

## Quantification of 1,25-Dihydroxyvitamin D<sub>2</sub> and D<sub>3</sub> From Human Serum Using Immunopurification, ACQUITY UPLC, and Xevo TQ-S micro

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### Abstract

This application note demonstrates, a robust, selective, and sensitive analytical method for the quantification of 1,25 (OH)<sub>2</sub> Vitamin D<sub>2</sub> and D<sub>3</sub> from human serum was developed using the Waters ACQUITY UPLC System and Xevo TQ-S micro Mass Spectrometer. The Xevo TQ-S micro has the ideal combination of sensitivity, reproducibility, and versatility to provide an excellent option for all types of bioanalytical labs.

### Benefits

- Highly sensitive and robust method
  - Ability to detect 5 pg/mL using Waters ACQUITY UPLC and Xevo TQ-S micro systems
  - Accuracy and precision of <15% across the calibration range of 5–500 pg/mL
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### Introduction

Vitamin D is an important fat-soluble vitamin, which helps maintain bone health. Vitamin D exists in two primary forms: Vitamin D<sub>3</sub> (cholecalciferol) synthesized from 7-dehydrocholesterol when the skin is exposed to UV radiation from sunlight, and Vitamin D<sub>2</sub> (ergocalciferol) produced by plants and fungi through solar irradiation of ergosterol. Vitamin D is converted first to 25-hydroxyl (OH) Vitamin D by the liver via the CYP family of enzymes (Figure 1). 25 (OH) 25 (OH) Vitamin D is then hydroxylated into its biologically-active form: 1,25-dihydroxy (OH)<sub>2</sub> Vitamin D in the kidneys.

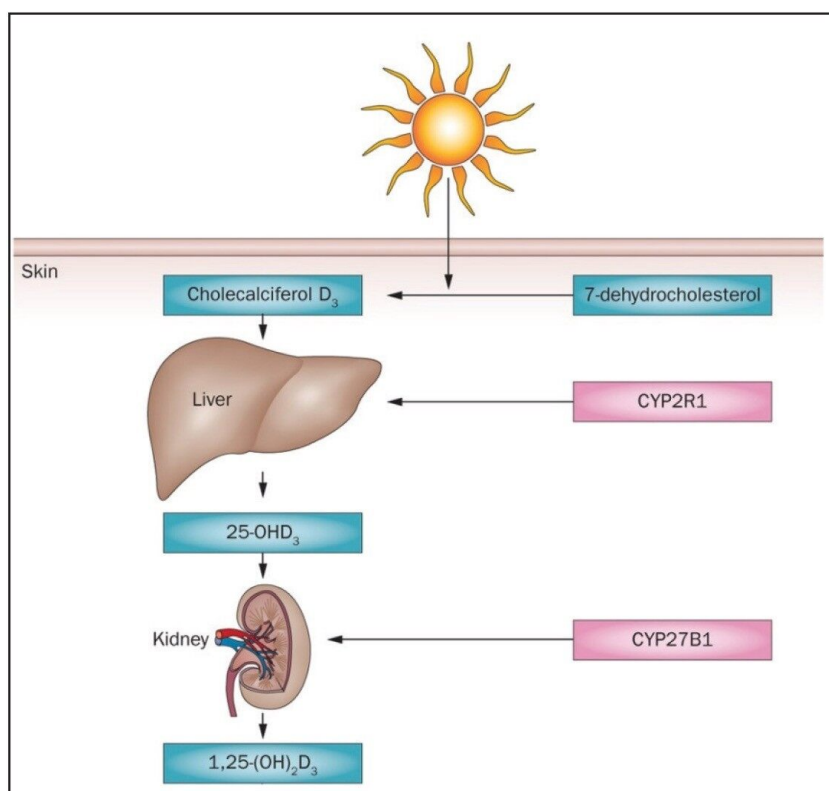


Figure 1. Process for conversion of 7-dehydrocholesterol to 1,25 (OH)<sub>2</sub> Vitamin D<sub>3</sub>.<sup>2</sup>

