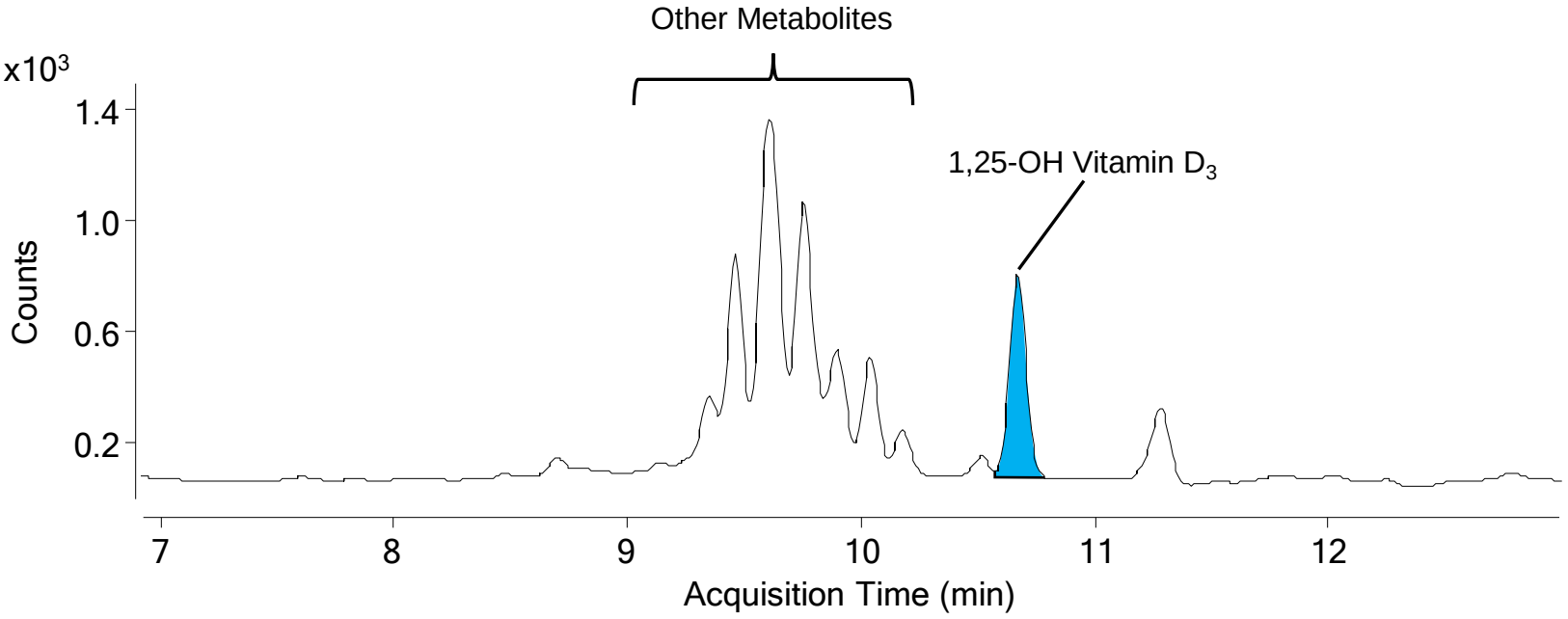
Capture up to 10 x more ions

Liquid-Liquid Extraction for 1,25-$(OH)_2$ Metabolite Analysis

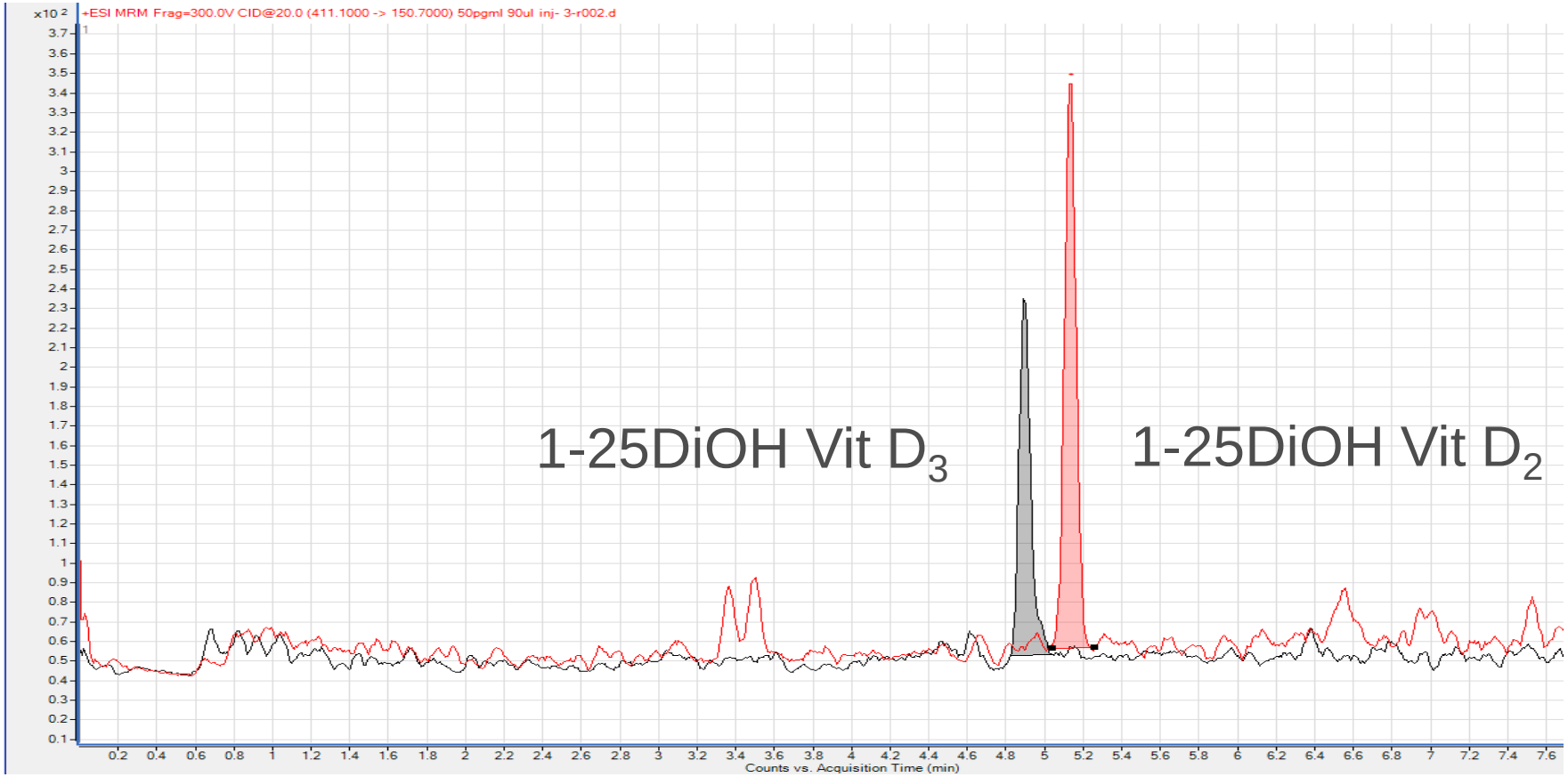


