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Fully Automated Sample Preparation for the Determination of Vitamin D on Biomek i7 Hybrid Workstation using Advanced Sample Cleanup with Phree Phospholipid Removal Cartridges

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Abstract

Vitamin D is a crucial component of metabolism. Vitamin D deficiency leads to several disease conditions such as rickets and osteomalacia. Therefore, researchers are interested in identification of the role of vitamin D in treating and prevention of metabolic abnormalities. To do so, they often quantify the level of vitamin D in serum using LC-MS based methods. The sample preparation for these analytical methods are labor-heavy and prone to human error. In this application note we evaluate the feasibility of automating the sample prep for vitamin D determination on the Biomek i7 hybrid workstation. We used Phree phospholipid removal cartridges for the protein precipitation prior the sample cleanup by solid phase extraction.

Introduction

The quantitative determination of 25(OH)D_2 and 25(OH)D_3 in blood plasma and blood serum is becoming increasingly important to understand the effects of overdosing and underdosing of vitamins. Due to the complicated matrix, the sample preparation for vitamin D analysis is extensive, including protein precipitation and extraction steps. The protein precipitation and the separation of the metabolites from the vitamin D-binding proteins is usually carried out by adding acetonitrile, methanol, mixtures of acetonitrile and methanol or 2-propanol followed by a centrifugation step [1]. In addition to the proteins, there are other substances in biological matrices, in particular phospholipids (components of the cell membrane), whose highly ionic nature interferes with the ionization in the mass spectrometer [2]. In addition, phospholipids can cause accelerated column breakdown through enrichment in the mobile phase, which leads to changes in the separation properties and an increase in the baseline noise [3]. Therefore, the removal of the phospholipids is an important step in the sample preparation. The main methods of removing phospholipids are solid phase extraction (SPE) with strong cation exchangers, SPE and liquid-liquid extraction (LLE). However, performing these methods increases the time it takes to complete the analytical measurement process.

To reduce sample preparation time, different manufacturers have developed plates that combine the advantages of protein precipitation, removal of phospholipids and extraction, so that only a single plate or cartridge can be used [4]. In this application note we automated a sample preparation method for vitamin D determination that uses Phree phospholipid removal cartridges and solid phase extraction.

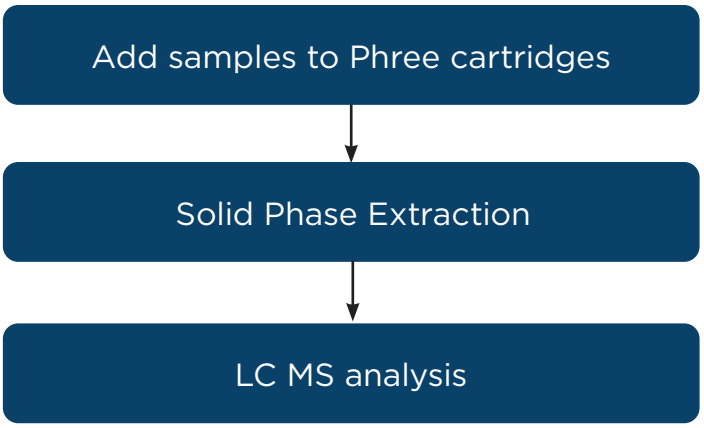

Figure 1. The sample preparation workflow. All steps except LC-MS analysis was done on a Biomek i7 hybrid workstation with integrated devices.

Materials and Methods

The automated method was based on a published vendor protocol on the usage of Phree Phospholipid Removal Cartridges (Phenomenex, Torrance, US). Pig blood serum was used for the method development and evaluation (Source: Leibniz Institute for Farm Animal Biology, Dummerstorf, DE). Materials used in the test procedures were purchased from commercial sources as listed in the tables 2-4. The Biomek i7 hybrid deck layout shown in Figure 2 is used for the automated sample processing. The deck was optimized to process 144 samples during a single run. Sample cleanups were carried out using the Positive Pressure Extractor (amplius GmbH, Rostock, DE) integrated on the right side of the Biomek i7 deck. A self-refilling reservoir (amplius GmbH, Rostock, DE) was integrated to facilitate the delivery of large volumes (e.g. 127 mL acetonitrile for protein precipitation).

