

**Sample Preparation  
and Ionization Mode Comparison Study  
for the Quantification of  
25-Hydroxy Vitamin D2/D3  
by LC/MS/MS for Clinical Research**

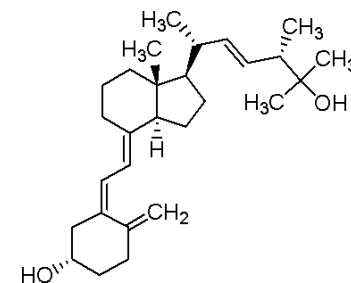
Rory Doyle  
Senior LC/MS Applications Scientist  
Agilent Technologies

# Objectives

Comparison Study between Electrospray (ESI) and Atmospheric Pressure (APCI) ionization modes

Comparison Study between 2-dimensional and 1-dimensional liquid chromatography (LC) modes

Sample Preparation comparison between Liquid-Liquid Extraction (LLE), Protein Precipitation (PPT), and Solid Phase Extraction (SPE)



# Materials- Reagents

## Biological Solutions

Human Serum Double Charcoal Stripped, Delipidized  
Bovine Serum Albumin 10%

Golden West Biologicals  
Golden West Biologicals

## Solvents

Methanol  
Acetonitrile  
Formic Acid

Honeywell  
Honeywell  
Sigma-Aldrich

## Phosphate Buffer Saline

Sodium Chloride  
Sodium Phosphate Dibasic/Monobasic

Sigma-Aldrich  
Sigma-Aldrich

## Other

Zinc Sulphate

Sigma-Aldrich

# Materials- Standards

25-Hydroxy-Vitamin D3

Isosciences

25-Hydroxy-Vitamin D2

25-Hydroxy-Vitamin D<sub>3</sub>-<sup>2</sup>H<sub>3</sub>

25-Hydroxy-Vitamin D<sub>2</sub>-<sup>2</sup>H<sub>3</sub>

Calibrators from 0.01 to 1000 ng/ml made up in 5% BSA/0.1M PBS solution

UTAK Vitamin D PLUS Controls Low, 1 and 2

NIST Standards SRM 972- Levels 1 to 4

# Method 1D: Agilent 1200 RR-HPLC SL System

**HPLC:** binary pump, well plate sampler, thermostatic column compartment

**Column:** Poroshell 120 EC-C18, 2.1 x 50mm, 2.7  $\mu\text{m}$

**Column temperature:** 50°C

**Injection volume:** 10  $\mu\text{L}$

**Auto sampler temp:** 5°C

**Needle wash:** flush port (50:25:25, IPA:MeOH:H<sub>2</sub>O) 5 seconds

**Mobile phase:**  
A = H<sub>2</sub>O + 0.1% Formic Acid  
B = MeOH + 0.1% Formic Acid

**Flow rate:** 0.5 mL/min

**Isocratic Analysis:** A = 20% B = 80%

**Analysis Time:** 5.0 min



# Method 2D: Agilent 1200 RR-HPLC SL System

**HPLC:** 2 x binary pump, well plate sampler, thermostatic column compartment

**Column A:** Eclipse Plus-C18 Guard Column 2.1 x 12.5mm, 5  $\mu$ m

**Column B:** Poroshell 120 EC-C18, 2.1 x 50mm, 2.7  $\mu$ m

**Column temperature:** 50°C

**Injection volume:** 10  $\mu$ L

**Auto sampler temp:** 5°C

**Needle wash:** flush port (50:25:25, IPA:MeOH:H<sub>2</sub>O) 5 seconds

**Mobile phase:**  
A = H<sub>2</sub>O + 0.1% Formic Acid  
B = MeOH + 0.1% Formic Acid

**Flow rate:** 0.5 mL/min

**Isocratic Analysis:** A = 15% B = 85%

**Analysis Time:** 5.0 min



# Solvent Parameters

## 1D

### Eluting Column-20%/80%

| Time | B% |
|------|----|
| 0    | 80 |
| 2.3  | 80 |
| 2.4  | 98 |
| 3.9  | 98 |
| 4    | 80 |

## 2D

### Loading Column 50%/50%

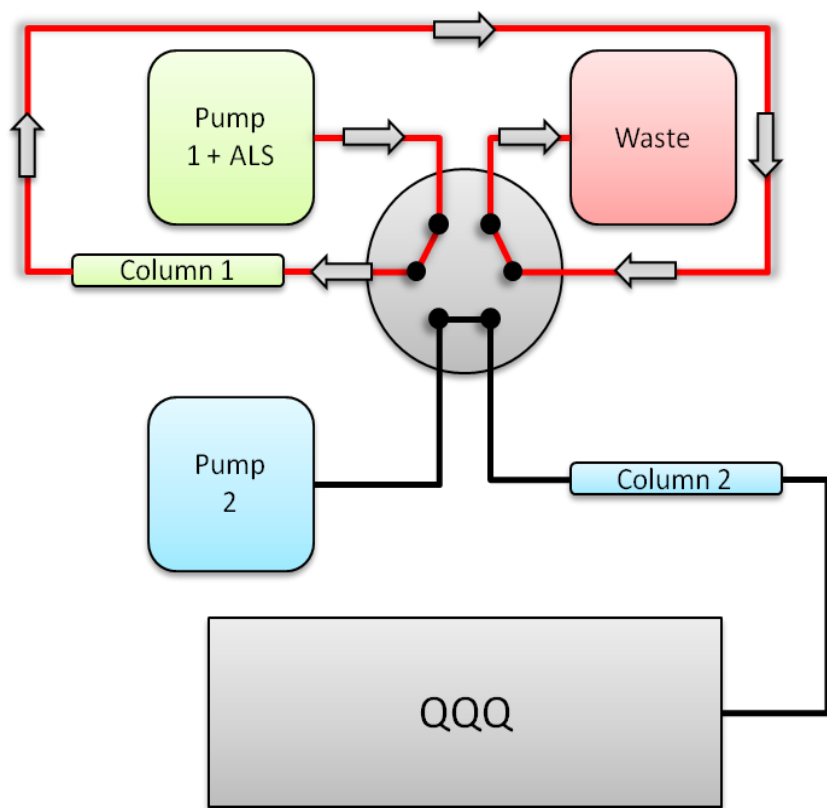
| Time | B% |
|------|----|
| 1    | 90 |
| 2.3  | 90 |
| 2.4  | 98 |
| 3.3  | 98 |
| 3.4  | 50 |

### Elutting Column-15%/85%

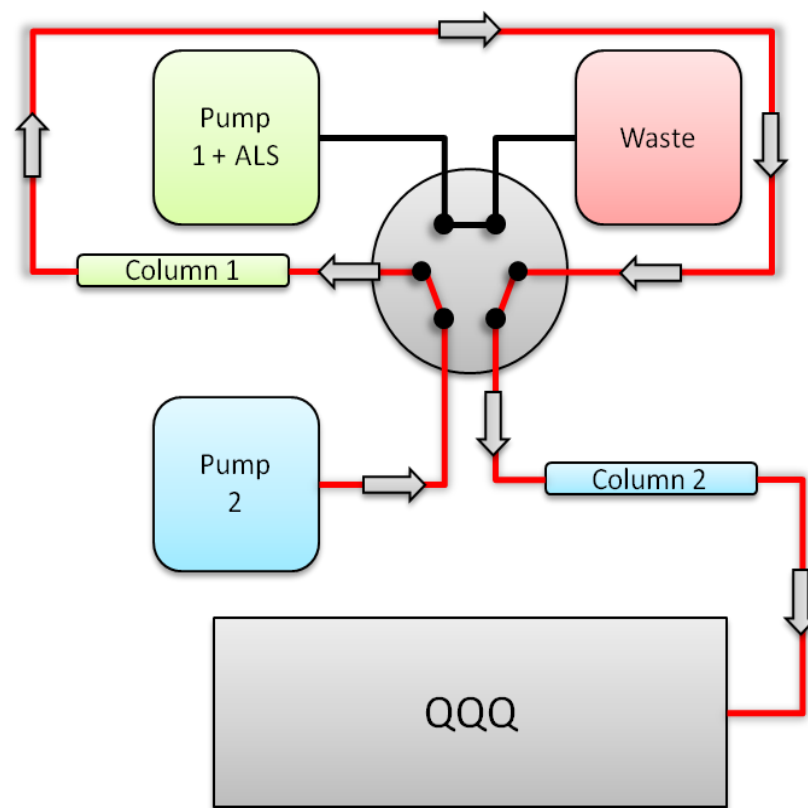
|      |    |
|------|----|
| 3.2  | 85 |
| 3.3  | 98 |
| 3.8  | 98 |
| 3.81 | 85 |
| 5    | 85 |

# On-line Sample Clean-up (2D) Flow Diagram

## Position 1: 1.9 mins



## Position 2: 2.4 mins





# Methods: MS Parameters Used

Parameters used in this comparison

**MS:** Agilent 6400 Series HS Triple Quadrupole LC/MS/MS

## QQQ MS Conditions-6430

Ion Mode: Positive APCI ESI

### Source conditions:

Drying Gas Temp: 350 °C 275 °C

Vaporizer Temp: 250 °C ---

Drying Gas Flow: 5 L/min 10 L/min (5)

Nebulizer Pressure: 25 psi 50 psi (45)

Capillary Voltage 3000 V 5000 V

Corona Current: 2 µA ---

Charging Voltage: --- ---

**MRM acquisition: (Q1 peak width = 0.7 and Q2 peak width = 0.70 m/z)**

Delta EMV 200 to 400V

Note- 6460 Sheath Gas temp- 325 °C/ Gas Flow- 11 L/min



# Methods: Other MS Parameters

Other parameters used in previous Agilent studies

**MS:** Agilent 6400 Series HS Triple Quadrupole LC/MS/MS

## QQQ MS Conditions

| Ion Mode: Positive | APPI* | MM-APCI | MM-ESI | MM-ESI + APCI |
|--------------------|-------|---------|--------|---------------|
|--------------------|-------|---------|--------|---------------|

### Source conditions:

|                     |         |         |         |         |
|---------------------|---------|---------|---------|---------|
| Drying Gas Temp:    | 350 °C  | 250 °C  | 250 °C  | 250 °C  |
| Vaporizer Temp:     | 250 °C  | 170 °C  | 170 °C  | 170 °C  |
| Drying Gas Flow:    | 5 L/min | 5 L/min | 5 L/min | 5 L/min |
| Nebulizer Pressure: | 20 psi  | 60 psi  | 60 psi  | 60 psi  |

|                   |        |        |        |        |
|-------------------|--------|--------|--------|--------|
| Capillary Voltage | 3000 V | 3000 V | 3000 V | 3000 V |
| Corona Current:   | ---    | 2 µA   | ---    | 2 µA   |
| Charging Voltage: | ---    | ---    | 2000 V | 2000 V |

**MRM acquisition: (Q1 peak width = 0.7 and Q2 peak width = 0.70 m/z)**

Delta EMV            200 to 400V

\*APPI- Post column addition of 5% Toluene

# MS MRM Transition Ions- ESI Mode

| Compound   | MRM           | Fragmentor (V) | Dwell (msec) | Collision Energy (V) |
|--|---------------|----------------|--------------|----------------------|
| 25-Hydroxy Vitamin D <sub>3</sub>                                  | 401.3 > 383.2 | 106            | 100          | 4                    |
|  | 401.3 > 159.1 | 106            | 100          | 24                   |
| 25-Hydroxy Vitamin D <sub>2</sub>                                  | 413.3 > 395.3 | 106            | 100          | 4                    |
|  | 413.4 > 355.2 | 106            | 100          | 4                    |
| IS-25-Hydroxy Vitamin D <sub>3</sub> - <sup>2</sup> H <sub>3</sub> | 404.4 > 386.3 | 106            | 100          | 4                    |
| IS-25-Hydroxy Vitamin D <sub>2</sub> - <sup>2</sup> H <sub>3</sub> | 416.4 > 398.3 | 106            | 100          | 4                    |

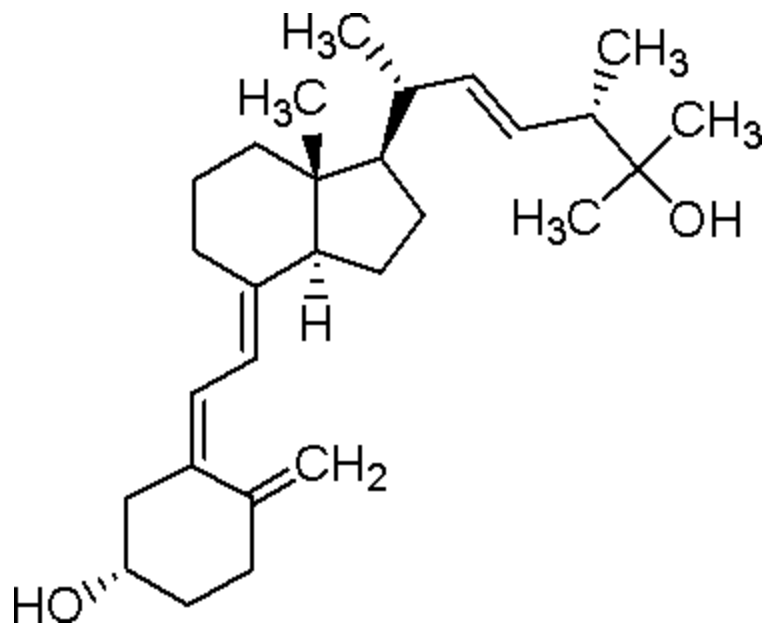
# MS MRM Transition Ions- APCI Mode

| Compound   | MRM           | Fragmentor (V) | Dwell (msec) | Collision Energy (V) |
|--|---------------|----------------|--------------|----------------------|
| 25-Hydroxy Vitamin D <sub>3</sub>                                  | 401.3 > 383.4 | 122            | 100          | 4                    |
|  | 401.3 > 159.1 | 122            | 100          | 28                   |
| 25-Hydroxy Vitamin D <sub>2</sub>                                  | 413.3 > 395.3 | 82             | 100          | 4                    |
|  | 413.3 > 355.3 | 82             | 100          | 4                    |
| IS-25-Hydroxy Vitamin D <sub>3</sub> - <sup>2</sup> H <sub>3</sub> | 404.4 > 386.4 | 82             | 100          | 4                    |
| IS-25-Hydroxy Vitamin D <sub>2</sub> - <sup>2</sup> H <sub>3</sub> | 416.4 > 398.5 | 82             | 100          | 4                    |

# Other MS MRM Transition Ions- APCI/ESI Mode

| Compound   | MRM           | Fragmentor (V) | Dwell (msec) | Collision Energy (V) |
|--|---------------|----------------|--------------|----------------------|
| 25-Hydroxy Vitamin D <sub>3</sub>                                  | 383.3 > 365.4 | 152            | 100          | 8                    |
|  | 383.3 > 211.1 | 152            | 100          | 20                   |
| 25-Hydroxy Vitamin D <sub>2</sub>                                  | 395.3 > 269.1 | 152            | 100          | 16                   |
|  | 395.3 > 211.1 | 152            | 100          | 24                   |
| IS-25-Hydroxy Vitamin D <sub>3</sub> - <sup>2</sup> H <sub>3</sub> | 386.3 > 368.4 | 152            | 100          | 12                   |
| IS-25-Hydroxy Vitamin D <sub>2</sub> - <sup>2</sup> H <sub>3</sub> | 398.4 > 272.2 | 152            | 100          | 20                   |