This conversion is tightly controlled through a cascade pathway which involves calcium, phosphorous, parathyroid hormone, and Vitamin D receptors. Regulated by a feedback mechanism process, 1,25 (OH)<sub>2</sub> Vitamin D circulates in the pg/mL-level range in serum. Main application areas for the measurement of Vitamin D are in nutrition, pharmacokinetic studies, clinical studies, and quality control for foods and supplements.<sup>1</sup>

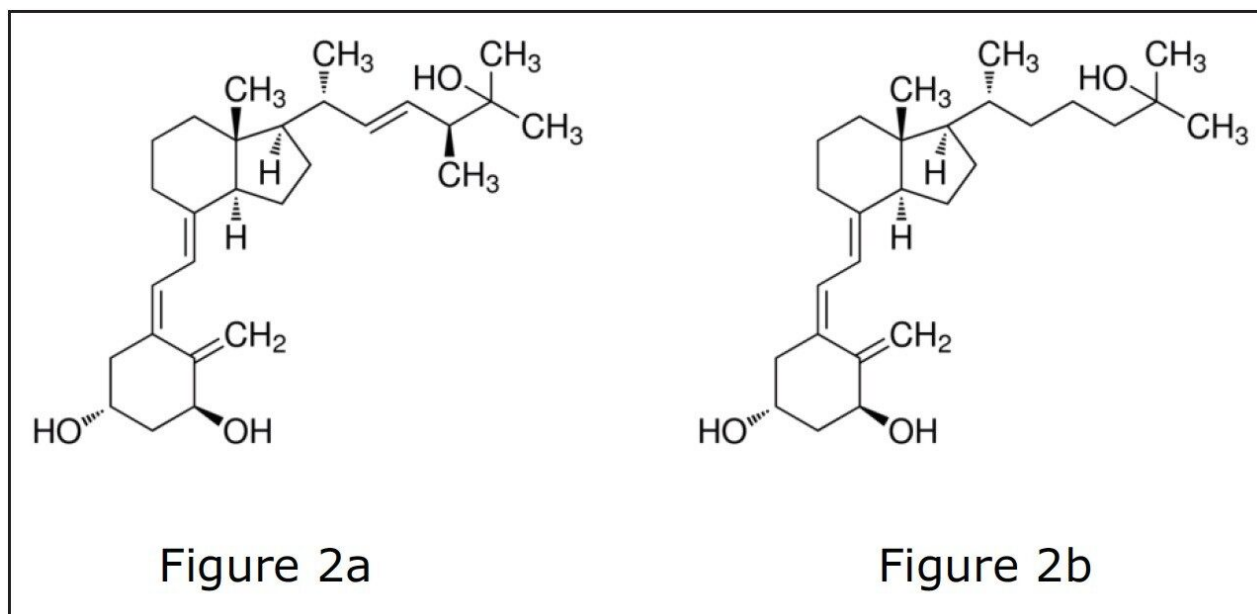


Figure 2a. 1,25 di-hydroxy Vitamin D<sub>2</sub>. Figure 2b. 1,25 di-hydroxy Vitamin D<sub>3</sub>.

In recent years, LC-MS/MS assays – compared to immunoassays – have gained popularity as the method of choice for quantification of 1,25 (OH)<sub>2</sub> Vitamin D. LC-MS/MS assays provide orthogonal selectivity. The identity of every compound is based upon its retention time as well as its unique MRM transition. The addition of immunoaffinity using specific antibodies in sample cleanup adds an extra layer of selectivity to the assay.

LC-MS/MS is accepted by the FDA as the gold standard analytical technique for pharmacokinetic studies of small molecules. The FDA has set specific guidelines to be followed while developing and validating LC-MS/MS assays. These guidelines involve intra-day (within the same day) and inter-day (across multiple days) accuracy and precision studies, linearity, and reproducibility. The method presented here followed the Bioanalytical Method Validation guidelines set out by the FDA. Accuracy, precision, linear range, and reproducibility of the method was evaluated, and the method met the criteria detailed in the FDA guidelines.<sup>3</sup>

The data presented in this application note was generated using a Waters ACQUITY UPLC System and Xevo TQ-S micro Mass Spectrometer.