Analysis of 1,25-$(OH)_2$ Vitamin D

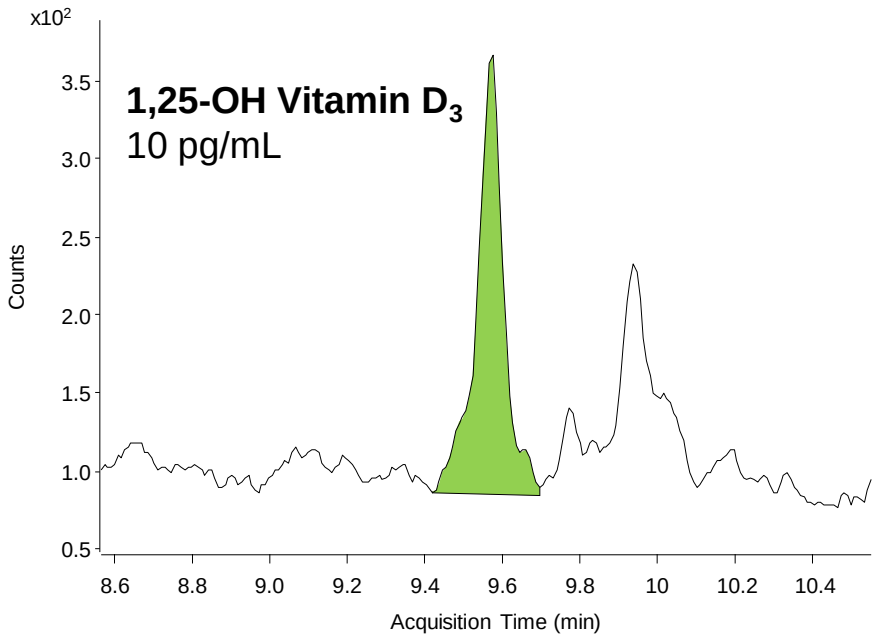
- LOQ of 5 pg/mL
- Chromatographic separation from isobaric metabolites
- Possible to quantitate other metabolites for research