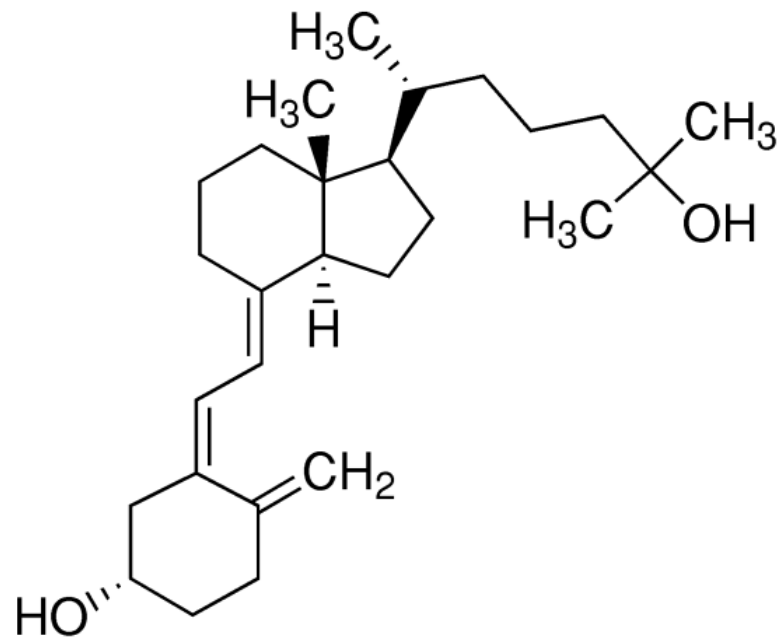
# Structures



**25-Hydroxyvitamin D<sub>2</sub>**

**C<sub>28</sub>H<sub>44</sub>O<sub>2</sub>**

**MW 412.3 g/mol**

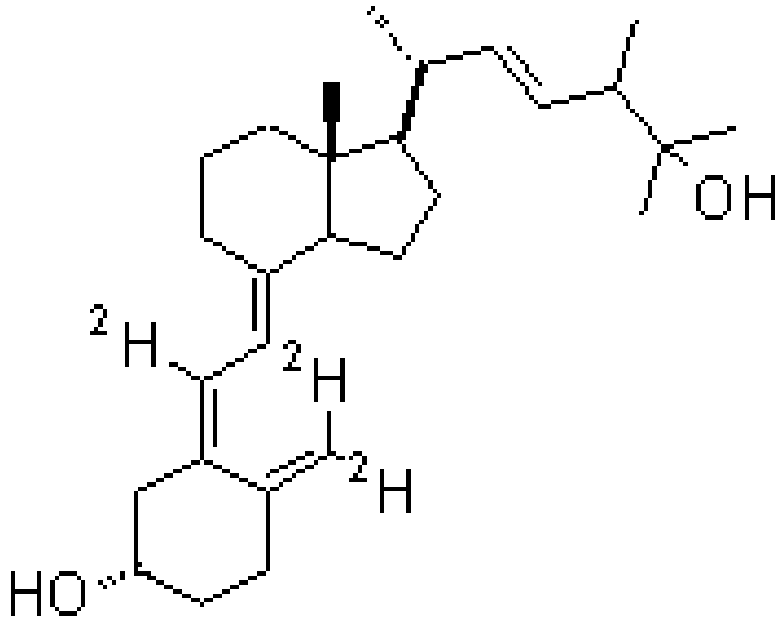


**25-Hydroxyvitamin D<sub>3</sub>**

**C<sub>27</sub>H<sub>44</sub>O<sub>2</sub>**

**MW 400.3 g/mol**

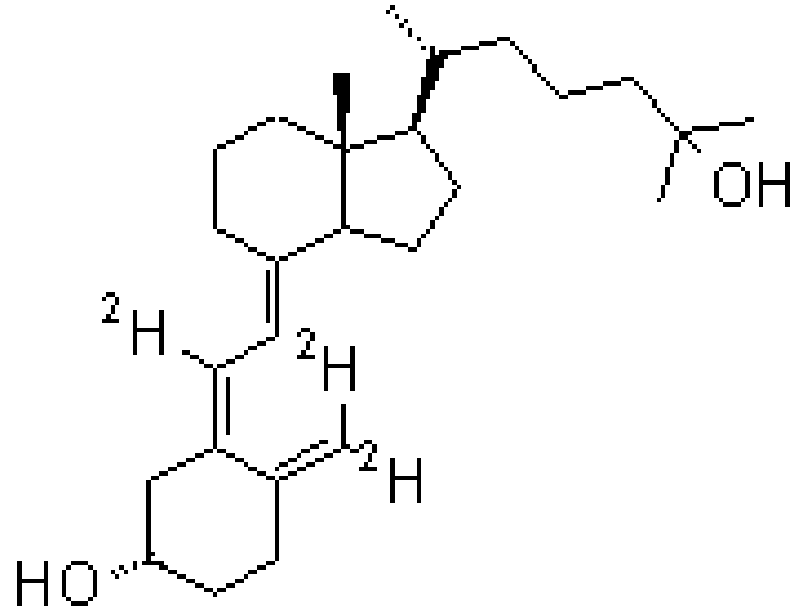
# Internal Standard Structures



**25-Hydroxyvitamin D<sub>2</sub>-<sup>2</sup>H<sub>3</sub>**

**C<sub>28</sub>H<sub>41</sub>D<sub>3</sub>O<sub>2</sub>**

**MW 415.3 g/mol**

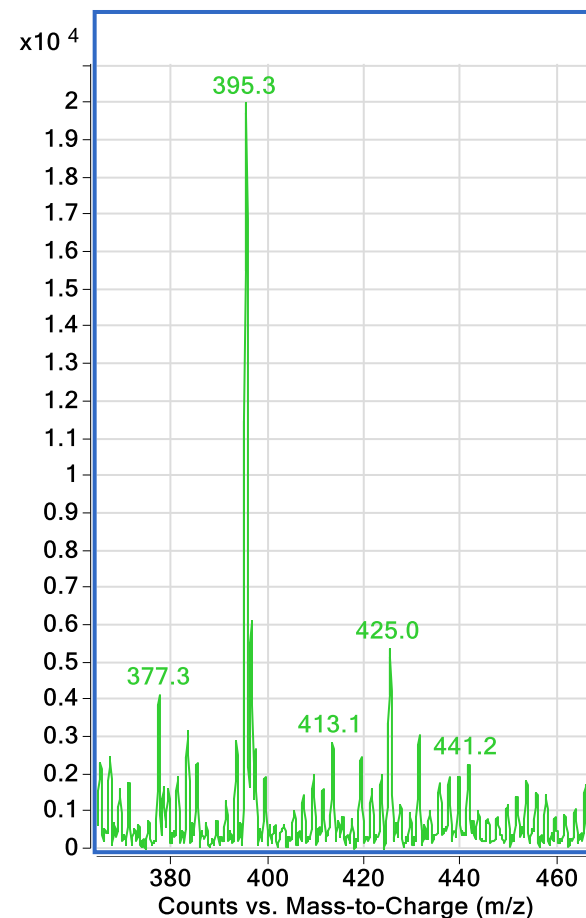
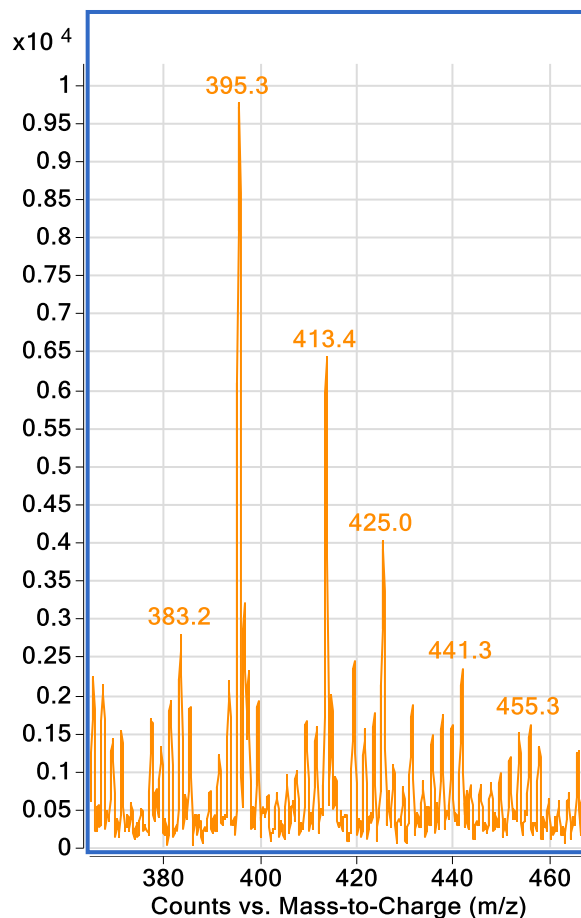
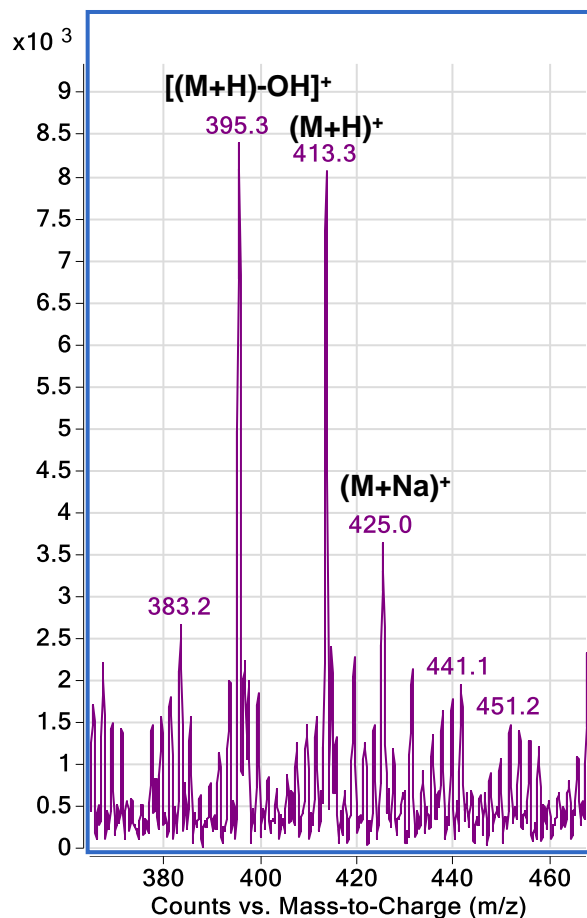


**25-Hydroxyvitamin D<sub>3</sub>-<sup>2</sup>H<sub>3</sub>**

**C<sub>27</sub>H<sub>41</sub>D<sub>3</sub>O<sub>2</sub>**

**MW 403.3 g/mol**

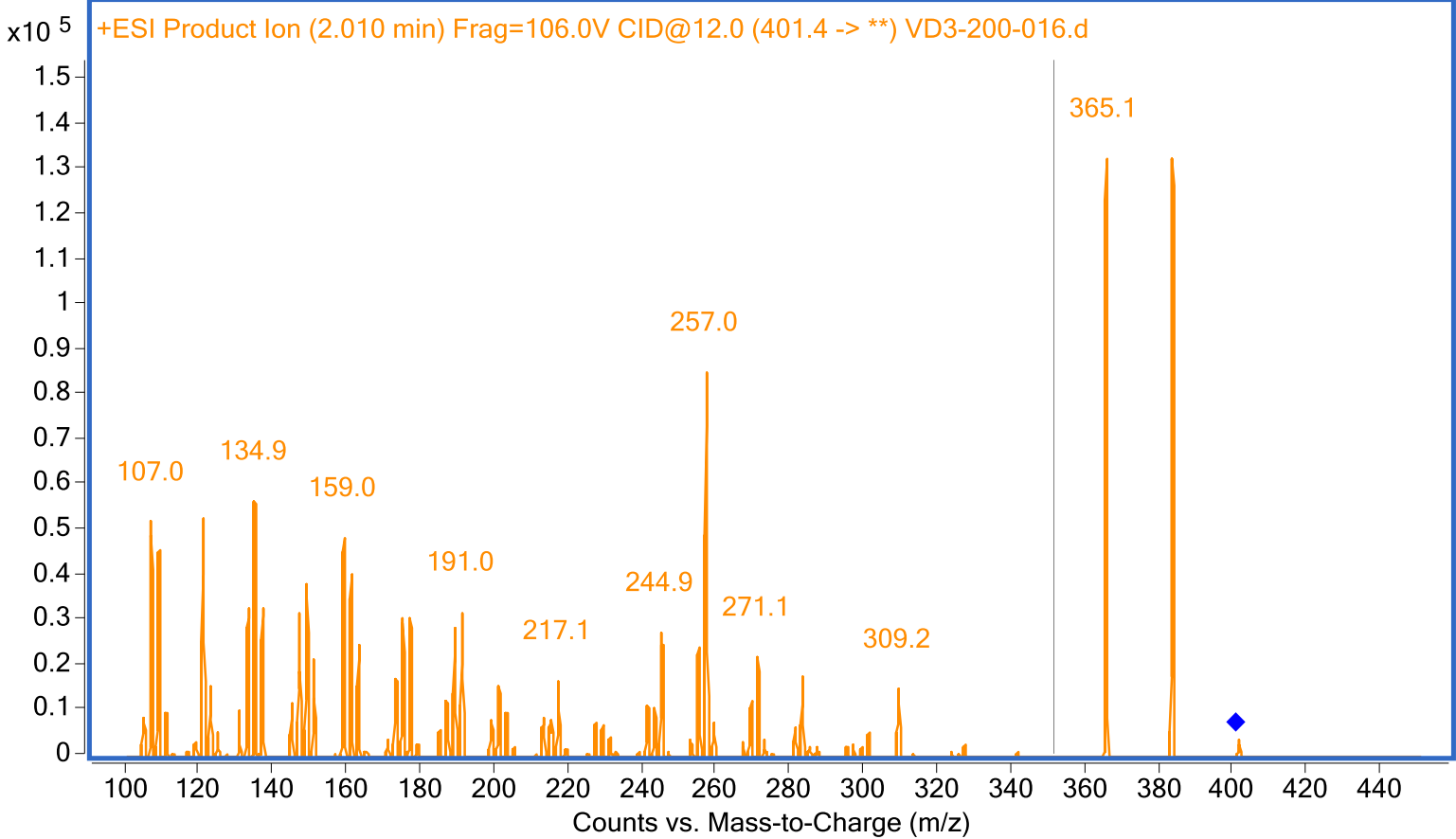
# Effect of temperature on (M+H)<sup>+</sup> Formation