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## Experimental

## Sample description

Commercially-available immunopurification kits were purchased from ALPCO Diagnostics and used to extract 1,25 (OH)<sub>2</sub> Vitamin D from 500 µL of human serum. The eluate from the last step was evaporated to dryness. Samples were derivatized with PTAD (4-phenyl-1,2,4-triazoline-3,5-dione) using 100 µL of 0.75 mg/mL PTAD in acetonitrile, which was added to each tube and allowed to incubate in the dark – at room temperature – for one hour. PTAD was then evaporated using a CryoVac system. The contents of the tubes were reconstituted using 50 µL of 50:50 water–methanol mix. This solution was then transferred to LC MS/MS vials, and 20 µL were injected into a column.

## LC conditions

|                   |   |
|-------------------|---|
| Instrument:       | Waters ACQUITY UPLC   |
| Column:           | ACQUITY UPLC BEH C <sub>18</sub> , 1.7 µm, 2.1 mm x 50 mm (P/N 1860002350)  |
| Column temp.:     | 60 °C   |
| Sample temp.:     | 4 °C  |
| Injection volume: | 20 µL   |
| Flow rate:        | 0.500 mL  |
| Mobile phase A:   | 100% water, 0.1% formic acid, 2 mM methylamine  |
| Mobile phase B:   | 100% methanol, 0.1% formic acid, 2 mM methylamine   |
| Gradient:         | Start with 50% A and hold for two minutes.<br>Change to 80% B between 2–4 minutes.<br>Followed by one minute of flushing and one minute of equilibration. |

## MS conditions

|                    |  |
|--------------------|--|
| Instrument:        | Xevo TQ-S micro  |
| Ionization mode:   | ESI+   |
| Transitions:       | 1,25 (OH) <sub>2</sub> Vit D <sub>3</sub> – 574.2>314.1<br>1,25 (OH) <sub>2</sub> Vit D <sub>2</sub> – 635.3>314.1<br>1,25 (OH) <sub>2</sub> Vit D <sub>3</sub> Int std – 580.3>314.1<br>1,25 (OH) <sub>2</sub> Vit D <sub>2</sub> Int std – 641.3>314.1 |
| Capillary voltage: | 2.5 kV   |
| Cone voltage:      | 25 V   |
| Desolvation temp.: | 500 °C   |
| Desolvation gas:   | 1000 L/Hr  |
| Cone gas:          | 25 L/Hr  |

## Data management

MassLynx 4.1

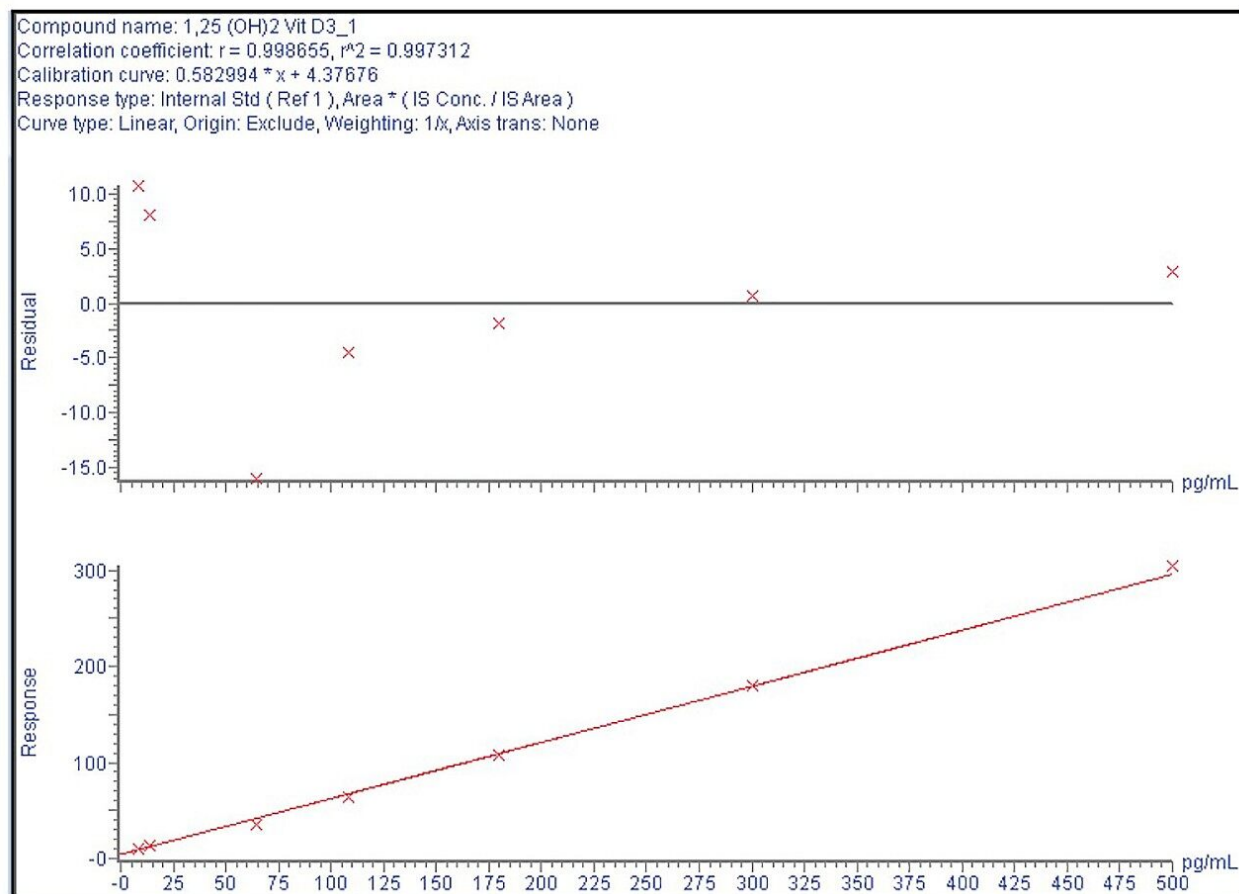
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## Results and Discussion