The samples were placed on the deck in 1.5 mL Eppendorf safe-lock vials (Eppendorf, Hamburg, DE) using a special adapter (CELISCA, Rostock, DE) (see Figure 4) on the i7 deck and were processed according to Table 1. Serum samples with concentrations of 1 µg/mL 25(OH)D_2 and 25(OH)D_3 were used for the method validation. 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 flow rate 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.

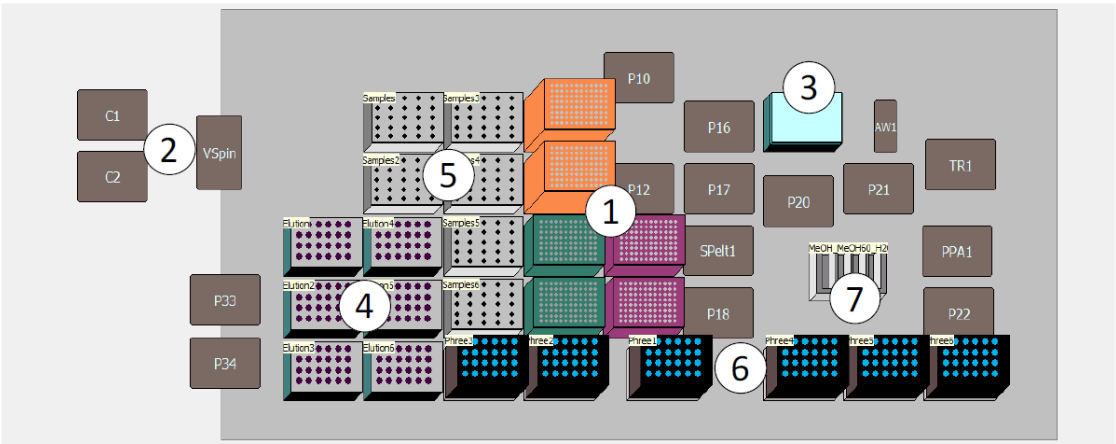

Figure 2. Deck layout for vitamin D determination using Phree phospholipid removal cartridges - (1) Tip boxes 1070 µL, 230 µL and 930 µL, (2) Centrifuge, (3) Internal Standard (ISTD), (4) Adapter made of aluminum with elution vials, (5) Samples, (6) Phree phospholipid removal cartridges, (7) Quarter self-refilling reservoir. Note: The integrated centrifuge is not used in the protocol.