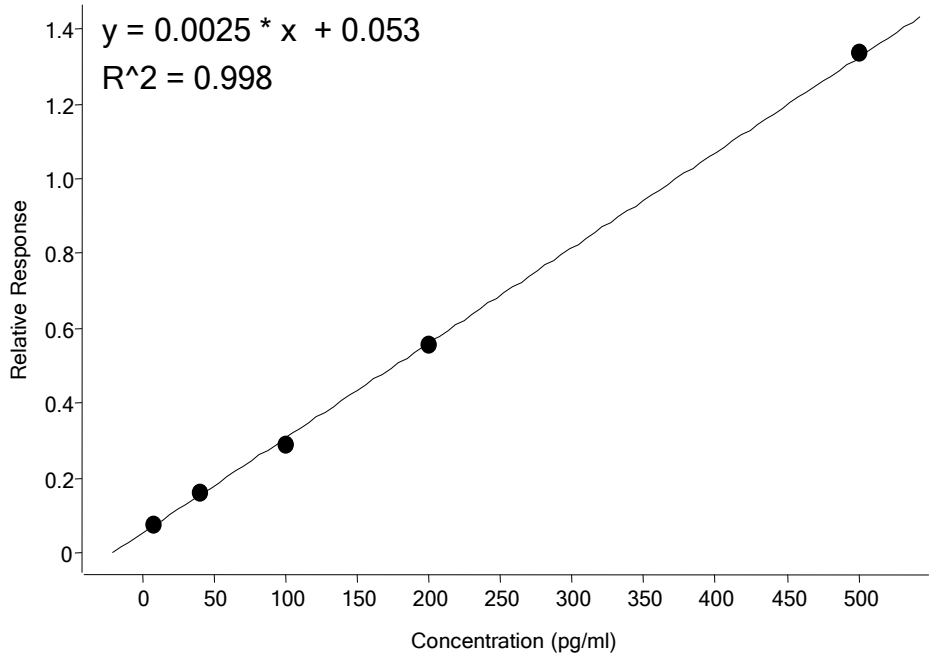


Immuno-cleanup of 1,25-Dihydroxy-Vitamin D3/D2



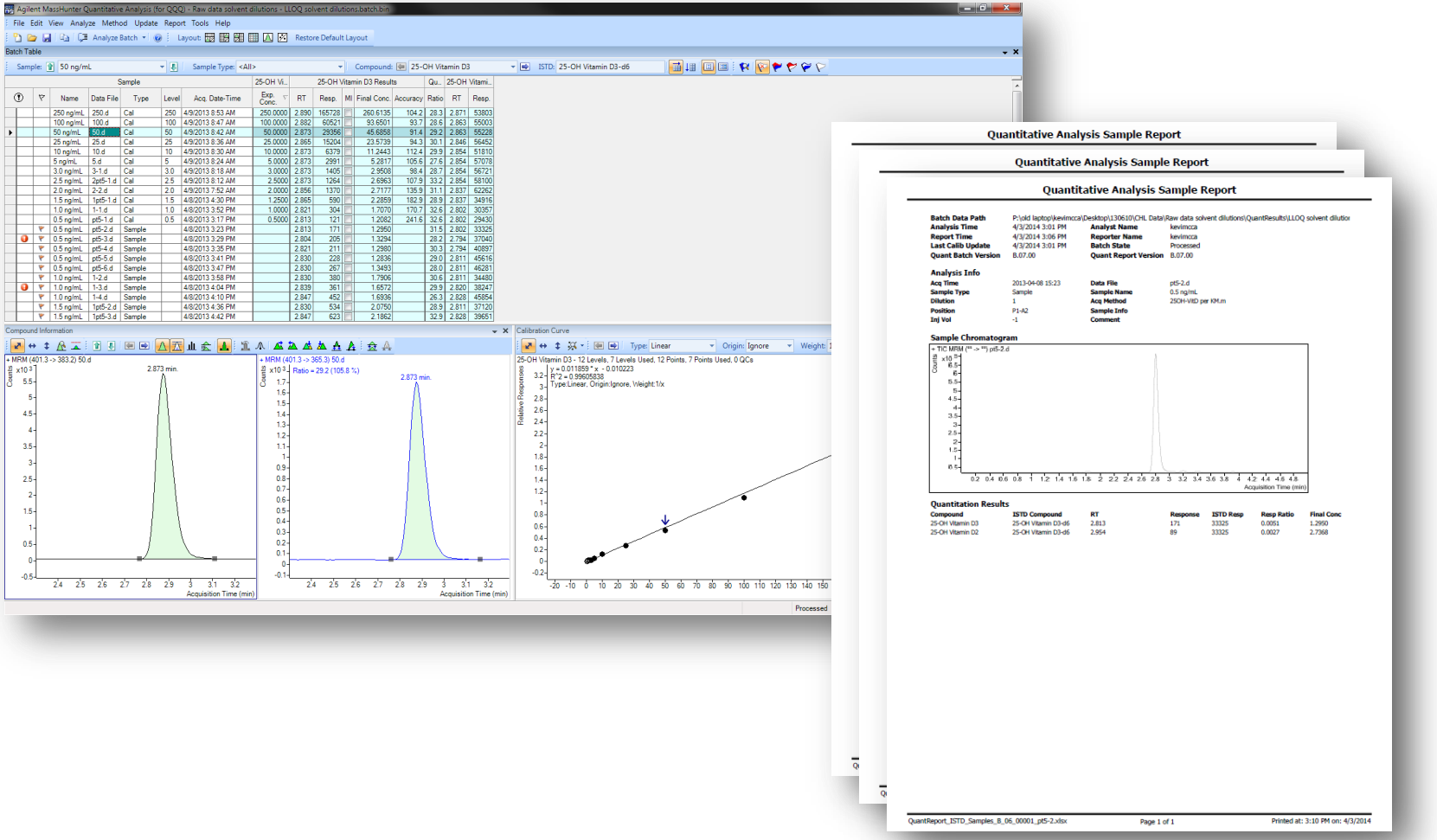
Accurate Quantitation of 1,25-$(OH)_2$ Vitamin D for Research





MassHunter Quantitative Analysis for Research

Batch at a Glance and Reporting



Conclusions

- Agilent offers robust solutions to enable the reliable quantification of abundant and low-level Vitamin D metabolites for research
- Proven technology reduces your workload by minimizing hands-on sample preparation
- Agilent’s MassHunter software is optimized for researchers' data review and reporting workflow

Multiply LC/MS Productivity

The New Agilent StreamSelect LC/MS System

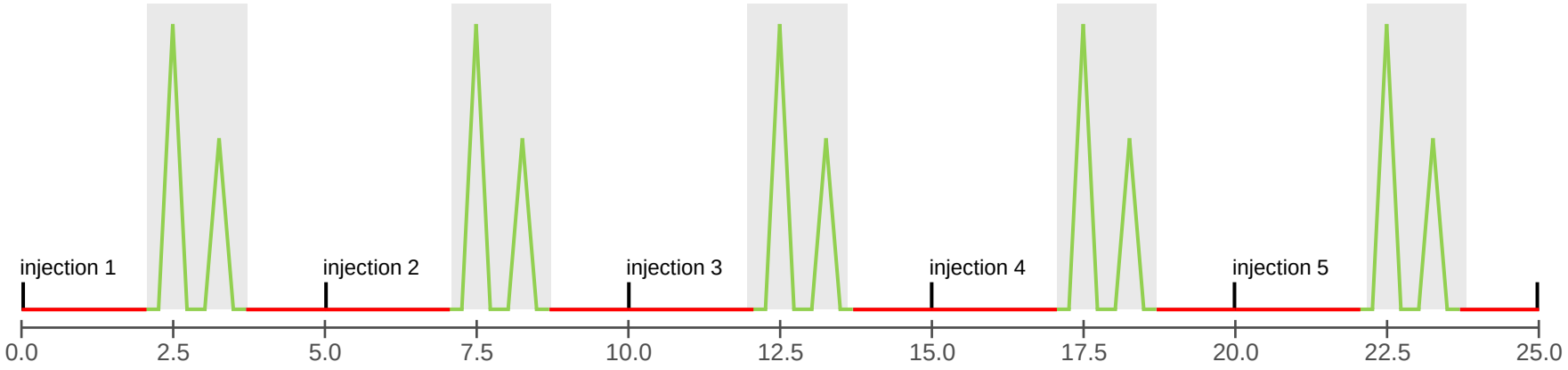
Maria VanDamme
StreamSelect Product Manager
Agilent Technologies, Inc.

Multiply LC/MS Productivity
Agilent StreamSelect LC/MS System

- Dual-stream capacity increases throughput up to two-fold
- More samples on fewer instruments through efficient MS utilization
- Excellent analytical performance for accurate & reproducible quantitation for research
- Simple implementation with intuitive automation software
- Fully integrated solution allows intelligent error handling