1,25 (OH)<sub>2</sub> Vitamin D is an important biomarker tested routinely in clinical and bioanalytical laboratories. The extremely low circulating levels of this molecule, coupled with lack of ionization in electrospray ionization mode, make this a challenging assay. The method described here combines affinity-based sample preparation combined with derivatization to increase ionization as an elegant solution for this analytically-difficult molecule.

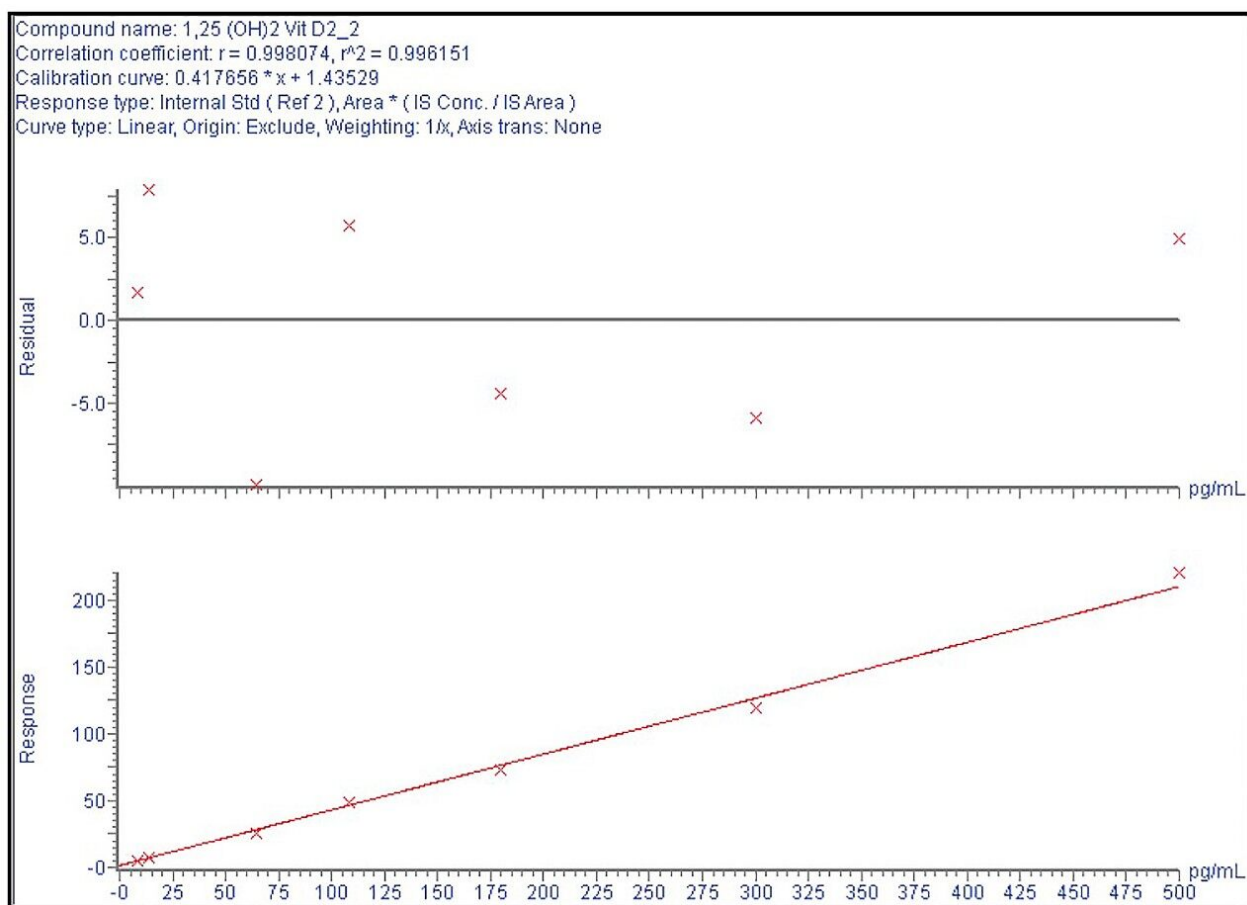
## Calibration curve and residual plots

Calibration curve from 5–500 pg/mL is linear and % bias across the range is <15% for both 1,25 (OH)<sub>2</sub> Vit D<sub>2</sub> and D<sub>3</sub> as shown in Figure 3a and 3b.



3a

Figure 3a. Residual plot and calibration curve for 1,25 (OH)<sub>2</sub> Vit D<sub>3</sub>.



3b

Figure 3b. Residual plot and calibration curve for 1,25 (OH)<sub>2</sub> Vit D<sub>2</sub>.

### Inter-day accuracy and precision

Six replicates at low (30 pg/mL), mid (150 pg/mL), and high (300 pg/mL) QC levels were extracted and injected across three days. The intra-day and inter-day precision and accuracy were <15% at all levels as shown in Tables 1–4.

| <b>1a</b>         | Mean  | Std dev | % CV  | % Bias | <b>1b</b>           | Mean   | Std dev | % CV  | % Bias | <b>1c</b>           | Mean   | Std dev | % CV | % Bias |
|-------------------|-------|---------|-------|--------|---------------------|--------|---------|-------|--------|---------------------|--------|---------|------|--------|
| 30 25.8           |       |         |       |        | 150 160.7           |        |         |       |        | 300 270.4           |        |         |      |        |
| 30 31.4           |       |         |       |        | 150 138.9           |        |         |       |        | 300 280             |        |         |      |        |
| 30 26.4           | 29.18 | 2.89    | 9.91  | -2.72  | 150 160.4           | 144.73 | 14.16   | 9.78  | -3.51  | 300 260.6           | 273.82 | 9.95    | 3.63 | -8.73  |
| 30 28.5           |       |         |       |        | 150 128.1           |        |         |       |        | 300 268.6           |        |         |      |        |
| 30 33.3           |       |         |       |        | 150 131.5           |        |         |       |        | 300 289.4           |        |         |      |        |