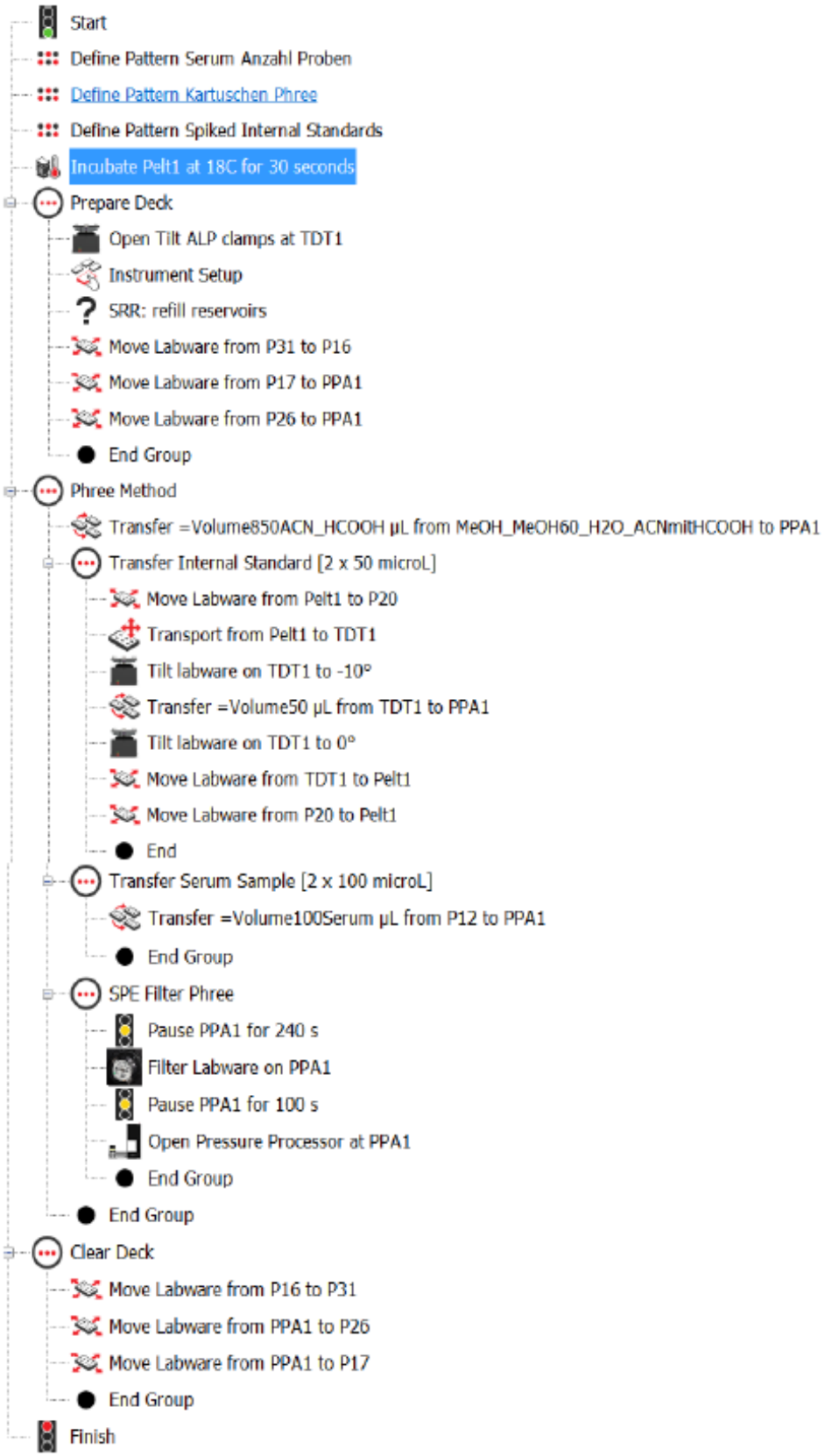

Figure 3. Biomek i7 method for vitamin D determination – Sample cleanup using Phree phospholipid removal cartridges

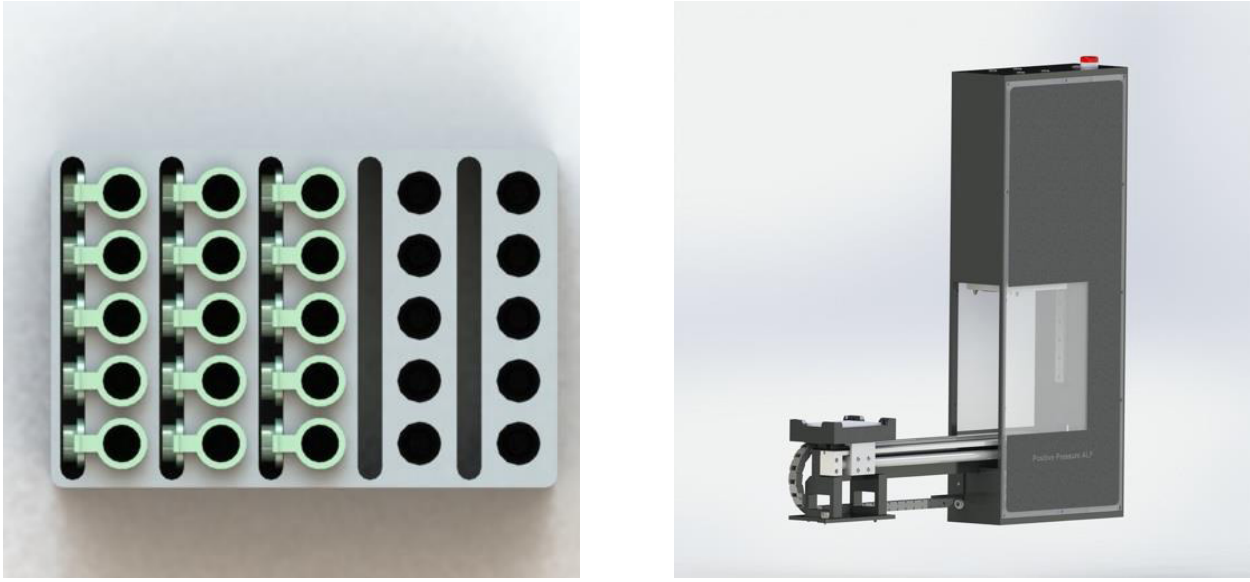

Figure 4. (a) Storage for samples vials (CELISCA, Rostock, DE), (b) Positive Pressure Extractor (amplius GmbH, Rostock, DE)

