Vitamin D biomarkers in plasma

Sensitive, reproducible quantitation with SOLA HRP 30 mg SPE for clinical research

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Goal

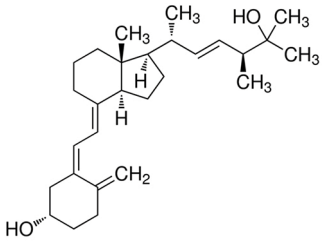
Maximizing the sensitivity of vitamin D biomarker quantitation assay in human plasma by leveraging Thermo Scientific™ SOLA™ HRP solid phase extraction (SPE). Analysis carried out on the Thermo Scientific™ Vanquish™ Horizon UHPLC system and Thermo Scientific™ TSQ Quantiva™ mass spectrometer with the Thermo Scientific™ Hypersil GOLD™ VANQUISH™ C18 UHPLC column, 1.9 µm, 2.1 × 50 mm, for developing a fast, robust, reliable LC-MS method.

Introduction

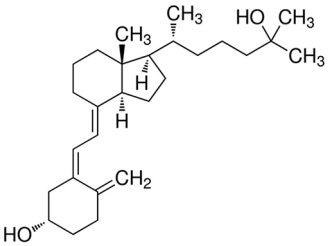
Solid-phase extraction (SPE) is an efficient extraction technique to separate critical analytes that are dissolved or suspended in a liquid mixture according to their physical and chemical properties. The extraction detailed here from human plasma is carried out using SOLA HRP 30 mg cartridges. Its revolutionary design is specifically tailored for processing biological samples and helps reduce blocking and sample failures.

Vitamin D is widely monitored and used as an indicator for skeletal health in both children and adults. The active form of the hormone controls the concentration of calcium and phosphorous in the bloodstream, with deficiencies resulting in both rickets in children and osteoporosis in adults. Vitamin D occurs in two forms: vitamin D_2 (ergocalciferol) is found naturally in plants and is commonly used as a dietary supplement; vitamin D_3 (cholecalciferol) occurs naturally in mammals and is formed in the skin by exposure to sunlight.

Vitamin D_2 and vitamin D_3 are hydroxylated in the liver to form 25-hydroxyvitamin D_2 (OH-Vit D_2) and 25-hydroxyvitamin D_3 (OH-Vit D_3), Figure 1. These two forms of the compounds are then released into the circulation and transported via the vitamin D binding protein.



25-hydroxyvitamin D_2 (OH-Vit D_2)



25-hydroxyvitamin D_3 (OH-Vit D_3)

Figure 1. Vitamin D biomarkers

Further hydroxylation occurs in the kidneys to form the active hormone 1,25-dihydroxyvitamin D (1,25(OH)2D). Although it is possible to monitor the active hormone, the limits of detection required to quantify this are challenging as 1,25(OH)2D occurs at picogram levels per mL within the body. It has, therefore, been readily accepted that the measurement of OH-Vit D_2 and OH-Vit D_3 is the preferred method for assessing a subject's total vitamin D status due to the higher circulating concentrations (ng/mL) and greater serum half-life.

Suggested optimum concentration ranges within the body for 25-OH-D differ between organizations, although generally a range of 20–100 ng/mL is deemed sufficient, while anything below this level is classified as deficient.[1,2] The dynamic range of this assay, 2.5 to 1000 ng/mL and 5.0 to 1000 ng/mL, allows quantification of OH-Vit D_3 and OH-Vit D_2 levels, respectively, within a sample to show the vitamin D status of the subject.

This first-in-class SPE product introduces next generation, innovative technological advancements, giving unparalleled performance characteristics compared to conventional SPE, phospholipid, and protein precipitation products. These include:

- Higher levels of reproducibility
- Higher levels of extract cleanliness
- Reduced sample and solvent requirements
- Increased sensitivity

Additionally, a higher bed size of 30 mg enables:

- More sample loading without analyte breakthrough
- An excellent approach to increase sensitivity when faced with a complex matrix

SOLA products provide significant advantages for the analyst when processing compounds in complex matrices, particularly in high-throughput bioanalytical and clinical research laboratories where reduced failure rate, higher analysis speed, and lower sample/solvent requirements are critical. Increased performance from SOLA products gives higher confidence in analytical results and lowers cost without compromising ease of use or requiring complex method development.

