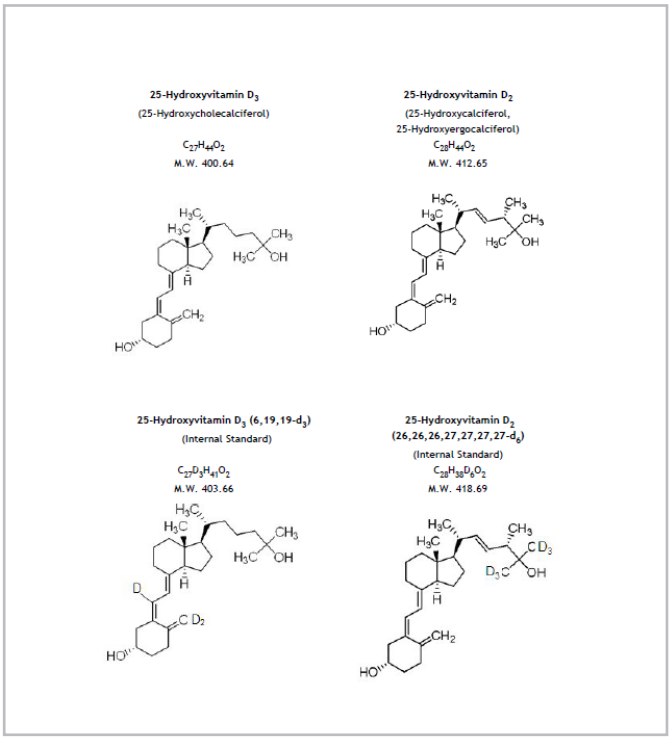
Converting a Liquid-Liquid Extraction Method for Vitamin D to a 96-Well Plate Supported Liquid Extraction Format

Tested on Patient Samples

Leveraging Reduced Workflow Solutions in High Throughput Clinical Laboratories

Cindy Gordon[1], Katerina Sadikova[1], Jane Dickerson[1], Frank Kero[2], Victor Vandell[2], Michael Yu[2], Elena Gairloch[2], Lee Williams[3]

1. *Seattle Children's Hospital, 4800 Sand Point Way NE Seattle WA 98105*
2. *Biotage, 10430 Harris Oaks Blvd, Suite C, Charlotte, NC 28269*
3. *Biotage GB Limited, Dyffryn Business Park, Ystrad Mynach, CF82 7TS, UK*



Introduction

The objective of this study was to determine feasibility of transferring a validated liquid-liquid extraction method to a supported liquid extraction method using Biotage ISOLUTE® SLE+ 96-well plates (400µL sample capacity). The laboratory supporting this study processes ~550 samples per month supporting vitamin D analysis in plasma and serum (mixed samples / not separated). During this evaluation, it was determined that sample processing time was reduced by ~50% for a de-identified patient set (n=30, split and processed by both methods). Towards high throughput analysis platforms, ISOLUTE SLE+ was demonstrated as a viable solution in the sample preparation workflow for vitamin D applications.

Experimental Overview

The strategy for this development workflow began with the liquid-liquid extraction procedure and the direct transfer to supported liquid extraction was evaluated. To optimize the method for analyte recovery, pH adjustment was evaluated in the sample pre-treatment. Elution volumes were also optimized for analyte recovery. Once optimized, the proof-of-concept was determined on a real patient sample set. The samples were split and process by each method for verification.

Analytical Conditions

Reagents (all obtained from Fisher Scientific (LC-MS grade))

Internal standard	d_6-25-hydroxyvitamin D_2, d_3-25-hydroxyvitamin D_3. 200 ng/mL in MeOH
Sample pre-treatment mix (SLE)	5M NaOH/H_2O/IPA (50:50:100, v/v/v)
Aqueous mobile phase	2 mM ammonium acetate, 0.1% formic acid in H_2O
Methanol mobile phase	2 mM ammonium acetate, 0.1% formic acid in MeOH

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Equipment

Instrument : Waters QuattroMicro triple quadrupole tandem mass spectrometer
Electrospray source (+)
Agilent 1100 or Waters 2795 Alliance HT HPLC