Traditional LC/MS

Analyses in Series for Researchers

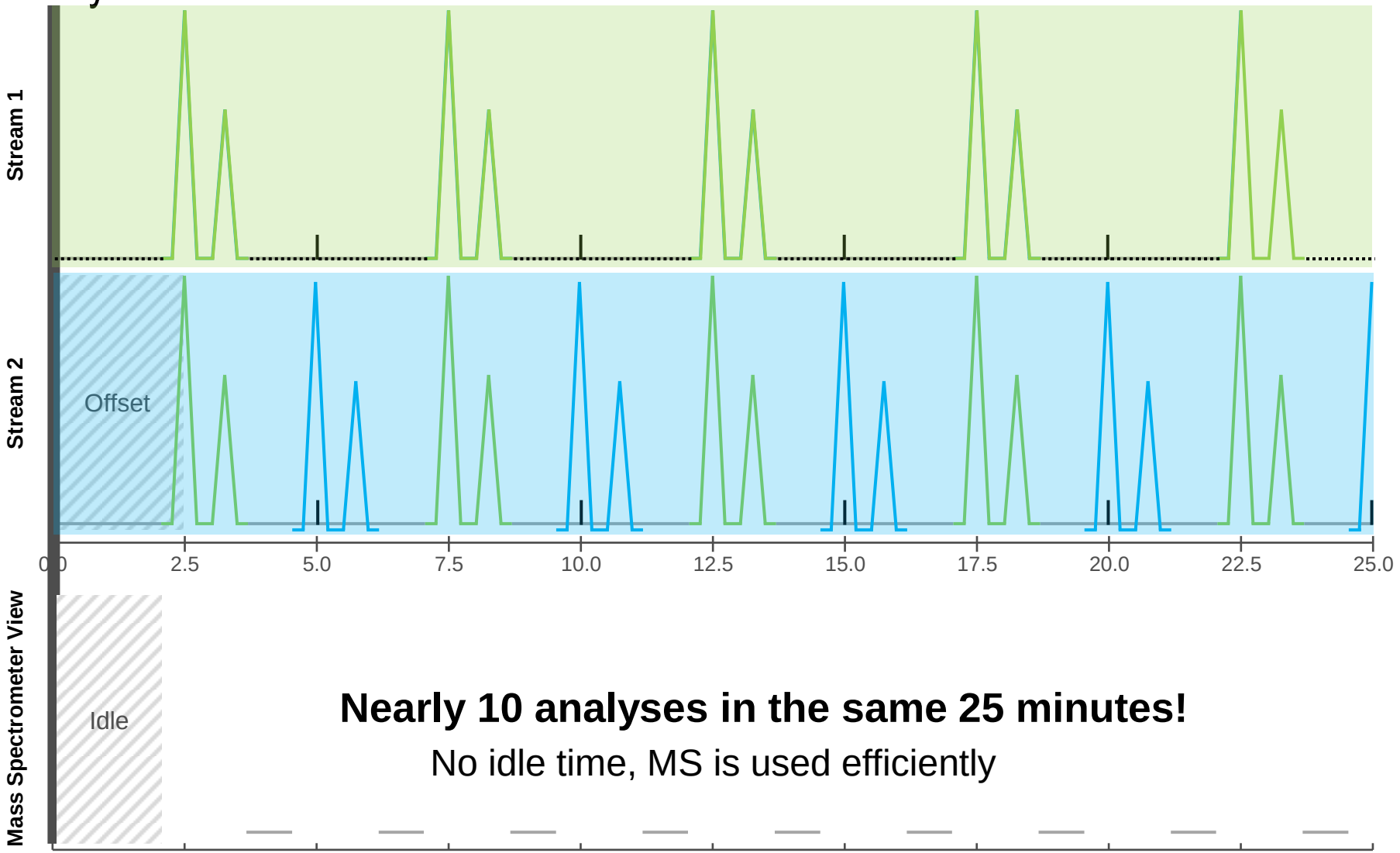


5 analyses in 25 minutes

A significant portion of the chromatographic run contains no peaks of interest

Agilent StreamSelect LC/MS Solution

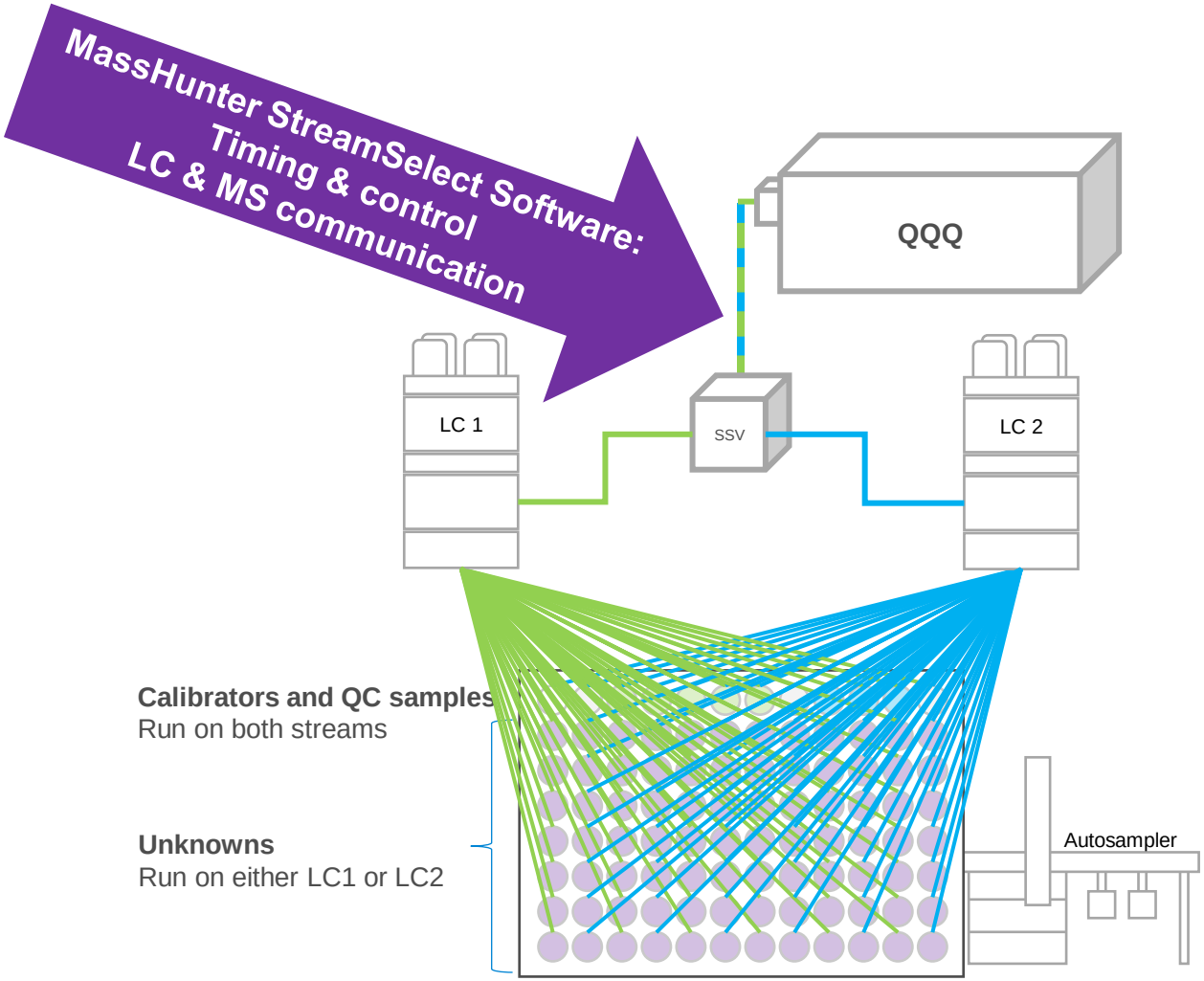
Analyses in Parallel for Researchers



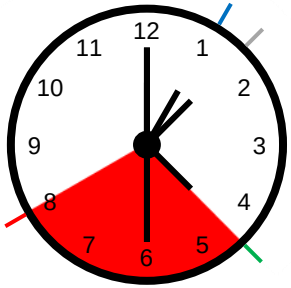
Nearly 10 analyses in the same 25 minutes!