| 30 29.7           |       |         |       |        | 150 148.8           |        |         |       |        | 300 273.9           |        |         |      |        |
| 30 34             |       |         |       |        | 150 145.3           |        |         |       |        | 300 295.7           |        |         |      |        |
| 30 26.9           |       |         |       |        | 150 159.5           |        |         |       |        | 300 333.1           |        |         |      |        |
| Intra-day 30 32.3 | 32.83 | 4.77    | 14.51 | 9.44   | Intra-day 150 130.2 | 148.08 | 14.44   | 9.75  | -1.28  | Intra-day 300 296.6 | 303.38 | 17.24   | 5.68 | 1.13   |
| 30 29.3           |       |         |       |        | 150 135.1           |        |         |       |        | 300 314.1           |        |         |      |        |
| 30 40.8           |       |         |       |        | 150 168.4           |        |         |       |        | 300 295.2           |        |         |      |        |
| 30 33.7           |       |         |       |        | 150 150             |        |         |       |        | 300 285.6           |        |         |      |        |
| 30 25.6           |       |         |       |        | 150 156.4           |        |         |       |        | 300 269.5           |        |         |      |        |
| 30 32.1           |       |         |       |        | 150 134.2           |        |         |       |        | 300 329.1           |        |         |      |        |
| 30 30.5           | 28.75 | 3.27    | 11.37 | -4.17  | 150 165.6           | 151.63 | 15.16   | 10.00 | 1.09   | 300 276.2           | 294.27 | 23.47   | 7.98 | -1.91  |
| 30 32.4           |       |         |       |        | 150 130.9           |        |         |       |        | 300 304.7           |        |         |      |        |
| 30 25.6           |       |         |       |        | 150 159.2           |        |         |       |        | 300 308.7           |        |         |      |        |
| 30 26.3           |       |         |       |        | 150 163.5           |        |         |       |        | 300 277.4           |        |         |      |        |