Column: Phenomenex Kinetex 2.6 µm PFP 100A
100 mm x 3 mm

Pre-column: Phenomenex SecurityGuard ULTRA cartridges
UHPLC PFP for 3.0mm ID columns
SecurityGuard ULTRA holder

UPLC Conditions

Pump	Flow (mL/min)	0.5
	Run time (min)	10.0

Table 1. Pump Gradient

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow (mL/min)
0.00	24.0	76.0	0.5
4.00	24.0	76.0	0.5
4.10	5.0	95.0	0.5
5.40	5.0	95.0	0.5
5.50*	24.0	76.0	0.5

*The analytical window runs to 5.5 min and then the column equilibrates for additional time.
The total time is 10 min.

Mass Spectrometry Conditions

Source: ES+

Capillary (kV): 0.4

Source temperature (°C): 120

Desolvation temperature (°C): 450

Collision gas (mbar): 1.1×10^{-3}

Dwell (s): 0.2

Delay (s): 0.1

Table 2. MS/MS Conditions

Compound	Type of use	Transition	Cone (V)	Collision (eV)
25-(OH)D_3	Primary	383.3>257.2	28	18
25-(OH)D_3	Secondary	401.3>365.2	18	10
d_3-25-(OH)D_3	Internal std	404.4>368.2	18	10
25-(OH)D_2	Primary	413.3>355.2	18	10
25-(OH)D_2	Secondary	395.3>269.2	28	20
D_6-25-(OH)D_2	Internal std	419.4>355.2	18	10

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Sample Preparation Methodology

Method 1: Gold standard referee method – validated liquid-liquid extraction method

1. Set up labeled 2 mL Eppendorf centrifuge tubes for each blank, standard, control, and patient sample.
2. Vortex patient specimens, controls, and standards for 10 seconds. Centrifuge the tube if thevolume is close to needed volume (200 µL).
3. Pipette 200 µL of sample into the 2 mL Eppendorf tube.
4. Add 50 µL NaOH (5 M/L). Mix on Mix-Mate at 2000 RPM for 1 minute. Tubes are OK uncapped for this step.
5. Pipette 200 µL of internal standard into each tube. MixMate for 3 minutes.
6. Extract by adding 1.5 mL of hexane to each tube. Cap and vortex for 10 seconds. MixMate for 4 minutes.
7. Centrifuge in micro-centrifuge at 13,000 rpm for 5 min.
8. Cool in -20 °C freezer for 20 min. Use tube racks previously pre-cooled in the freezer.
9. Using a transfer pipette, carefully transfer as much of the upper solvent layer as possiblewithout removing any of the bottom layer to a 2 mL labeled LCMS autosampler vial.
10. Dry down extracts under nitrogen in Reacti-therm Evaporator with the bath temperature set up at 35 °C. Before the evaporation, clean nozzles with paper towel wetted by 20% methanol or isopropanol wipe.
11. Reconstitute with 80 µL 70% MeOH 30%H2O. Vortex well.
12. Transfer all into a glass insert. Return the insert to the original vial and cap .
13. Centrifuge for 10 min at 2,500 rpm.
14. Inject 20 µL into LCMS.

Total time for processing 30 samples **>4 hours**

Method 2: Candidate supported liquid extraction procedure using ISOLUTE® SLE+ plates

1. Label 0.6 mL microfuge tubes.
2. Add 200 µL patient sample (plasma or serum) to each tube.
3. Add 30 µL IS to each tube.
4. Add 200 µL sample pre-treatment mix to each tube.
5. Cap, mix on MixMate for 5 seconds.
6. Transfer entire sample to ISOLUTE SLE+ 400µL plate.
7. Wait 5 minutes.
8. Add 750 µL hexane to wells.
9. Wait 5 minutes. If solvent isn't completely eluted, pulse with pressure using a Biotage® PRESSURE+ 96. Repeat step 8 and 9 with a further aliquot of hexane.
10. Dry under nitrogen, 45 °C.
11. Reconstitute with 80 µL 70% MeOH 30%H2O.
12. Mix on MixMate 5 seconds.
13. Transfer to vial inserts.
14. Inject 20 µL into LCMS.