Step Description	Manufacturer
Step 1	Transfer 850 µL acetonitrile to Phree cartridges
Step 2	Transfer 50 µL internal standard (ISTD) to Phree cartridges for all samples
Step 3	Transfer 100 µL serum sample to Phree cartridges
Step 4	Mix the solution with multiple aspiration/dispensing
Step 5	Incubation at room temperature for 4 min
Step 6	Apply positive pressure (100 sec., 500 mbar)
Step 7	Measurement using LC/MS or LC/MS/MS

Table 1. Sample processing protocol for the vitamin D determination using Phree cartridges

Results

The measurements were calibrated between 0.01 ppm and 2 ppm for 25(OH)D_2 and 25(OH)D_3 (see Figure 5). The recovery rates were determined with values of 90.95%-106.21% for 25(OH)D2 and 94.61%-115.50 % for 25(OH)D_3. The high recovery rates indicate optimal removal of phospholipids. The coefficient of variation (CV) of 4.36% (25(OH)D_2) and 5.1% (25(OH)D_3) and was determined with 10 samples. To determine between-experiment precision, the experiment was repeated on 5 days with 10 samples each resulting in a CV between 2.68% and 8.36% (25(OH)D_2) and 1.69-5.51% (25(OH)D_3). The limit of detection (LOD) and limit of quantification (LOQ) were determined at 5.91 ng/mL (LOD) and 12.54 ng/mL (LOQ) for 25(OH)D_2 as well as 26 ng/mL and 73.2 ng/mL for 25(OH)D_3. The detection limits can be further improved by optimizing the LC/MS/MS system. The results obtained were comparable to the results of classical methods in which protein precipitation is carried out with centrifugation.

Summary

In this application note we automated sample processing for LC-MS based vitamin D analysis on a Biomek i7 hybrid workstation. The use of Phree phospholipid removal cartridges for combined protein precipitation and sample clean-up reduced the number of processing steps and the processing time. The high recovery rate is indicative of efficient removal of phospholipids. The low CV values (2-9%) of the automated workflow indicates high repeatability.

