# Comparison of Biotage® Extrahera™ vs. Manual Sample Processing Using a Vacuum Manifold

## Extraction of 25-OH Vitamin D from Plasma Using ISOLUTE® SLE+

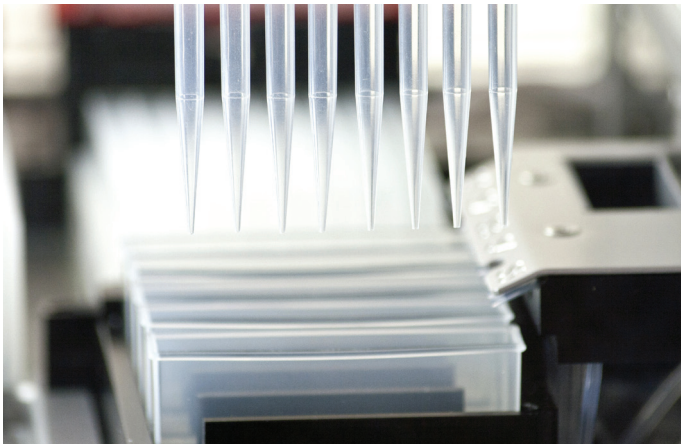
Automated sample preparation using the Biotage® Extrahera™ was compared to an equivalent manual method utilizing a vacuum manifold. Analytes were extracted from pooled stripped plasma using a supported liquid extraction procedure. ISOLUTE® SLE+ 400 µL sample volume plates, part number 820-0400-P01 were used for extraction.

Resulting extracts from both sample preparation methods were subsequently analyzed by LC-MS/MS.


## Procedure

A pooled plasma sample was prepared in a sufficient quantity to run a full 96-well plate for each processing method. This pooled plasma sample was fortified with 25-OH Vitamin D2 and D3 at a concentration of 30 ng/mL respectively. 25-OH Vitamin D3-d6 was added as an internal standard at a concentration of 30 ng/mL.

From this pooled plasma sample 200 µL was transferred to all wells of two 96-well plates.



**All subsequent aspects of sample preparation were performed in duplicate on two separate plates utilizing either Extrahera or manual preparation using a calibrated air-displacement pipette.**

The pooled plasma sample was then pre-treated 1:1 (v/v) with Water:Propan-2-ol 1:1 (v/v) (200 µL).

After pre-wetting the pipette tips via aspirate/dispense cycling and to mix the samples, 300 µL of the pre-treated sample was loaded to each well of the ISOLUTE® SLE+ plates. Flows were initiated using a pulse of positive pressure (Extrahera) or vacuum (manual method).

After leaving for 5 minutes to allow the sample to completely absorb into the plates, elution was performed by the application of 2 x 750 µL of Heptane to the ISOLUTE® SLE+ plates.

The extracts were collected in 2 mL 96-well collection plates under gravity elution, and as a final step to recover all available solvent from the media, by applying a pulse of positive pressure (Extrahera) or vacuum (manual method).

The extracts were evaporated to dryness in a TurboVap® 96 at 37 °C or a SPE Dry at 40 °C and reconstituted in 100 µL of 30:70 (v/v) water/methanol solution.

The plates were mixed on an orbital shaker for 10 minutes.



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## HPLC Conditions

**Instrument:** Waters Aquity UHPLC

**Column:** Restek Pinnacle DB BiPhenyl, 50 mm x 2.1 mm 1.9 µm

**Mobile Phase:** 80:20 (v/v) 2mM ammonium formate with 0.1 % formic acid (aq.)/ Methanol with 0.1 % formic acid at 0.4 mL/min

**Injection Volume:** 15 µL

## Experimental Precautions

The following precautions were performed to minimize differences between the manual and Extrahera extracted plates.

- Both plates were evaporated side by side on the same evaporation instrument (TurboVap® 96).
- During analysis on the LC-MS system samples were injected alternately from the two plates to reduce the effect of any sample stability issues.
- The same batch/bottles of samples, reagents and solvents were used for both methods.