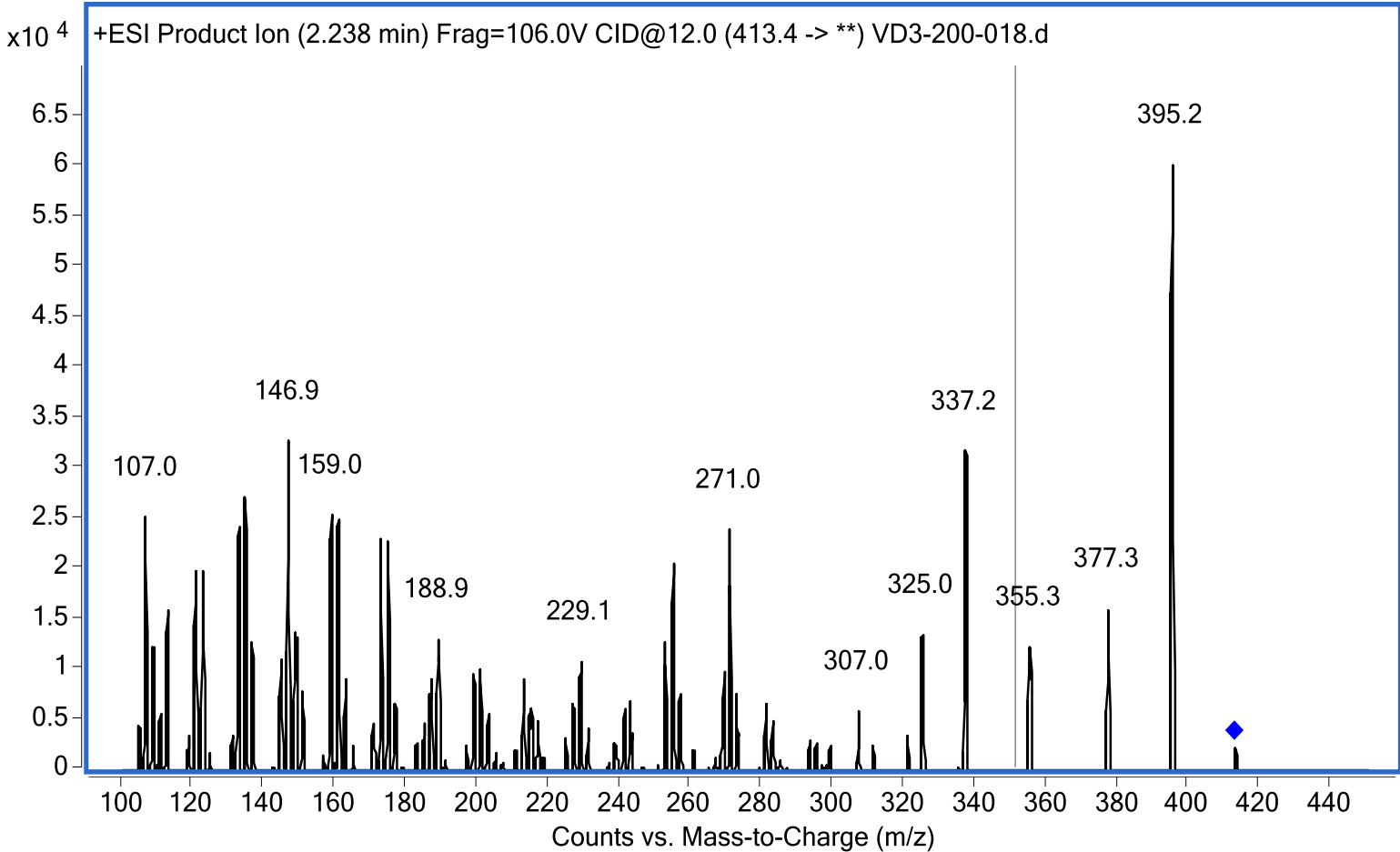
ESI-D<sub>2</sub> Drying Gas 200°      275°      350°  
APCI- Vaporization temperature more important



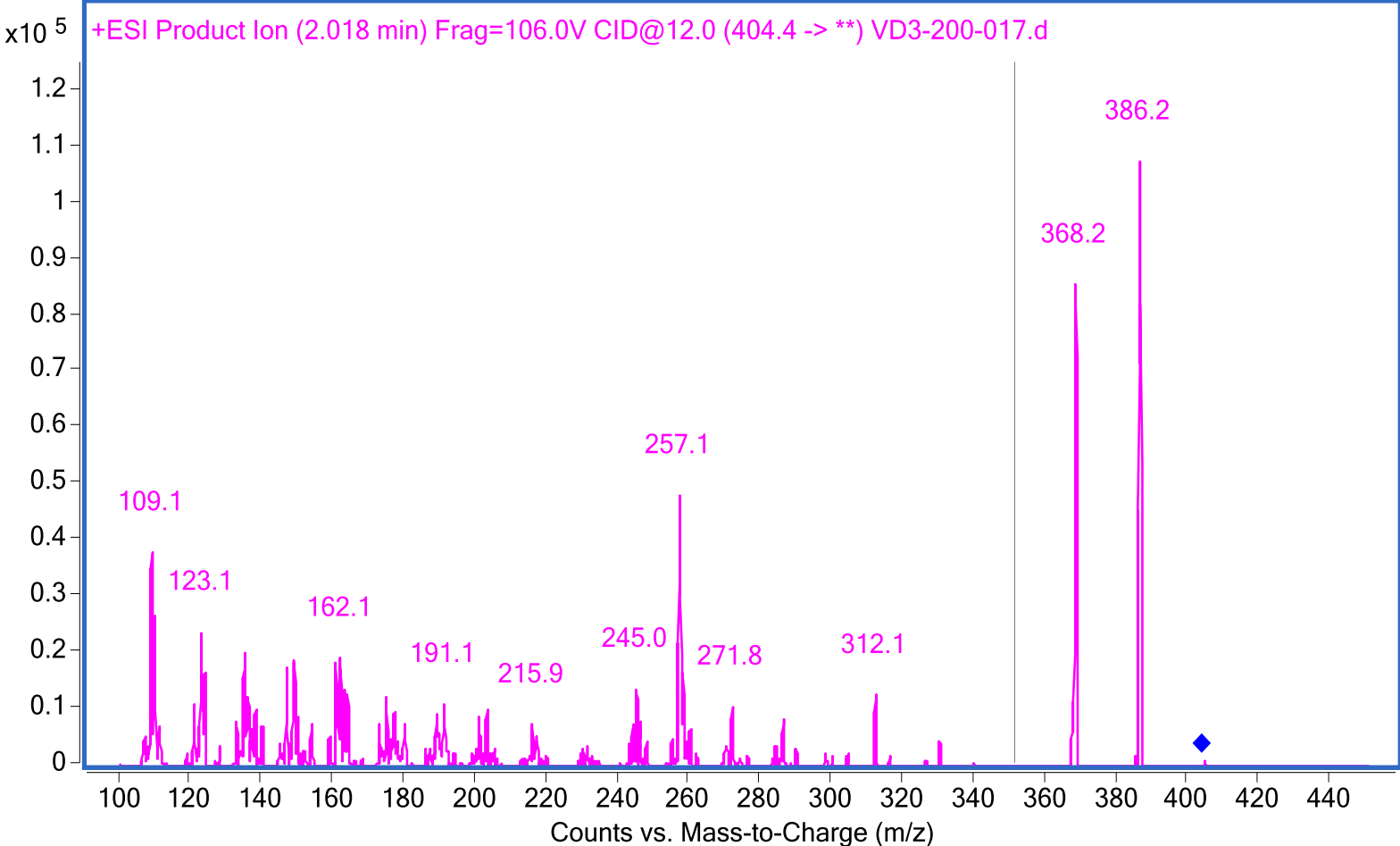
# Product Ion: MRM Ion Selection-D3



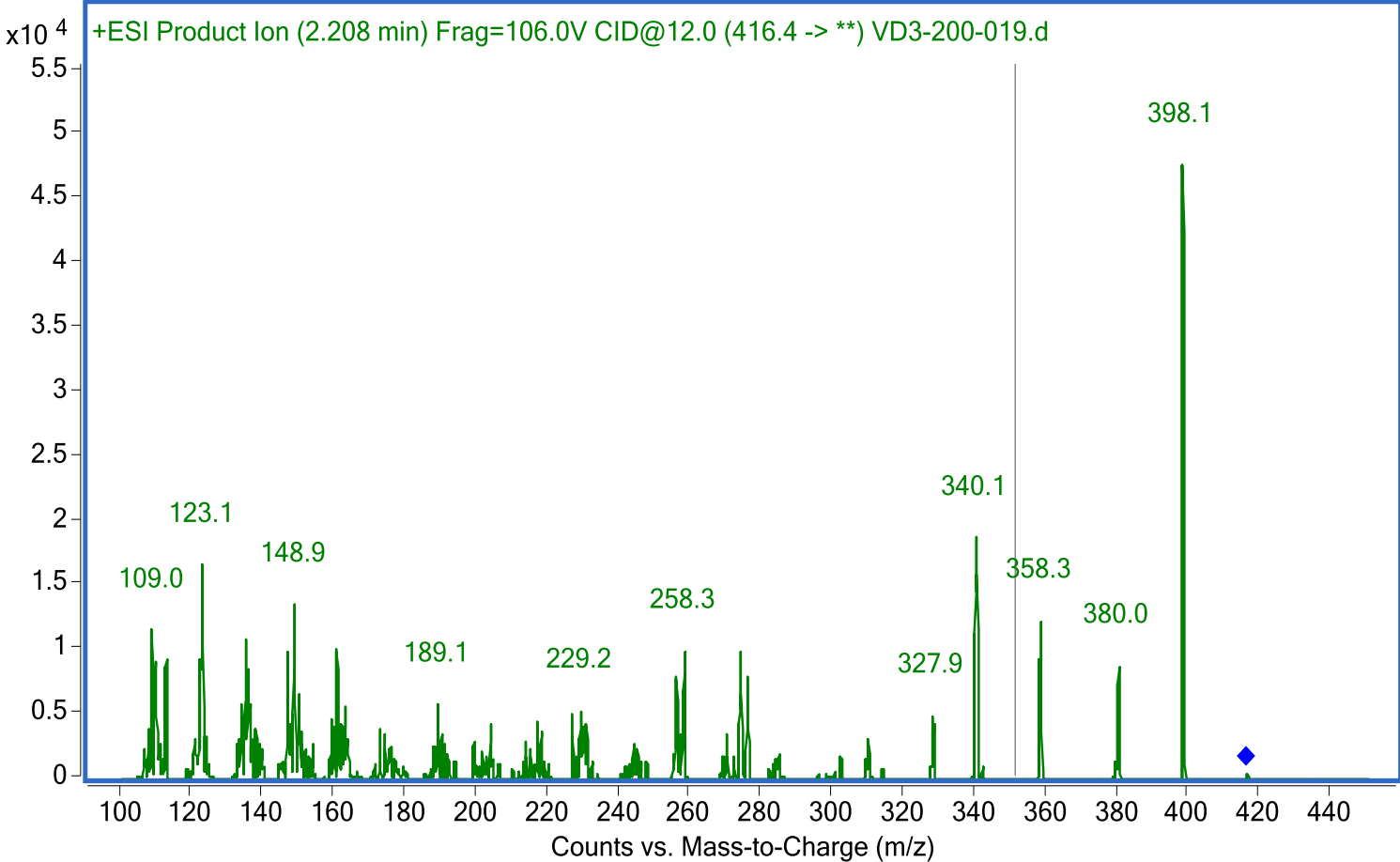
# Product Ion: MRM Ion Selection-D2



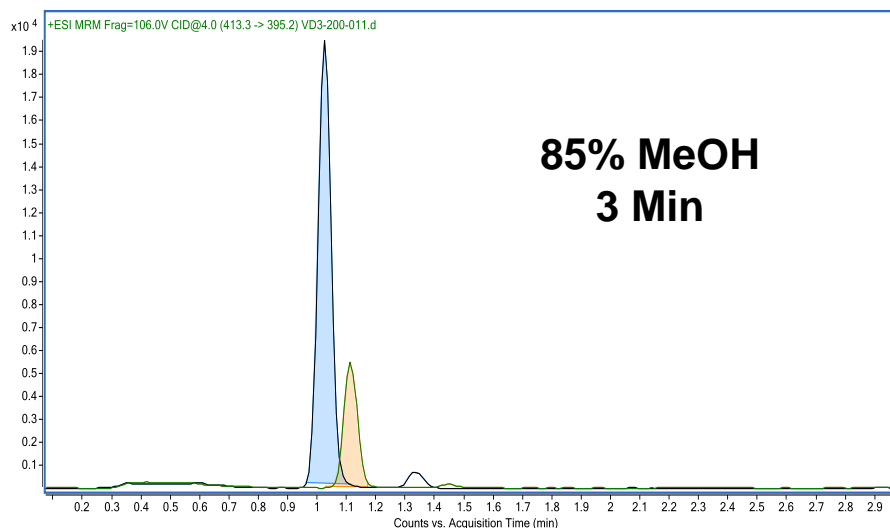
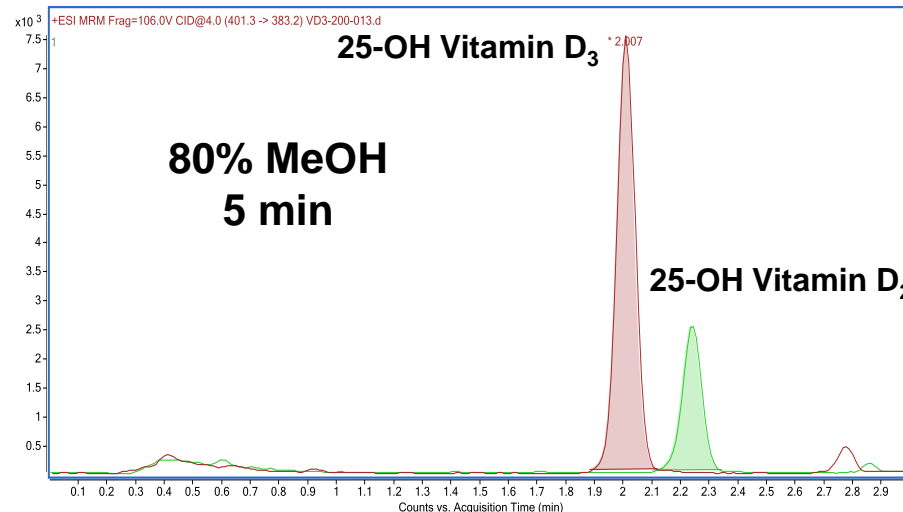
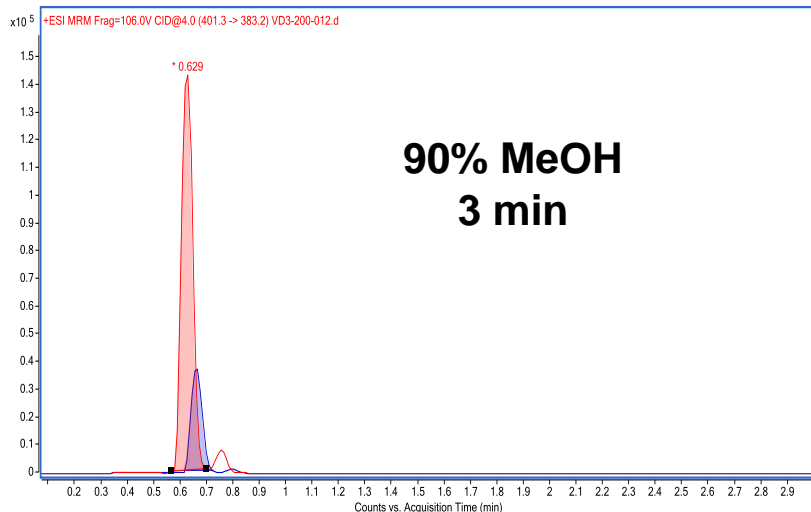
# Product Ion: MRM Ion Selection-D3 ISTD