No idle time, MS is used efficiently

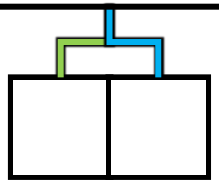
StreamSelect Research Workflow



Dramatically Increase Throughput for Researchers

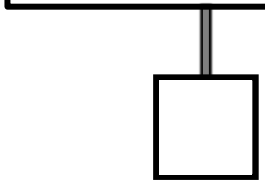


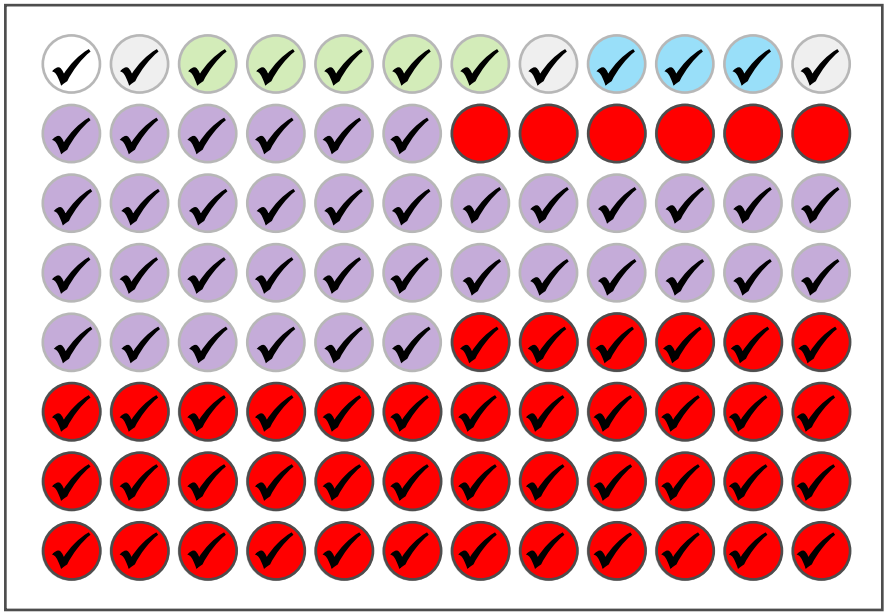
StreamSelect LC/MS



4.5 hours to complete the batch

Standard LC/MS

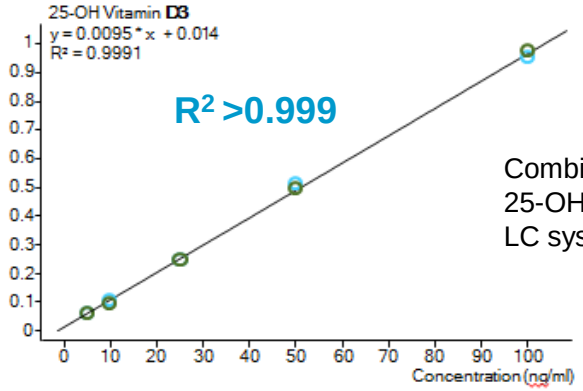




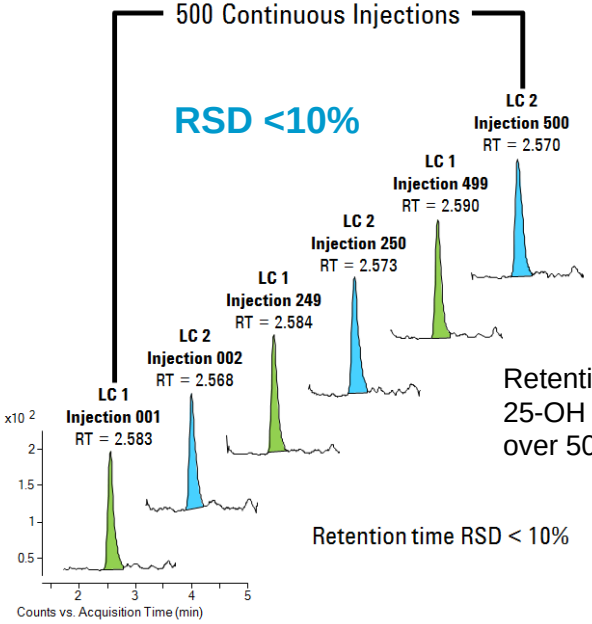
8 hours to complete the batch

Quantitative and Retention Time Reproducibility

Results



Combined calibration curves for 25-OH Vitamin D3 across both LC systems



Retention time reproducibility of 25-OH Vitamin D3 for research over 500 injections across both LCs

Experimental

Trapping column	Agilent Eclipse Plus C18 Guard Column, 2.1 × 12.5 mm, 5 µm
Analytical column	Agilent Pursuit PFP, 2.0 × 50 mm, 3.0 µm
Column temperature	50 °C
Injection volume	10 µL
Needle wash	Flush port, 50:25:25 IPA:MeOH:H_2O, 5 seconds
Mobile phase A	H_2O + 0.1 % formic acid
Mobile phase B	MeOH + 0.1 % formic acid
Flow rate	0.5 mL/min
Stop time	5.0 minutes