This application achieves rapid separation of 25-hydroxyvitamin D_2 and D_3 using a Hypersil GOLD VANQUISH C18 UHPLC column. These Validated-for-Vanquish columns were designed to fully leverage the robustness and extended pressure capabilities of the new Thermo Scientific™ Vanquish™ UHPLC platform. This work results in development of a robust, fast, sensitive method with high-throughput capabilities that addresses critical analytical challenges of analyzing complex matrices using liquid chromatography (LC) and liquid chromatography-mass spectrometry (LC-MS).

Hypersil GOLD VANQUISH UHPLC columns deliver:

- Better separations: High efficiency enables separation of very complex mixtures, even at high flow rates
- Easier interactions: Seamless workflow solution, for simple and easy separations

This application method was assessed intra-day to determine linearity, accuracy and precision, carryover, recovery, and matrix effects, as presented in Table 1.

Table 1. Summary of assay

Parameter	Value	
Analytes	25-hydroxyvitamin D_2 (OH-Vit D_2)	25-hydroxyvitamin D_3 (OH-Vit D_3)
Analytical matrix	Human plasma	
Injection volume	5 µL	
Calibration range	5.00–1000 ng/mL	2.50–1000 ng/mL
Lower limit of quantification (LLOQ)	5.00 ng/mL	2.50 ng/mL
Calibration model	Linear regression, R^2>0.99	
Weighting factor	$1/x^2$	
Carryover	None observed	
Accuracy (bias) and precision	-8.2–3.7% (CV% 3.1–7.8%)	-6.9–6.2% (CV% 3.5–9.6%)
Recovery	100.2%	101.2%
Matrix factor (IStd normalized)	0.961 (CV% 2.4)	1.08 (CV% 1.0)

Experimental

Instrumentation

- Vanquish Horizon UHPLC system consisting of the following:
  - Vanquish system base (P/N VH-S01-A)
  - Binary pump H (P/N VH-P10-A)
  - Split sampler HT (P/N VH-A10-A)
  - Column compartment H (P/N VH-C10-A)
  - Active pre-heater (P/N 6732.0110)
- Thermo Scientific™ TSQ Quantiva™ Triple-Stage Quadrupole Mass Spectrometer (IQLAAEGAAXFAOUMZZZ)

Consumables

- Hypersil GOLD VANQUISH C18 UHPLC Column, 1.9 µm, 2.1 × 50 mm (P/N 25002-052130-V)
- SOLA HRP 30 mg/2 mL 96-well plate, 1 pk (P/N 60509-001)
- SOLA HRP 30 mg/3 mL cartridges, 50 pk (P/N 60409-001)
- Thermo Scientific™ WebSeal™ certified 96 square well plate, 2 mL (P/N 60180-P202)
- Thermo Scientific™ PTFE/Silicone sealing mat for 96 square well plates, 8 mm, flat base plugs, large penetration area, pre-slit (P/N 60180-M122)

Reagents

- Thermo Scientific™ UHPLC-MS grade water (P/N W8-1)
- Thermo Scientific™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Fisher Chemical™ Optima™ UHPLC-MS grade formic acid (P/N A117-50)

Sample pretreatment

The extraction of analytes from plasma was carried out using a SOLA HRP plate, which retained the compound by hydrophobic reversed phase interactions. As OH-Vit D_2 and OH-Vit D_3 are transported around the body by the vitamin D binding protein, it is necessary during sample pretreatment to disrupt the protein binding prior to extraction to ensure maximum compound recovery (Table 2). Typically, this is carried out using 100% acetonitrile. However, as the sample is retained on the sorbent by a hydrophobic mechanism, it is important not to load in conditions that contain too much organic solvent as this will result in compound breakthrough. Therefore, acetonitrile was added to the sample prior to loading to disrupt the protein binding (ensuring that the final ratio did not exceed 1:1).

Table 2. Sample pretreatment procedure

Step	Procedure
1	To 200 µL of matrix*, 180 µL acetonitrile were added.
2	For standards, QCs, and single blanks, 10 µL of internal standard solu-tion were added.
3	For standards and QCs, 10 µL of spiking solution were added.
4	For single blanks, 10 µL acetonitrile were added.
5	For double blanks and matrix effect overspikes, 20 µL acetonitrile were added only.
6	All samples were vortexed for 30 seconds and then centrifuged for 10 minutes at 4000 × g.

*For calibration standards and one set of OH-Vit D_3 QC_{LLOQ} samples, matrix equals 5% BSA/PBS; for all remaining QC samples, the matrix was blank human plasma.