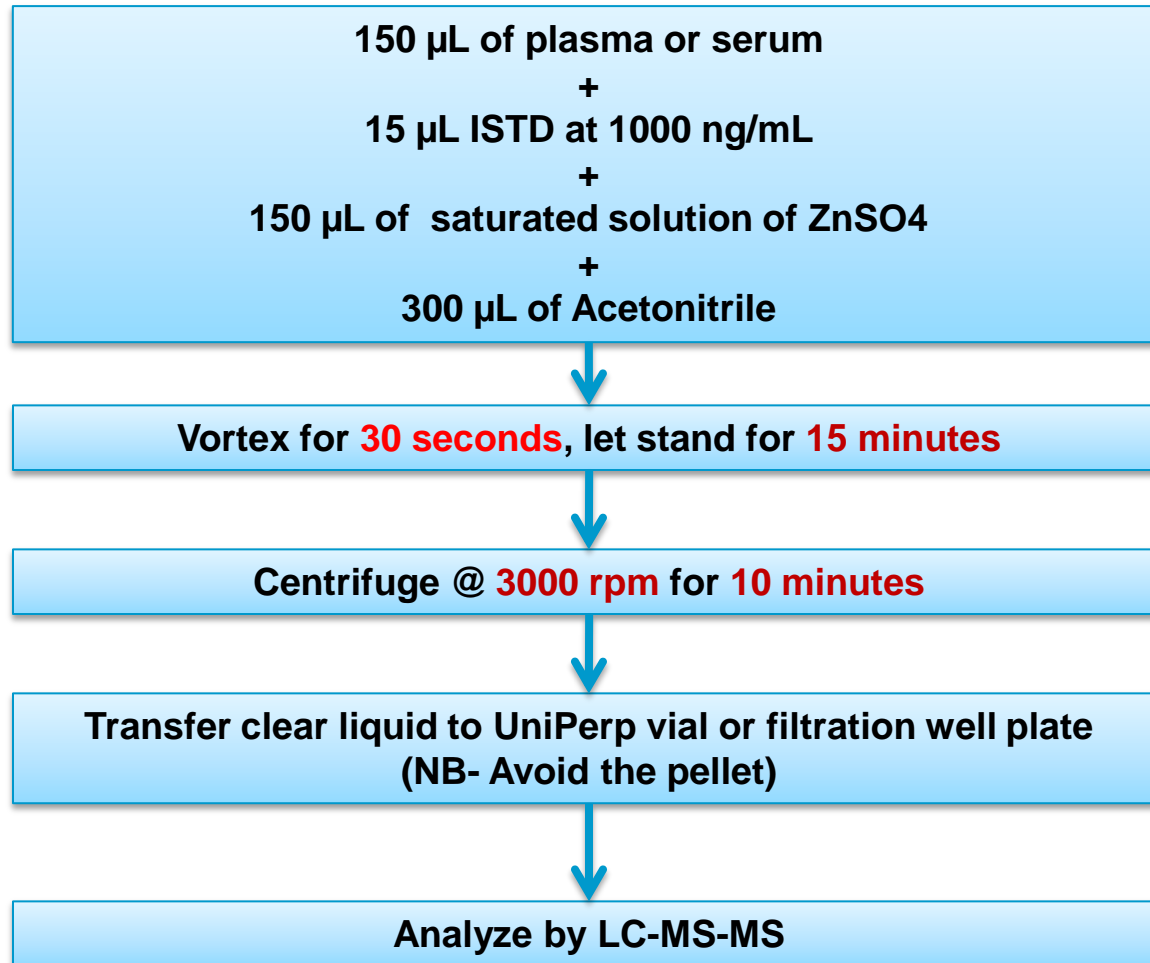
# Product Ion: MRM Ion Selection- D2 ISTD



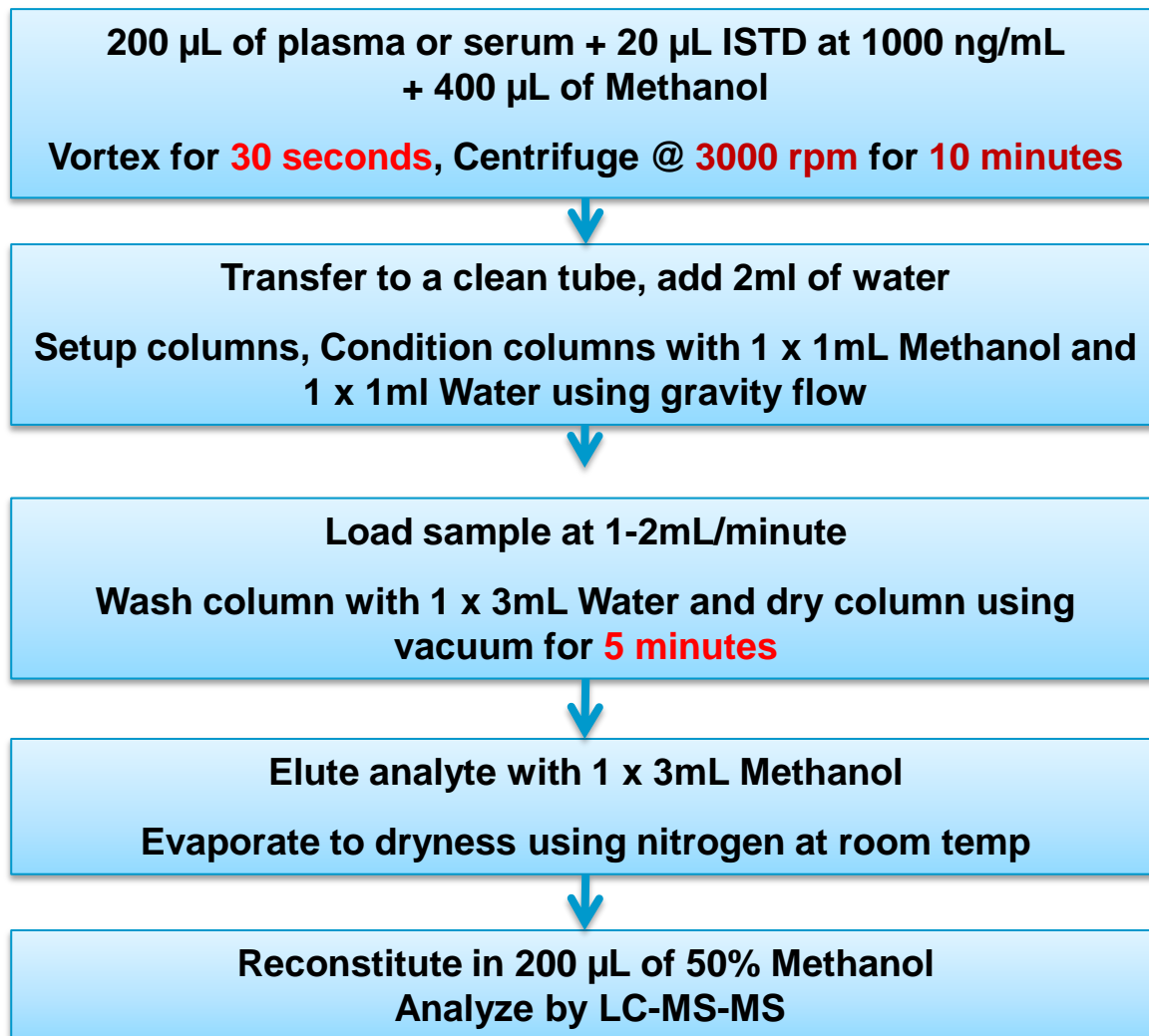
# Chromatography: Speed vs. Resolution 1D



# Sample Preparation- Protein Crash



# Sample Preparation- Solid Phase Extraction Using Bond Elut



# Sample Preparation- Liquid/Liquid Extraction

150  $\mu$ L of plasma or serum + 15  $\mu$ L ISTD at 1000ng/ml  
Vortex **30 seconds**, let it stand 10 minutes in room temp

150  $\mu$ L of 0.2M of ZnSO<sub>4</sub> + 300  $\mu$ L Methanol  
Vortex **30 seconds**

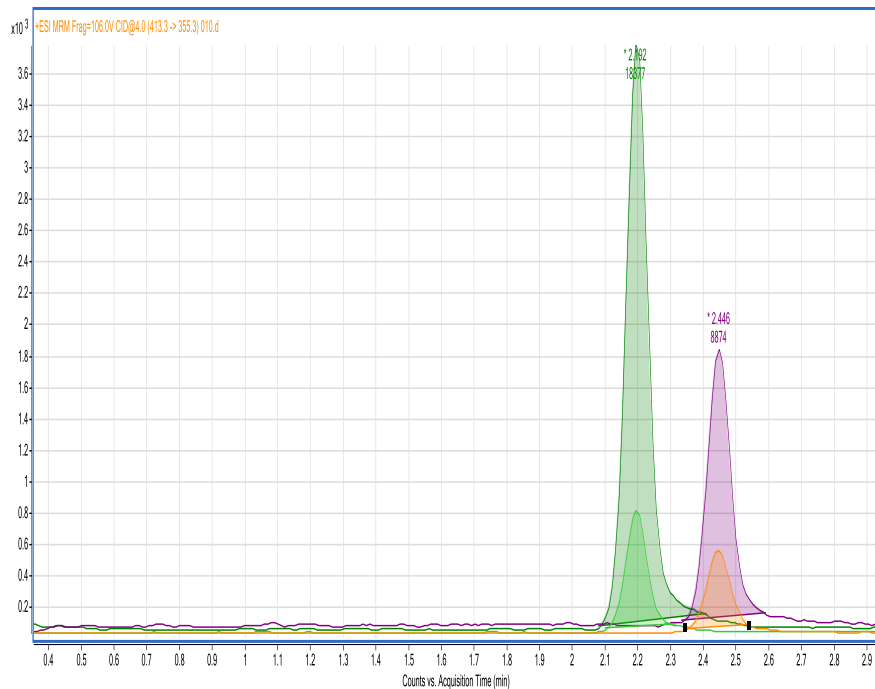
750  $\mu$ L Hexane  
Vortex **30 seconds**  
Centrifuge @ **13000 rpm** for **5 minutes**

Transfer Hexane layer (top) to a tube  
then evaporate under N<sub>2</sub>

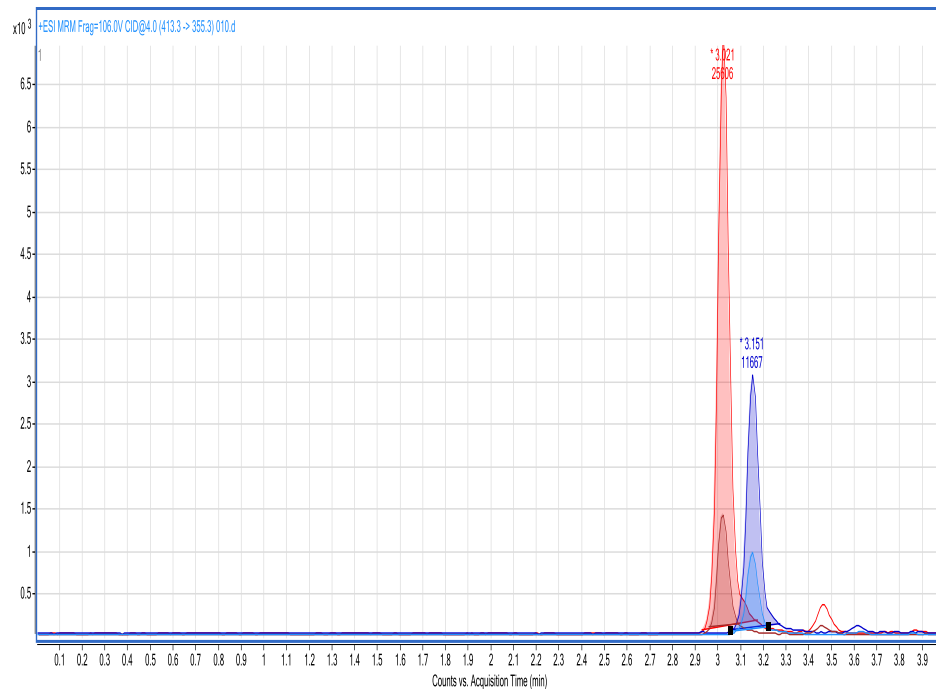
Reconstitute in 200  $\mu$ L in 50%Methanol  
Analyze by LC-MS-MS



