

Analysis of 25-Hydroxyvitamin D In Serum Using Automated On Line Sample Preparation Technique With High Resolution Orbitrap Mass Spectrometer

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Overview

Purpose: A high throughput method for the quantitation of vitamin D using online sample preparation and high resolution, accurate mass (HRAM) quantitation with an Exactive Plus orbitrap mass spectrometer for clinical research use.

Methods: Solvent precipitated serum is injected onto an extraction column under turbulent flow conditions. A 200 microliter loop containing organic solvent is used to transfer the Vitamin D to a solid core HPLC column for separation and analysis using an Exactive Plus Orbitrap MS.

Results: The method provided reliable quantitation of 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ levels in serum with a lower limit of quantitation of 2 ng/mL.

Introduction

Blood levels of 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ are commonly tested by clinical researchers. In the last decade, liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS) has become a popular technique for vitamin D testing. Due to their higher resolving power over triple stage quadrupole mass spectrometers, high-resolution benchtop orbitrap mass spectrometers are better able to resolve analytes from sample matrix. In addition, the ease of initial method set up and daily use provide an advantage over triple quadrupole mass spectrometers for clinical research. A method has been created that allows the precipitated serum to be injected onto an HPLC system with minimal sample preparation and analyzed by an Exactive Plus benchtop Orbitrap mass spectrometer. Total method time is 7.75 minutes on a Transcend TLX-1 system. Throughput can be increased to a sample every 3.7 minutes by using a Transcend TLX-2 multiplexed UHPLC system or 1.9 minutes for a Transcend TLX-4 system.

Methods

Standards

Standard solutions of 25-hydroxyvitamin D₂, 25-hydroxyvitamin D₃, and deuterated 25-hydroxyvitamin D₃ internal standard were obtained from Cerilliant, Inc.

Six calibrators at 2, 5, 10, 25, 50 and 100 ng/mL and three QCs at 5 ng/mL, 40 ng/mL and 80 ng/mL were prepared by fortifying bovine serum albumin diluent with 200 ng/mL 25-hydroxyvitamin D₂ and D₃ standard mix. Precipitating reagent was prepared by adding deuterated D₃-25-hydroxyvitamin D₃ to acetonitrile for a final concentration of 75 ng/mL. In addition, pooled patient serum samples were crashed 2 to 1 with acetonitrile and spiked with analytes for a final concentration of 20 ng/mL for 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃, and 50 ng/mL of D₃ 25-hydroxyvitamin D₃ internal standard.

Sample Preparation

1. Aliquot 100 µL of calibrators and controls into centrifuge tubes.
2. Add 200 µL of precipitating reagent containing internal standard to each tube
3. Vortex each tube for 30 seconds.
3. Centrifuge tubes at 5,000 RCF for 10 minutes.
4. Aliquot supernatants into autosampler vials for analysis. The supernatant should contain internal standard at a concentration of 50 ng/mL.

Liquid Chromatography

Transcend™ TLX-2 system in focus mode configuration (Figure 2).

Extraction column: Thermo Scientific TurboFlow™ XL C-18P 0.5x 50 mm

Separation column: Thermo Scientific Accucore™ C18 3 x 50 mm 2.6µM

Injection Volume: 50µL

Mobile Phases:

Loading A, Eluting A: Water 0.1% Formic Acid

Loading B, Eluting B: Acetonitrile 0.1% Formic Acid

Loading C: 40% Acetonitrile, 40% Isopropanol, 20% Acetone

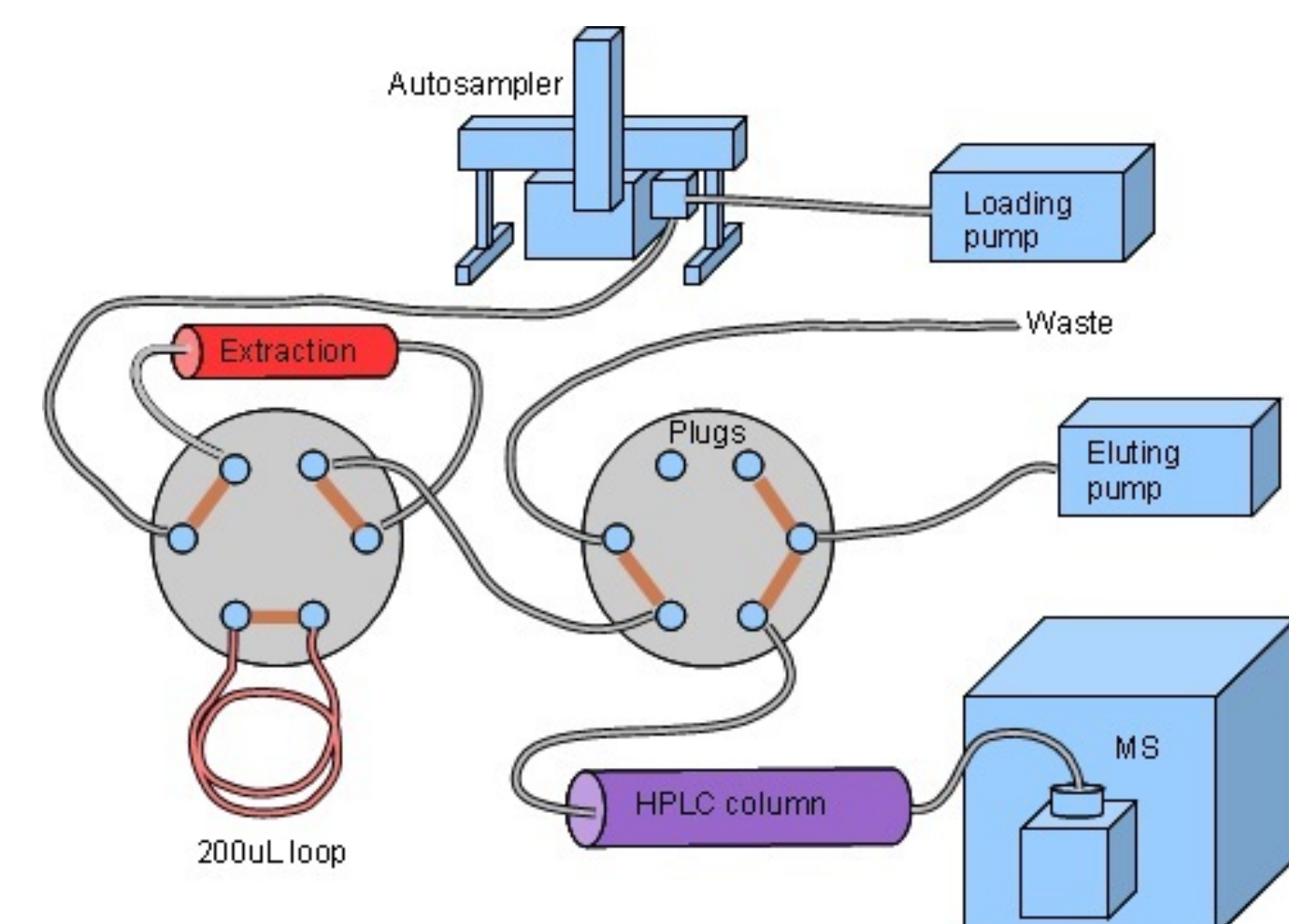
Total method time: 7.75 minutes

Cycle time when multiplexed: 3.7 minutes

FIGURE 1. Liquid Chromatography Method

| Step | Start | Sec | Flow | Grad | %A | %B | %C | %D | Tee | Loop | Flow | Grad | %A | %B | %C | %D |
|------|-------|-----|------|------|-------|------|-------|----|-------|------|------|------|------|-------|----|----|
| 1 | 0.00 | 30 | 2.00 | Step | 80.0 | 20.0 | - | - | ===== | out | 1.00 | Step | 70.0 | 30.0 | - | - |
| 2 | 0.50 | 5 | 1.00 | Step | 80.0 | 20.0 | - | - | ===== | out | 0.80 | Step | 70.0 | 30.0 | - | - |
| 3 | 0.58 | 10 | 1.00 | Step | 80.0 | 20.0 | - | - | ===== | in | 0.80 | Step | 70.0 | 30.0 | - | - |
| 4 | 0.75 | 90 | 0.20 | Step | 100.0 | - | - | - | T | in | 0.80 | Step | 70.0 | 30.0 | - | - |
| 5 | 2.25 | 30 | 2.00 | Step | - | - | 100.0 | - | ===== | out | 1.00 | Step | 40.0 | 60.0 | - | - |
| 6 | 2.75 | 60 | 2.00 | Step | 100.0 | - | - | - | ===== | out | 1.00 | Step | 10.0 | 90.0 | - | - |
| 7 | 3.75 | 90 | 2.00 | Step | - | - | 100.0 | - | ===== | in | 1.00 | Step | 10.0 | 90.0 | - | - |
| 8 | 5.25 | 30 | 2.00 | Step | - | - | 100.0 | - | ===== | in | 1.50 | Step | - | 100.0 | - | - |
| 9 | 5.75 | 120 | 2.00 | Step | 80.0 | 20.0 | - | - | ===== | out | 1.00 | Step | 70.0 | 30.0 | - | - |

FIGURE 2. Plumbing Diagram for online TurboFlow Extraction



Mass Spectrometry

The Thermo Scientific Exactive Plus benchtop orbitrap mass spectrometer was used with an APCI source in positive ionization mode. Full scan data was collected from 350 to 425 m/z, using a lock mass of 391.28429 (Di-iso-octyl Phthalate). Lock mass was added in order to maintain mass accuracy during the batch runs.

Mass Spectrometer Parameters:

| | |
|---------------------------|--------------------------------|
| Scan Mode: Full | Ion Source: APCI |
| Scan Range: m/z 350 – 425 | Sheath Gas Flow Rate: 30 units |
| Fragmentation: None | Aux Gas Flow Rate: 5 units |
| Resolution: 70,000 | Sweep Gas Flow Rate: 1 unit |
| Polarity: Positive | Discharge Current: 3.5 µA |
