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

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Abstract

Vitamin D is a steroid derivative that exists in several forms including Vitamin D_2 (Ergocalciferol) and Vitamin D_3 (Cholecalciferol). Determining the level of vitamin D in blood serum provide crucial information about metabolism. The main methodology for vitamin D determination is LC-MS based. The sample preparation for LC-MS is time consuming and prone to human-error. In this application note we automate the sample preparation for LC-MS based vitamin D analysis on the Biomek i7 hybrid. The method uses protein precipitation plates (Phenomenex, Torrance, US) and sample cleanup by solid phase extraction.

Introduction

Vitamin D is a crucial component of bone and mineral metabolism. Therefore, quantitation of vitamin D_2 and D_3 (25(OH)D_2 and 25(OH)D_3) in blood plasma and blood serum is becoming increasingly important among researchers to understand the serious health effects of underdosing and overdosing 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[1].

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[1]. Some authors reported that adding zinc sulfate prior to the precipitationstep results in a better dissolution of the vitamin D metabolites from the vitamin-binding proteins[2]. Other authors described the use of sodium hydroxide to break the protein bonds before the actual precipitation with acetonitrile/methanol solution[3]. We recently reported automation of a centrifuge-based LC-MS sample preparation method[4]. However, this workflow requires integration of a centrifuge. This increases the cost of the overall system and requires additional space on the automation deck. Therefore, in this application note we aimed to develop an automated method for determining the vitamin D metabolites 25(OH)D_3 and 25(OH)D_2 from blood serum, without a centrifugation. We use Biomek i7 hybrid workstation with integrated Positive Pressure Extractor (amplius GmbH, D).

To avoid centrifugation, we used the Impact protein precipitation plate (Phenomenex, Torrance, US) in the workflow (See Figure 1 and Figure 2)[5]. 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[6].

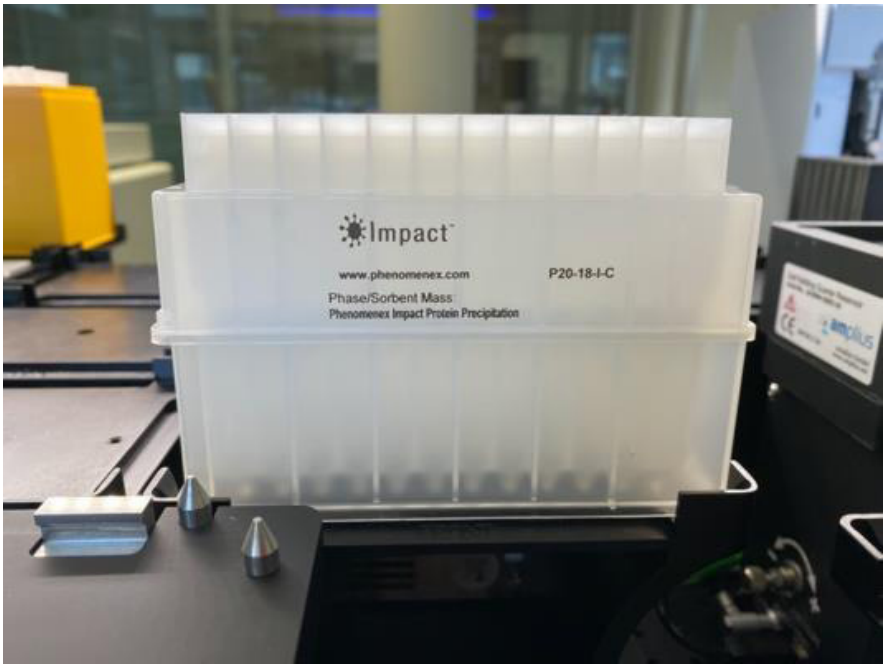

Figure 1. Impact protein precipitation plate (Phenomenex, Torrance, US) with a deep well collecting plate on the Biomek i7 hybrid deck