# ESI PPT at 100 ng/mL

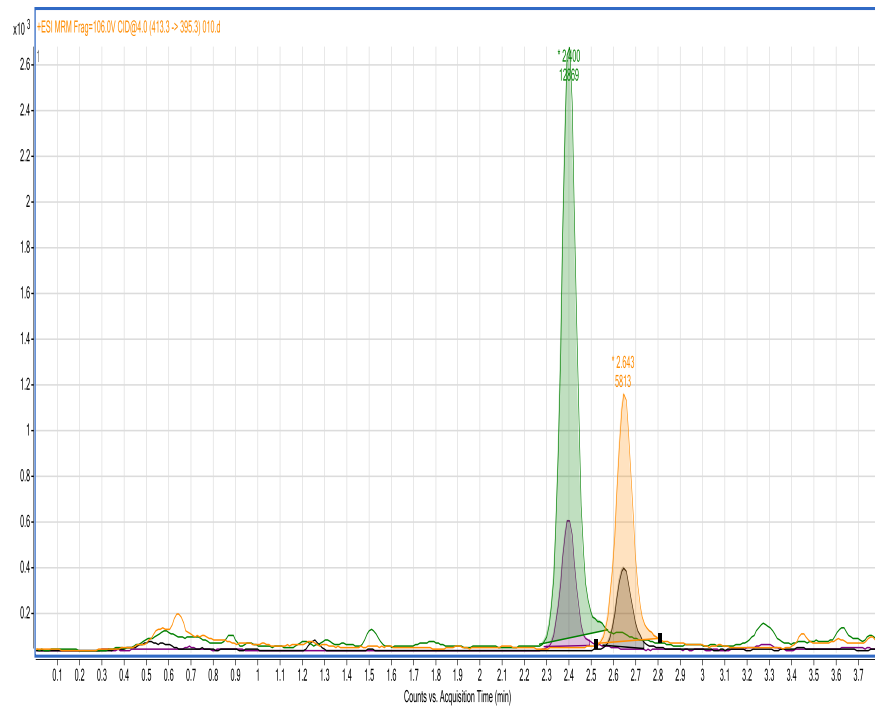


**1D Counts 3600**

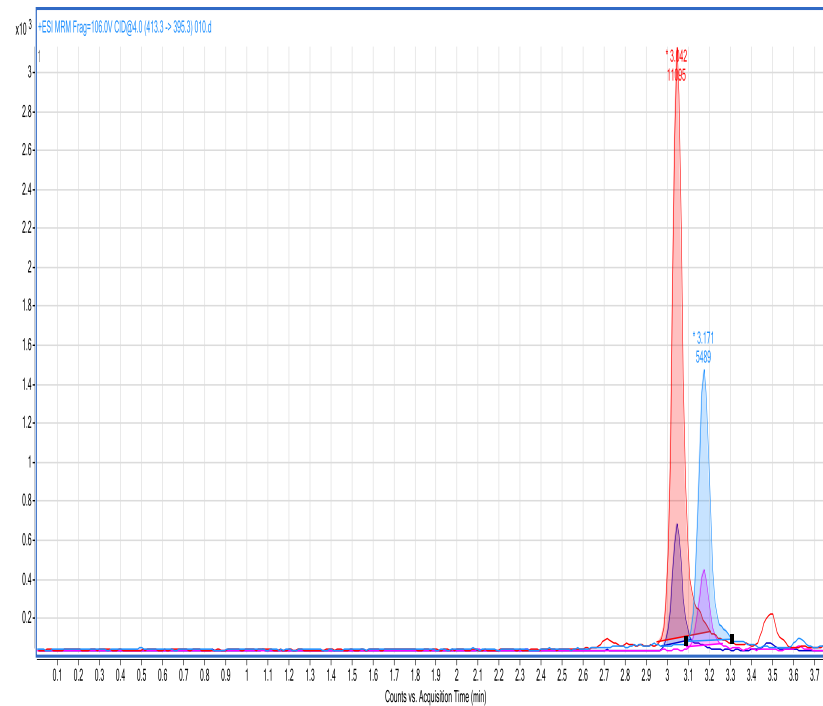


**2D Counts 6500**

# ESI SPE at 100 ng/mL

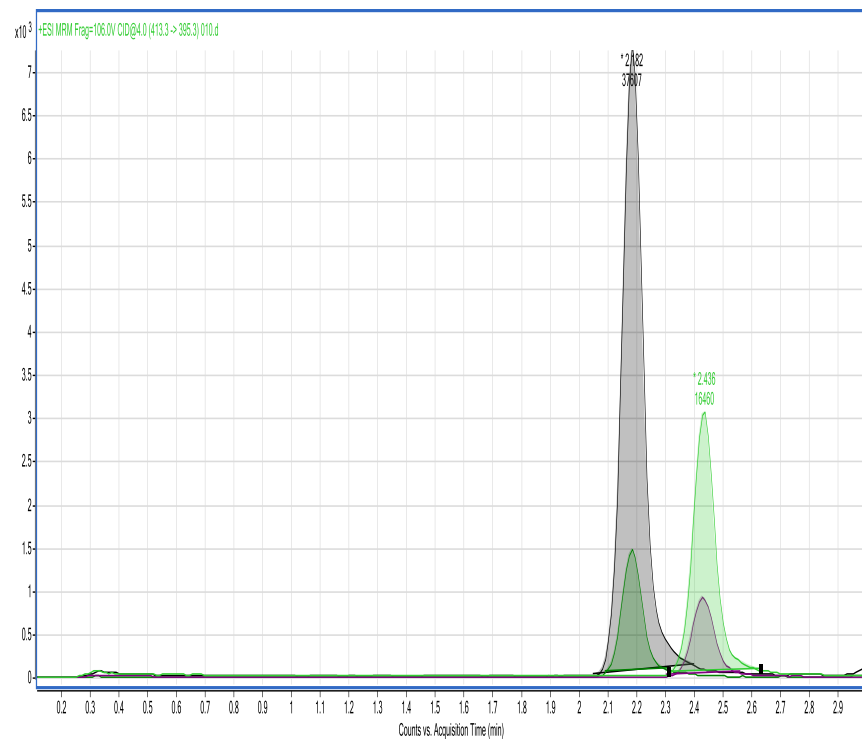


**1D Counts 2800**

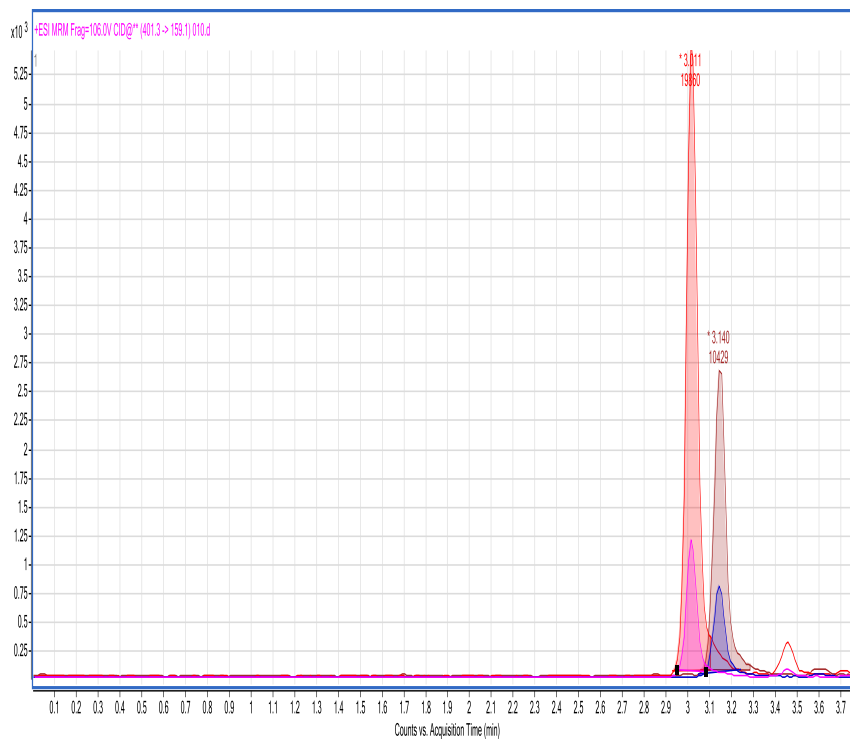


**2D Counts 3000**

# ESI LLE at 100 ng/mL

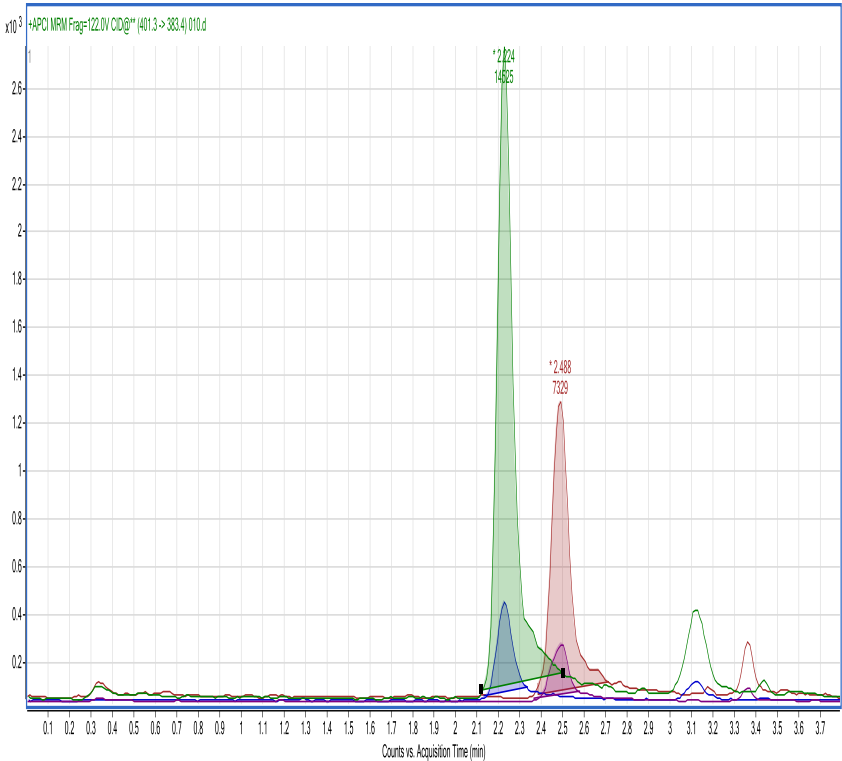


**1D Counts 7000**

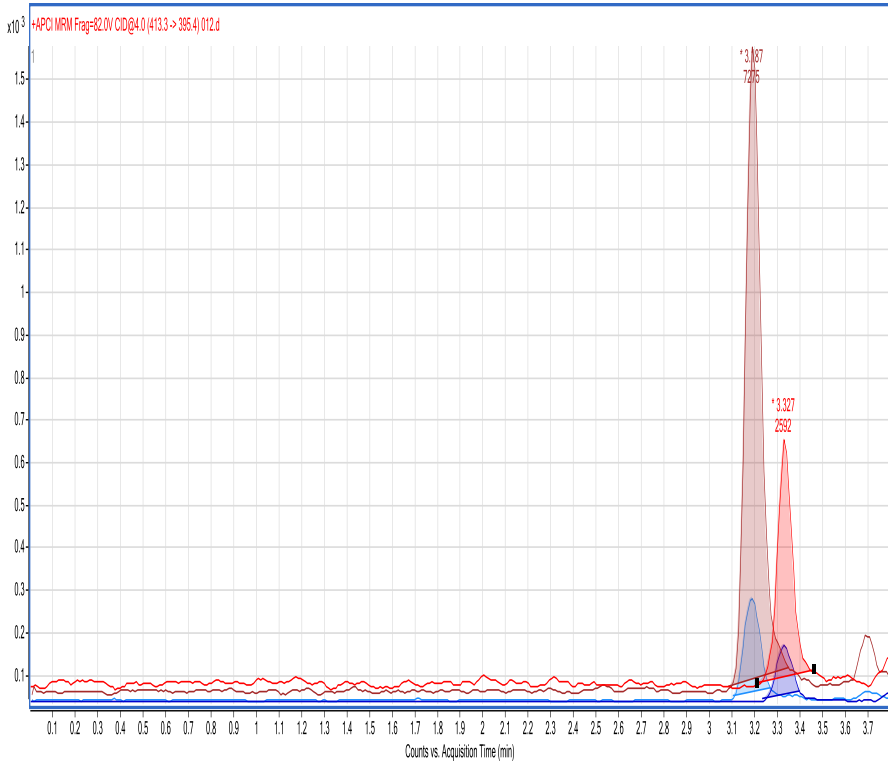


**2D Counts 6000**

# APCI LLE at 100 ng/mL

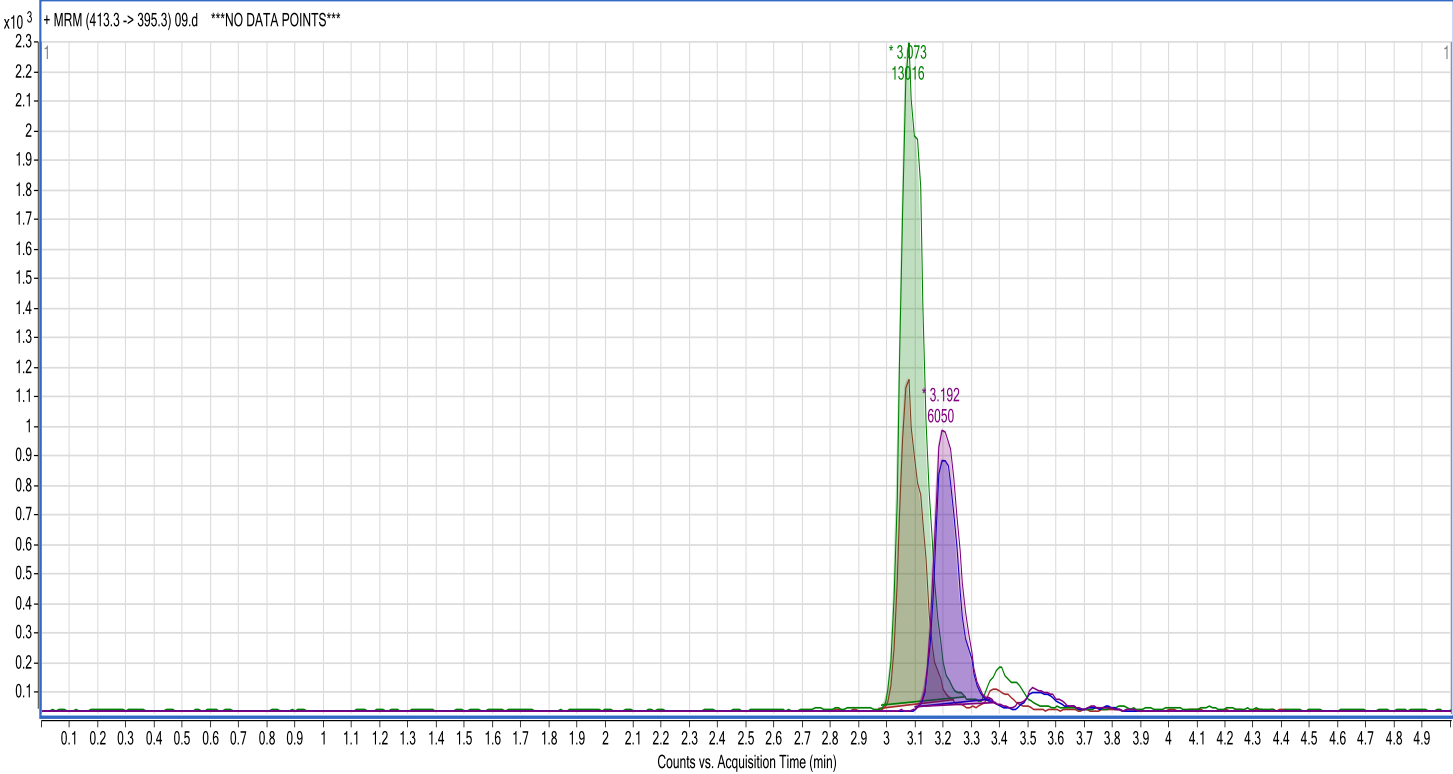