| Microscans: 1 | Capillary Temperature: 250 °C |
| AGC Target: 3e6 | S-Lens RF Level: 60 |
| Maximum Inject Time: 200 | Vaporizer Temp: 500 °C |

Data Analysis

Calibration curves and QCs were run in triplicate each day across four days. In addition, 800 pooled serum sample replicates containing 20 ng/mL 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ and 50 ng/mL of D₃-25-hydroxyvitamin D₃ internal standard were injected in order to test robustness of the method. Thermo Scientific Xcalibur™ software was used to collect data and analyze the results.

Results

Summary

The lower limit of quantitation (LLOQ) was determined to be 2 ng/mL for both analytes in BSA as indicated in Figure 3. Limits of quantitation (LOQs) were estimated from the triplicate injections of the standard solutions. The signal to noise was greater than 10 and the coefficient of variation (CV) values were less than 5 percent at the LLOQ of 2 ng/mL for both 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃. This is an improvement over the LLOQ of 10 ng/mL that was previously achieved on the Exactive Orbitrap MS. The correlation coefficients obtained using 1/X weighted linear regression analysis of the standard curves were greater than 0.99 for both analytes (Figure 6). A relative standard deviation (%RSD) test was performed in pooled human serum fortified with analytes at 20 ng/mL and crashed with internal standard solution for a total internal standard concentration of 50 ng/mL. The RSDs of ten replicate injections were less than 10 percent for both analytes. A recovery study was also performed using a neat standard of 20 ng/mL 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ with 50 ng/mL D₃-25-hydroxyvitamin D₃. The standard was injected ten times on the TurboFlow column and analytical column, and ten times on the analytical column only, and area counts were compared. The relative recoveries were 97 percent and 99 percent for 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃, respectively. Consistent results were achieved with pooled serum containing 20 ng/mL of 25-Hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ and 50 ng/mL D₃-25-hydroxyvitamin D₃ up to about 600 injections as indicated in Figure 7.

