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Sensitive Measurement of Hydroxy Metabolites of Vitamin D and Respective Epimers using LC-MS/MS which Overcomes Challenges of Chemiluminescent Immunoassay

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Introduction

Vitamin D is a group of lipophilic secosteroids that have been associated with several pathologies. Vitamin D status is assessed by measuring two main circulating metabolites: 25-hydroxyvitamin D2 [25(OH)D2] and 25-hydroxyvitamin D3 [25(OH)D3].

LC-MS/MS analysis typically offers specific estimation of 25(OH)D2 and 25(OH)D3. However, the presence of epimeric forms of 25(OH) can interfere with identification and quantitation, resulting in potential overestimation of 25(OH)D2 and/or 25(OH)D3.

Epimers differ only by the spatial arrangement of the C3-hydroxyl, which makes them isobaric (therefore indistinguishable by MS) and difficult to separate chromatographically.

The LC-MS/MS method described here allows simultaneous, baseline separation and sensitive quantification of 25(OH)D and epimers for both D2 and D3 .

Experimental Details

Instrumentation

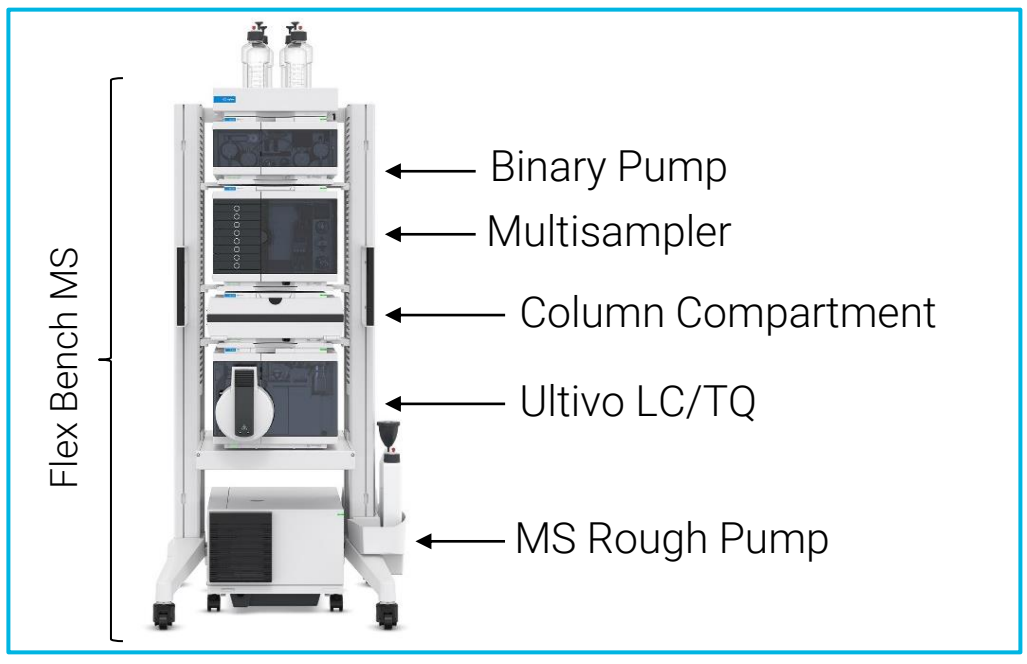

Figure 1. Ultivo triple quadrupole LC/MS system

- Agilent 1260 Infinity II pump
- Agilent 1260 Infinity II Multisampler with 0.3 µm inline filter
- Agilent 1260 Infinity II multicolumn compartment
- Agilent Ultivo LC/TQ equipped with Agilent Jet Stream (AJS) source

Experimental Details

Table 1: 1260 Infinity II LC parameters

Parameter	Value	
Analytical column	Agilent InfinityLab Poroshell 120 PFP, 3.0x100 mm 2.7 µm (p/n: 695975-308), 0.550 mL/minute, at 20 °C	
Mobile phase A	Water with 0.1% Formic acid	
Mobile phase B	Methanol with 0.1% Formic acid	
Gradient	Time (min)	%B
	0.00	65
	1.00	75
	6.00	80
	6.50	95
	8.40	95
	8.50	65
	10.00	65