Quaternary pump gradient

Time (min)	% B
0.00	50
1.00	90
2.30	90
2.40	98
3.90	98
3.91	50

Binary pump gradient

Time (min)	% B
0.00	85
3.20	85
3.80	98
3.81	85

Valve timing

Time (min)	Position
0.00	1
1.90	2
2.30	1

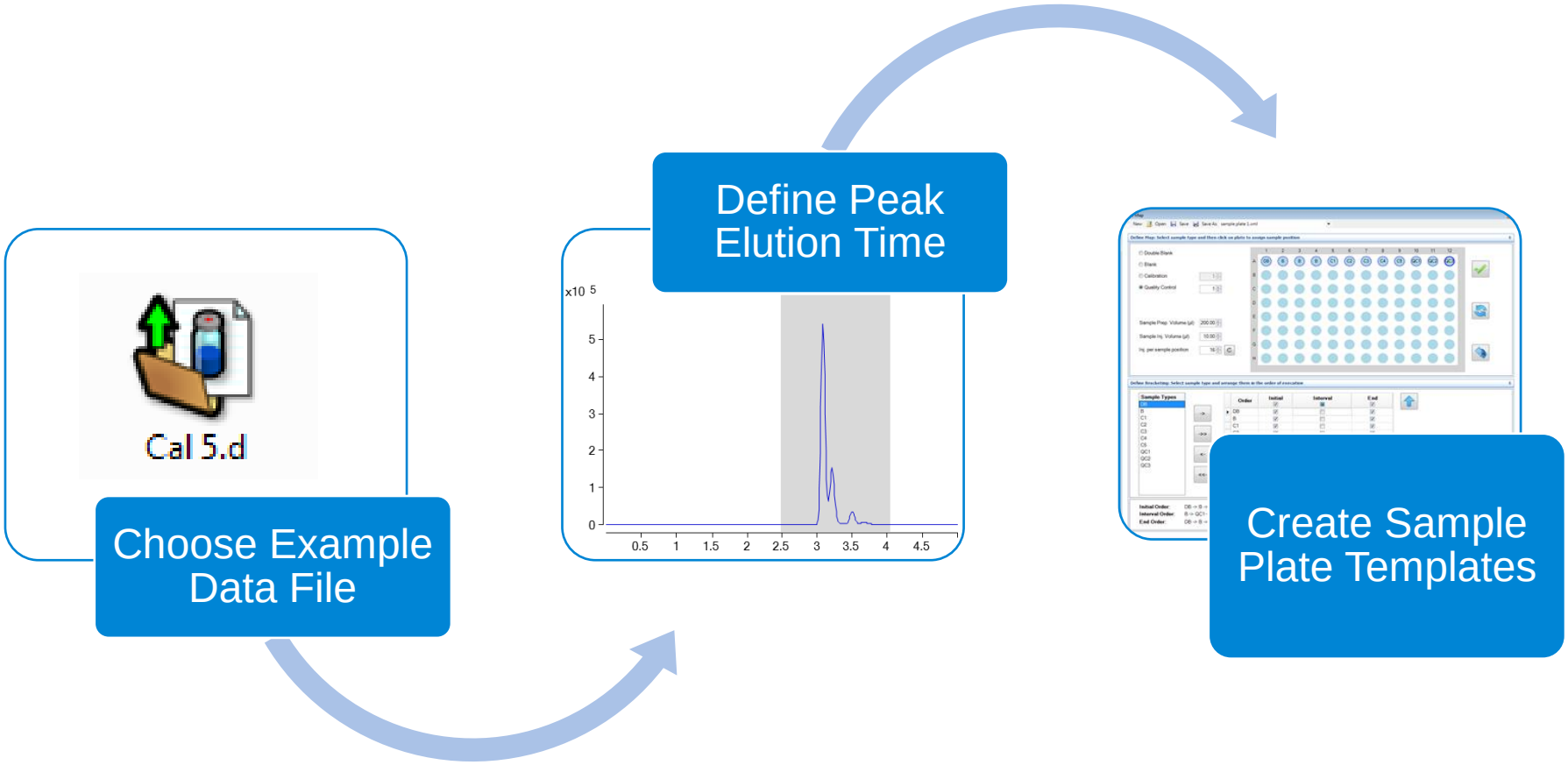
Application Note 5991-2900EN

Batch Submission

Simple 3-Step Process

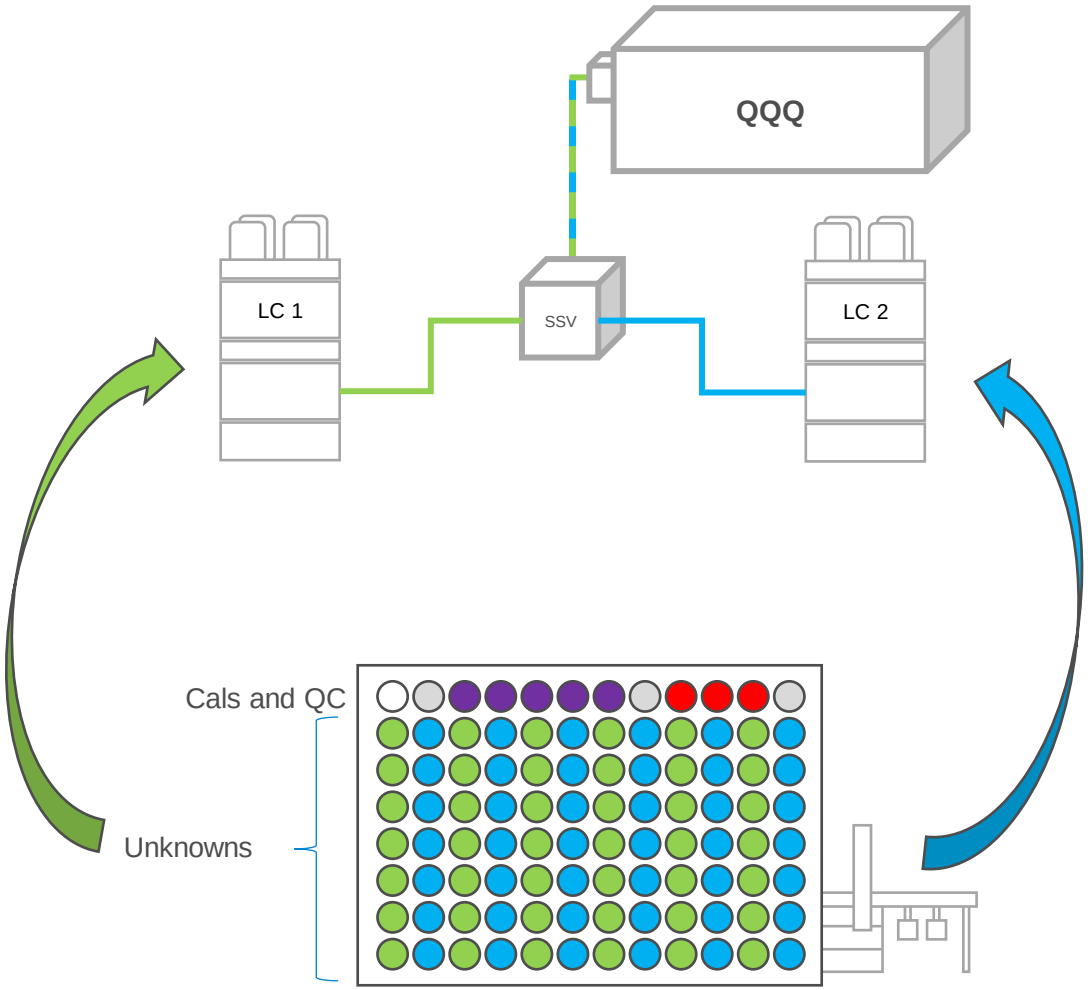
The simulation shows what a researcher would do to submit a batch