Total time for processing 30 samples **<2 hours**

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Results

Example chromatograms for samples prepared using **a)** original liquid-liquid extraction method and **b)** supported liquid extraction method are shown in **Figures 2 and 3** below:

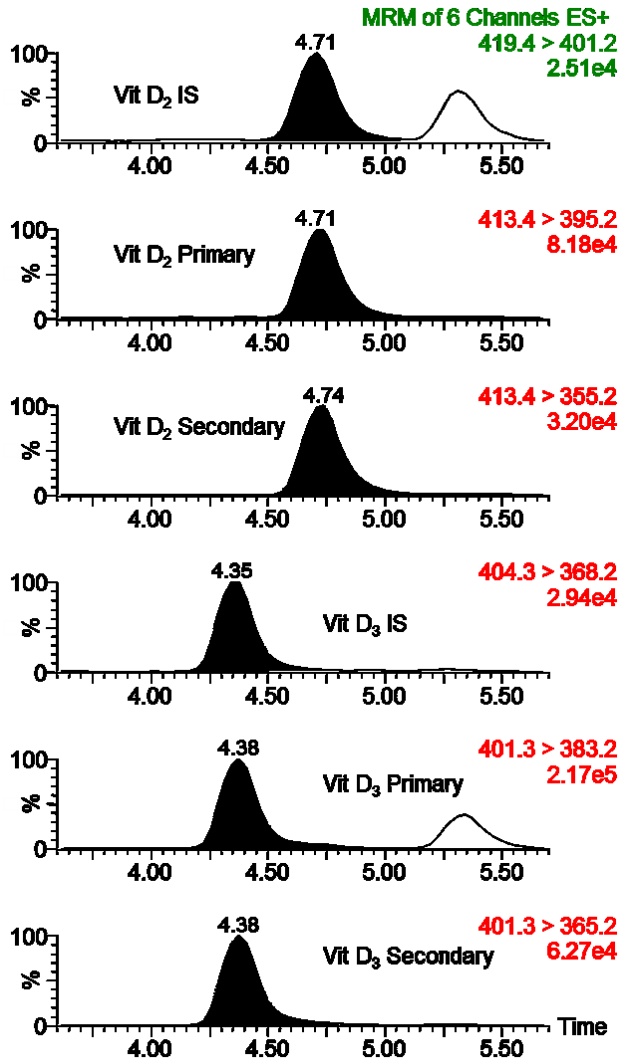

Figure 2. Example chromatogram for vitamin D metabolites extracted using original liquid-liquid extraction method.