Table 2: Ultivo LC/TQ and AJS Parameters

Parameter	Value
Ionization mode	Positive
MS/MS mode	MRM
Drying gas temperature	250 °C
Drying gas flow	7 L/min
Nebulizer pressure	45 psi
Sheath gas temperature	325 °C
Sheath gas flow	11 L/min
Nozzle voltage	500 V
Capillary voltage	4000 V
Diverter valve to waste	at 7 minute
Software	MassHunter Acquisition (V 1.2) and MassHunter quantitative analysis (V: 10.0)

Table 3: MRM parameters, Dwell: 100 (ms), Polarity: Positive

Analyte	Precursor (m/z)	Product (m/z)	Frag (V)	CE (V)
25(OH)D2 + epi (Quant)	413.3	355.2	105	4
25(OH)D2 + epi (Qual)	413.3	395.3	105	4
25(OH)D3 + epi (Quant)	401.3	383.3	107	4
25(OH)D3 + epi (Qual)	401.3	365.3	107	8
25(OH)D2 d3 + epi d3 (1)	416.4	358.2	96	4
25(OH)D2 d3 + epi d3 (2)	416.4	398.4	96	4
25(OH)D3 d3 + epi d3 (1)	404.4	386.3	94	4
25(OH)D3 d3 + epi d3 (2)	404.4	368.3	94	8

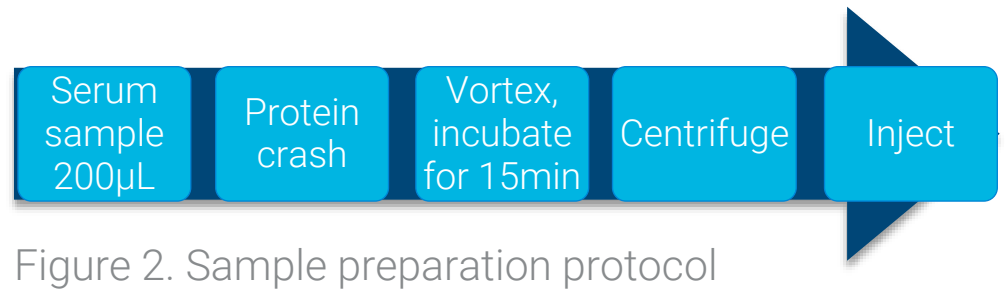

Figure 2. Sample preparation protocol

Analytical sensitivity and Linearity

Nine calibrators (range: 0.4 to 200 ng/mL) in serum matrix were included in the study. Internal standard (ISTD) concentration was 8 ng/mL. The overlay of blank, LOD, and LOQ levels of all three analytes are included in figure 3. Linearity curves were plotted for all analytes from the LOQ to the highest spiked calibration level with internal standard correction. All three targets displayed a linear response with R2 values > 0.999 (Figure 4).

Precision and Accuracy

Good RT and response precision values for all analytes were obtained, with % RSD of <0.3% and <5.0%, respectively. The average accuracy value for all three analytes across calibration range were within 95 to 115%.

Recovery and repeatability

Recovery was assessed using four technical preparations of three levels of QC samples (LQC: 4 ng/mL, MQC:10 ng/mL, and HQC: 20 ng/mL). The intra-batch recovery repeatability was measured as % RSD of recovery values calculated using four technical preparations. Recoveries for overall analytes were within 91 to 111% with intra-batch RSD ≤ 2%.

Matrix effect (ME)

To assess the ME, the response of 100ng/mL serum spiked calibrator level was compared with that from corresponding neat standard. All three analytes showed ME of > 87%, indicating minor matrix suppression.