StreamSelect Profile Development



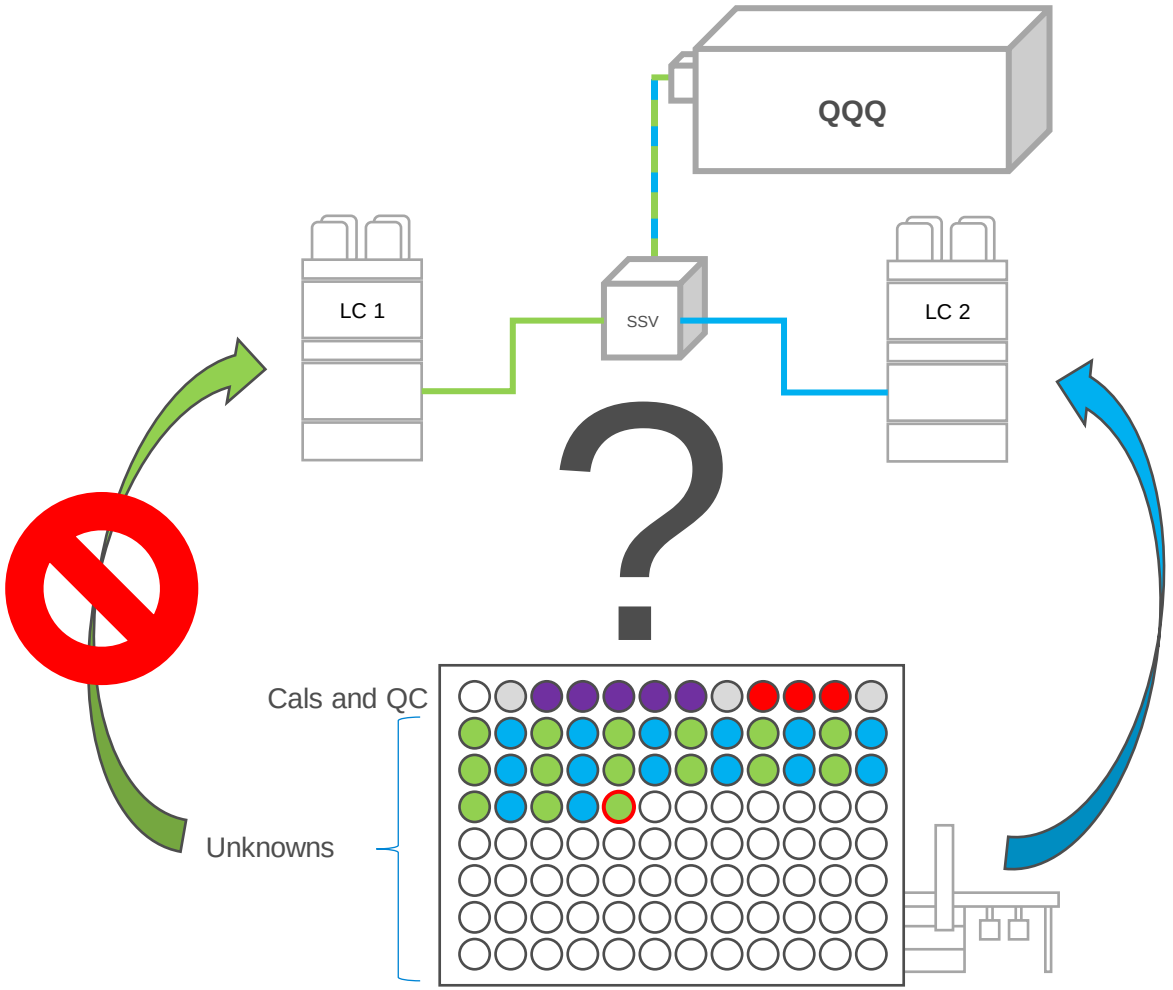
Intelligent Operation
Constant Communication

- Direct, bi-directional communication between the LCs and the MS eliminates sample loss.



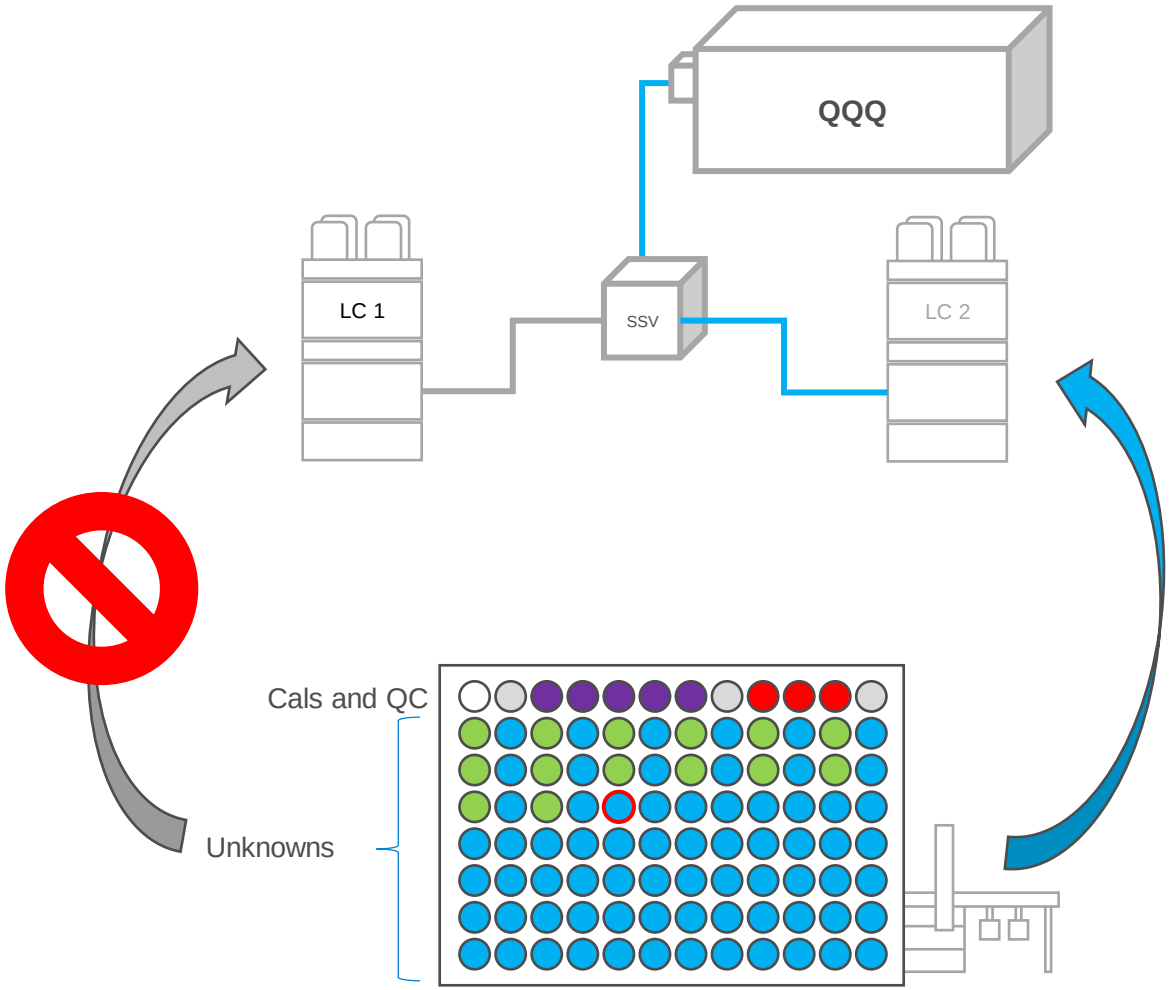
Intelligent Operation
Constant Communication

