Extraction of 25-hydroxy Vitamin D from Serum Using ISOLUTE® PLD+ prior to LC-MS/MS Analysis

This application note describes the extraction of 25-hydroxy vitamin D from serum, prior to LC-MS/MS analysis.

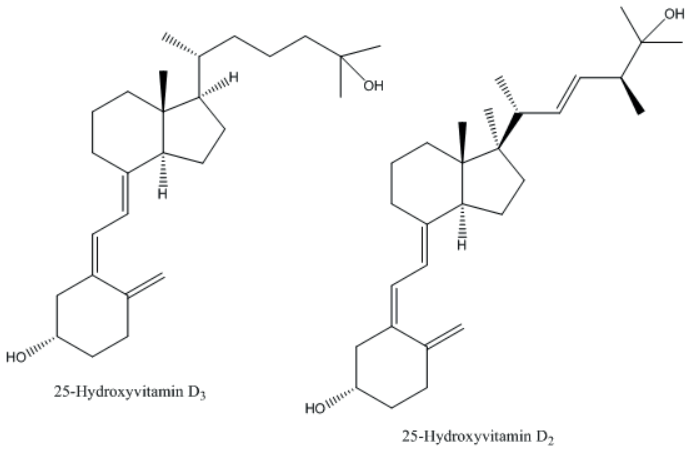

Figure 1. Structures of 25-hydroxy Vitamin D

Introduction

ISOLUTE® PLD+ Protein and Phospholipid Removal plates offer a substantial improvement in extract cleanliness compared to traditional protein precipitation techniques for bioanalytical sample preparation.

This application note describes a simple, effective ISOLUTE® PLD+ protocol for the extraction of 25-hydroxy vitamin D from serum, demonstrating high, reproducible analyte recoveries with low protein and phospholipid content in the extracts.

Analytes

25-hydroxy vitamin D2, 25-hydroxy vitamin D3 and d6-25-hydroxy vitamin D3 as the internal standard.

Sample Preparation Procedure

Format: ISOLUTE® PLD+ Protein and Phospholipid Removal plate, part number 918-0050-P01

Sample Pre-treatment

Add 10 µL of ISTD (equivalent to 30 ng/mL) to the serum sample. Mix. Allow to stand for ~1 hour for binding to occur.

Solvent Application

Apply 400 µL of Acetonitrile (MeCN) to each well of the ISOLUTE® PLD+ plate.

Sample Application

Add 100 µL of serum with ISTD and mix thoroughly via repeat aspirate/dispense steps.

Analyte Elution

Apply vacuum -0.2 bar or 3 psi positive pressure for approximately 5 minutes. For highly particulate laden samples increased pressure or vacuum conditions may be required.

Post Extraction

Dry the extract in a stream of air or nitrogen using a SPE Dry (40 °C at 40 L/min) or TurboVap (40 °C at 1.0 bar).

Reconstitution

Apply 100 µL of 30/70 2 mM Ammonium Formate, 0.1% formic acid aq/MeOH.

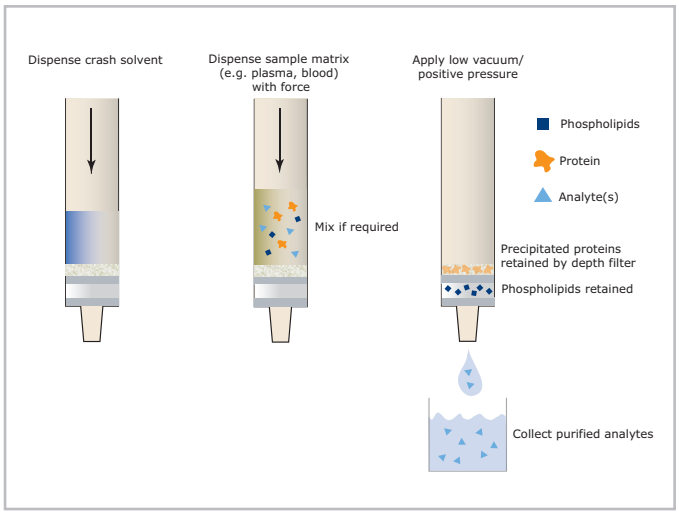

Figure 2. Typical ISOLUTE® PLD+ procedure

UPLC Conditions

Instrument
Waters Acquity UPLC (Waters Assoc., Milford, MA, USA)

Column
ACE EXCEL 2 C18-PFP, 100 mm x 2.1 mm id 2 µm, (ACT, UK)

Mobile Phase
A: 2 mM ammonium formate/0.1% formic acid (aq)
B: 2 mM ammonium formate/0.1% formic acid/MeOH

Flow Rate
0.4 mL/min

Table 1. UPLC Gradient Conditions.

Time	%A	%B	Curve
0	25	75	1
3	0	100	6
4	25	75	11

Curve 11: Conditions in line initiated immediately once time passed. i.e. 25:75 resumed at 4 minutes.