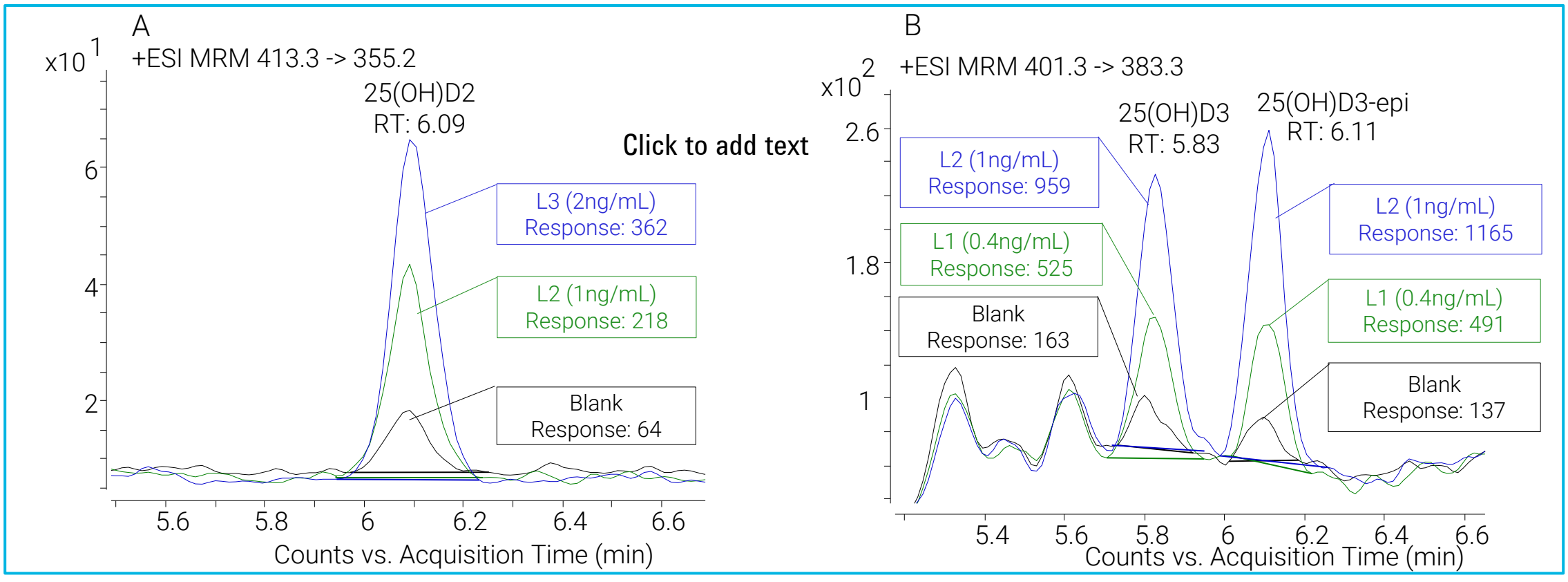

Figure 3. A: MRM trace of 25(OH)D2 in blank serum (black trace), LOD 1 ng/mL serum spike (green trace), and LOQ 2 ng/mL serum spike (blue trace). B: MRM trace of 25(OH)D3 and 25(OH)D3-epi in blank serum (black trace), LOD 0.4 ng/mL serum spike (green trace), and LOQ 1 ng/mL serum spike (blue trace).

