



## **Validation of Automated Liquid-Liquid Extraction of 25-hydroxy vitamin D from whole blood or spiked BioCell material (artificial blood) samples using VERSA Workstation, and its determination by LC/MS**

### **I. Introduction:**

It is well known that vitamin D is important to bone health and its deficiency is associated with osteoporosis and rickets, cardiovascular disease, diabetes, and autoimmune disorders to depression, stroke, chronic pain, osteoarthritis, and many forms of cancer<sup>1-3</sup>. Measurement of circulating levels of 25-hydroxy vitamin D is also important in the diagnosis of intestinal mal-absorption and vitamin D deficiency or intoxication in humans. Moreover, new research reports indicate that vitamin D deficiency is widespread in the general population<sup>4</sup>. This has led to exponential growth in clinical diagnosis tests for 25-hydroxyvitamin D also known as [25(OH)D] in blood serum. Biologically inert, vitamin D requires two successive hydroxylation reactions for activation. The first hydroxylation occurs in the liver forming 25(OH)D. A second hydroxylation reaction occurs primarily in the kidney to form the physiologically active 1,25-dihydroxyvitamin D [1,25(OH)2D]. However, circulating 1,25(OH)2D is not an acceptable indicator of vitamin D status because its level is often normal or elevated even when a person is actually vitamin D deficient<sup>5</sup>. Therefore, in clinical diagnosis, serum concentration of 25(OH)D is an accepted indicator of vitamin D status in the patient. However, this form of vitamin D in the blood has a half-life of four to six hours, and is tightly bound to a transport protein in circulation<sup>6</sup>. Therefore, a valid method should be able process the samples faster, and dislodge 25(OH)D from the bound proteins.

Several methods are available to assess vitamin D sufficiency through measurement of serum 25(OH)D<sup>7,8</sup>. Since there are two forms of vitamin D that contribute to the overall status of an individual (one derived primarily from vitamin D produced in the skin, and the other from diet or supplementation), it is important that both forms be measured equally. Antibody (ELISA) or other protein-binding assays are available, but proficiency testing and comparison studies have raised questions about the ability of most existing assays to accurately measure the different forms of 25(OH)D, and by extension, the total vitamin D value that these assays report. Some of the larger laboratories have developed assays using LC-MS/MS tandem mass spectrometry or HPLC. However, sample preparation to meet the requirements of these innovative methods, is a cumbersome process if performed manually. To assist the sample preparation, VERSA Workstation was developed for automation of liquid-liquid extraction (partition chromatography). The validation data is presented in this tech-note.

### **II. Materials & Methods:**

The following materials and protocol were used in the automated process:

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# **Validation of Vitamin D**



- a. Materials:** In the preliminary automation of liquid-liquid extraction of 25-hydroxy vitamin D from whole blood or spiked Biocell material (artificial blood) samples and its determination by LC/MS, 200ul, 200uL of the sample was transferred from the primary sample tube to the extraction vial on the shaker.
- b. Protocol:** The steps of the protocol followed for both the automation, and manual extraction are as follows:
1. Add 150µL of serum to a 2mL extraction vial
  2. Add 10µL IS standard : 250ng/mL d<sub>6</sub>-25(OH)Vit D<sub>3</sub> (80% MeOH/20% IPA)
  3. Shake at 1300 rpm for 30 sec
  4. Add 150µL 0.2M ZnSO<sub>4</sub> aq
  5. Shake at 1300 rpm for 30 sec
  6. Add 300µL MeOH
  7. Shake at 1300 rpm for 30 sec
  8. Add 750µL hexane
  9. Shake at 1300 rpm 30 sec
  10. Remove 650µL (87%) of the top organic layer (hexane) into 2mL Waters maximum recovery vial
  11. Dry down under nitrogen at 50 °C
  12. Reconstitute in 75µL MeOH/water (70/30 v/v)
  13. Shake at 1300 rpm for 10secs
  14. Sample ready for injection for analysis on LC/MS.

### III. Results:

Peak height data reflecting target compound concentration from LC/MS was compared among replicates of manual and automated liquid handling methods. The performance of automated extraction (Figure 1) and manual procedure (Figure 2) were compared for extraction of this analyte from BioCell, and artificial blood material. VERSA performance of the isolation of the biomolecule from human blood sample is presented in Figure 3.

