Fully Automated Sample Preparation for the Determination of Vitamin D on the Biomek® i7 Hybrid Using the Impact™ Protein Precipitation Plate Combined with Positive Pressure Solid Phase Extraction

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Overview

Quantitation of vitamin D_2 and D_3 (25(OH)D_2 and 25(OH)D_3) is becoming increasingly important among researchers to understand the effects of varying amounts of vitamins. The manual sample preparation for LC-MS based vitamin D analysis is time consuming and prone to human errors. Consequently, automation of the complex sample preparation workflow is important to carry out vitamin D analysis in a high-throughput manner.

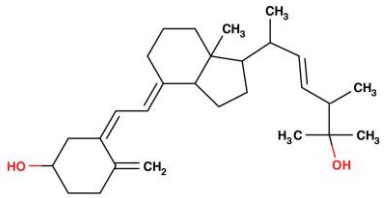
The protein precipitation and the separation of the metabolites from the vitamin D binding proteins is usually carried out by adding acetonitrile, methanol, as well as mixtures of acetonitrile and methanol or 2-propanol. The samples are then vortexed for 1 min, followed by centrifugation. Thereafter, supernatant is removed, and the solvent is evaporated in a gentle stream of nitrogen. Subsequent redissolution of the samples in a suitable solvent is required prior to analysis. In this application note, we aimed to develop an automated method for determining the vitamin D metabolites 25(OH)D_2 and 25(OH)D_3 without centrifugation. We used the Biomek i7 hybrid workstation with integrated Positive Pressure Extractor (Amplius GmbH, D). To avoid centrifugation, we used the Impact protein precipitation plate kit (Part No. CE0-8201, Phenomenex, Torrance, US) in the workflow. Once the samples and the reagents are added to the Impact protein precipitation plate, the protein precipitation takes place in the plate. After mixing, the sample is moved through the filter by applying positive pressure or vacuum, separating vitamin D metabolites from the protein aggregates. The resulting filtrate can be further cleaned-up using solid phase extraction (SPE) Strata® C8 Cartridges with a sorbent mass of 30 mg/1 mL (Part No. 8B-S005-TAK, Phenomenex, Torrance, US).

We automated the complete workflow by integrating the Positive Pressure Extractor and the Impact protein precipitation plate. This automated workflow is less costly as it eliminates the centrifugation steps. We analyzed the processed samples using LC-MS and generated calibration curves to identify repeatability of the automated sample preparation (**Figure 2**). The low CV values (2 % -6 %) indicated high repeatability of the automated method. The results were comparable with manual sample preparation.

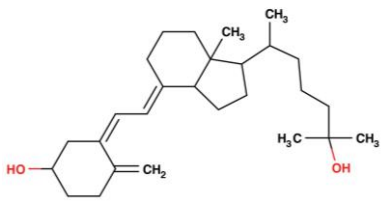
Sample Preparation

Pre-treatment:	Transfer 500 µL Methanol/Water (60:40, v/v) followed by 200 µL Zinc Sulfate (0.2 M)
Add:	40 µL internal standard followed by 100 µL serum sample
Shake:	Plate for 3 min to enable suitable protein precipitation
Incubate:	Plate for 3 min at room temperature
Apply:	Positive pressure for 10 min (1500 bar) using the PP ALP
Condition:	Strata C8 cartridge with 250 µL of Methanol
Equilibrate:	Strata C8 cartridge with 500 µL Water
Load:	500 µL sample onto the Strata C8 Cartridge, apply pressure
Wash:	Strata C8 cartridge with 1 mL Methanol/Water (60:40, v/v)
Dry:	Strata C8 cartridge for 10 min
Elute:	Vitamin D components twice with 100 µL Methanol
Measure:	Samples using LC-MS or LC-MS/MS

Figure 1. Structures of 25(OH)D_2 and 25(OH)D_3



25-hydroxyvitamin-D2



25-hydroxyvitamin-D3

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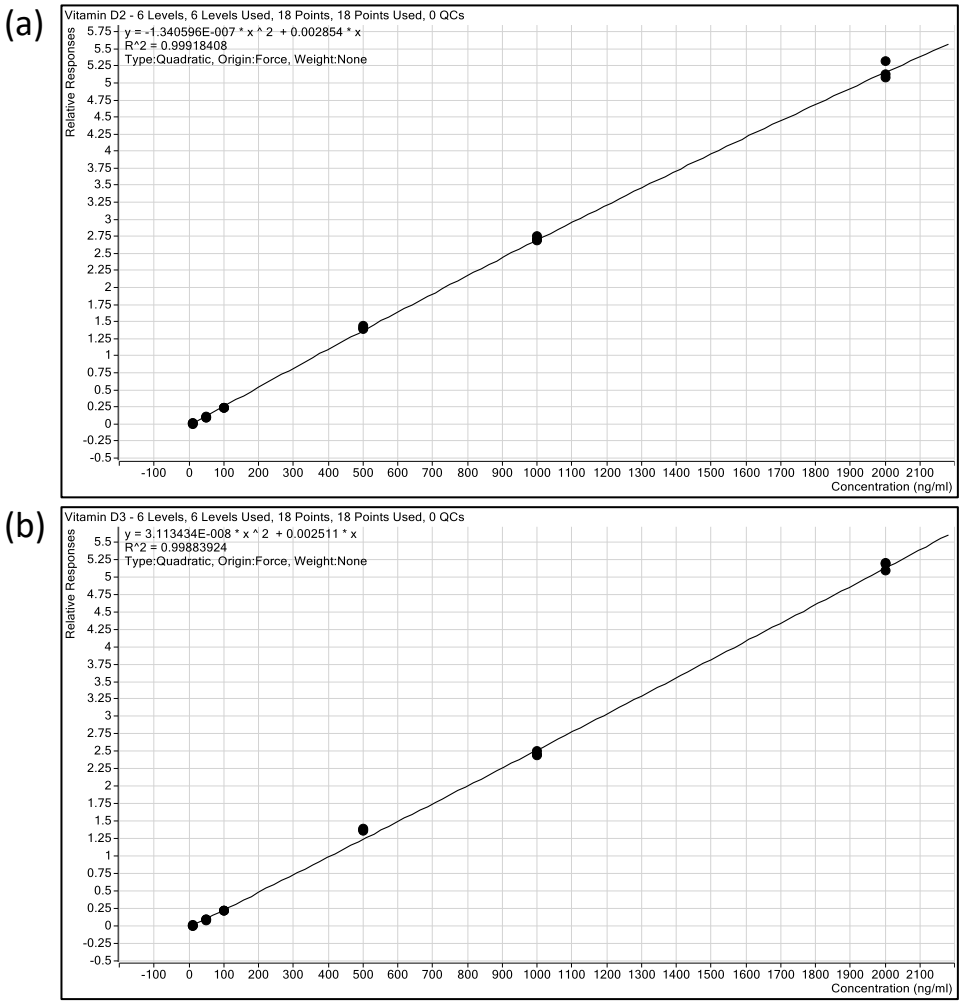
Figure 2. Calibration Curves for (a) 25(OH)D_2 and (b) 25(OH)D_3



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