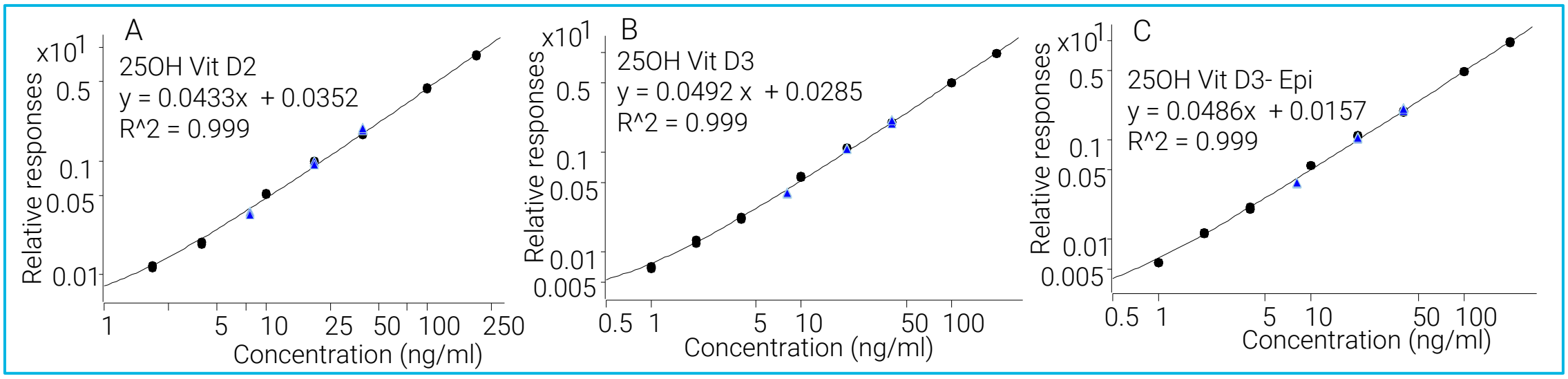

Figure 4. The calibration; A: 25(OH)D2, B: 25(OH)D3, & C:25(OH)D3-epi. The three QC levels are marked in a blue triangle. (calibration model Type: Linear, Origin: Ignore, Weight: 1/x)

Results and Discussion

Robustness

The method robustness was assessed from 500 continuous injections of the MQC sample. The calculated concentration and RT were monitored for all three targets over time and % RSD values were calculated to assess the robustness. The data acquisition was continuous (run time: 3.5 days), and the Ultivo LC/TQ was operated without readjusting any tune parameters.

The elution profile was extremely consistent over 500 injections. Good reproducibility of calculated concentration with RSD < 5.0% and RT RSD < 0.2% were observed for all three targets (Figure 5).

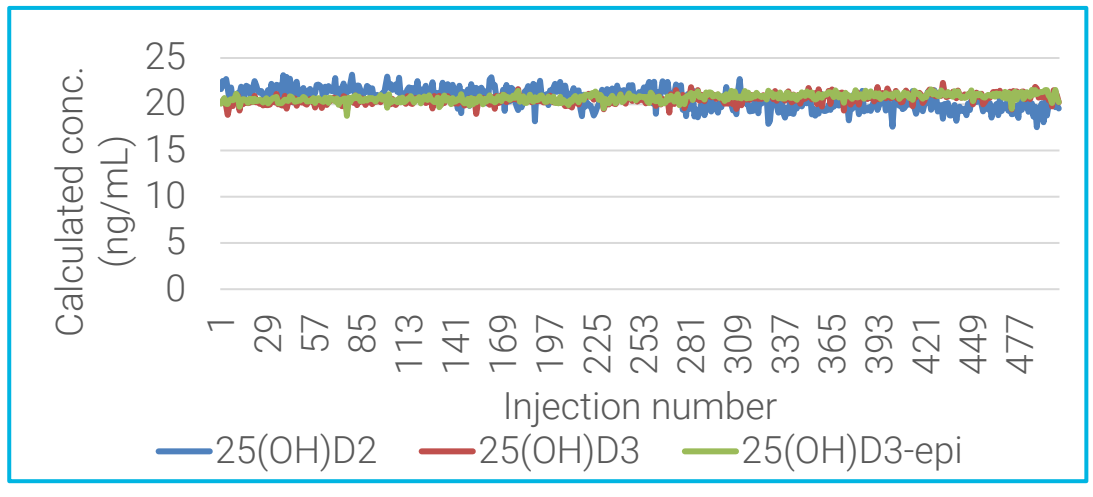

Figure 5. The calculated concentration of all three targets over 500 injections using MQC sample

Performance testing using Chromsystems reference standards

Chromsystems blank, calibrators, and four technical preparations of each QC level were processed using the in-house developed sample preparation protocol and acquired using the newly developed Ultivo LC/TQ method.

Observed good separation between 25(OH)D3 and 25(OH)D3-epi in the reference standards (Figure 6A).

In addition, although 25(OH)D2-epi was not included in our initial method development, its presence in reference standards demonstrated that the method also achieves baseline separation of 25(OH)D2 and 25(OH)D2-epi (Figure 6B).

The linearity curves were plotted using the 3-level calibrators and all four targets displayed a linear response with R2 values > 0.999. The average recovery of all four targets using both QC levels was within 97 to 109%, with a repeatability RSD of ≤4%.