Table 1a. Intra-day: 1,25 (OH)<sub>2</sub> D<sub>2</sub> – LQC (30 pg/mL). Table 1b. Intra-day: 1,25 (OH)<sub>2</sub> D<sub>2</sub> – MQC (150 pg/mL).

Table 1c. Intra-day: 1,25 (OH)<sub>2</sub> D<sub>2</sub> – HQC (300 pg/mL).

|           |         | LQC   | MQC    | HQC    |
|-----------|---------|-------|--------|--------|
|           | Mean    | 30.26 | 148.15 | 290.49 |
| Intra-day | Std dev | 3.98  | 14.01  | 20.99  |
|           | % CV    | 13.15 | 9.46   | 7.22   |
|           | % Bias  | 0.85  | -1.23  | -3.17  |

Table 2. Inter-day precision data: 1,25 (OH)<sub>2</sub> Vitamin D<sub>2</sub>.



| 3a                | Mean  | Std dev | % CV  | % Bias | 3b                  | Mean   | Std dev | % CV | % Bias | 3c                  | Mean   | Std dev | % CV | % Bias |
|-------------------|-------|---------|-------|--------|---------------------|--------|---------|------|--------|---------------------|--------|---------|------|--------|
| 30 32             |       |         |       |        | 150 168.4           |        |         |      |        | 300 334.3           |        |         |      |        |
| 30 27.2           |       |         |       |        | 150 170.8           |        |         |      |        | 300 336.3           |        |         |      |        |
| 30 27.7           | 31.47 | 3.21    | 10.20 | 4.89   | 150 167.8           | 166.90 | 3.48    | 2.09 | -11.27 | 300 342.5           | 332.25 | 12.83   | 3.86 | -10.75 |
| 30 34.2           |       |         |       |        | 150 161.8           |        |         |      |        | 300 332.2           |        |         |      |        |
| 30 33.8           |       |         |       |        | 150 169.1           |        |         |      |        | 300 307.3           |        |         |      |        |
| 30 33.9           |       |         |       |        | 150 163.5           |        |         |      |        | 300 340.9           |        |         |      |        |
| 30 26.5           |       |         |       |        | 150 162.3           |        |         |      |        | 300 314.9           |        |         |      |        |
| 30 31.7           |       |         |       |        | 150 166.3           |        |         |      |        | 300 313.5           |        |         |      |        |
| Intra-day 30 33.6 | 29.75 | 3.92    | 13.19 | -0.83  | Intra-day 150 144.6 | 153.17 | 10.86   | 7.09 | -2.11  | Intra-day 300 321.5 | 316.30 | 22.89   | 7.24 | -5.43  |
| 30 34.4           |       |         |       |        | 150 145.2           |        |         |      |        | 300 274.9           |        |         |      |        |
| 30 25.8           |       |         |       |        | 150 140.8           |        |         |      |        | 300 341.6           |        |         |      |        |
| 30 26.5           |       |         |       |        | 150 159.8           |        |         |      |        | 300 331.4           |        |         |      |        |
| 30 26.5           |       |         |       |        | 150 157.4           |        |         |      |        | 300 260.1           |        |         |      |        |
| 30 25.8           |       |         |       |        | 150 154             |        |         |      |        | 300 316.8           |        |         |      |        |
| 30 29.5           | 28.72 | 2.62    | 9.13  | -4.28  | 150 154.8           | 156.10 | 3.85    | 2.47 | -4.07  | 300 307.5           | 293.32 | 27.33   | 9.32 | 2.23   |
| 30 29             |       |         |       |        | 150 151             |        |         |      |        | 300 256.5           |        |         |      |        |
| 30 28.3           |       |         |       |        | 150 157             |        |         |      |        | 300 309.1           |        |         |      |        |
| 30 33.2           |       |         |       |        | 150 162.4           |        |         |      |        | 300 309.9           |        |         |      |        |