FIGURE 3. Chromatograms at LLOQ of 2 ng/mL with 50 ng/mL internal standard

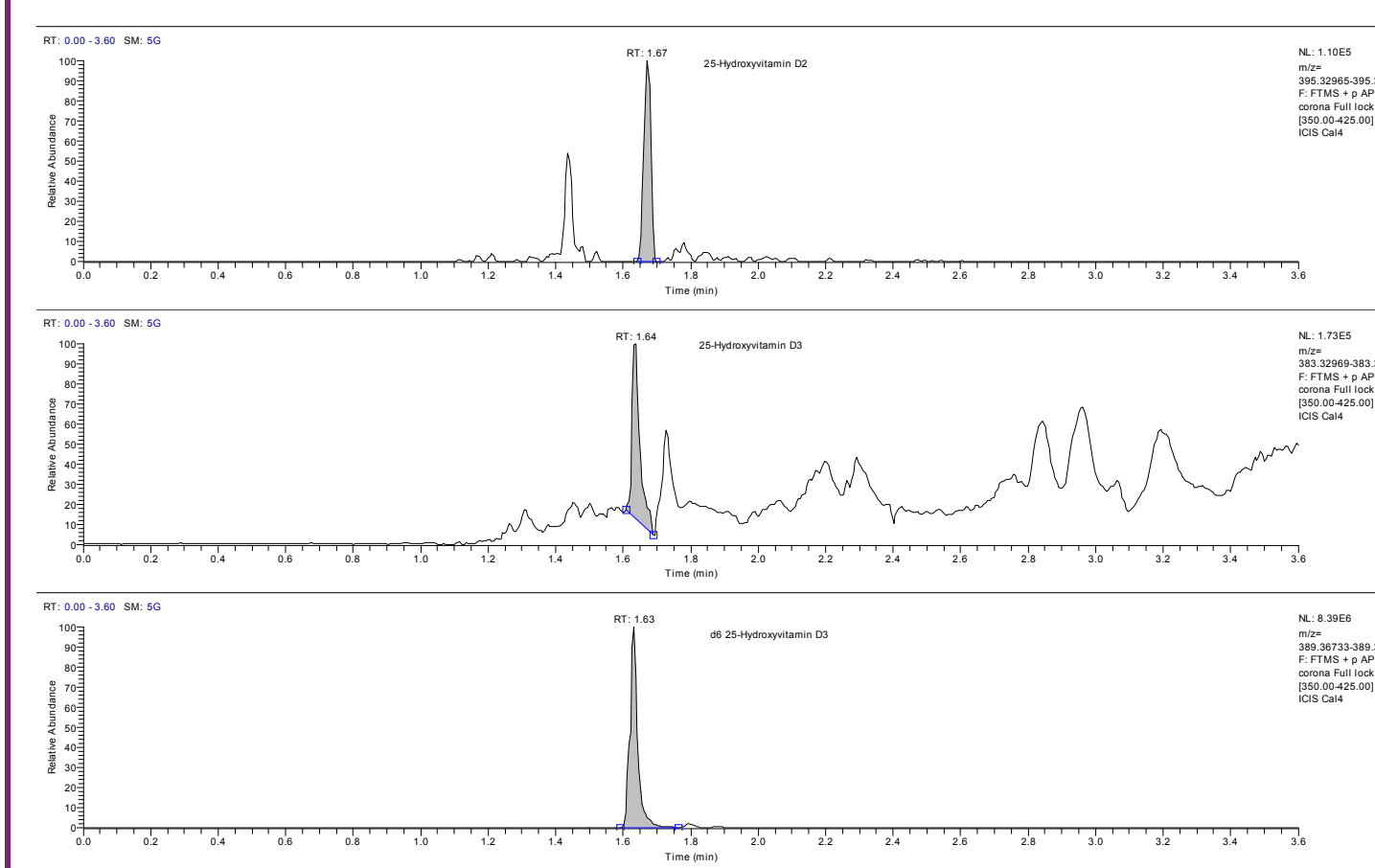


FIGURE 4. Chromatograms of 40 ng/mL QC injection

