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

VITAMIN D

APPLICATION NOTE

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 (ToT) filtration technology is coupled to dispersive pipette extraction technology on an automated liquid handling system (ALH) for rapid and efficient sample preparation.

DPX ToT Filtration technology automates the traditional vortexing and centrifugation steps used to protein precipitate serum samples. Efficiency is enhanced as ToT Filtration may be performed in a 96-sample format. Following filtration, dSPE+S tips containing weak anion exchange (WAX) sorbent and the salt necessary for SALLE are used to rapidly remove matrix interferences. This method processes 96 samples in less than ten minutes.

WORKFLOW

1	***Load platform***
2	***Protein Crash*** *Add ACN and Mix*
3	***Filter*** *Tip-on-Tip*
4	***Aspirate & Dispense Filtrate 3x*** *Tips with WAX sorbent + Salt (WAX-S Tips)*
5	***Transfer*** *Upper Layer*
6	***Inject***

Well plates containing 100 µL of serum and internal standard are loaded onto an ALH platform. Wide bore tips (1 mL) are used to transfer 300 µL of acetonitrile to the serum followed by thorough mixing to allow for extensive protein precipitation. The wide bore tips aspirate the precipitated solution, then pressure fit onto a 300 µL tip with two filters of different porosities (Refer to DPX ToT Technical Note, DPXTN001). The filtrate is aspirated and dispensed three times using 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 3.3σ/m, where σ 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 10σ/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 ToT Filtration. The two protein precipitation steps were compared by preparing patient samples with each method, followed by dispersive pipette extraction and LC-MS/MS analysis. The comparison produced an average percent difference of 2%. The similarity in results confirms the viability of our alternative ToT protein precipitation method.

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%

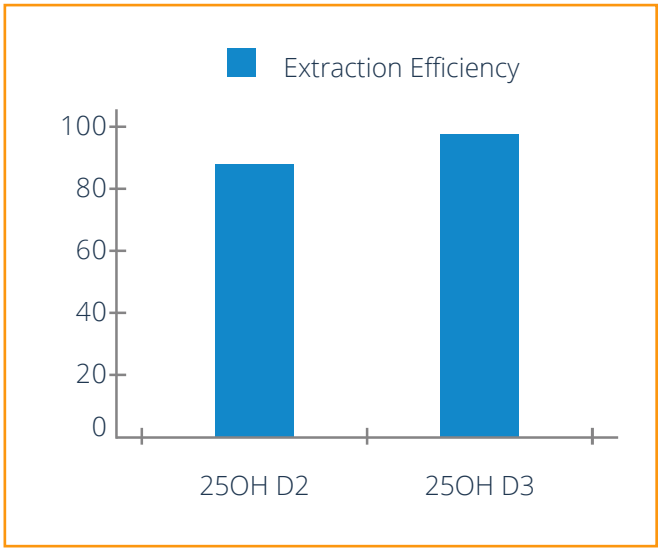

Figure 1. The average extraction efficiencies for 25-hydroxyvitamin D2 (25OHD2) and 25-hydroxyvitamin D3 (25OHD3).