Table 3a. Intra-day: 1,25 (OH)<sub>2</sub> D<sub>3</sub> – LQC (30 pg/mL). Table 3b. Intra-day: 1,25 (OH)<sub>2</sub> D<sub>3</sub> – MQC (150 pg/mL).  
Table 3c. Intra-day: 1,25 (OH)<sub>2</sub> D<sub>3</sub> – HQC (300 pg/mL).

|           |         | LQC   | MQC    | HQC    |
|-----------|---------|-------|--------|--------|
|           | Mean    | 29.98 | 158.72 | 313.96 |
| Intra-day | Std dev | 3.31  | 8.92   | 26.32  |
|           | %CV     | 11.03 | 5.62   | 8.38   |
|           | % Bias  | -0.07 | -5.81  | -4.65  |

Table 4. Inter-day precision data: 1,25 (OH)<sub>2</sub> Vitamin D<sub>3</sub>.

## Injection reproducibility

The Waters ACQUITY UPLC and Xevo TQ-S micro displayed robust injection reproducibility as shown below. The % CV for the retention times was 0.32% (Figure 4a) and the % CV for analyte area counts was 5.21% (Figure 4b) – both of which are well within the acceptable criteria.

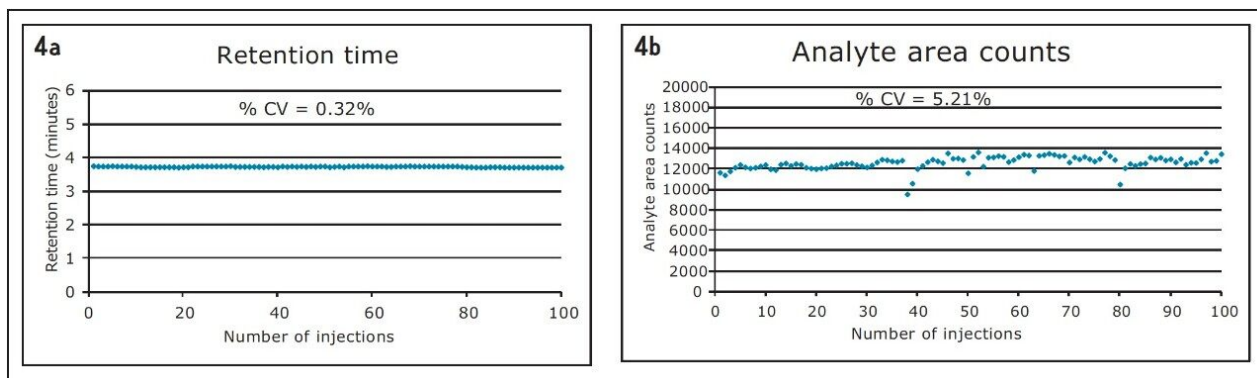


Figure 4a. % CV for retention time over 100 injections. Figure 4b. % CV for analyte area counts over 100 injections.

### Instrument dynamic range

The analytical range for this method is from 5–500 pg/mL and covers the relevant concentrations typically found in serum. 1,25 (OH)<sub>2</sub> Vitamin D showed linearity from 50 pg/mL–1 µg/mL using the ACQUITY UPLC and TQ-S micro for the method described here.