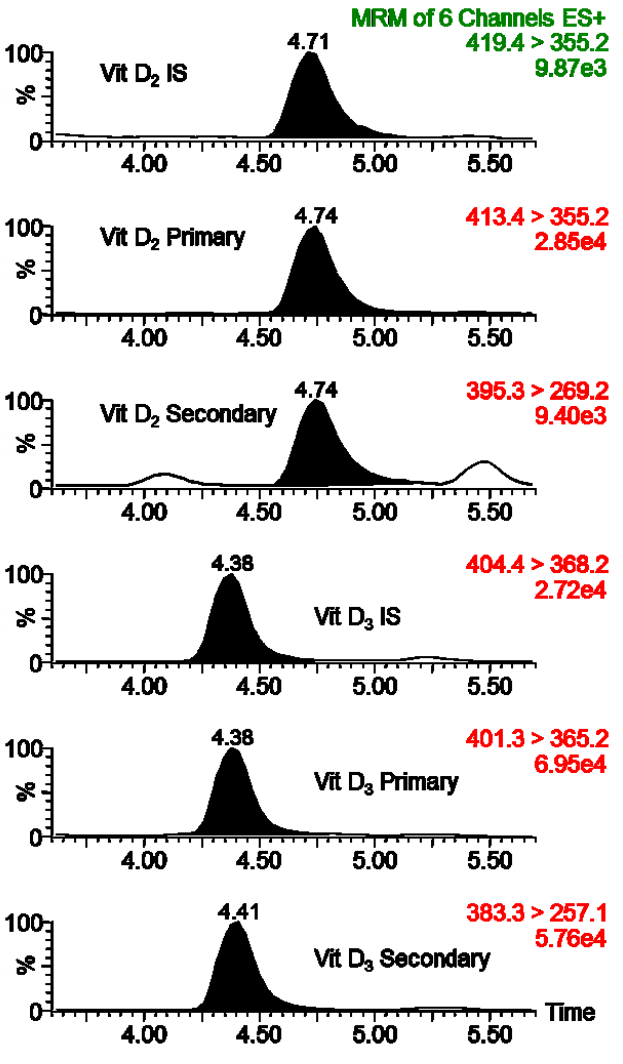

Figure 3. Example chromatogram for vitamin D metabolites extracted using supported liquid extraction method on ISOLUTE®SLE+ plate.

Levels of Vitamin D metabolites from split samples extracted using each technique are shown in **Table 3**, and correlation plots for each metabolite are shown in **Figures 4 and 5**.

Table 2. Nominal data comparison, de-identified split sample patient set.

Patient Sample ID	D3-Liquid Extraction ng/mL	D3-ISOLUTE® SLE+ ng/mL	Relative Error (%)
1	14.0	14.0	0.0
2	13.0	13.4	-3.1
3	56.0	57.8	-3.2
4	41.0	46.2	-12.7
5	17.0	19.1	-12.4
6	20.0	22.6	-13.0
7	54.0	57.0	-5.6
8	35.0	35.8	-2.3
9	12.4	14.3	-15.3
10	17.4	19.3	-10.9
11	26.9	27.7	-3.0
12	27.1	30.2	-11.4
13	4.5	4.30	4.4
14	21.7	24.8	-14.3
15	15.6	16.6	-6.4
16	32.0	38.6	-20.6
17	39.0	42.6	-9.3
18	15.0	15.2	-1.3
19	15.0	17.4	-16.3
20	41.0	50.5	-23.1
21	13.0	14.1	-8.5
22	25.0	27.8	-11.0
23	13.0	13.4	-3.1
24	8.0	9.3	-16.3
25	37.0	40.4	-9.2
26	36.0	40.3	-11.9
27	18.0	18.5	-2.8
28	31.0	29.7	4.2
29	33.0	36.1	-9.4
30	19.0	18.9	0.5

Patient Sample ID	D2-Liquid Extraction ng/mL	D2-ISOLUTE® SLE+ ng/mL	Relative Error (%)
1	41.0	40.7	0.7
2	3.0	2.2	26.7
3	<2	<2	N/A
4	<2	<2	N/A
5	3.0	2.4	20.0
6	6.0	6.1	-1.7
7	<2	<2	N/A
8	<2	<2	N/A
9	<2	<2	N/A
10	2.2	2.1	4.5
11	<2	<2	N/A
12	<2	<2	N/A
13	13.3	15.5	-16.5
14	<2	<2	N/A
15	<2	<2	N/A
16	<2	<2	N/A
17	<2	<2	N/A
18	21.0	20.5	2.5
19	<2	<2	N/A
20	<2	<2	N/A
21	<2	<2	N/A
22	<2	<2	N/A
23	7.0	5.3	24.3
24	8.0	6.2	22.5
25	<2	<2	N/A
26	<2	<2	N/A
27	4.0	3.1	22.5
28	3.0	2.6	13.3
29	3.0	2.4	20.0
30	6.0	4.6	23.3