Due to endogenous levels of OH-Vit D_3 in the sample, the calibration model was established using 5% bovine serum albumin in phosphate buffer saline (0.01 M) [PBS] as a surrogate matrix. Samples were fortified with internal standard solution, 25-hydroxyvitamin D_3-23,24,25,26,27-$^{13}C_5$, for a final matrix concentration of 50 ng/mL.

Sample preparation

During method development, it was observed that up to 50% methanol could be applied to the SPE plate without causing the compounds to be eluted. Therefore, the wash solution used to remove matrix interferences was 40% methanol. To elute the compounds of interest, 100% methanol was applied to ensure maximum recovery (Table 3). Full chromatographic and mass spectrometer conditions are laid out in Tables 4–7.

Table 3. Solid-phase extraction procedure

Parameter	Value
Compounds	25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_3-$^{13}C_5$ (IS)
Matrix	Human plasma; 5% bovine serum albumin in phosphate buffered saline (0.01 M)
Plate type	SOLA HRP 30 mg/2 mL (P/N 60509-001)
Conditioning	1 mL methanol, 1 mL water
Load	200 µL of sample supernatant
Wash	200 µL water / methanol (60:40 v/v)
Elution	2 × 200 µL methanol
Dry down	Under nitrogen without heat
Reconstitute	In 100 µL water/ acetonitrile (35:65 v/v), cap and vortex well

Chromatography and separation conditions

Table 4. UHPLC conditions

Parameter	Value
Mobile phase A	Water/formic acid (100/0.1, v/v)
Mobile phase B	Acetonitrile/formic acid (100/0.1, v/v)
Flow rate	0.8 mL/min
Run time	7.6 min
Column temperature	40°C, active preheating and still air mode
Injection volume	5 µL
Gradient	Linear

Table 5. LC gradient

Time (min)	B % Methanol
0.00	65
0.10	65
2.10	95
2.10	100
4.85	100
4.85	65
7.60	65

Mass spectrometry conditions

Table 6. MS/MS source parameters

Parameter	Value
Source	Thermo Scientific™ Ion Max source with HESI-II probe
Polarity	Positive ionization
Spray voltage	3500 V
Vaporizer temperature	460 °C
Sheath gas pressure	56 Arb
Aux gas pressure	18 Arb
Sweep gas pressure	2 Arb
Ion transfer tube temperature	368 °C
CID gas pressure	1.5 mTorr

Table 7. Compound transition details

Parameter	Value
25-hydroxyvitamin D_3 (OH-Vit D_3)	
Retention time	1.05 min
Polarity	Positive
Precursor	383.2 *m/z*
Product	365.3[a] and 211.2[b] *m/z*
Collision energy	15.9 and 26.4 V
25-hydroxyvitamin D_2 (OH-Vit D_2)	
Retention time	1.17 min
Polarity	Positive
Precursor	395.4 *m/z*
Product	269.2[a] and 209.1[b] *m/z*
Collision energy	20.7 and 26.5 V
25-hydroxyvitamin D_3-$^{13}C_5$ (OH-Vit D_3-$^{13}C_5$)	
Retention time	1.05 min
Polarity	Positive
Precursor	388.4 *m/z*
Product	370.3 *m/z*
Collision energy	13.7 V

[a]quantitation ion, [b]confirmation ion

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.10, was used for LC-MS system control, data acquisition, and data analysis.

Results and discussion

Calibration model, linearity, and range

A calibration line for 25-hydroxyvitamin D_2 was produced using nine non-zero calibrators analyzed in duplicate intra-batch (Figure 2 and Table 8). 25-hydroxyvitamin D_3 was established using the same model with a tenth calibration level, 2.50 ng/mL (Figure 3). Calibration standards were freshly prepared in surrogate matrix and subjected to SPE. Linearity was achieved for both analytes as detailed in Figures 2 and 3 and Table 8.