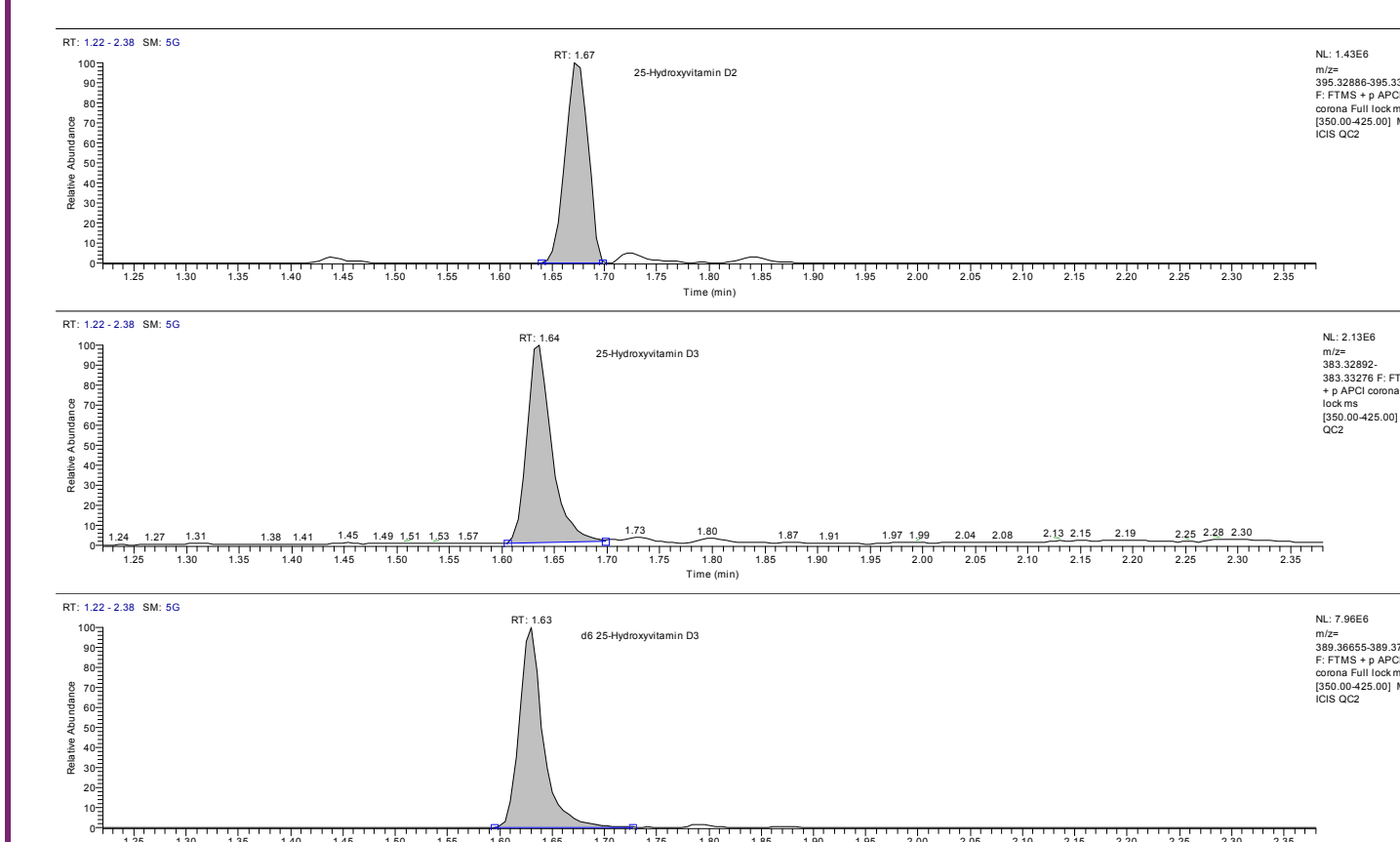


FIGURE 5. QC Replicate Injections. Three replicates of each QC level were run each day over four days and results compared.

