# Comparison of 25-hydroxy Vitamin D Extraction Using Supported Liquid Extraction and Phospholipid Depletion Plate Technology Using Manual and Automated Sample Preparation Prior to LC/MS Analysis

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## Introduction

Vitamin D deficiency can result in various health issues such as osteoporosis, liver and kidney problems and is associated with increased risk of cancers and multiple sclerosis. From this standpoint vitamin D analysis has extremely important clinical relevance. Many sample preparation approaches to the extraction of 25-hydroxy vitamin D have been employed prior to LC-MS/MS analysis. Simple protein precipitation, supported liquid extraction and complex SPE methodology are all in routine use. This poster compares the use of supported liquid extraction (ISOLUTE® SLE+) and a novel protein and phospholipid depletion plate (ISOLUTE® PLD+), for the extraction of 25-hydroxy vitamin D. The extraction protocols were ultimately transferred to an SPE automation platform and method performance versus manual processing compared.

## Experimental

### Reagents

Formic acid (FA), ammonium formate and 25-hydroxy vitamin D metabolites and internal standards were purchased from Sigma Chemical Co. (Dorset, UK). Human serum and bovine serum albumin was obtained through Sera Labs International (West Sussex, UK). 25-hydroxyvitamin D serum calibrators were purchased from Chromsystems (Munich, Germany). Test serum samples were obtained from the DEQAS scheme, Imperial College London. All solvents were HPLC grade from Fisher Scientific (Loughborough, UK) and Milli-Q (Merck Millipore, Germany) water used throughout.

### Sample Preparation

#### ISOLUTE® PLD+ Optimized Procedure (Figure 1.)

**96-well Plates:** ISOLUTE® PLD+ 50mg; P/N: 918-0050-P01.

**Apply Solvent:** Apply 400 µL of MeCN into the wells.

**Sample Application:** Apply 100 µL of serum with 10 µL ISTD and mix thoroughly via repeat aspirate/dispense steps.

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

**ISTD: d6 25-OH D3:** 30 ng/mL.

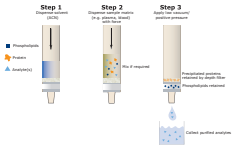


***Figure 1.*** *Schematic of ISOLUTE® PLD+ Phospholipid Depletion Plate Procedure.*

#### ISOLUTE® SLE+ Optimized Procedure (Figure 2.)

**96-well Plates:** ISOLUTE® SLE+ 400 µL capacity;

P/N: 820-0400-P01.

**Sample pre-treatment:** Apply 10 µL of ISTD into a deep well collection plate followed by 200 µL of serum. Pre-treat with 200 µL of 50/50 $H_2O$/IPA. Mix with vortex or repeat aspirate/dispense.

**Sample Application:** 300 µL was taken from the collection plate and pipetted onto the SLE+ plate. A pulse of vacuum was applied to initiate flow and the samples were left to absorb for 5 minutes.

**Analyte Extraction:** 750 µL of heptane was applied to the plate and allowed to flow under gravity for 5 minutes.
A second aliquot of heptane was applied to the plate and allowed to flow under gravity for 5 minutes. A 5 second application of positive pressure was applied remove the remaining aliqout.

**Post Extraction:** The eluate was evaporated to dryness and reconstituted in 100 µL of 30/70 2 mM ammonium formate (aq) with 0.1% FA / 2 mM Ammonium Formate (99% MeOH 1% aq) with 0.1% FA.

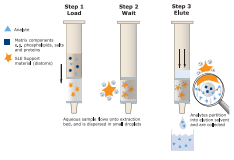


***Figure 2.*** *Schematic of ISOLUTE® SLE+ Supported Liquid Extraction procedure.*

### Extrahera™ Automated Sample Preparation Platform

The optimized extracton protocols were transferred to an automated sample preparation platform equipped with an 8 channel pipetting head and positive pressure processing functionality. The Extrahera™ platform is shown in ***Figure 3***. Elution from the plates was afforded using 0.3 bar pressure for 6 minutes with ISOLUTE® PLD+ and gravity followed by 5 seconds of 5 ar pressure with the ISOLUTE® SLE+ plate.

***Figure 3.*** *Picture of the Extrahera™ automated sample preparation platform.*

### UPLC Conditons

**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 (aq) with 0.1% FA
**Mobile Phase B:** 2 mM ammonium formate with 0.1% FA
**Flow Rate:** 0.4 mL/min
**Gradient:** 75-100% B over 3 min; hold 1 min: resume 75% B at 4 min
**Injection Volume:** 15 µL
**Column Temperature:** 40 °C

### Mass Spectrometry

**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 Gas Pressure:** 3.5 x $10^{-3}$ mbar

## Results

Initial method development involved optimization of organic solvent composition and crash ratio for efficient precipitation of proteins, phospholipid removal and maximum analyte recovery using the ISOLUTE® PLD+. ***Figure 4.*** demonstrates vitamin D recoveries using MeOH as the crash solvent. Lower recoveries, higher RSDs and a less efficient precipitation were obtained using MeOH compared to MeCN as the solvent.