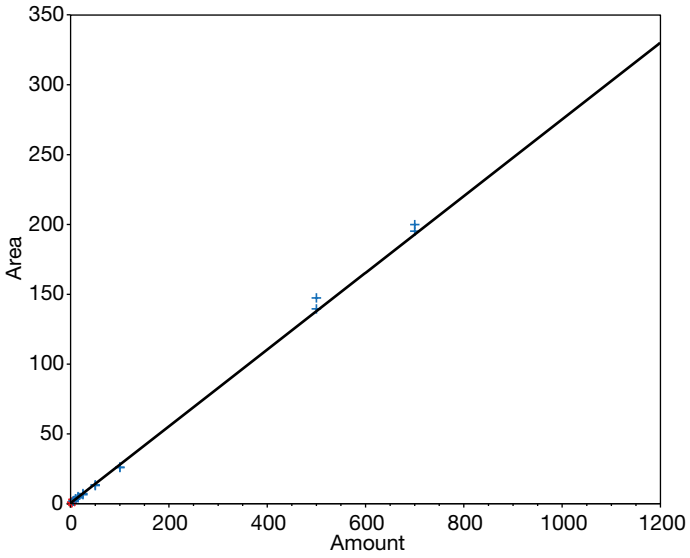


Figure 2. Linearity of 25-hydroxyvitamin D_2 over the dynamic range 5.0–1000 ng/mL

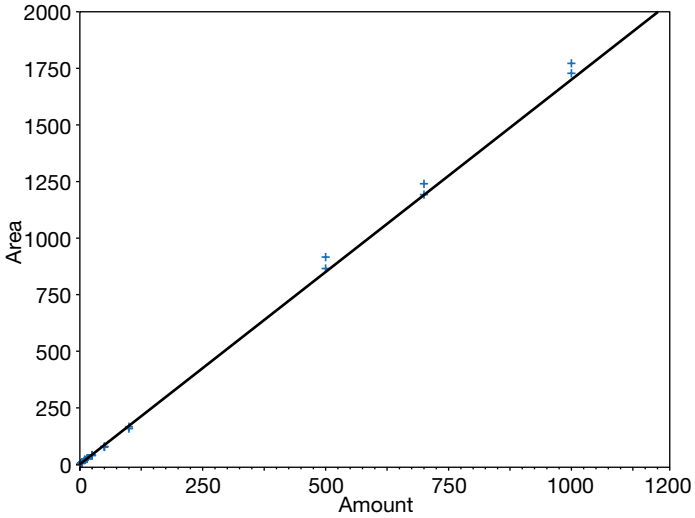


Figure 3. Linearity of 25-hydroxyvitamin D_3 over the dynamic range 2.5–1000 ng/mL

Table 8. Calibration data for 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3

Standard (n=2)	Analyte	Specified conc. (ng/mL)	Calculated conc. (ng/mL)	Bias (%)	CV (%)
S1	OH-Vit D_2	5.0	4.91	-1.8	1.2
S2	OH-Vit D_2	10	10.6	6.3	4.9
S3	OH-Vit D_2	15	15.4	2.9	13.5
S4	OH-Vit D_2	25	23.3	-7.0	8.2
S5	OH-Vit D_2	50	46.7	-6.7	3.0
S6	OH-Vit D_2	100	93.3	-6.7	0.7
S7	OH-Vit D_2	500	520	4.1	3.9
S8	OH-Vit D_2	700	717	2.5	1.7
S9	OH-Vit D_2	1000	1065	6.5	0.6
S1	OH-Vit D_3	2.5	2.45	-2.2	0.9
S2	OH-Vit D_3	5.0	5.03	0.5	7.2
S3	OH-Vit D_3	10	11.4	14.0	0.8
S4	OH-Vit D_3	15	14.8	-1.5	10.1
S5	OH-Vit D_3	25	23.3	-7.0	4.5
S6	OH-Vit D_3	50	45.7	-8.7	2.3
S7	OH-Vit D_3	100	95.1	-4.9	3.8
S8	OH-Vit D_3	500	524	4.7	4.0
S9	OH-Vit D_3	700	715	2.2	2.8
S10	OH-Vit D_3	1000	1030	3.0	1.8

Accuracy and precision

Accuracy and precision (A&P) were evaluated via analysis of quality control (QC) samples in replicate (n=6) at four concentration levels over the calibration range. The endogenous concentration of OH-Vit D_3 in the human plasma source was quantified from six single blank samples (spiked with internal standard, 50 ng/mL) analyzed in triplicate using the calibration model. This native concentration, 19.07 ng/mL, was considered when processing OH-Vit D_3 QC samples and is reflected in the data. For this reason, an additional set of QC_{LLOQ} samples for OH-Vit D_3 was created in surrogate matrix. All other QC samples for both analytes were prepared in human plasma.