Curve 6: Linear Gradient

Injection Volume
15 µL (partial loop with overfill)

Sample Temperature
20 °C

Column Temperature
40 °C

Note: alternative UPLC conditions may be suitable. Check for good chromatographic separation of isobaric interferences to ensure accurate analyte quantitation

Mass Spectrometry Conditions

Instrument
Quattro Premier XE triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface for mass analysis

Desolvation Temperature
450 °C

Ion Source Temperature
150 °C

Collision Cell Pressure
3.76 e^{-3} mbar

Positive ions acquired in the multiple reaction monitoring (MRM) mode:

Table 2. Quattro Premier XE MRM parameters (Qualifier ion details shown in parenthesis).

Analyte	MRM Transition	Cone V	Collision Energy eV
25-OH D_2	395.5 > 269.5 (395.5 > 119.2)	30	18 26
25-OH D_3	383.5 > 257.5 (383.5 > 107.2)	30	17 25
d6-25-OH D_3	389.6 > 263.5	30	16

Results

Chromatography
Good chromatographic separation of 25-hydroxy vitamin D2 and D3 was achieved in less than 3 minutes, as shown in **Figure 3.**

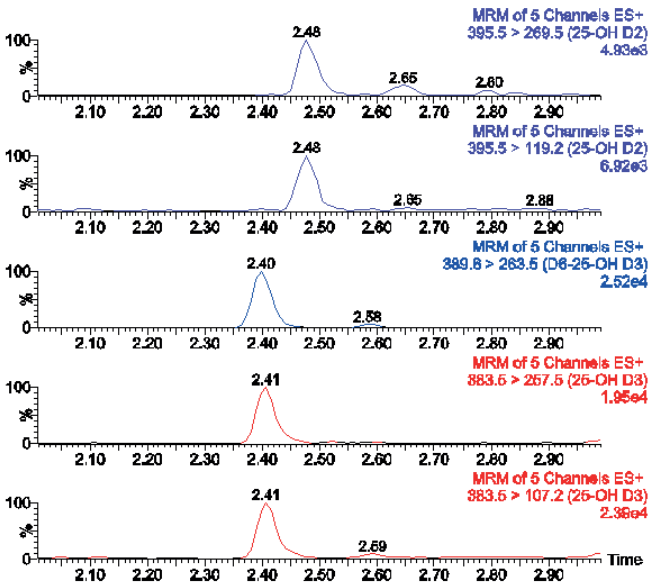

Figure 3. Chromatographic separation of 25-hydroxy vitamin D2 and D3 from Chromsystems calibrated serum at 14.8 and 19.6 ng/mL respectively. ISTD at 30 ng/mL.

Recovery

Serum and stripped serum was spiked at various concentrations from 2–100 ng/mL. High reproducible recoveries > 70% with corresponding RSDs < 10% were demonstrated. Typical recovery data is shown in **Figure 4.**

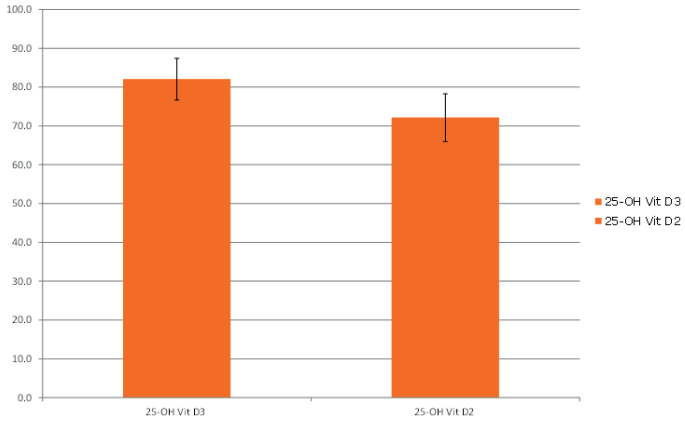

Figure 4. Recovery profile for 25-hydroxy vitamin D extracted at 50 ng/mL.

PBS/BSA Calibration Curves

Calibration curves were generated using PBS/BSA spiked at concentrations from 1–100 ng/mL. Good coefficients of determination were obtained for 25-hydroxy vitamin D2 and D3 (r^2 > 0.99).

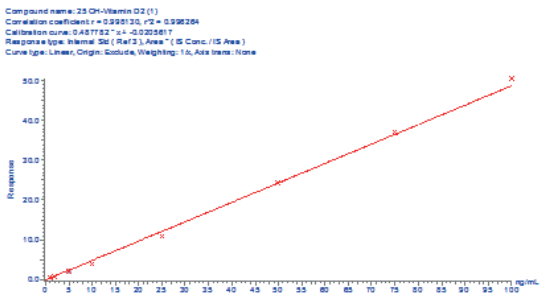

Figure 5. PBS/BSA Calibration Curve for 25-OH vitamin D2 constructed from 1–100 ng/mL.