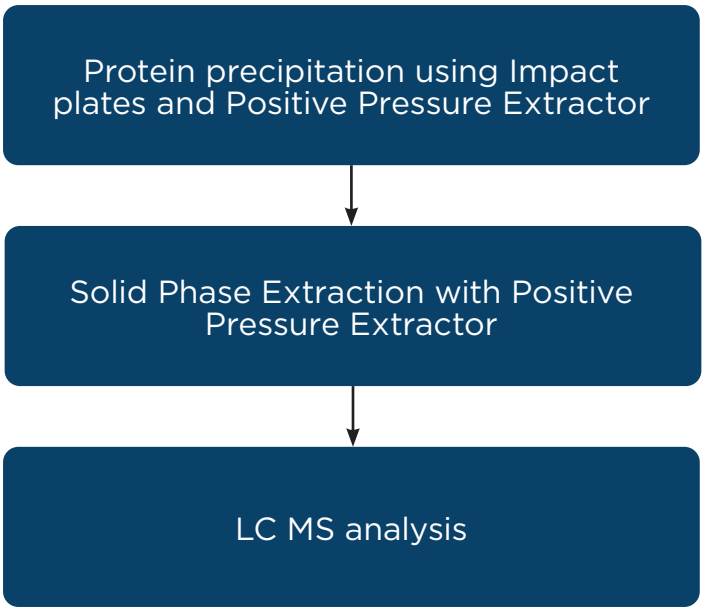

Figure 2. The sample preparation workflow. All steps except LC-MS analysis were done on a Biomek i7 hybrid workstation with integrated devices.

Materials and Methods

A previously published protocol of Vitamin D metabolite determination was automated on a Biomek i7 hybrid workstation (Figures 3 & 4, Table 1)[2]. Pig blood serum was used for the method development and evaluation (Source: Leibniz Institute for Farm Animal Biology, Dummerstorf, DE). The reagents, consumables and instruments used are listed in the tables 2-4. The vitamin D detection protocol was automated on the Biomek deck layout shown in Figure 3. The integrated Positive Pressure Extractor (amplius GmbH, D) was used for the solid phase extraction. The deck was optimized for the processing of up to 96 samples. The method required large volumes of liquids (e.g. 96 mL methanol/water (60/40, v/v)) for protein precipitation and column conditioning. Therefore, a quarter self-refilling reservoir (amplius GmbH, D) was integrated to facilitate solvent delivery.

The specific steps of the automated protocol are listed in table 1. The samples were provided in 1.5 mL Eppendorf safe-lock vials (Eppendorf, Hamburg, DE) and they were placed on the Biomek using a special adapter (CELISCA, Rostock, DE) (see Figure 5a). Serum samples with a concentration of 1 µg/mL of 25(OH)D_2 and 25(OH)D_3 were used for the method validation. Four Impact protein precipitation plates are used for the processing of 96 samples. To mix the sample and the precipitation solvents, each Impact plate and a deep well collecting plate was placed on a shaking Peltier (INHECO Industrial Heating & Cooling GmbH, Martinsried, DE). After mixing and 3 minute incubation, the Impact plates were transported to integrated the Positive Pressure Extractor (amplius GmbH, D) for solid phase extraction and cleanup (see Figure 5b). Standard 2 mL vials were used in final elution. After extraction, the samples were analyzed by injecting 10 µL of the sample into an LC/TOF-MS system (Agilent Technologies, Santa Clara, US) with a flowrate of 0.5 mL / min. The system was calibrated according to the internal standard method in the range of 0.01 to 2 ppm 25(OH)D_2 and 25(OH)D_3.