Accuracy was reported as bias (%) according to criteria of ±15% for low, mid, and high QC levels and ±20% for LLOQ level, while precision was assessed under the same criteria as coefficient of variation (CV %). Intra-batch A&P was acceptable for both analytes with bias <8.2% and CV <9.6% at each of the four QC levels, as presented in Tables 9 and 10.

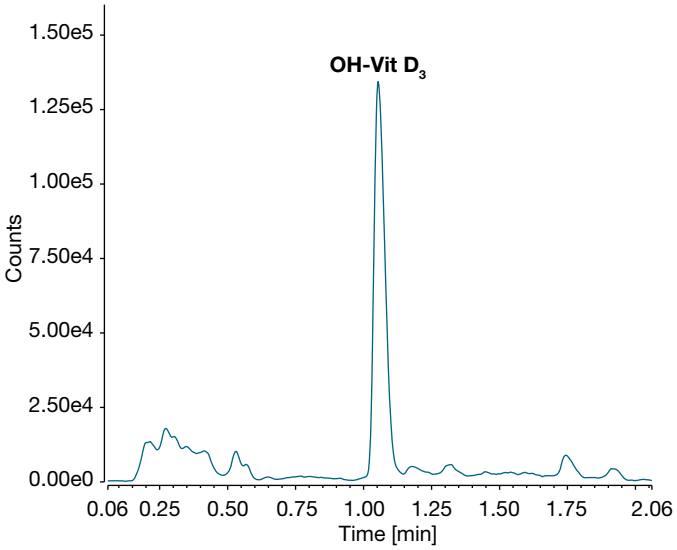


Figure 4. Representative chromatogram of 25-hydroxyvitamin D_3 at 150 ng/mL, the mid quality control (MQC) level

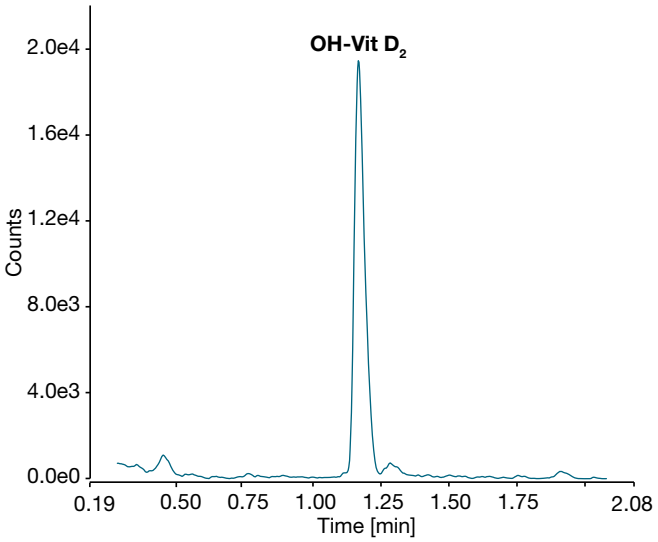


Figure 5. Representative chromatogram of 25-hydroxyvitamin D_2 at 150 ng/mL, the mid quality control (MQC) level

Table 9. QC data for 25-hydroxyvitamin D_2. All QC samples analyzed in human plasma.

	OH-Vit D_2 ng/mL			
	LLOQ	Low	Medium	High
Target	5.00	15.00	150.00	600.00
Mean (n=6)	4.59	14.68	154.25	622.20
Bias %	-8.2	-2.1	2.8	3.7
CV %	4.5	7.8	7.7	3.1

Table 10. QC data for 25-hydroxyvitamin D_3. All QC levels were analyzed in human plasma. A second set of LLOQ QC samples (n=6) was also prepared and analyzed in surrogate matrix (denoted in Table 10 as LLOQ*).

	OH-Vit D_3 ng/mL				
	LLOQ*	LLOQ	Low	Medium	High
Target	2.50	2.50	5.00	150.00	600.00
Mean (n=6)	2.33	2.61	5.22	159.37	633.50
Bias %	-6.9	4.6	4.3	6.2	5.6
CV %	9.6	7.5	5.6	8.2	3.5

Carryover

The carryover was assessed with the injection of two double blank samples following an injection of the highest calibration sample (ULOQ), Figure 6.