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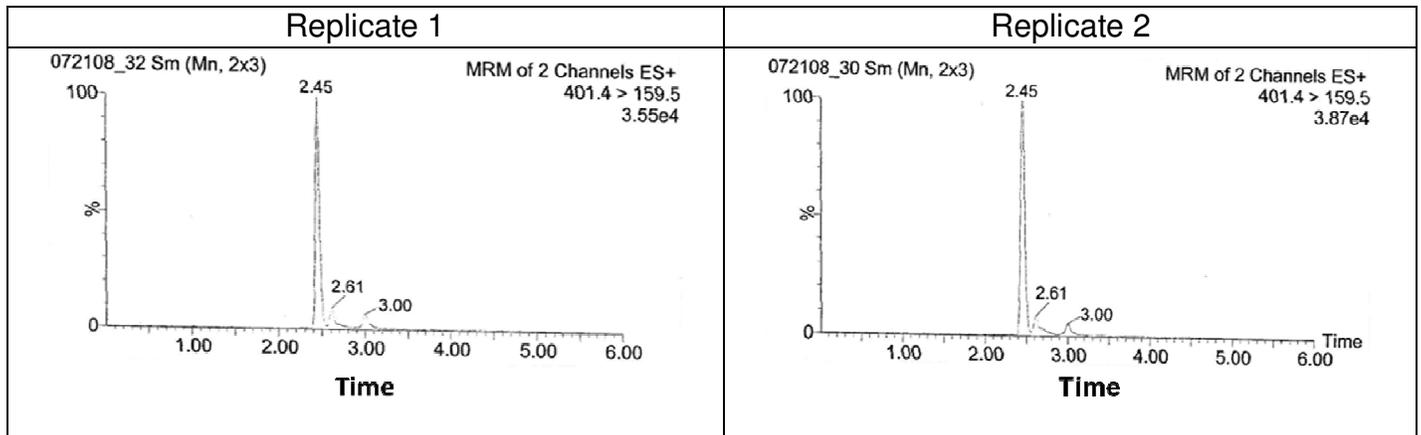
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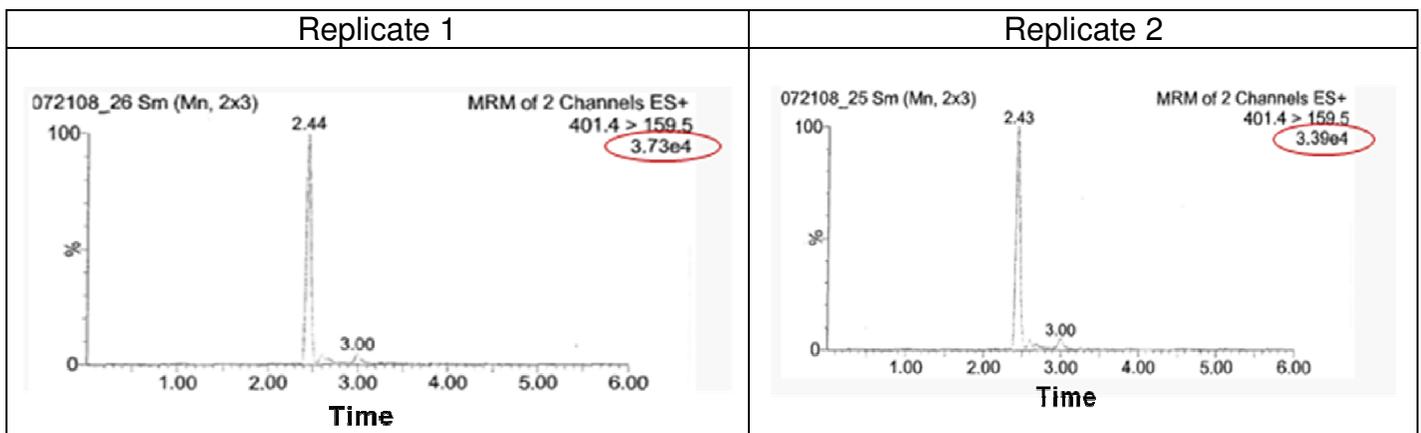
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**Figure 1.** Automated extraction (n=2) of 25-hydroxy vitamin D spiked in BioCell (artificial blood) samples using VERSA Workstation.



**Figure 2.** Manual extraction (n=2) of 25-hydroxy Vitamin D spiked in BioCell.(artificial blood).

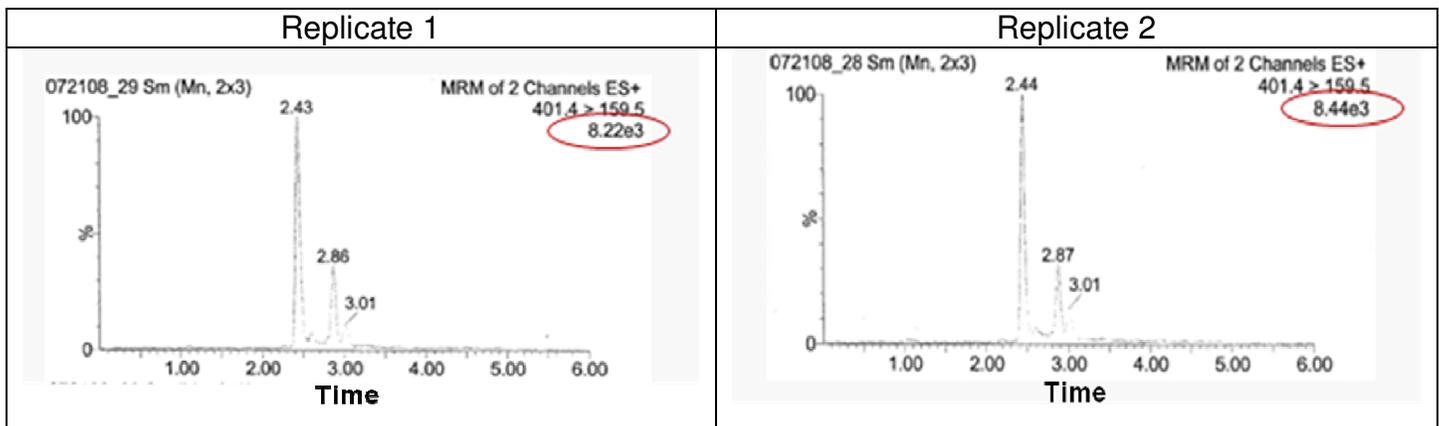
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**Figure 3.** Automated extraction (n=2) of 25-hydroxy Vitamin D from human blood samples using VERSA Workstation.

Reliable reproducibility of peak height data from the prototype unit suggests that biomolecules like 25(OH)D can be effectively extracted using this automation within acceptable values. Moreover, the automated process takes 60 minutes for a set of 96 samples with no detectable cross contamination. The extraction process took approximately 17 minutes less than the manual procedure.

**IV. Conclusion:** The process of liquid-liquid extraction assisted by automation with VERSA Workstation appears to be a promising alternative to the cumbersome separatory funnel for isolation of such compounds.

**V. References:**

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