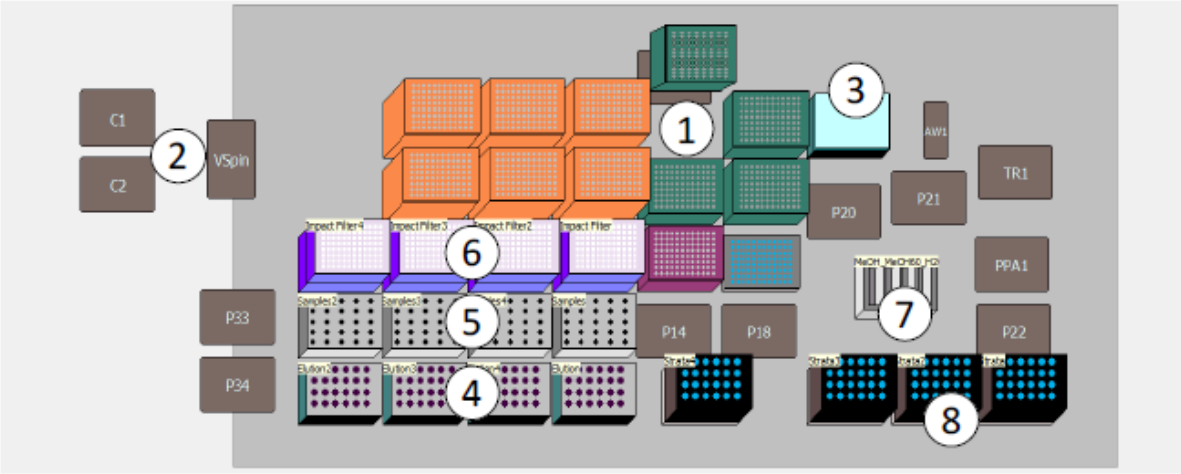

Figure 3. Deck layout for vitamin D determination using Impact protein precipitation plates - (1) Tip boxes 1070 µL, 230 µL and 930 µL, (2) Centrifuge, (3) Internal Standard (ISTD), (4) Adapter made of aluminum with elution vials, (5) Samples, (6) Impact protein precipitation plates with deep-well collecting plates, (7) Quarter self-refilling reservoir, (8) Adapter for SPE cartridges. Note: The on-deck centrifuge is not used in the current workflow.

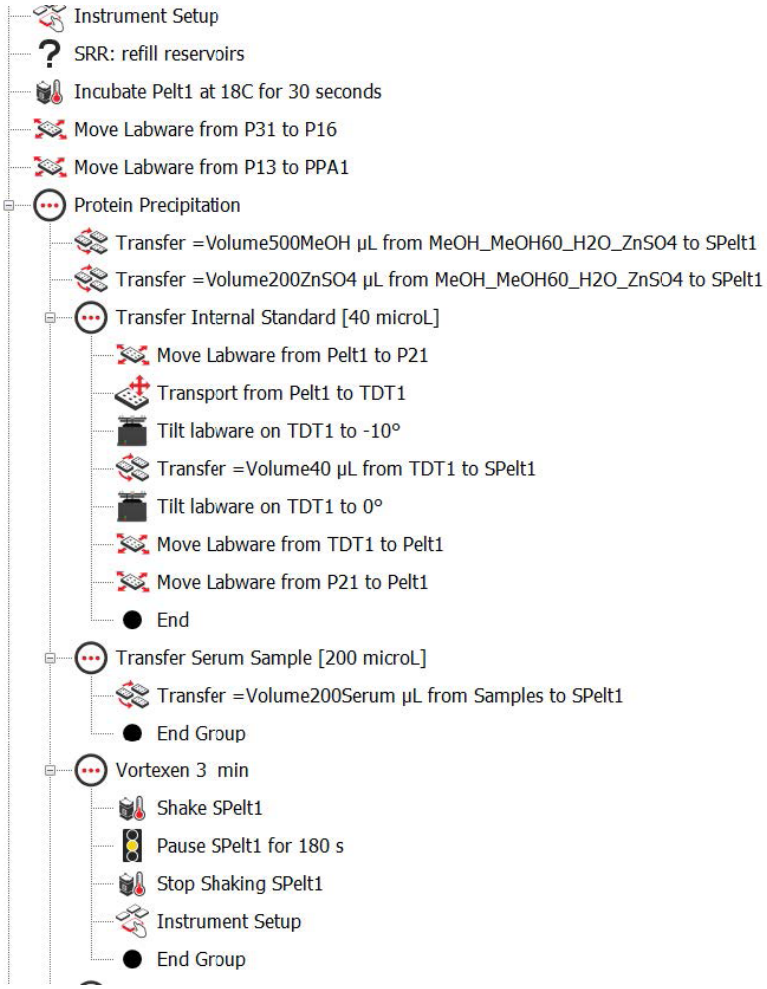

Figure 4. Biomek i7 hybrid method for vitamin D determination – protein precipitation using Impact plates.