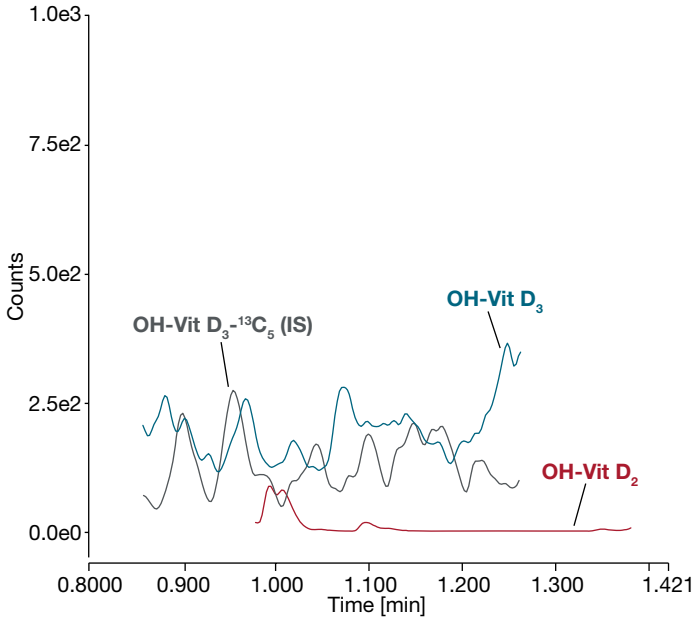


Figure 6. Chromatogram of a double blank sample following a ULOQ injection. No carryover was observed with the Vanquish Horizon UHPLC system.

Matrix effects

Matrix effects were evaluated from a single pooled source of human plasma. Six extracted double blank samples were fortified post-extraction with a standard solution of both analytes and internal standard at the Mid QC level: 150 ng/mL and 50 ng/mL, respectively. The mean analyte peak area from these samples was compared against the mean analyte peak area of non-extracted samples of the same concentration in standard solution. The resulting analyte matrix factor was normalized with the equivalent IS response to deliver an internal standard normalized matrix factor of 0.961 and 1.08 for OH-Vit D_2 and OH-Vit D_3, respectively.

Recovery

Assay recovery was assessed at the mid QC level. While no specific acceptance criteria were applied, recovery using the SOLA HRP 30 mg/2 mL plate was high for all compounds, as shown in Table 11.

Table 11. Recovery data for 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, and 25-hydroxyvitamin D_3-$^{13}C_5$ (IS) at the MQC level

Sample	Mean response	CV%	Recovery
OH-Vit D_2 MQC extract	55874 (n=6)	4.9	100.2
OH-Vit D_2 overspike	55778 (n=17)	3.3	
OH-Vit D_3 MQC extract	396534 (n=6)	3.3	101.2
OH-Vit D_3 MQC overspike	391920 (n=18)	2.0	
OH-Vit D_3-$^{13}C_5$ MQC extract	131133 (n=6)	9.8	90.2
OH-Vit D_3-$^{13}C_5$ MQC over-spike	145382 (n=18)	1.6	

Sample loading capacity

The increased loading capacity of SOLA 30 mg against a smaller bed size is shown in Figures 7 and 8, where 1000 µL plasma was spiked at the MQC level and extracted using 10 mg and 30 mg bed weight cartridges. When scaling up an assay, the larger loading capacity of SOLA 30 mg allows it to retain far more analyte, even in the presence of more matrix interferences associated with the higher loading volume. Higher sample loading volumes can also be used to boost analyte signal (Figure 7), and this benefit could be used to reach lower quantifiable levels if required.

A higher SPE bed weight such as SOLA 30 mg can also prevent analyte breakthrough, which can happen if the loading capacity of the sorbent is insufficient. This is shown in Figure 8, where a higher recovery of analyte is demonstrated with SOLA 30 mg in comparison to a smaller bed size.

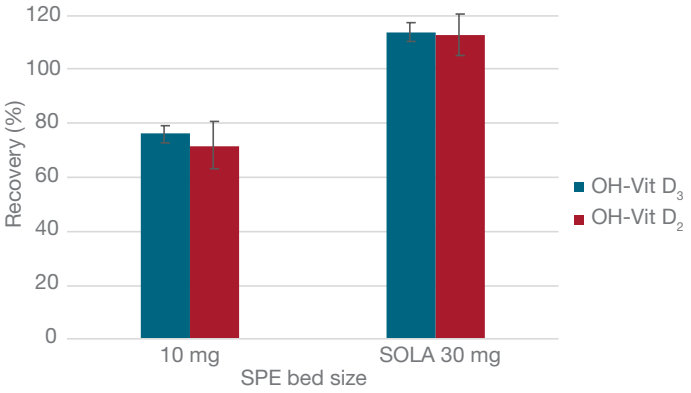


Figure 8. Loading capacity as recovery between 10 mg and 30 mg bed sizes using 1 mL matrix. Human plasma (1 mL) was spiked at the MQC level and loaded onto each phase.

