



## AUTOMATED HIGH THROUGHPUT DISPERSIVE PIPETTE EXTRACTION OF SERUM SAMPLES FOR 25-OH-VITAMIN D3 AND D2 BY LC-MS/MS

VITAMIN D

Vitamin D is an essential prohormone that regulates calcium and phosphate homeostasis. Vitamin D deficiency is a growing problem, manifesting as calcium malabsorption, secondary hyperthyroidism, muscle weakness, and/or osteoporosis/osteomalacia. With an estimated 50% of the global population being deficient in vitamin D, the extent of clinical testing continues to rise, with an increased emphasis on accuracy. Vitamin D assessment is performed by quantitation of 25-hydroxyvitamin D in serum and/or plasma. 25-hydroxyvitamin D has two forms: 25-hydroxy-vitamin D2 (25OHD2) from vitamin D2, a plant-based supplement and 25-hydroxy-vitamin D3 (25OHD3) from vitamin D3, which is produced in the skin as a result of photochemical reaction.

Both 25OHD2 and D3 can be accurately and efficiently analyzed using LC-MS/MS. However, removal of the sample matrix is required to ensure that interferences do not adversely affect quantitative analysis. Therefore, most applications require protein precipitation followed by solid-phase extraction (SPE) prior to chromatographic analysis. In the current study, a patent-pending automated Tip-On-Tip filtration method is coupled to dispersive pipette extraction (DPX) on an automated liquid handling system (ALH) for rapid and efficient sample preparation.

Our Tip-On-Tip methodology automates the traditional vortexing and centrifugation steps used to protein precipitate serum samples. Efficiency is enhanced as Tip-On-Tip filtration may be performed in a 96-sample format. Following filtration, DPX tips containing weak anion exchange sorbent with salts (WAX-S) are used to rapidly remove matrix interferences. This method processes 96 samples in less than ten minutes.

### WORKFLOW

Well plates containing 100  $\mu$ L of serum and internal standard are loaded onto an ALH platform. Wide bore tips (1 mL) are used to transfer 300  $\mu$ L of acetonitrile to the serum followed by thorough mixing to allow for extensive protein precipitation. The

1	<i>Load platform</i>
2	<i>Protein Crash Add ACN and Mix</i>
3	<i>Filter Tip-on-Tip</i>
4	<i>Aspirate &amp; Dispense Filtrate 3x DPX WAX-S Tips</i>
5	<i>Transfer Upper Layer</i>
6	<i>Inject</i>

wide bore tips aspirate the precipitated solution, then pressure fit onto a 300  $\mu$ L tip with two filters of different porosities (Refer to DPX Tip-On-Tip Technical Note, DPXTN001). The filtrate is aspirated and dispensed three times using DPX WAX-S tips to produce separation between the aqueous and acetonitrile phases. The hydrophobic analytes remain in the clean acetonitrile layer for injection by LC-APCI-MS/MS analysis.

### RESULTS AND DISCUSSION

This method was evaluated for linearity, precision, accuracy, and limits of detection and quantitation. Calibration plots spanning 5–100 ng/mL for 25OHD2 and D3 resulted in correlation coefficients greater than 0.99. Precision and accuracy values were determined using serum controls at 10, 30/26, and 73 ng/mL (Utak Laboratories, Valencia, CA). Accuracy and precision were monitored in triplicate over three days. Accuracy values ranged from 93.2–97.7% for 25OHD3 and 91.8–108.4% for 25OHD2. 25OHD3 and

25OHD2 total imprecision (%RSD) ranged from 7.4–7.8% and 9.6–14.0%, respectively. Limits of detection (LOD) were calculated as  $3.3\sigma/m$ , where  $\sigma$  is the standard deviation of the lowest non-zero calibrator (5 ng/mL) and  $m$  is the slope of the calibration line. Limit of quantitation (LOQ) was  $10\sigma/m$ . LODs and LOQs were 5.5 and 16.4 ng/mL and 3.4 and 10.3 ng/mL for 25OHD2 and 25OHD3, respectively.