- Direct, bi-directional communication between the LCs and the MS eliminates sample loss.
- In this example, the LC running Stream 1 fails during the analysis of Sample 29. What happens to Sample 29?

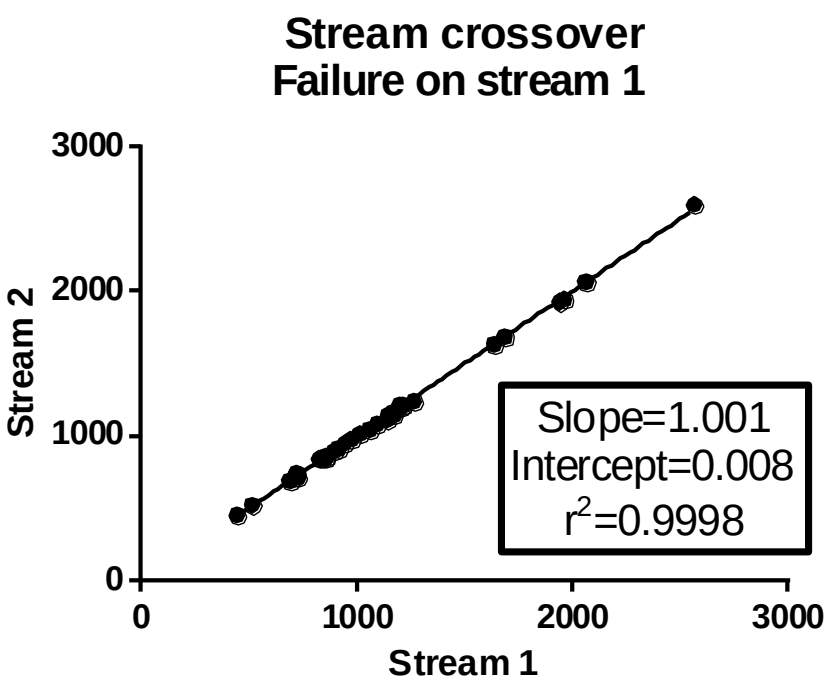


Intelligent Operation
Constant Communication

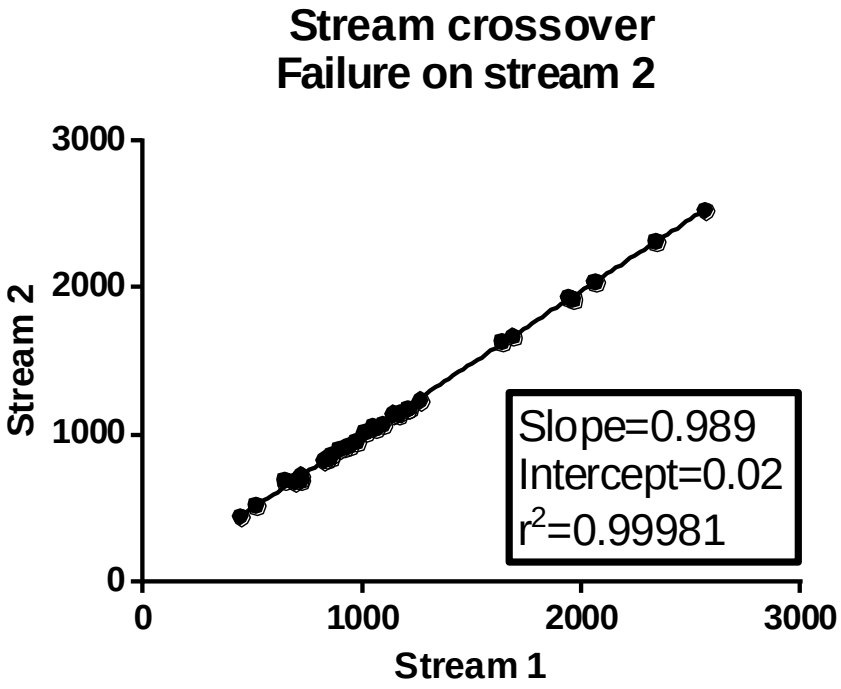
- Direct, bi-directional communication between the LCs and the MS eliminates sample loss.
- In this example, the LC running Stream 1 fails during the analysis of Sample 29. What happens to Sample 29?
- Sample 29 and all further samples are seamlessly re-assigned for analysis on Stream 2, assuring continued productivity without any loss of data.



Intelligent Operation
Stream Equivalency



Simulated Leak on Stream 1, samples diverted to Stream 2, -> equivalent results



Simulated Overpressure on Stream 2, samples diverted to Stream 1 -> equivalent results

Conclusions

- Agilent's StreamSelect LC/MS Solution is an efficient way for researchers to increase productivity without sacrificing quantitative performance
- Intuitive StreamSelect Software is part of a fully integrated system that provides a simple and robust solution to your research laboratory's needs
- Straightforward implementation based on existing methods and efficient system utilization lower overall costs

Questions?

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