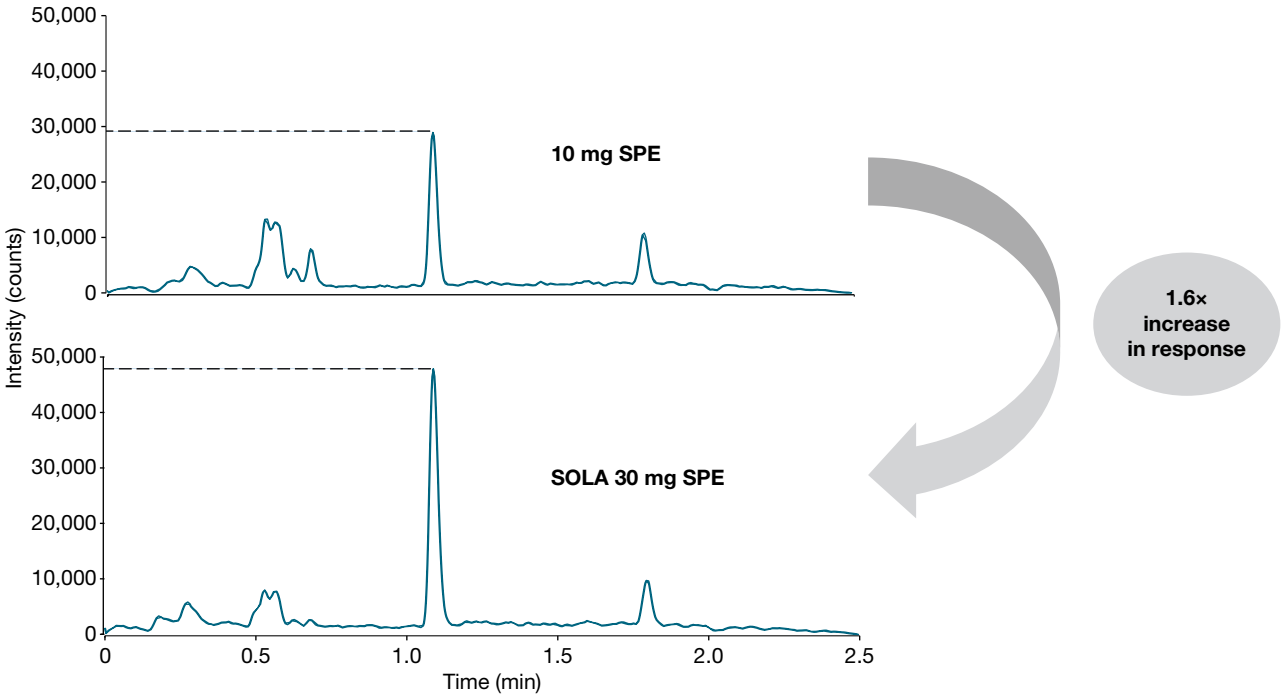


Figure 7. The high loading capacity of SOLA HRP 30 mg also improves signal response when compared to smaller bed weights. The chromatograms show an improved signal response of OH-Vit D_3 for SOLA HRP 30 mg after human plasma (1 mL) was spiked at MQC level and loaded onto each bed weight.

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Conclusion

- This report highlights a robust, reliable method with excellent sensitivity achieved by using SOLA HRP 30 mg SPE plates along with the Vanquish UHPLC system and TSQ Quantiva triple quadrupole mass spectrometer for quantitation of 5.0 and 2.5 ng/mL for 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3, respectively
- Extraction recovery from plasma was high at 100.2% and 100.7% for OH-Vit D_2 and OH-Vit D_3, respectively
- The method showed good accuracy and precision for both compounds with bias (%) and CV% of <8.2% and <9.6%, respectively, owing to the excellent reproducibility of SOLA processing of biological samples
- The use of SOLA HRP 30 mg SPE and the Hypersil GOLD Vanquish UHPLC column allows for simple extraction and rapid quantitation of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 from human plasma
- The calibration model was established in surrogate matrix and successfully applied to human plasma QC samples, from LLOQ to HQC

References

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