According to Bickle, et al., approximately 88% of 25-hydroxyvitamin D is protein bound, primarily to vitamin D binding protein and to a lesser extent, albumin. In order to release the 25-hydroxyvitamin D, thorough protein precipitation is necessary for accurate analysis. Protein precipitation practices most commonly employ acetonitrile crash with vortex mixing, followed by centrifugation. To eliminate this labor intensive approach, we implemented an automated wide bore mixing step followed by Tip-On-Tip filtration. The two protein precipitation steps were compared by preparing patient samples with each method, followed by DPX and LC-MS/MS analysis. The comparison produced an average percent difference of 2%. The similarity in results confirms the viability of our alternative Tip-On-Tip protein precipitation procedure.

It should be emphasized that LOD and LOQ are highly dependent on the sensitivity of the LC/MS instrumentation and method. If sensitivity needs to be improved to lower LODs and LOQs, solvent evaporation can be employed to concentrate the extracts. Alternatively, a larger volume of sample solution can also be extracted to provide lower LODs/LOQs.

## CONCLUSION

The method described herein provides the necessary speed, sensitivity, and reproducibility for a reliable sample preparation method in a high throughput setting. Human error is minimized by automating the entire process. Clean extracts minimize instrument downtime. Reduced turnaround time and increased throughput are essential to minimize costs and increase efficiency.

Table 1. The limit of detection (LOD) in ng/mL, limit of quantitation (LOQ) in ng/mL, and the coefficient of determination ( $R^2$ ) for 25-hydroxyvitamin D2 (25OHD2) and 25-hydroxyvitamin D3 (25OHD3)

Analyte	Equation	$R^2$	LOD (ng/mL)	LOQ (ng/mL)
Vitamin D2	$y = 1.0095x - 0.5698$	0.9973	5.5	16.4
Vitamin D3	$y = 0.9929x + 0.4503$	0.9939	3.4	10.3

Table 2. Accuracy and precision parameters calculated according to Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines.

25OHD2 (ng/mL)			
Precision (%RSD)	10	26	73
Intraday	6.5	13.6	13.8
Interday	7.0	3.1	0
Total	9.6	14.0	13.8
Accuracy	108.4%	91.8%	99.1%
25OHD3 (ng/mL)			
Precision (%RSD)	10	30	73
Intraday	4.7	5.9	5.1
Interday	5.7	5.1	6.0
Total	7.4	7.8	7.8
Accuracy	97.7%	93.2%	94.8%

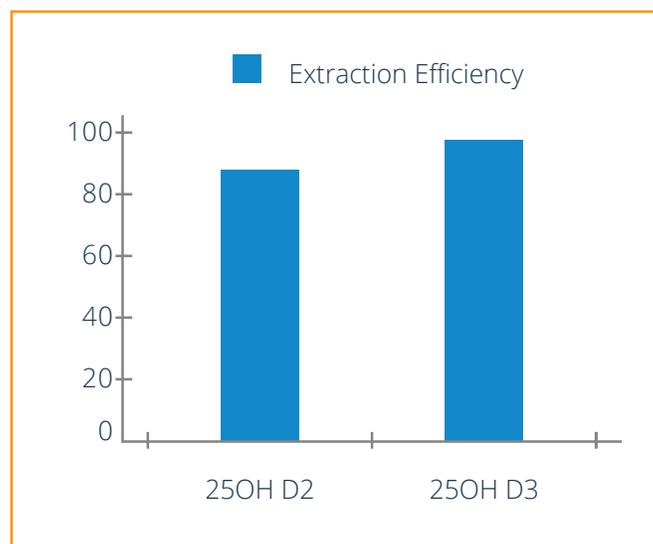


Figure 1. The average extraction efficiencies for 25-hydroxyvitamin D2 (25OHD2) and 25-hydroxyvitamin D3 (25OHD3).