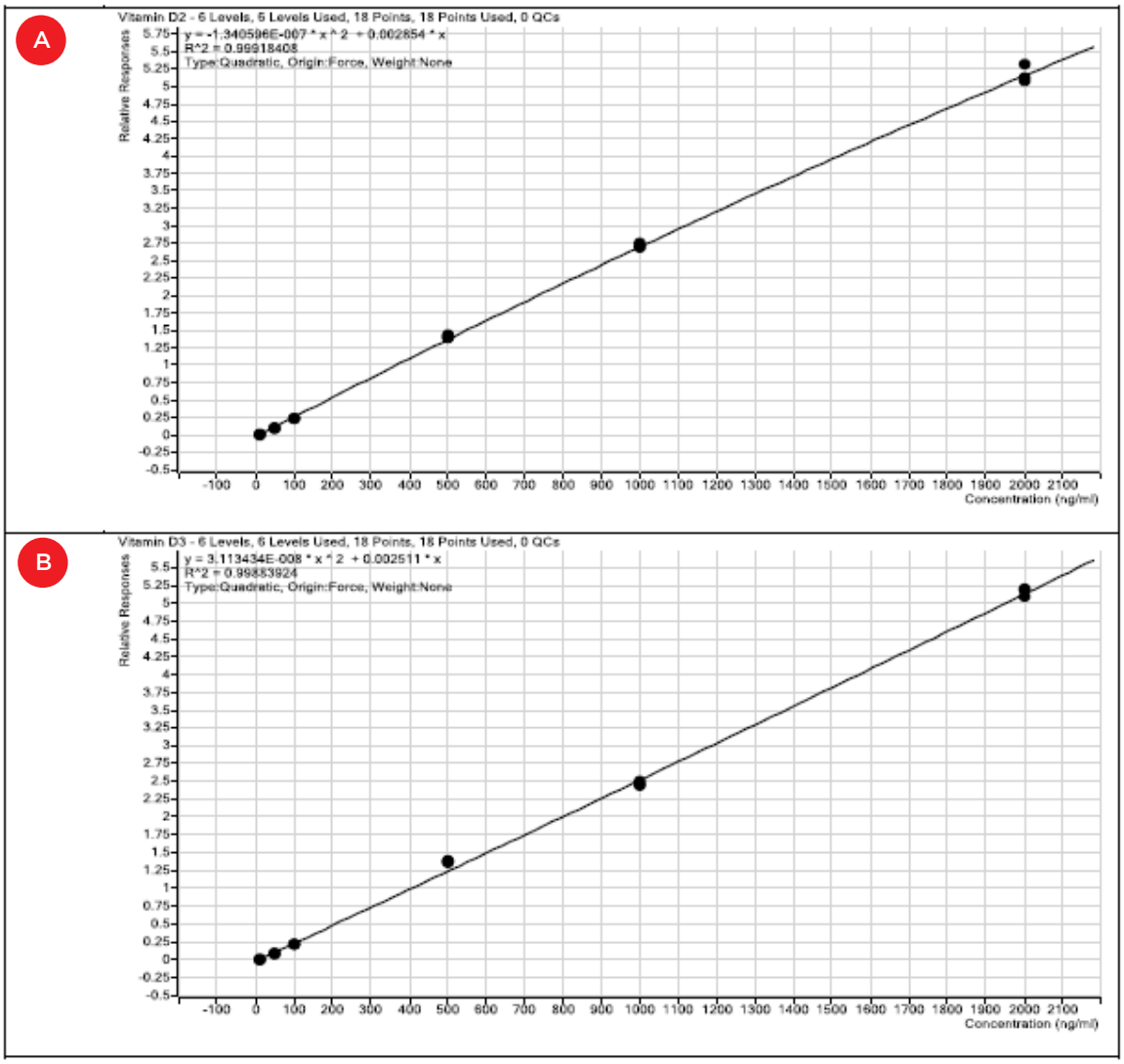

Figure 5. Calibration for (a) 25(OH)D_2 and (b) 25(OH)D_3

References

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2. Pucci V, Di Palma S, Alfieri A, et al. A novel strategy for reducing phospholipids-based matrix effect in LC-ESI-MS bioanalysis by means of Hybrid SPE. J Pharm Biomed Anal 2009;50 (5) 867-871.
3. Tulipani S, Llorach R, Urpi-Sarda M, et al. Comparative analysis of sample preparation methods to handle the complexity of the blood fluid metabolome: When less is more. Anal Chem 2013;85(1): 341-348.
4. Huq S, Pike Safan E, Rapid and Effective Cleanup of Vitamin D2 and D3 from Human Plasma using PhreeTM Phospholipid Removal Plates and Kinetex®Core-Shell HPLC/UHPLC Columns. Application Note TN0061, Phenomenex Inc., 2013.

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 3

Reagents	Manufacturer	Part Number
D6-25(OH)D_3 (50 ppm)	Sigma Aldrich, St. Louis, USA	H-074
25(OH)D_2 (50 ppm)	Sigma Aldrich, St. Louis, USA	H-073
25(OH)D_3 (100 ppm)	Sigma Aldrich, St. Louis, USA	H-083
ACN mit HCOOH 0.1%	Sigma Aldrich, St. Louis, USA	1590024000

Table 3. Reagents Method 3

Consumables used per 144 samples			
Consumables	**Number**	**Manufacturer**	**Part number**
Biomek i-series tips 90 µL	144	Beckman Coulter Life Sciences, Indianapolis, USA	B85881
Biomek i-series tip 230 µL	144	Beckman Coulter Life Sciences, Indianapolis, USA	B85903
Biomek i-series tips 1070 µL	144	Beckman Coulter Life Sciences, Indianapolis, USA	B85971
Phree Phospholipid Removal 1mL Tube	144	Phenomenex, Torrance, USA	8B-S133-TAK
GC Vials 2 mL	144	Agilent Technologies, Santa Clara, USA	5182-0716
Eppendorf Vials 1.5 mL (Safe Lock)	144	Eppendorf AG, Hamburg, Germany	EP0030121880
Septum Lid	144	Agilent Technologies, Santa Clara, USA	5182-0731

Table 4. Consumables Method 3

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

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