**1D Counts 2600**



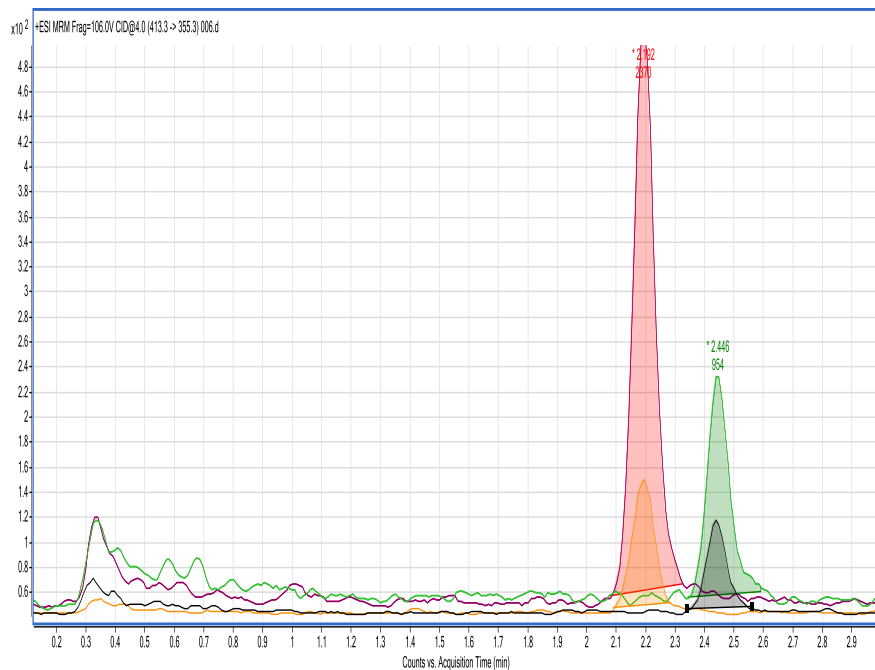
**2D Counts 1500**

# APCI LLE at 100 ng/mL- Hydroxylated Ions

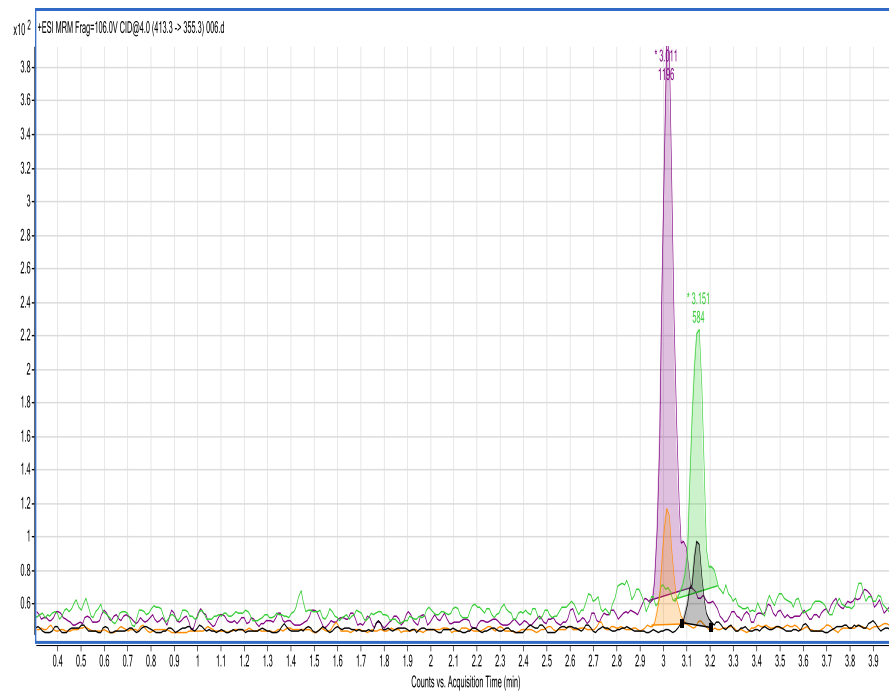


**2D Counts 2300**

# ESI LLE at 5 ng/mL

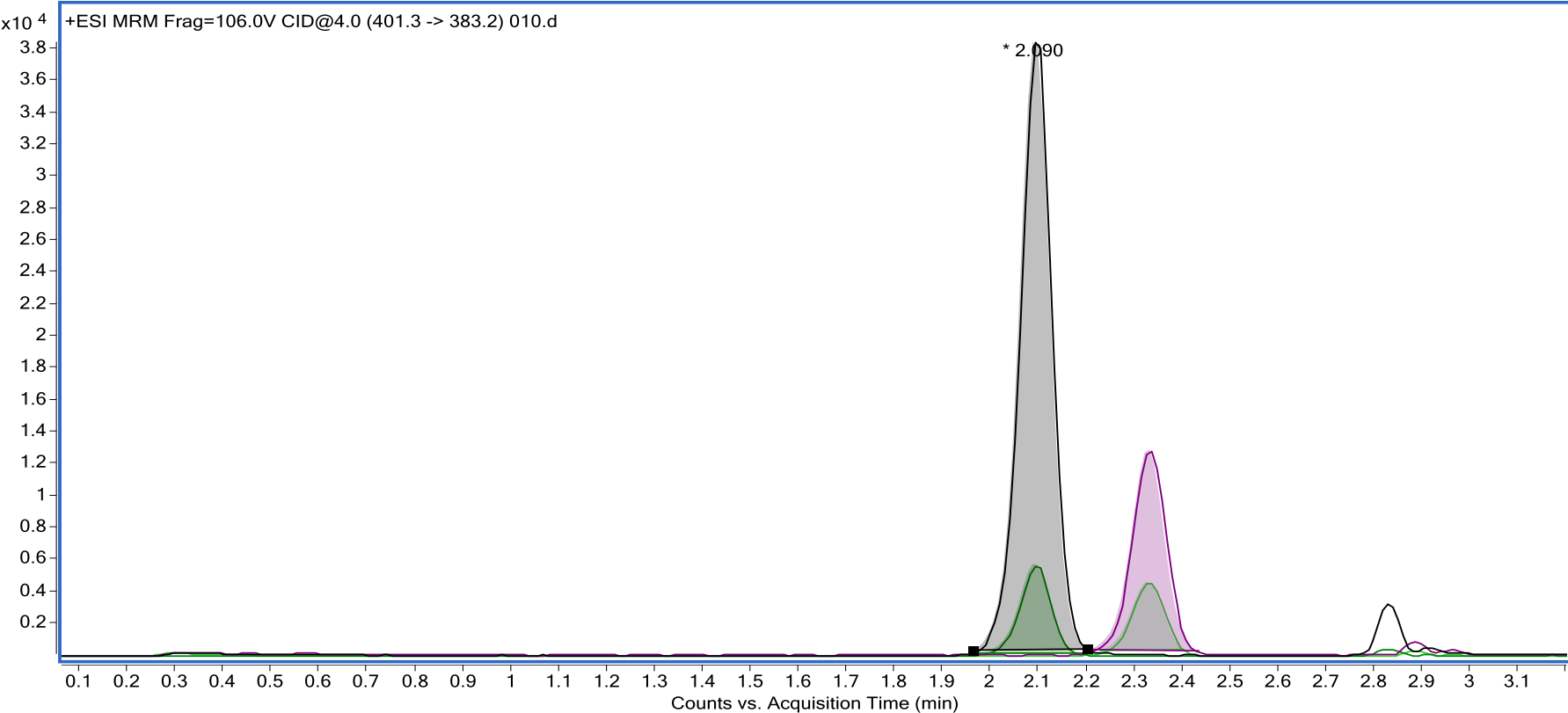


**1D Counts 480**



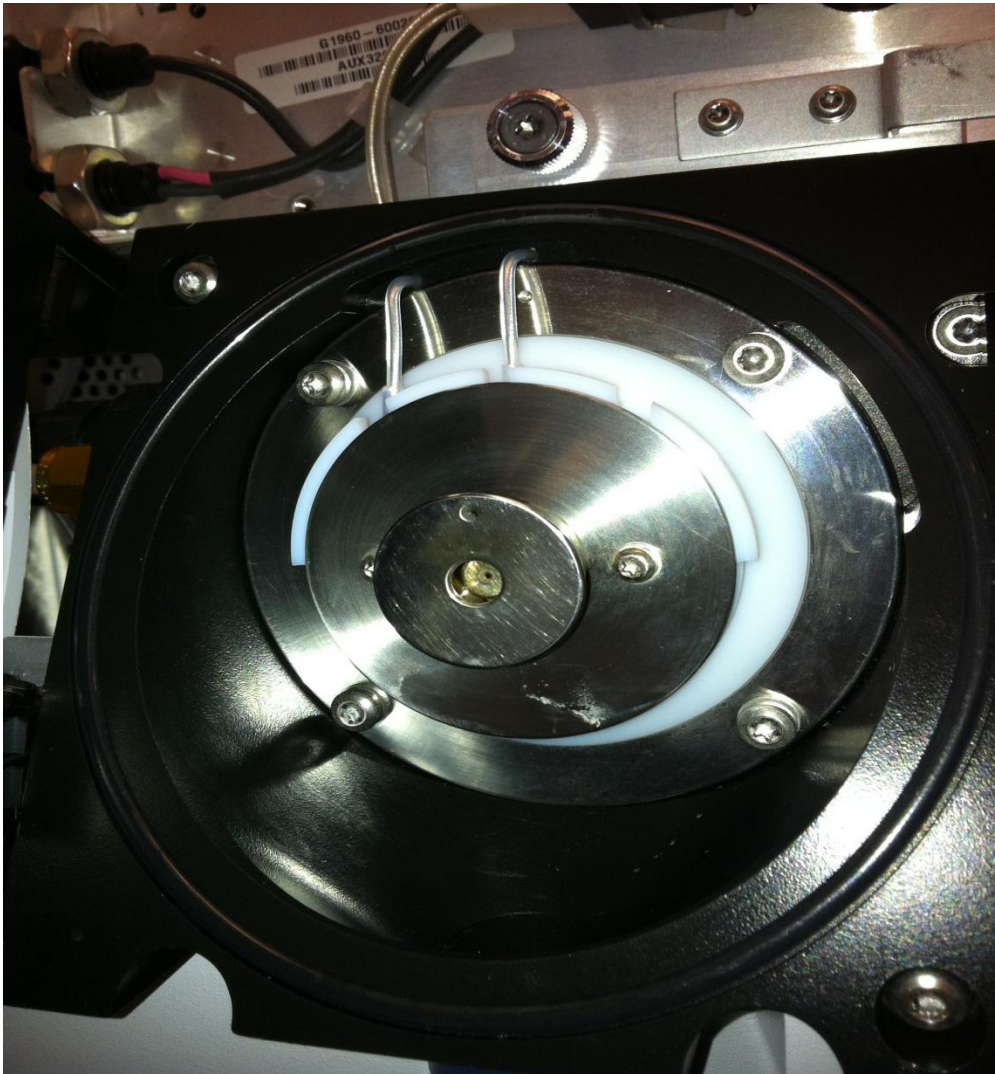
**2D Counts 400**

# AJS LLE at 100 ng/mL- 6460 QQQ



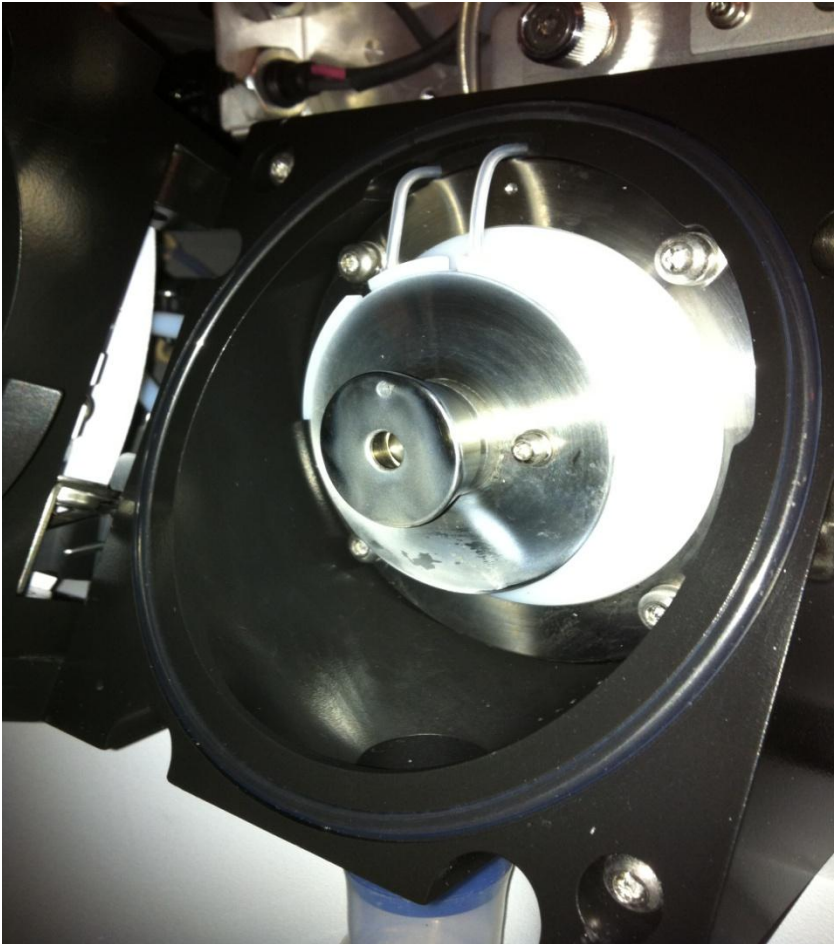
**1D Counts 38000**

# Clean 6430 Source

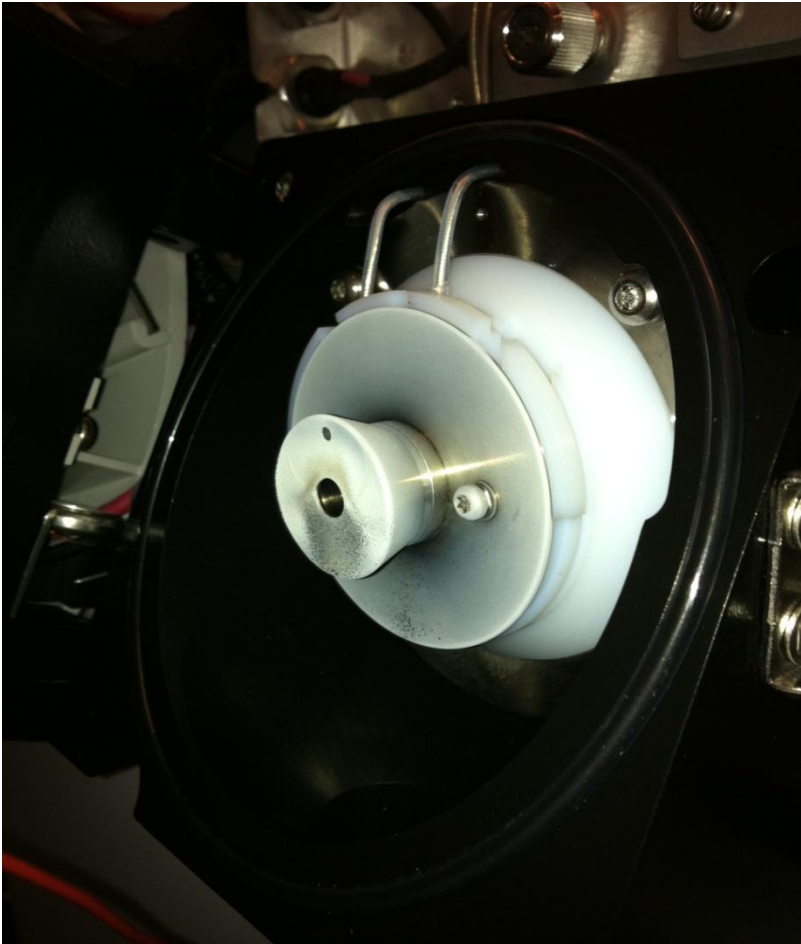