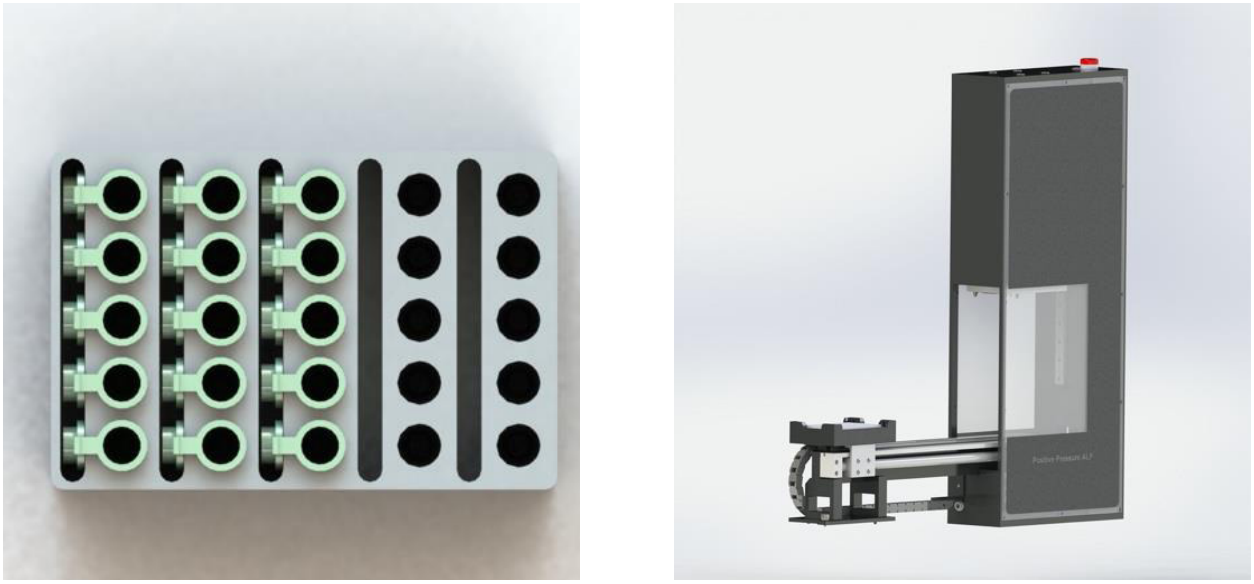

Figure 5. (a) Storage for samples vials (CELISCA, Rostock, DE), (b) Positive Pressure Extractor (amplius GmbH, Rostock, DE)

Step Description	Manufacturer
Step 1	Transfer 500 µL MeOH/water (60/40, v/v) to Impact plate for all samples
Step 2	Transfer 200 µL ZnSO4 (0.2 M) to Impact plate for all samples
Step 3	Transfer 40 µL internal standard (ISTD) to Impact plate for all samples
Step 4	Transfer 100 µL serum sample to Impact plate for all samples
Step 5	Shake the plate for 3 min to enable suitable protein precipitation
Step 6	Incubation of Impact plate at room temperature for 3 min
Step 7	Transfer Impact plate to PP ALP
Step 8	Apply positive pressure for 10 min (1500 mbar)
Step 9	Condition the Strata® C8 cartridge with 250 µL MeOH
Step 10	Equilibrate the Strata® C8 cartridge with 500 µL water
Step 11	Load 500 µL sample from step 8 onto the Strata® C8 cartridge, apply pressure
Step 12	Wash the Strata® C8 cartridge with 1 mL MeOH/water (60/40, v/v)
Step 13	Dry the Strata® C8 cartridge 10 min
Step 14	Elute the vitamin D components twice with 100 µL MeOH
Step 15	Measurement of samples using LC/MS or LC/MS/MS

Table 1. Automated sample processing protocol for the vitamin D determination using Impact protein precipitation plates and positive pressure solid phase extraction

Results

The calibration curves are shown in Figure 6. The recovery rates determined were in the range between 71.26% and 86.75% for 25(OH)D_2 and between 79.57% - 88.74% for 25(OH)D_3. The coefficient of variation (CV) of 4.7% for 25(OH)D_2 and 3.57% for 25(OH)D_3 determined with 10 samples indicates high repeatability. For the determination of the within-laboratory precision the experiment was repeated on 5 days with 10 samples resulting in CV values between 4.09% and 5.78%. for 25(OH)D_2 as well as 2.35% – 5% for 25(OH)D_3. The limits of detection (LOD) were determined at 4.78 ng/mL (25(OH)D_2) and 7.95 ng/mL (25(OH)D_3). The limits of quantification (LOQ) were found to be 11 ng/mL and 20.03 ng/mL respectively. Further improvement of the detection limits is possible using an LC/MS/MS system. These results obtained are comparable to those of classical methods[5,6].