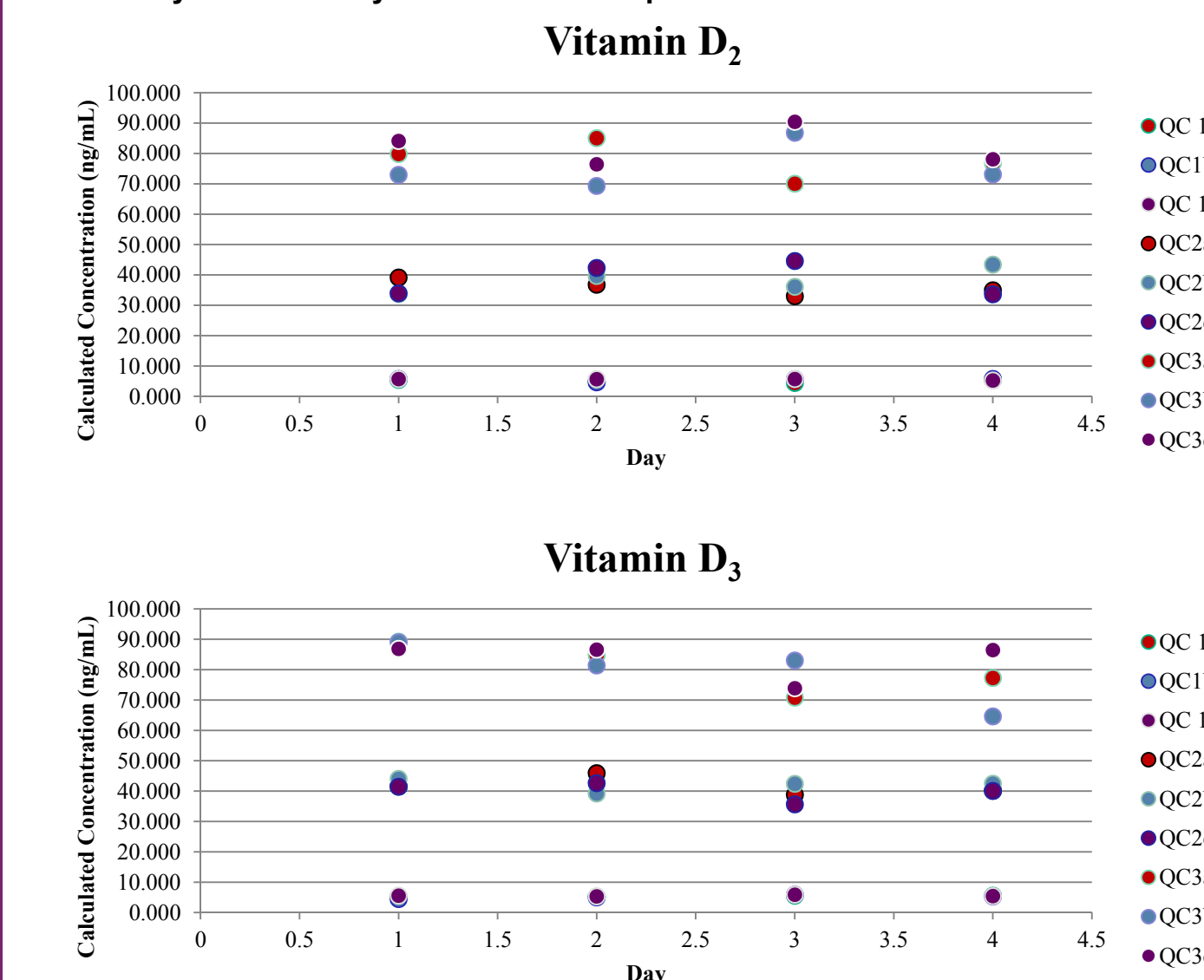


FIGURE 6. Calibration Curves

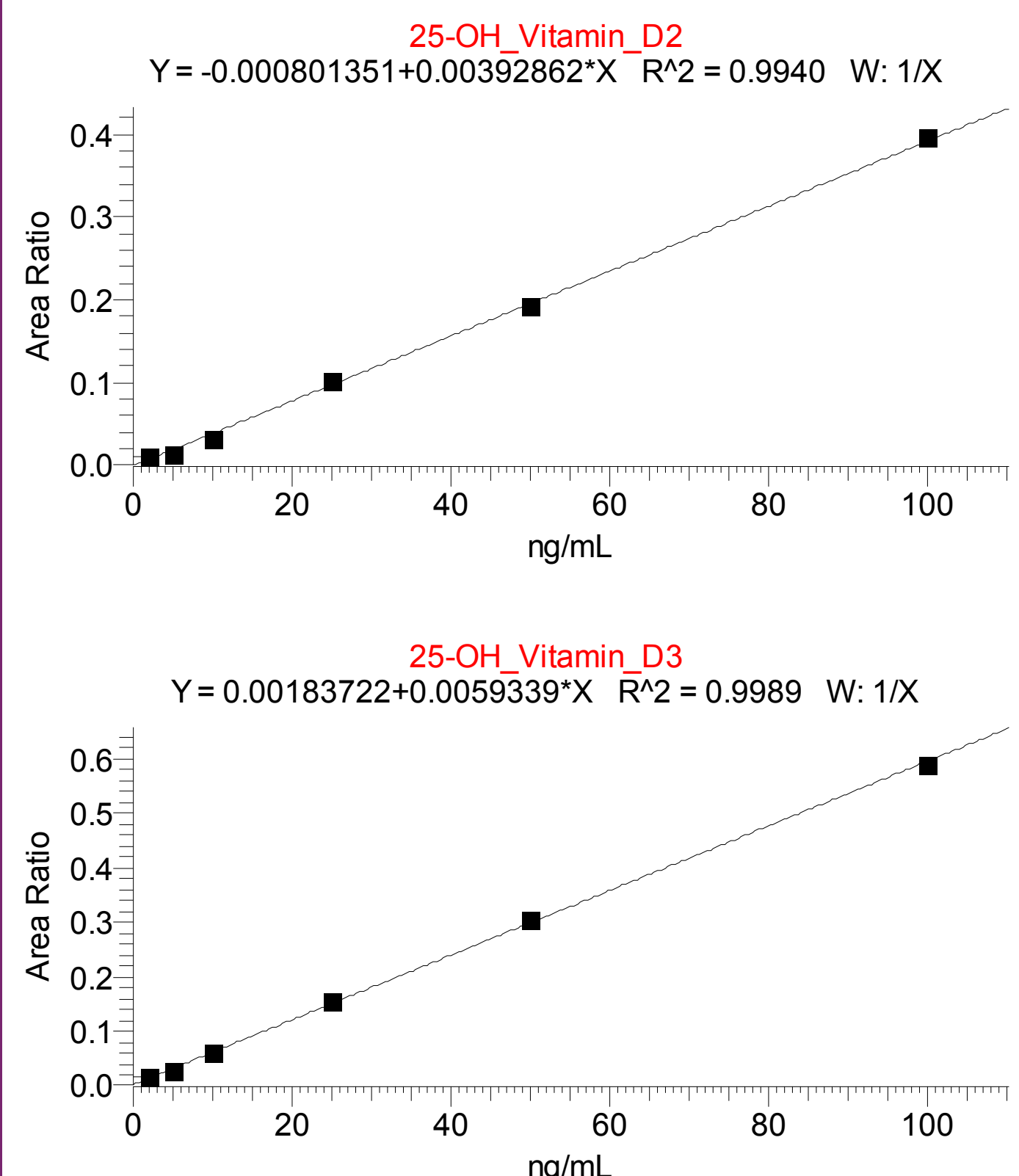
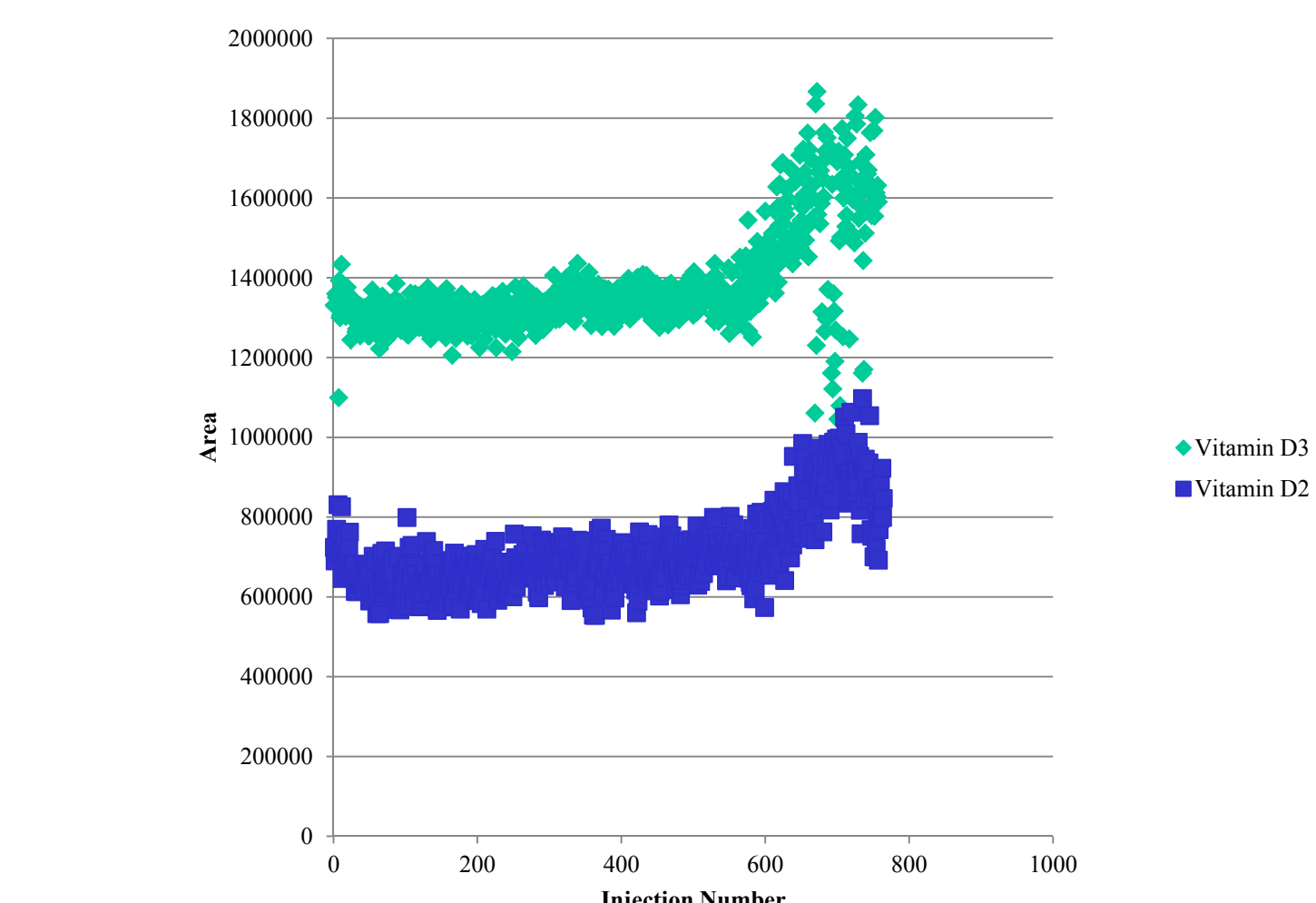


FIGURE 7. Serum injection replicates. Approximately 600 injections before variability increased.



Variability increased during replicate serum injections after approximately 600 injections. The system ran serum injections constantly during this time period with no blanks or washing in between injections. Increased variability could be due to the mass spectrometer source becoming dirty after so many serum injections with no cleaning. Future experiments will seek to extend the number of injections before variability occurs, possibly by increasing the amount of washing of the columns that occurs during the HPLC method, as well as steps to keep the MS source cleaner during the sample run.

Conclusions

- Reliable detection of 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ in serum by clinical researchers.
- Higher resolving power with Exactive+ orbitrap mass spectrometer
- Lower LLOQ with Exactive Plus
- Cycle time of 1.9 minutes when using a Transcend TLX-4 multiplexing system.
- Method robustness of approximately 600 serum injections between column changes.
- Ease of method set up compared to triple quadrupole MS methods.

References

- Shah I, James R, Barker J, Petroczi A, and Naughton D: Misleading measures in Vitamin D analysis: A novel LC-MS/MS assay to account for epimers and isobars. *Nutrition Journal* 2011, 10:46.

Acknowledgements

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