## Mass Spectrometry

**Instrument:** Waters Quattro Premier XE

### MRM Conditions

| Analyte | Transition | RT (min) | Dwell (sec) | Cone (V) | Col Energy (V) |
|---|---|---|---|---|---|
| 25-OH Vitamin D2 | 395.5 to 119.2 | 1.7 | 0.1 | 30 | 26 |
| 25-OH Vitamin D3 | 383.5 to 107.2 | 1.6 | 0.1 | 30 | 25 |
| 25-OH Vitamin D3-d6 | 389.6 to 263.5 | 1.5 | 0.1 | 30 | 16 |

## Results

Average peak area data was calculated for all three compounds to compare any improvements in analyte recovery between Biotage® Extrahera™ and manually processed samples.

Peak area ratio data was also generated for all samples by referencing the analyte vs. 25-OH Vitamin D3-d6. This provides standardized data to allow a comparison of the % RSD of the Extrahera vs. manual data sets.

| | Extrahera Peak Area Ratio Summary | Manual Peak Area Ratio Summary |
|---|---|---|
| Average 25-OH D2 Peak Area | 1697 | 1577 |
| Improvement (%) vs. Manual Method | 7.6 | - |
| | | |
| Average 25-OH D3-d6 Peak Area | 1441 | 1384 |
| Improvement (%) vs. Manual Method | 4.1 | - |
| | | |
| Average Peak Area Ratio | 1.1771 | 1.1421 |
| % RSD of Extrahera Extraction | 7.7 | 7.5 |
| Improvement (%) vs. Manual Method | -3.6 | - |

| | Extrahera Peak Area Ratio Summary | Manual Peak Area Ratio Summary |
|---|---|---|
| Average 25-OH D3 Peak Area | 4758 | 4528 |
| Improvement (%) vs. Manual Method | 5.1 | - |
| | | |
| Average 25-OH D3-d6 Peak Area | 1441 | 1384 |
| Improvement (%) vs. Manual Method | 4.1 | - |
| | | |
| Average Peak Area Ratio | 3.3051 | 3.2813 |
| % RSD of Extrahera Extraction | 5.9 | 6.7 |
| Improvement (%) vs. Manual Method | 11.8 | - |



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Additional experiments were also completed using serum as the sample matrix. The average recovery comparison data is presented in **Figure 1** below.

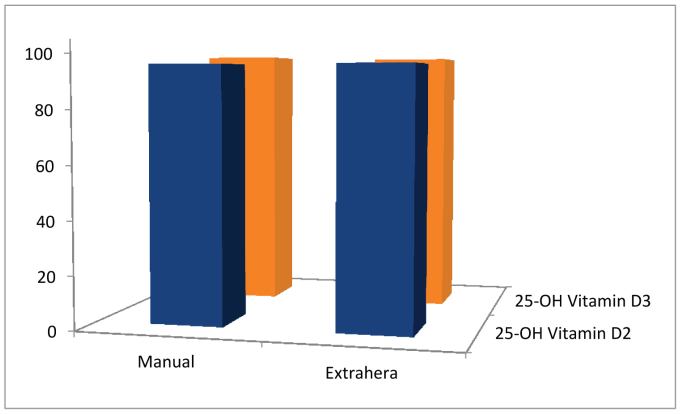



**Figure 1.** Average recovery (n=8) of 25-OH-Vitamin D2 and 25-OH-Vitamin D3 from serum

Calibration series samples prepared manually in Phosphate Buffered Saline with Bovine Serum Albumin (PBS-BSA) and those commercially available from Chromsystems were also processed and extracted using the procedure above with both Biotage® Extrahera™ and manual sample processing methods.

Calibration curves for both manual and Extrahera processed samples over concentration range 1–100 ng/mL for 25-OH Vitamin D2 and D3 in Phosphate Buffered Saline with Bovine Serum Albumin (PBS-BSA) are shown in **Figures 2 to 5**.