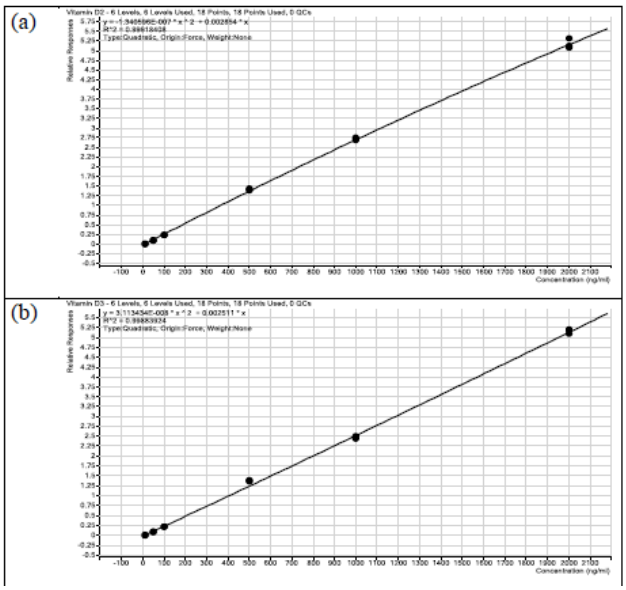

Figure 6. Calibration curves for (a) 25(OH)D_2 and (b) 25(OH)D_3

Summary

In this application note we successfully automated sample preparation for vitamin D detection on a Biomek i7 hybrid workstation. We automated the complete workflow by integrating the Positive Pressure Extractor (amplius GmbH, Rostock, DE). This automated workflow is less costly as it eliminates the centrifugation steps. We analyzed the processed samples using LCMS and generated calibration curves to identify repeatability of the automated sample preparation. The low CV values (2% -6%) indicated high repeatability of the automated method. The results were comparable with manual sample preparation.

References

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Materials

Equipment	Manufacturer
Biomek i7 Workstation	Beckman Coulter Life Sciences, Indianapolis, USA
Shaking Peltier for Biomek® 4000, FXp, NXp	INHECO Industrial Heating & Cooling GmbH, Martinsried, Germany
Static Peltier Biomek® 4000, FXp, NXp	INHECO Industrial Heating & Cooling GmbH, Martinsried, Germany
Positive Pressure Extractor	amplius GmbH, Rostock, Germany
3D Tilt ALP	amplius GmbH, Rostock, Germany
Self Refilling Quarter Reservoir	amplius GmbH, Rostock, Germany

Table 2. Instruments used

Reagents	Manufacturer	Part Number
D6-25(OH)D3 (50 ppm)	Sigma Aldrich, St. Louis, USA	H-074
25(OH)D2 (50 ppm)	Sigma Aldrich, St. Louis, USA	H-073
25(OH)D3 (100 ppm)	Sigma Aldrich, St. Louis, USA	H-083
MeOH	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	7342.1
ZnSO4 0,2 M	Sigma Aldrich, St. Louis, USA	83265
H20 Millipore	Sigma Aldrich, St. Louis, USA	W4502-10L

Table 3. Reagents used

Consumables	Number	Manufacturer	Part number
Biomek i-series tips 90 µL	96	Beckman Coulter Life Sciences, Indianapolis, USA	B85881
Biomek i-series tip 230 µL	384	Beckman Coulter Life Sciences, Indianapolis, USA	B85903
Biomek i-series tips 1070 µL	512	Beckman Coulter Life Sciences, Indianapolis, USA	B85971
Impact Protein Precipitation 2 mL 96-Square Well Filter Plate + 96-Deep Well Plate	1	Phenomenex, Torrance, USA	CE0-8201
GC Vials 300 µL	96	Agilent Technologies, Santa Clara, USA	9301-0978
Eppendorf Vials 1,5 mL (Safe Lock)	96	Eppendorf AG, Hamburg, Germany	EP0030121880
SPE Strata C8 Cartridges	96	Phenomenex, Torrance, USA	8B-S005-TAK
Septum Lid	96	Agilent Technologies, Santa Clara, USA	5182-0731

Table 4. Consumables used

Biomek i-Series Automated Workstations are not intended or validated for use in the diagnosis of disease or other conditions.

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