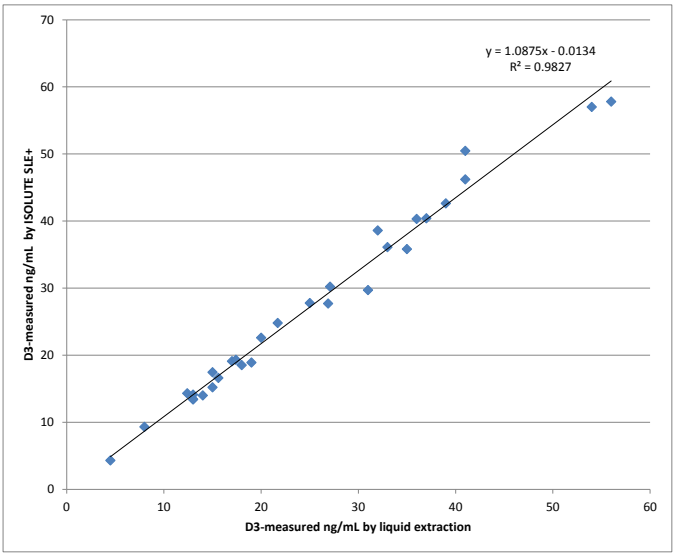

Figure 4. Correlation plot: Split patient sample study, determination of vitamin D3 in plasma and serum.

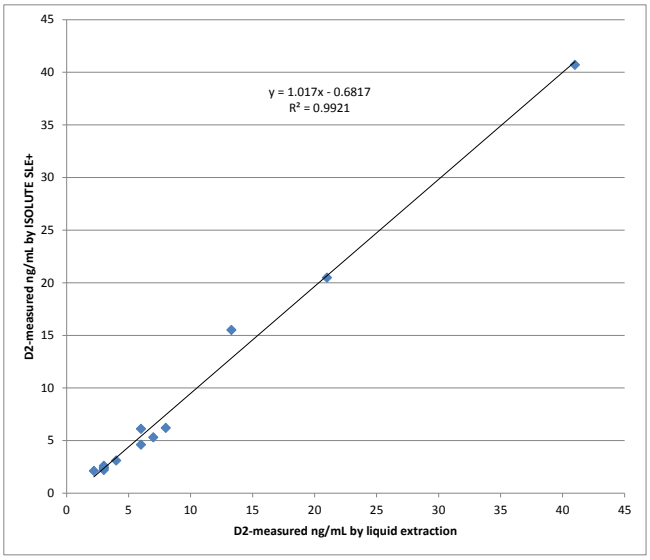

Figure 5. Correlation plot: Split patient sample study, determination of vitamin D2 in plasma and serum.

Conclusions

Transfer of methodology from a traditional liquid-liquid extraction method to a supported liquid extraction method was simple and straightforward, with minimal optimization required.

Good correlation between measured levels of vitamin D metabolites in split samples extracted using the original validated liquid-liquid extraction method and the optimized supported liquid extraction method using ISOLUTE SLE+ plates was achieved.

Time for processing a batch of 30 patient samples (serum and plasma) was approximately halved using the supported liquid extraction approach, from > 4 hours to < 2 hours.

Due to the cleaner nature of the extraction, centrifugation steps were not required in the supported liquid extraction method.

This study suggests that supported liquid extraction using ISOLUTE®SLE+ 96-well extraction plates is a viable alternative to liquid-liquid extraction in a busy clinical laboratory.

Ordering Information

Part Number	Description	Quantity
820-0400-P01	ISOLUTE® SLE+ 400 µL Supported Liquid Extraction Fixed Well Plate	1

EUROPE
Main Office: +46 18 565900
Toll Free: +800 18 565710
Fax: +46 18 591922
Order Tel: +46 18 565710
Order Fax: +46 18 565705
order@biotage.com
Support Tel: +46 18 56 59 11
Support Fax: + 46 18 56 57 11
eu-1-pointsupport@biotage.com

NORTH & LATIN AMERICA
Main Office: +1 704 654 4900
Toll Free: +1 800 446 4752
Fax: +1 704 654 4917
Order Tel: +1 704 654 4900
Order Fax: +1 434 296 8217
ordermailbox@biotage.com
Support Tel: +1 800 446 4752
Outside US: +1 704 654 4900
us-1-pointsupport@biotage.com

JAPAN
Tel: +81 3 5627 3123
Fax: +81 3 5627 3121
jp_order@biotage.com
jp-1-pointsupport@biotage.com

CHINA
Tel: +86 21 2898 6655
Fax: +86 21 2898 6153
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