Chromsystems calibration curves for both manual and Extrahera processed samples for 25-OH Vitamin D2 (15.8–61.6 ng/mL) and D3 (4.5–66.7 ng/mL) are shown in **Figures 6 to 9**.

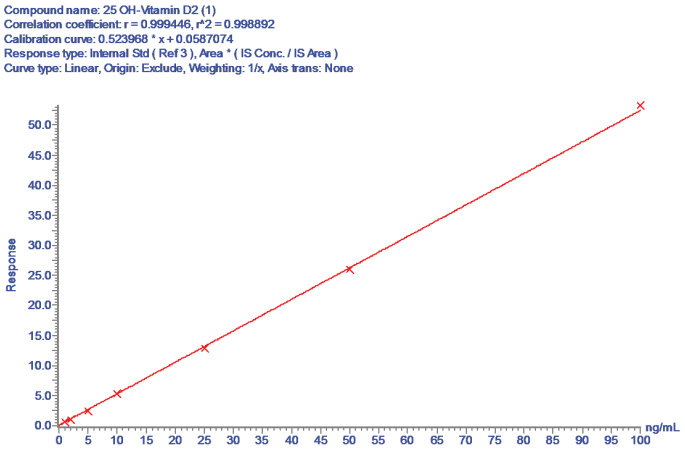



**Figure 2.** 25-OH Vitamin D2 in PBS-BSA – Biotage® Extrahera™

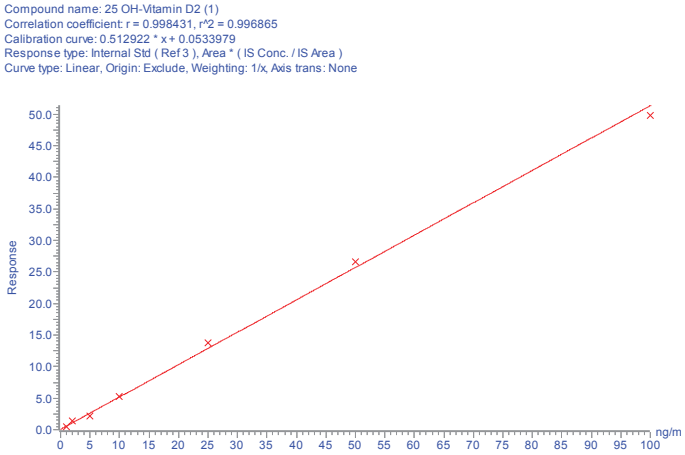



**Figure 3.** 25-OH Vitamin D2 in PBS-BSA – Manual

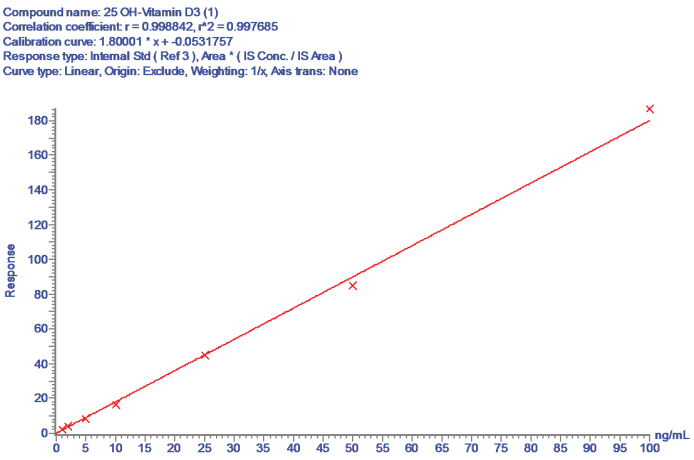



**Figure 4.** 25-OH Vitamin D3 in PBS-BSA – Biotage® Extrahera™

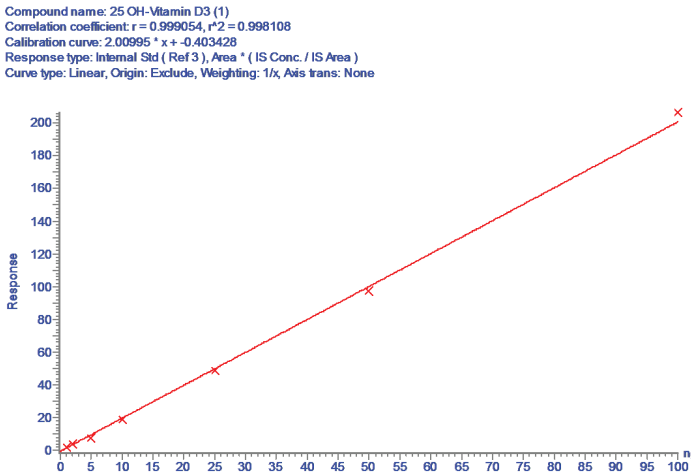



**Figure 5.** 25-OH Vitamin D3 in PBS-BSA - Manual

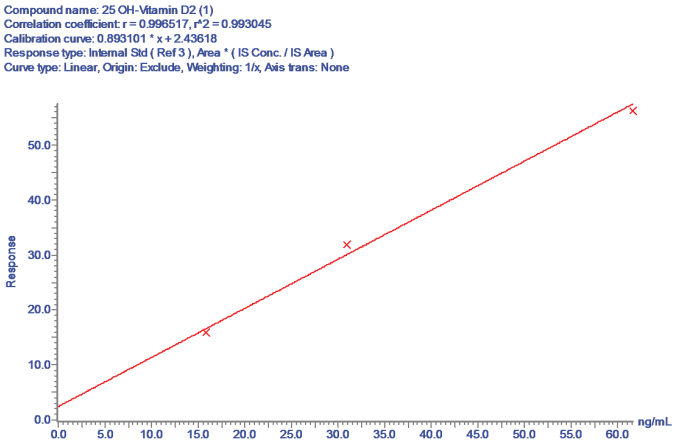



**Figure 6.** 25-OH Vitamin D2 Chromsystems – Biotage® Extrahera™

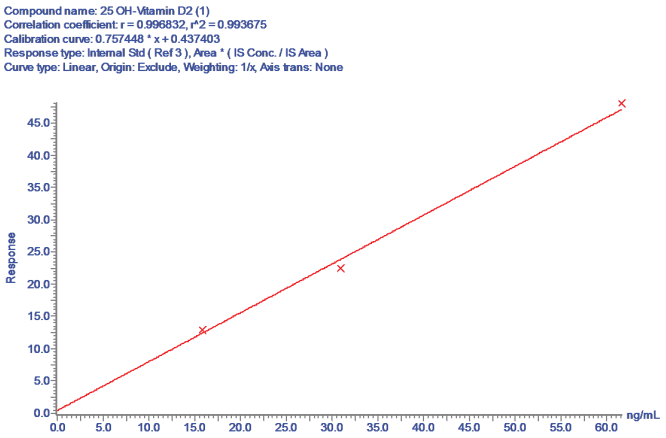



**Figure 7.** 25-OH Vitamin D2 Chromsystems – Manual

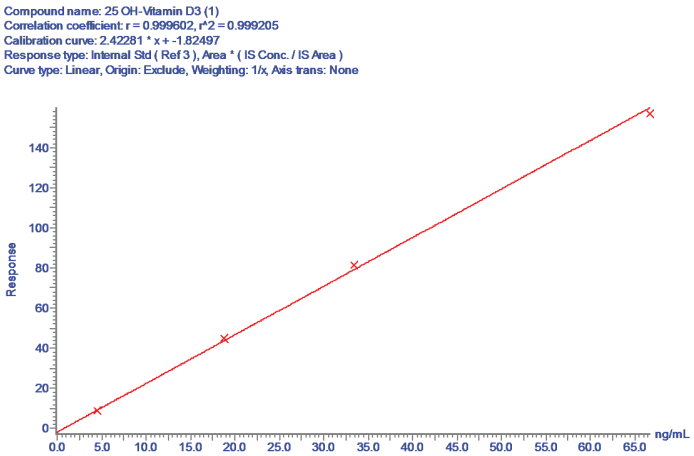



**Figure 8.** 25-OH Vitamin D3 Chromsystems – Biotage® Extrahera™

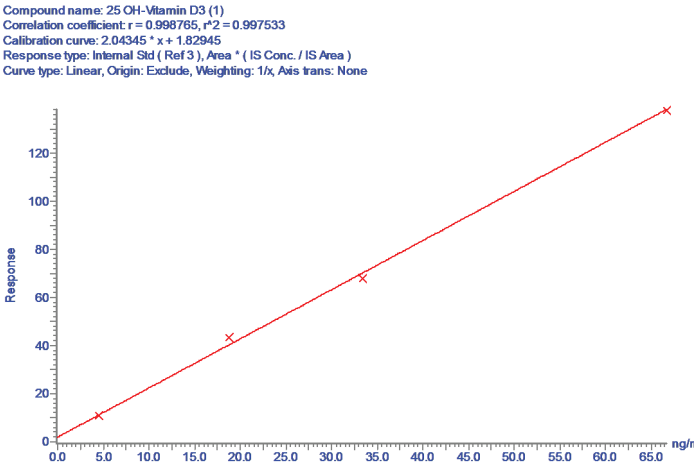