# 1D Issues- With Divert Valve

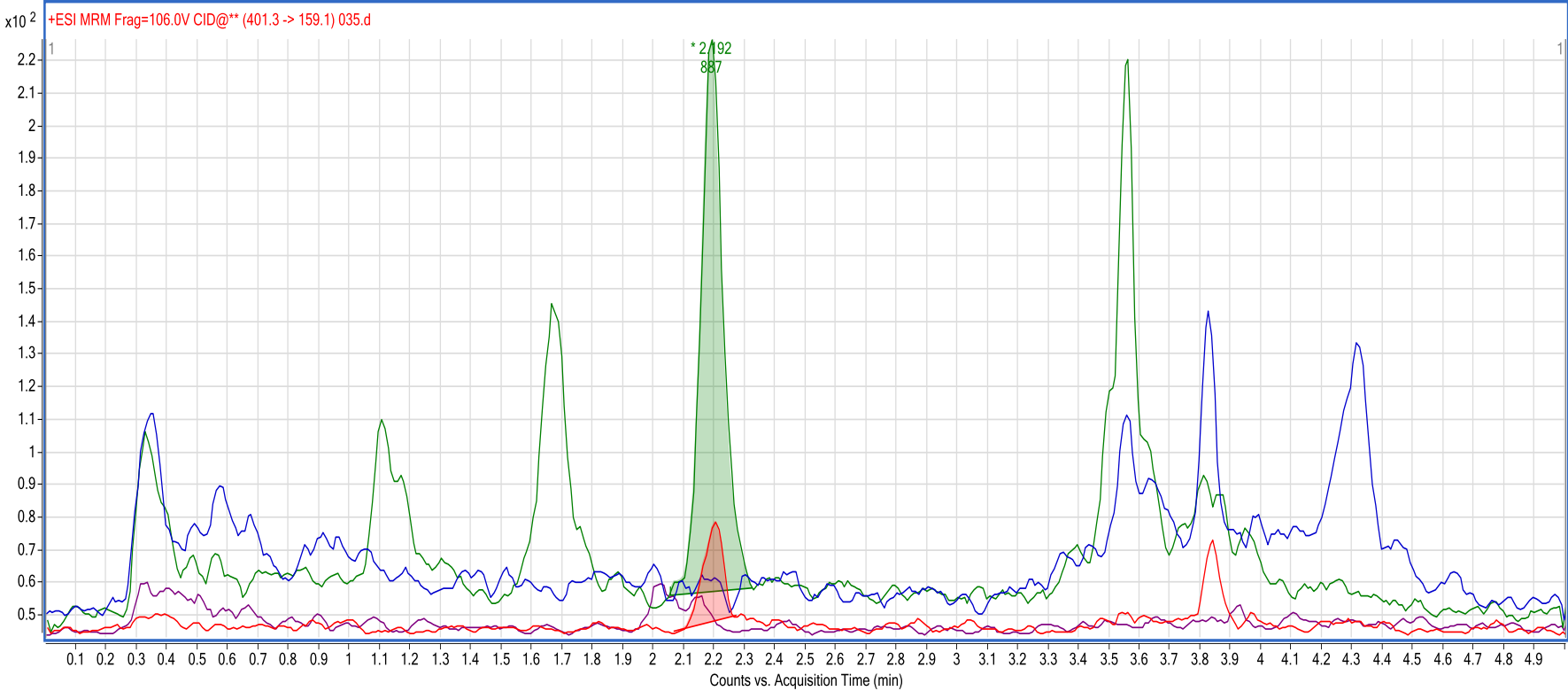


**After LLE/SPE**



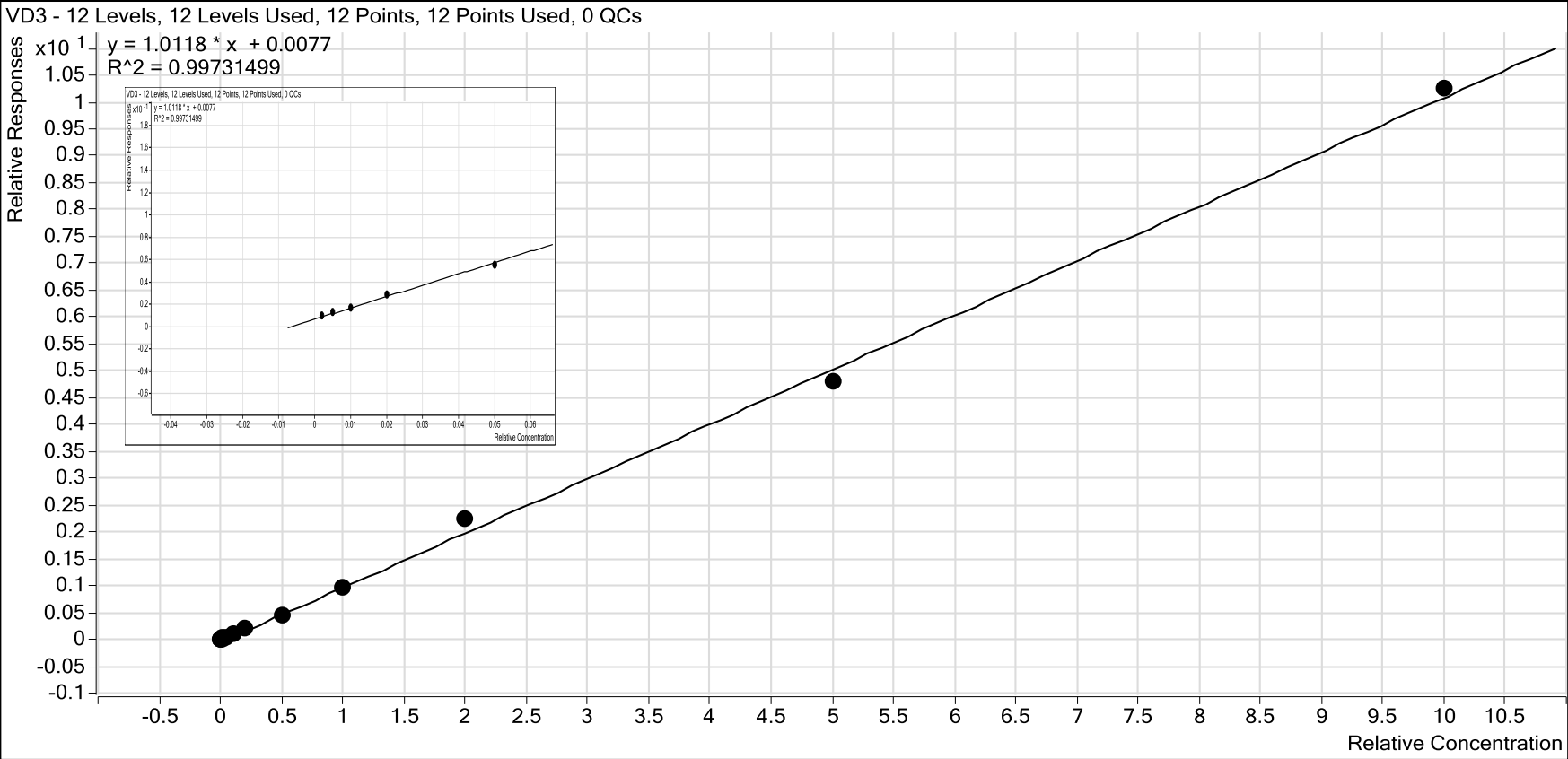
**After PPT**

# Challenges in Serum

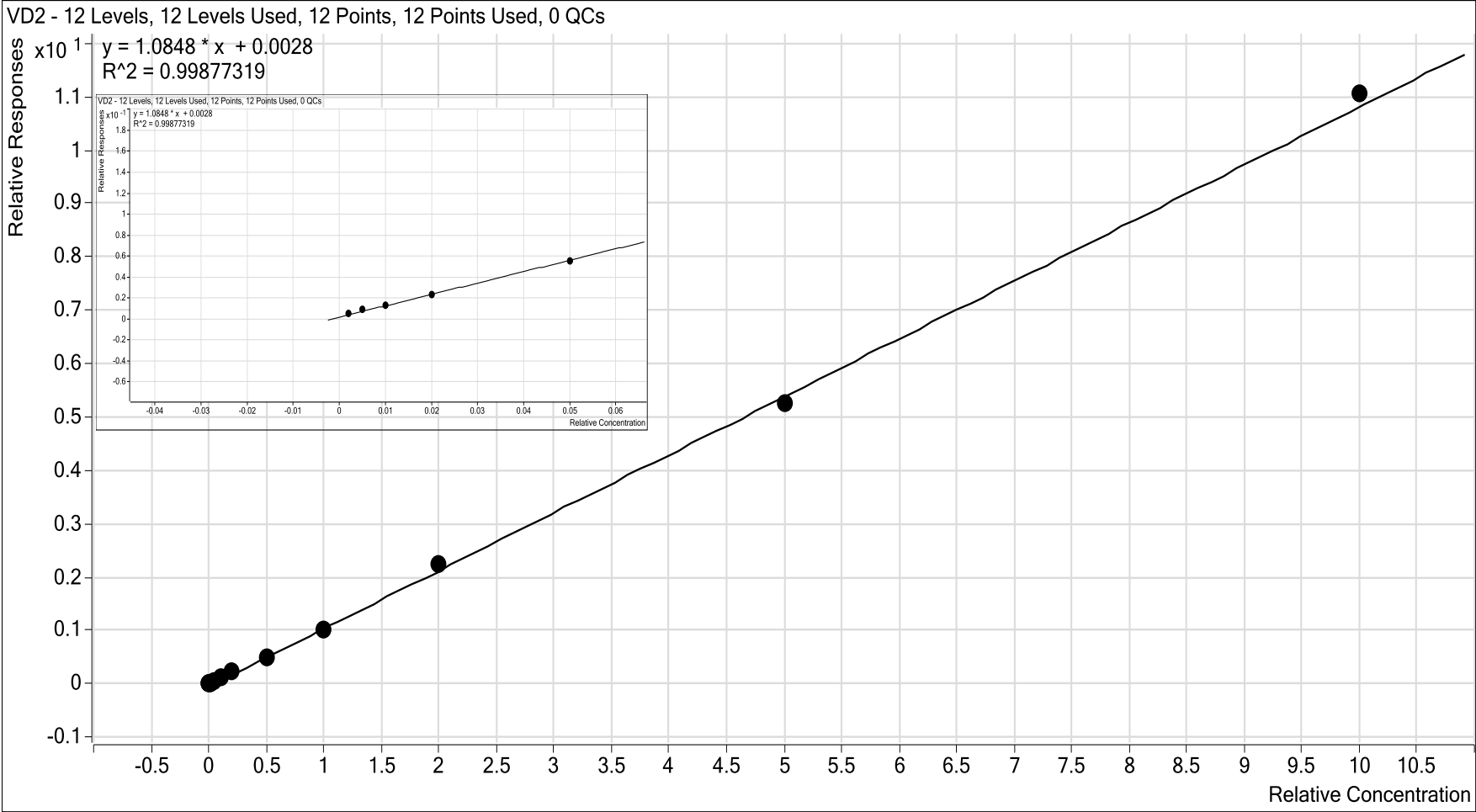


**25-Hydroxy-Vitamin D3 present in Serum at 0.5 to 2 ng/ml**

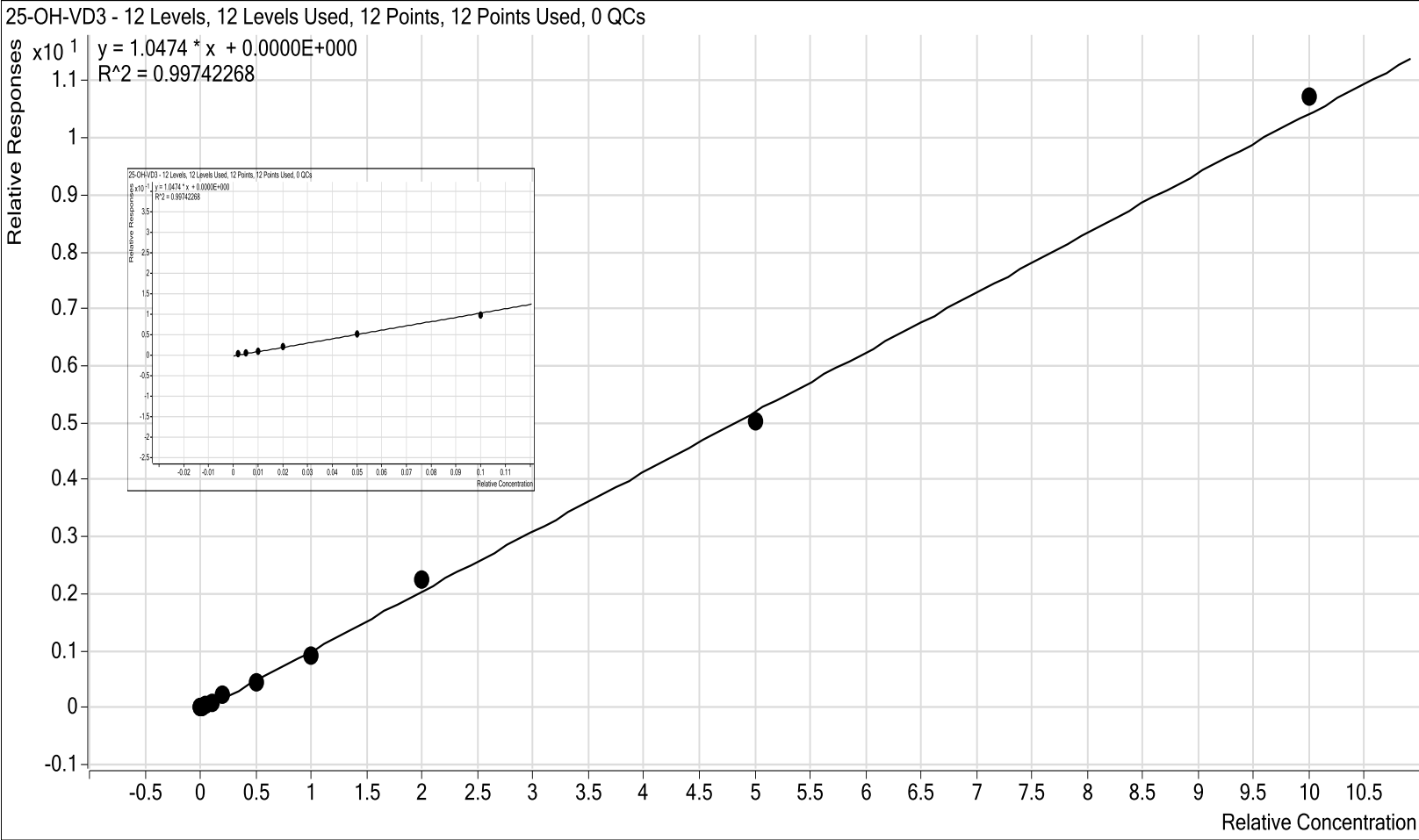
# Standard Curve: 25-OH vitamin D3- LLE 1D



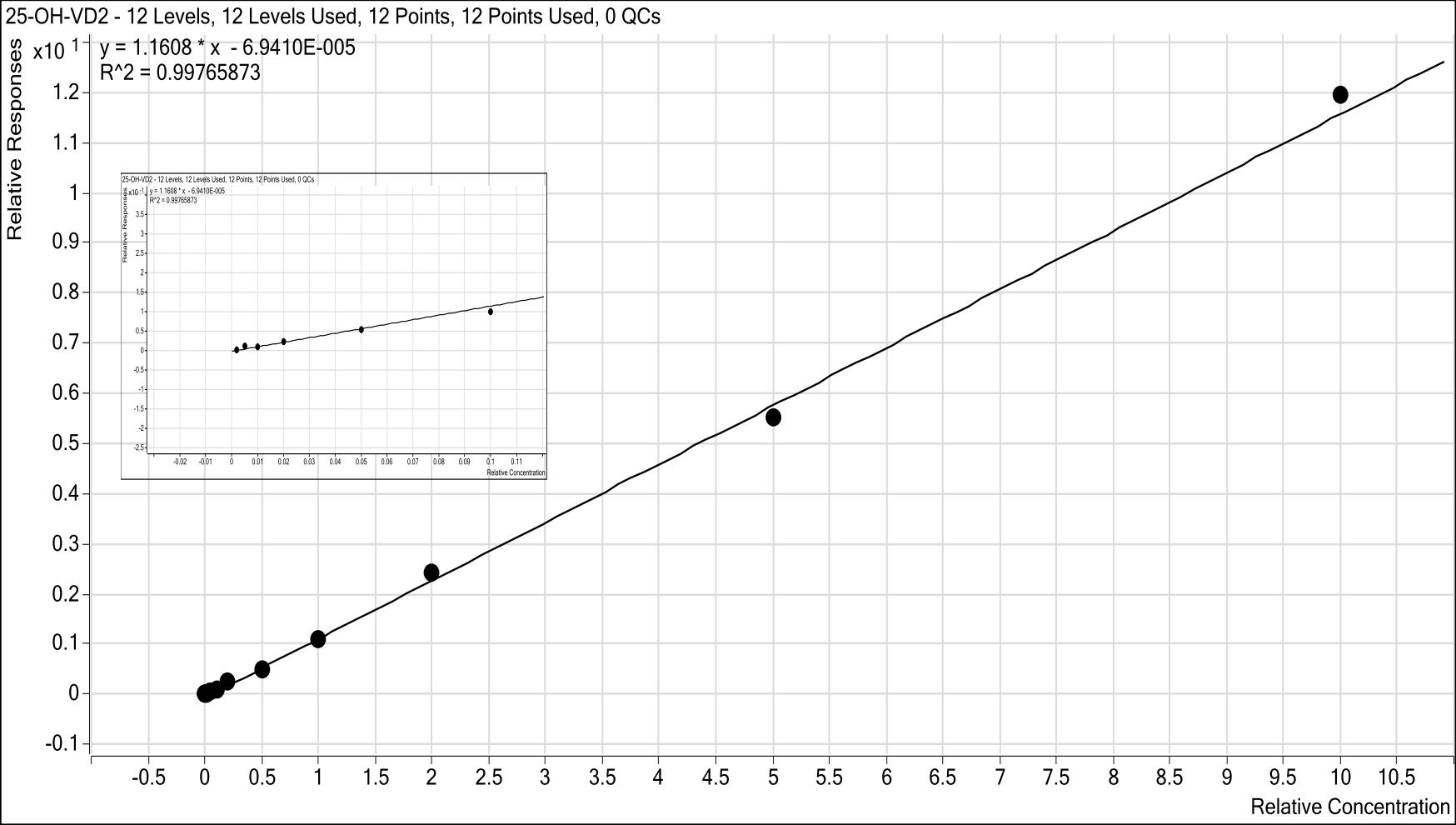
# Standard Curve: 25-OH vitamin D2-LLE 1D