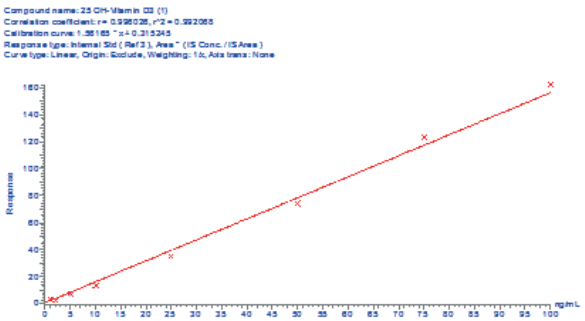

Figure 6. PBS/BSA Calibration Curve for 25-OH vitamin D3 constructed from 1–100 ng/mL.

Chromsystems Calibration Curves

Curves were also generated using calibrated serum standards (obtained from Chromsystems) spiked at concentrations from 0–69 ng/mL. Good coefficients of determination were obtained for 25-hydroxy vitamin D2 and D3 (r^2 > 0.99).

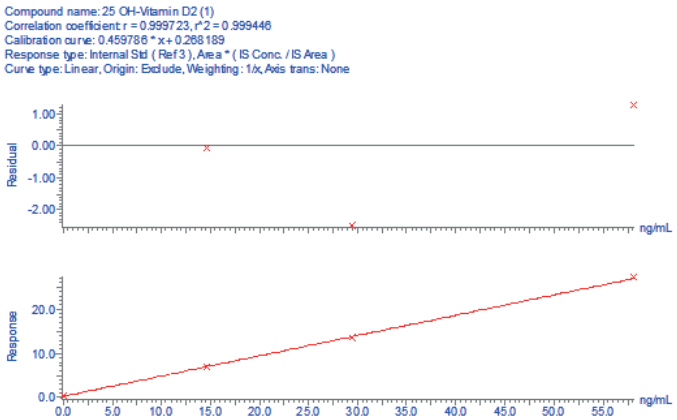

Figure 7. Chromsystems Calibrated Serum Curve for 25-OH vitamin D2 constructed from 4-69 ng/mL.

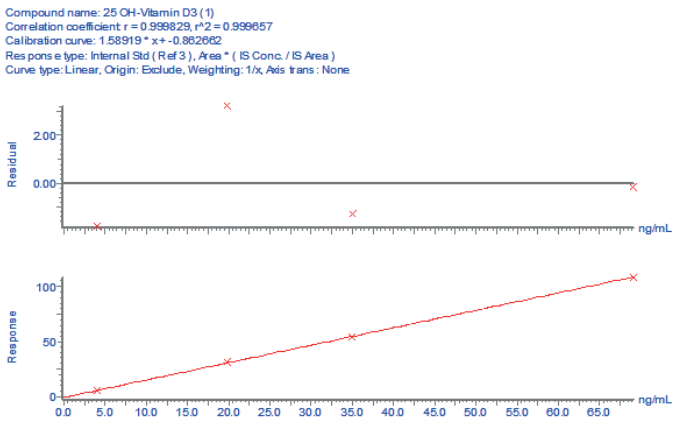

Figure 8. Chromsystems Calibrated Serum Curve for 25-OH vitamin D3 constructed from 0-58 ng/mL.

DEQAS External Quality Assessment Scheme

Final method testing was performed for 5 DEQAS serum samples extracted alongside the Chromsystems calibrators using the optimized method. The DEQAS criteria for acceptable performance is that at least 80% of results should fall within + or - 25% of the All Laboratory Trimmed Mean. Method performance is shown in **Table 3**. Units are quoted as ng/mL. All values fall within the accepted criteria.

Table 3. DEQAS 25-OH vitamin D results obtained using optimum method.

DEQAS Sample I.D.	DEQAS LC/MS Mean	ISOLUTE® PLD+
451	12.9	14.5
452	46.7	49.1
453	26.6	28.9
454	21.4	25.3
455	22.2	23.7

Ordering Information

Part Number	Description	Quantity
918-0050-P01	ISOLUTE® PLD+ Fixed Well Plate	1
121-9600	Biotage® VacMaster™-96 Sample Processing Manifold	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS-EU	Biotage® SPE Dry Sample Concentrator System 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry Sample Concentrator System 100/120 V	1
C103263	TurboVap®96, Evaporator 100/120V	1
C103264	TurboVap® 96, Evaporator 220/240V	1

Additional Notes

Buffer Preparation

1. 2 mM ammonium formate/0.1% formic acid (aq): Weigh 0.12612 g and dissolve in H_2O. Add 1 mL of formic acid and make up to 1 L in H_2O.
2. 2 mM ammonium formate/0.1% formic acid/MeOH: Weigh 0.12612 g and dissolve in MeOH. Add 1 mL of formic acid and make up to 1 L in MeOH.

Processing Conditions

Positive Pressure: Process at approximately 3 psi.

Vacuum Processing: Process at approximately -0.2 bar.

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