**Figure 9.** 25-OH Vitamin D3 Chromsystems - Manual

## Conclusion

A reduction to the % RSD was measured when using the Biotage® Extrahera™ for 25-OH Vitamin D3, the precision of the D2 data was slightly better when processed manually.

For 25-OH Vitamin D3 the % RSD improved by 11.8 %, for 25-OH Vitamin D2 there was a slight drop in the % RSD of 3.6 % to 7.74 vs. 7.47, this is not considered to be statistically significant.

The results suggest that methods performed on the Extrahera could give higher recoveries due to increases in the absolute average peak areas.

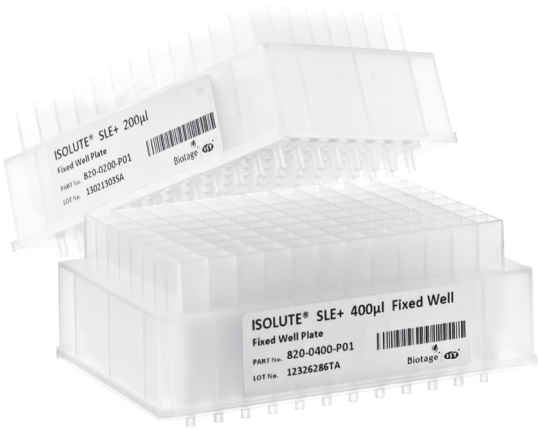
| % RSD | Manual Procedure (n=93) | Extrahera (n=92) |
|---|---|---|
| 25-OH Vitamin D2 | 7.47 | 7.74 |
| 25-OH Vitamin D3 | 6.70 | 5.91 |

| Average Peak Area | Manual Procedure (n=93) | Extrahera (n=962 |
|---|---|---|
| 25-OH Vitamin D2 | 1577 | 1697 |
| 25-OH Vitamin D3 | 4528 | 4758 |
| 25-OH VitaminD3-d6 | 1384 | 1441 |

All analytes returned average peak areas that were higher when sample extraction was performed using the Biotage® Extrahera™, than the manual method with an average peak area increase of 5 %. The greatest improvement was measured with 25-OH Vitamin D2 where the average peak area was increased by 7 %.

The serum samples extracted and analyzed under the same conditions showed near identical recoveries between Extrahera and manual processing, 99 % versus 100 % for 25-OH Vitamin D3 respectively and 97 % for 25-OH Vitamin D2 for both methods.

The calibration series data for samples prepared in PBS-BSA and the Chromsystems calibrators both returned good correlation coefficients with $r^2$ greater than 0.99 for samples prepared using Extrahera and processed manually.

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