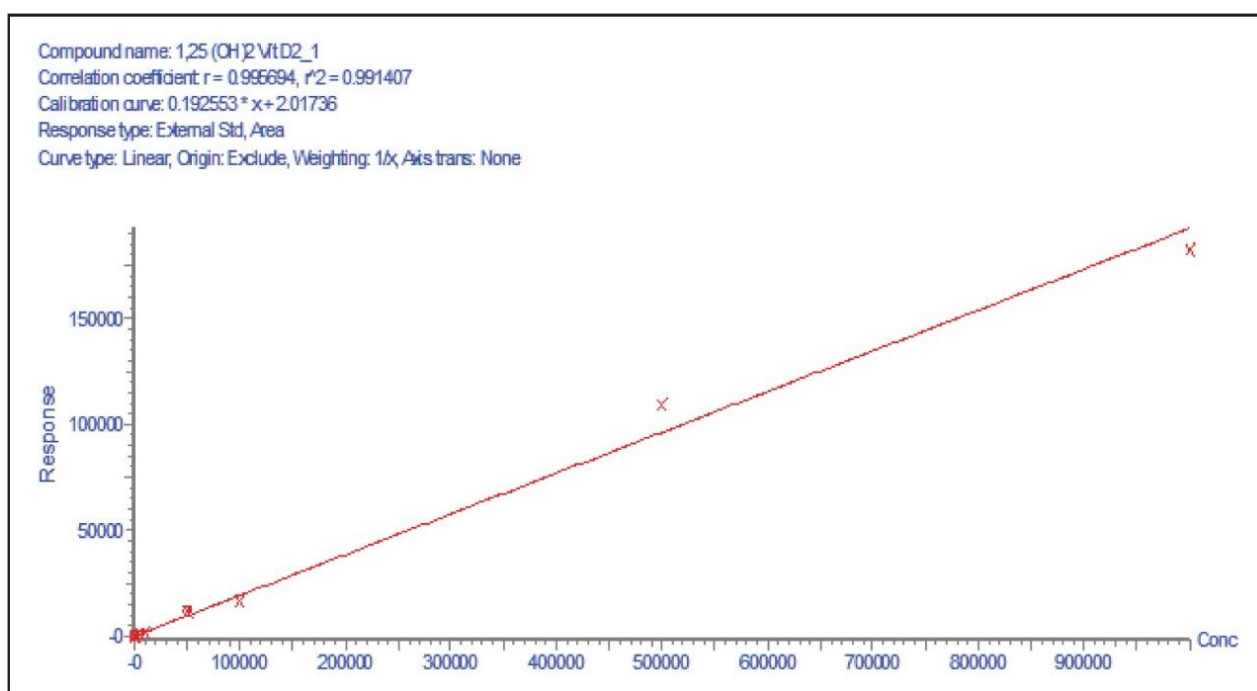


Figure 5. Linear dynamic range >4x.

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## Conclusion

A robust, selective, and sensitive analytical method for the quantification of 1,25 (OH)<sub>2</sub> Vitamin D<sub>2</sub> and D<sub>3</sub> from human serum was developed using the Waters ACQUITY UPLC System and Xevo TQ-S micro Mass Spectrometer. A limit of quantification of 5 pg/mL was readily achieved while maintaining excellent linearity. Calibration curves for both 1,25 (OH)<sub>2</sub> D<sub>2</sub> and D<sub>3</sub> were linear over the range of 5–500 pg/mL with  $r^2 > 0.99$ . Across three days, the intra- and inter-day CV as well as the % bias were <15% for both 1,25 (OH)<sub>2</sub> Vitamin D<sub>2</sub> and D<sub>3</sub>. Injection reproducibility was excellent with % CV <0.32% for retention time and <5.5% for area counts.

Today's analytical laboratories are becoming more diverse and multi-functional. Lab managers are expected to diversify their analytical platforms within limited lab spaces. The Xevo TQ-S micro has the ideal combination of sensitivity, reproducibility, and versatility to provide an excellent option for all types of bioanalytical labs.

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## References

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720005679, April 2016

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