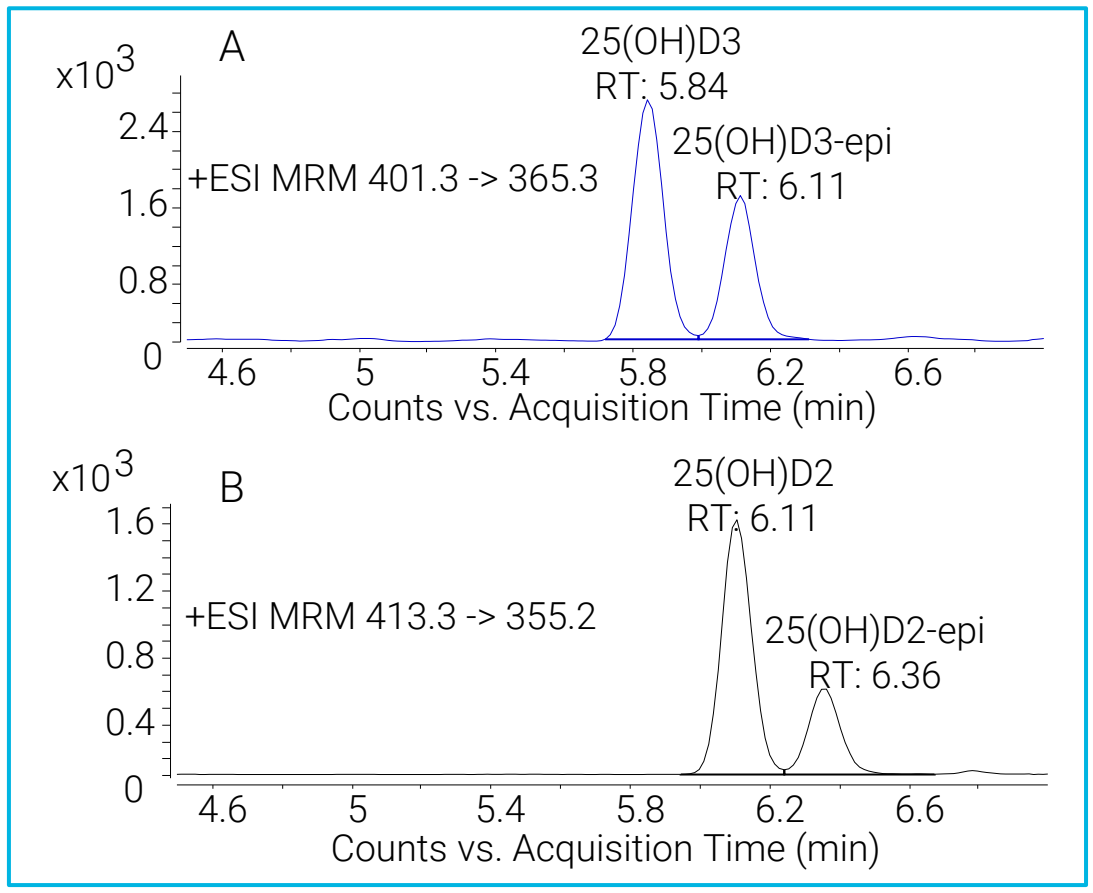

Figure 6. MRM trace of targets indicating baseline chromatographic separation of 25(OH)D and respective epimer. A: separation between 25(OH)D3 and 25(OH)D3-epi, B: separation between 25(OH)D2 and 25(OH)D2-epi.

Conclusions

- Developed a robust method for the simultaneous analysis of vitamin D hydroxy metabolites and epimers.
- Both 25(OH)D2 and 25(OH)D3 were chromatographically well resolved from their epimeric forms using a 10-minute gradient, adding specificity to the confident quantitation.
- The Ultivo triple quadrupole LC/MS offered excellent linearity, precision, and analytical sensitivity across the range of 1 to 200 ng/mL for 25(OH)D metabolites in serum.
- Using the newly developed method, Chromsystems reference samples were tested, and results were within the allowed range.
- The robustness results using 500 continuous injections illustrated the method reliability for day-to-day operation.

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