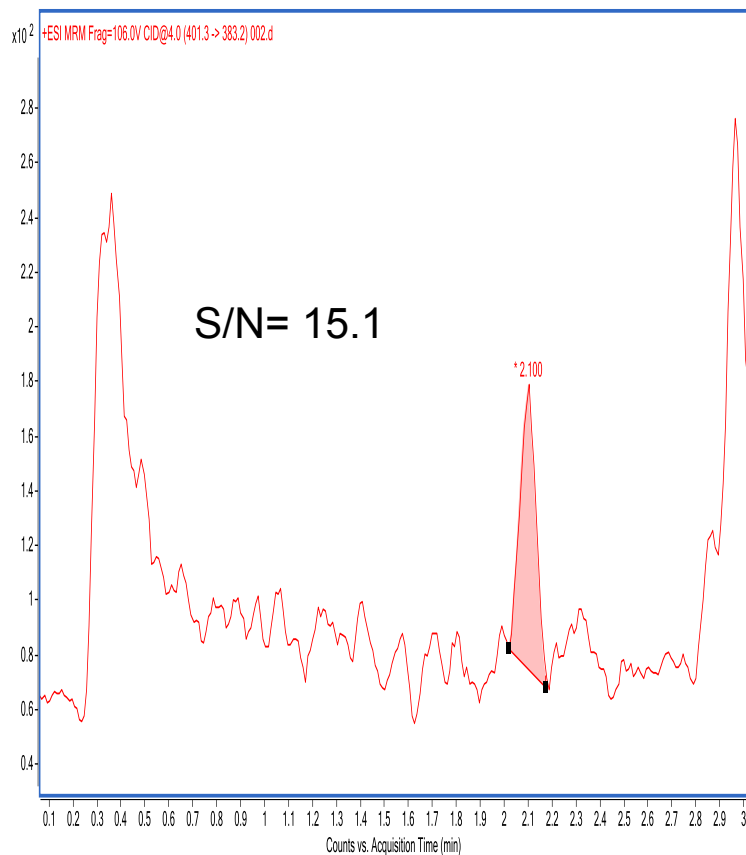
# Standard Curve: 25-OH vitamin D3-LLE 2D



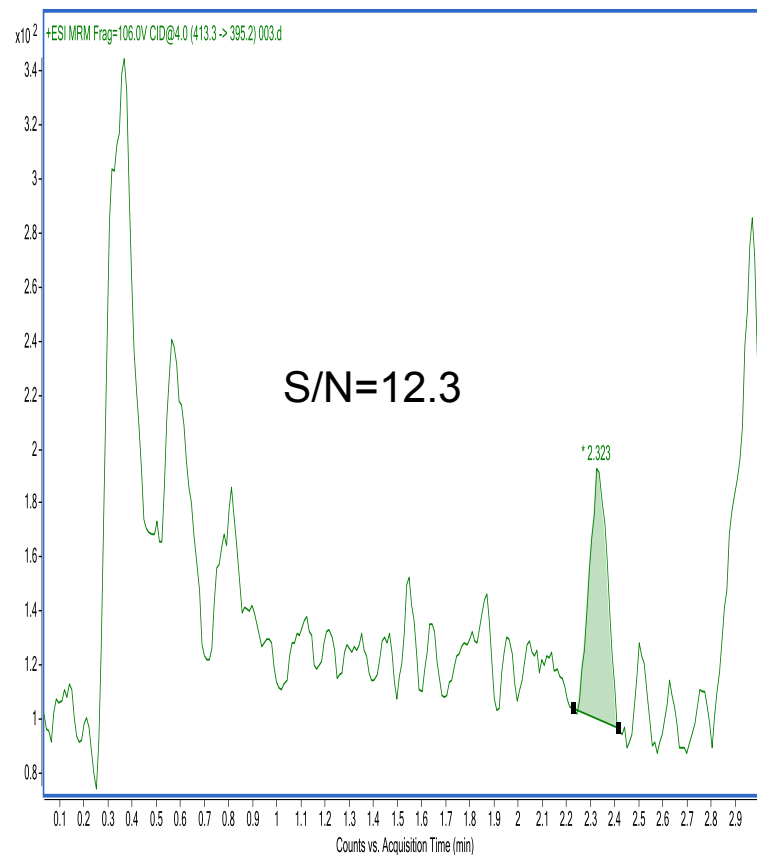
# Standard Curve: 25-OHvitamin D2-LLE 2D



# Limit of Detection- 6460 QQQ with AJS

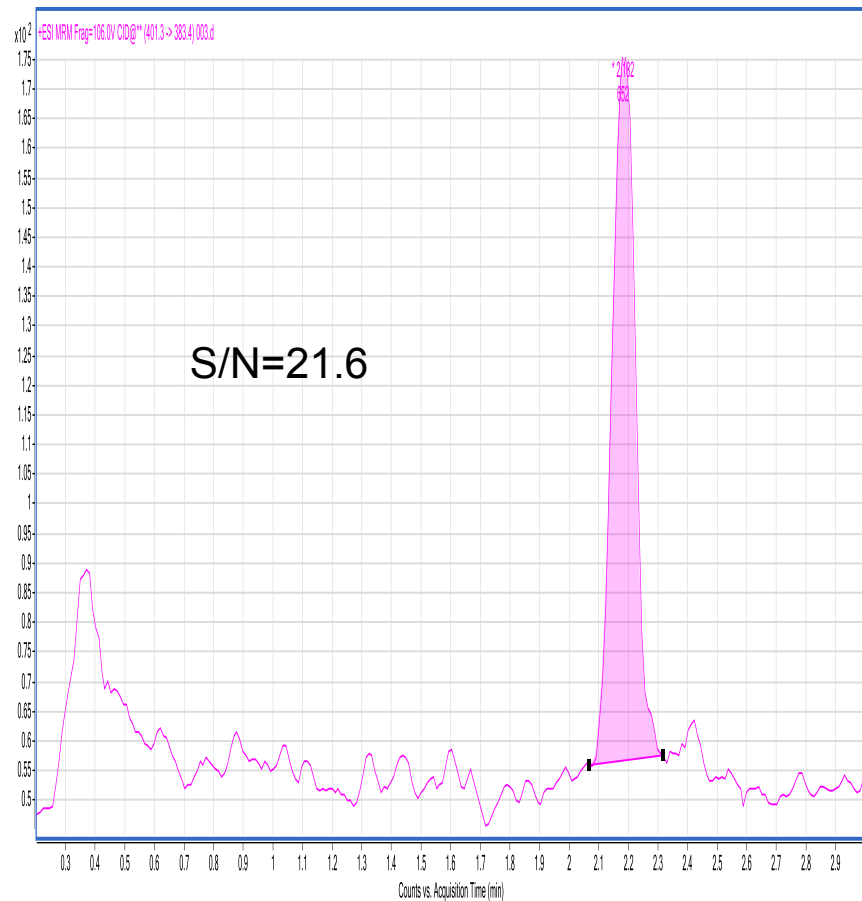


D3-100 pg/mL

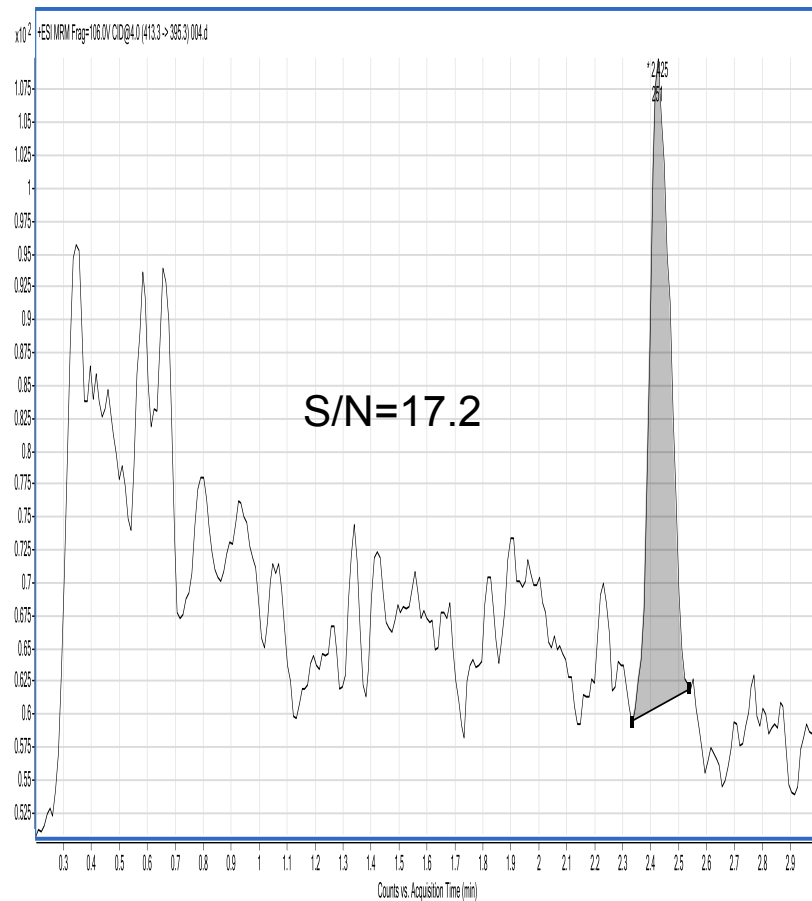


D2-500 pg/mL

# Limit of Detection- 6430 ESI



D3-1 ng/mL



D2-2 ng/mL



# UTAK Tri-Level Vitamin D Plus Controls

| Low            | Units | Expected Range | Target Value |
|----------------|-------|----------------|--------------|
| 25-OH-D2       | ng/mL | 9 – 13         | 11           |
| 25-OH-D3       | ng/mL | 10 - 14        | 12           |
| <b>Level 1</b> |       |                |              |
| 25-OH-D2       | ng/mL | 28 – 36        | 33           |
| 25-OH-D3       | ng/mL | 26 - 36        | 31           |
| <b>Level 2</b> |       |                |              |
| 25-OH-D2       | ng/mL | 65 - 89        | 77           |
| 25-OH-D3       | ng/mL | 65 - 89        | 77           |

# UTAK Tri-Level Vitamin D Plus Controls- ESI (n=10)

| %CV            | 1D PPT | 1D LLE | 1D SPE |
|----------------|--------|--------|--------|
| <b>Low</b>     |        |        |        |
| 25-OH-D2       | 5.764  | 4.848  | 6.987  |
| Mean           | 12.287 | 11.892 | 11.361 |
| 25-OH-D3       | 6.062  | 5.005  | 2.680  |
| Mean           | 12.492 | 12.865 | 11.753 |
| <b>Level 1</b> |        |        |        |
| 25-OH-D2       | 4.237  | 5.693  | 6.973  |
| Mean           | 35.675 | 35.494 | 33.221 |
| 25-OH-D3       | 6.504  | 3.632  | 4.929  |
| Mean           | 30.208 | 29.536 | 28.333 |
| <b>Level 2</b> |        |        |        |
| 25-OH-D2       | 7.251  | 5.314  | 4.579  |
| Mean           | 91.992 | 92.404 | 86.795 |
| 25-OH-D3       | 3.490  | 2.522  | 6.093  |
| Mean           | 87.869 | 87.095 | 86.422 |

# UTAK Tri-Level Vitamin D Plus Controls- ESI (n=10)

| %CV            | 2D PPT | 2D LLE | 2D SPE |
|----------------|--------|--------|--------|
| <b>Low</b>     |        |        |        |
| 25-OH-D2       | 2.840  | 5.787  | 7.447  |
| Mean           | 12.469 | 12.377 | 12.931 |
| 25-OH-D3       | 3.335  | 3.757  | 3.156  |
| Mean           | 12.462 | 12.131 | 12.408 |
| <b>Level 1</b> |        |        |        |
| 25-OH-D2       | 2.694  | 5.509  | 9.267  |
| Mean           | 35.683 | 35.296 | 34.892 |
| 25-OH-D3       | 2.736  | 3.598  | 5.273  |
| Mean           | 30.771 | 27.969 | 31.145 |
| <b>Level 2</b> |        |        |        |
| 25-OH-D2       | 7.962  | 2.220  | 7.574  |
| Mean           | 90.143 | 87.774 | 92.364 |
| 25-OH-D3       | 7.789  | 1.817  | 9.487  |
| Mean           | 87.442 | 88.160 | 88.104 |

# NIST Standard Control-ESI 2D

Units- ng/mL (N=4)

| Level 1   | 2D PPT | 2D LLE | 2D SPE | Expected | %CV   |
|-----------|--------|--------|--------|----------|-------|
| 25-OH-D2  |        |        |        |          |       |
| 25-OH-D3  | 24.50  | 23.43  | 24.36  | 23.9     | 4.48  |
| Level 2   |        |        |        |          |       |
| 25-OH-D2  | 2.68   | 2.34   | 2.60   | 1.71     | 13.71 |
| 25-OH-D3  | 12.03  | 12.37  | 12.49  | 12.3     | 3.88  |
| Level 3   |        |        |        |          |       |
| 25-OH-D2  | 28.09  | 28.34  | 26.69  | 26.4     | 6.42  |
| 25-OH-D3  | 20.46  | 19.36  | 20.25  | 18.5     | 5.83  |
| Level 4   |        |        |        |          |       |
| 25-OH-D2  | 2.87   | 2.46   | 2.61   | 2.4      | 15.68 |
| 25-OH-D3* | 85.78  | 85.33  | 85.95  | 70.7     | 0.75  |

\*Combination of 25-hydroxy Vitamin D3 and 3-Epi-25-hydroxy Vitamin D3

# Conclusions

- ESI mode is more sensitive than APCI
- 2D mode gives better results than 1D due to the on column clean up and concentration of the sample
- LLE is shown to be most sensitive and give consistent results and SPE has potential but needs fine tuning
- PPT and LLE can be easily automated while SPE is labor intensive
- PPT and LLE are good for high throughput clinical research laboratories

# Questions?