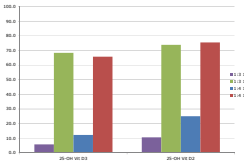

***Figure 4.*** *Recovery comparison using various ratios of MeOH as the crash solvent.*

The optimized MeCN crash ratio was deemed to be 1:4 serum:MeCN (data not shown). The optimized ISOLUTE® PLD+ method was then compared to a previously developed extraction protocol using ISOLUTE SLE+ and both methods transferred to the Extrahera™ automated sample preparation platform. ***Figure 5.*** demonstrates the recovery profile for manual and Extrahera processing of the ISOLUTE® SLE+ and PLD+ methods. The latter also compared 100 and 150 µL serum extraction.

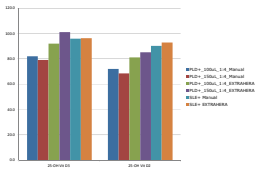

***Figure 5.*** *ISOLUTE® SLE+ and PLD+ final method recovery comparisons using manual and Extrahera™ processing.*

**Figure 6.** demonstrates the extract cleanliness of both techniques with respect to protein extraction. Very little protein was observed in the extracts for either sample preparation approach.

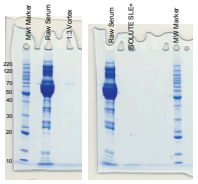


***Figure 6.*** *Gel Electrophoresis protein profiles for ISOLUTE® SLE+ and PLD+.*

The degree of phospholipid extraction for the optimized methods is demonstrated in ***Figure 7.*** Phospholipid levels for both ISOLUTE® SLE+ and PLD+ are extremely low when compared to simple protein precipitation techniques.

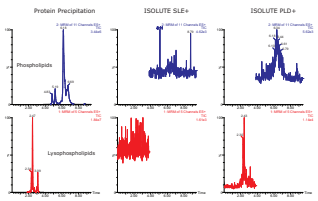


***Figure 7.*** *Phospholipid MRM TIC comparing ISOLUTE® SLE+ and PLD+ to protein precipitation.*

Linearity experiments were performed extracting PBS/BSA from 1-100 ng/mL. ISOLUTE® SLE+ and PLD+ optimized methods along with PLD+ methods extracting 150 µL of serum or using 1% formic acid as the crash solvent returned coefficients of determination > 0.99 (data not shown). Chromsystems calibrated serum lines were constructed for manual and Extrahera™ processing using ISOLUTE® SLE+ and PLD+ optimized methodology as demonstrated in ***Figures 8 and 9.***, respectively.

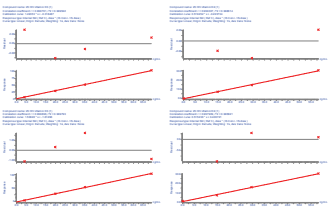

**Figure 8.** Chromsystems Calibration curves comparing Manual vs Extrahera™ processing using ISOLUTE® SLE+.

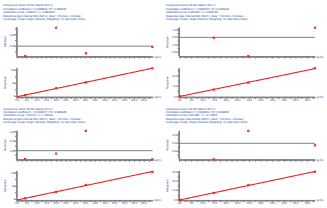

**Figure 9.** Chromsystems Calibration curves comparing Manual vs *Extrahera*™ processing using ISOLUTE® PLD+.

Final method testing was performed for 5 DEQAS serum samples extracted alongside the Chromsystems calibrators using the optimized methods. 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 2***. Units are quoted in ng/mL. All values for both extraction protocols fall within the acceptance criteria.

***Table 2.*** *DEQAS 25-OH vitamin D results obtained using optimum method.*

| DEQAS Sample I.D. | DEQAS LC/MS Mean | SLE+ Manual | SLE+ Extrahera™ | PLD+ Manual | PLD+ Extrahera™ |
|---|---|---|---|---|---|
| 451 | 12.9 | 13.5 | 12.4 | 14.5 | 13.1 |
| 452 | 46.7 | 46.5 | 46.9 | 49.1 | 46.8 |
| 453 | 26.6 | 28.5 | 26.2 | 28.9 | 26.6 |
| 454 | 21.4 | 22.7 | 21.4 | 25.3 | 21.8 |
| 455 | 22.2 | 21.1 | 23 | 23.7 | 24.1 |

## Conclusion

- This poster demonstrates the applicability of ISOLUTE® SLE+ and PLD+ for the extraction of 25-hydroxy vitamin D from serum.
- Good extraction efficiency, protein and phospholipid removal was afforded by both techniques.
- Excellent linearity, and $r^2$ values from PBS/BSA and Chromsystems calibrated serum along with good correlation data to DEQAS reported patient samples.
- Both techniques were successfully transferred on to the Extrahera™ automated sample preparation platform.



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