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A Simplified and Automated Extraction Method for the Determination of 25-OH Vitamin D_2/D_3 in Human Serum Using a Strata® RP On-Line SPE Column

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Overview

In this technical note, we explore the effectiveness of an automated on-line Solid Phase Extraction (SPE) method with a Thermo Cohesive system ran in TX mode coupled with MS/MS analysis for the characterization of 25-OH Vitamin D_2/D_3 from human serum. The relationship between the on-line extraction and LC column was investigated in the context of Aria® based software. The assay includes a shorter 30 x 3.0 mm Kinetex® 2.6 µm C18 LC analytical column and a 50 x 0.5 mm Strata RP on-line SPE column, which reduces the total run time to under 6 minutes including the system equilibration. The assay shows the accuracy and precision, including LLOQ (n=6), and the resulting method is assessed for a linear dynamic range from 2-100 ng/mL. **Figure 1** displays the Aria based software overview, while **Table 1** provides the on-line SPE conditions and **Table 2** shows the LC methodology.

Materials

25-OH Vitamin D2 and D3 standards were purchased from Cerilliant® (Round Rock, TX). Double charcoal stripped human serum was purchased from BioreclamationIVT® (Westbury, NY) All other reagents and chemicals were obtained from Sigma - Aldrich®.

Experimental Conditions

Sample Pre-treatment

1. Dilute 150 µL of human serum* with 200 µL of Precipitating Reagent**
2. Add 10 µL of 25-OH Vitamin-D_3-$_2H_6$ (1 µg/mL), mix for one minute
3. Centrifuge at 14,000 RPM for 10 minutes
4. Transfer 200 µL supernatant to autosampler injection vial

*Double Charcoal-stripped human serum was used to prepare all Standards and QCs

**Precipitating Reagent prepared as (5:2:1) Methanol/Acetonitrile/Zinc Sulfate

On-Line Solid Phases Extraction

On-line SPE Column: Strata RP
Dimensions: 50 x 0.5 mm
Part No.: 00B-S326-AF

LC Conditions

Column: Kinetex® 2.6 µm C18
Dimensions: 30 x 3.0 mm
Part No.: 00A-4462-Y0
Guard Column: SecurityGuard™ ULTRA Cartridges
Guard Part No.: AJ0-8775
Mobile Phase: A: 0.1 % Formic acid in Water
B: 0.1 % Formic acid in Methanol
Gradient: See Table 2
Flow Rate: See Table 2
Needle Wash 1: Methanol/Water (50:50)
Needle Wash 2: 0.1 % Formic acid in Water
Instrument: Cohesive System run in TX Mode: Agilent® 1260 with Leap Technologies PAL autosampler LX-2
Instrument: MS/MS (SCIEX 4000 QTRAP®) APCI+

Figure 1.
Screenshot of Method from Aria Software
Extraction column conditions (blue)
Analytical column conditions (pink)

Table 1.
On-line SPE Conditions

Step	Time (min)	Flow Rate (mL/min)	0.1 % Formic acid in Water (A)	0.1 % Formic acid in Methanol (B)	Comments
1	0	0.75	30	70	Extract sample
2	0.5	0.35	30	70	Slow down pumps
3	0.58	0.35	2	98	Transfer analytes
4	1.58	1.50	0	100	Separate analytes, wash extraction column
5	2.08	1.50	0	100	Elutes analytes, wash extraction column
6	3.58	0.75	30	70	Wash columns and valves
7	4.08	0.75	30	70	Fill transfer loop, equilibrate HPLC column
8	4.58	0.75	30	70	Equilibrate columns

Table 2.
LC Conditions

Step	Time (min)	Flow Rate (mL/min)	0.1 % Formic acid in Water (A)	0.1 % Formic acid in Methanol (B)
1	0	0.75	30	70
2	0.5	0.35	30	70
3	0.58	0.35	30	70
4	1.58	0.70	10	90
5	2.08	0.70	10	90
6	3.58	0.70	10	90
7	4.08	0.75	30	70
8	4.58	0.75	30	70

Table 3.
MRM Transitions

ID	Q1 Mass (DA)	Q3 Mass (DA)	Dwell (msec)	CE
25-OH D_2 1	395.4	209	100	36
25-OH D_2 2	395.4	269.1	100	28
25-OH D_3 1	383.6	257.2	100	23
25-OH D_3 2	383.6	229.4	100	28
D_6-25-OH D_3 1	389.5	263.3	100	23
D_6-25-OH D_3 2	389.5	229.4	100	28

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Table 4.
Assay Recovery

Serum Spiked at 100 ng	25-OH Vitamin D_2	25-OH Vitamin D_3
Average Area Ratio (n=4)	4.48E-01	3.01E-01
% CV (n=4)	0.74	1.28

Neat Solution at 100 ng	25-OH Vitamin D_2	25-OH Vitamin D_3
Average Area Ratio (n=4)	4.69E-01	3.23E-01
% CV (n=4)	3.09	2.10

Assay Recovery	25-OH Vitamin D_2	25-OH Vitamin D_3
Spiked Serum/Neat Solution	95.5	93.2

Figure 2.
Representative of chromatograms of LLOQ at 2 ng/mL in human serum

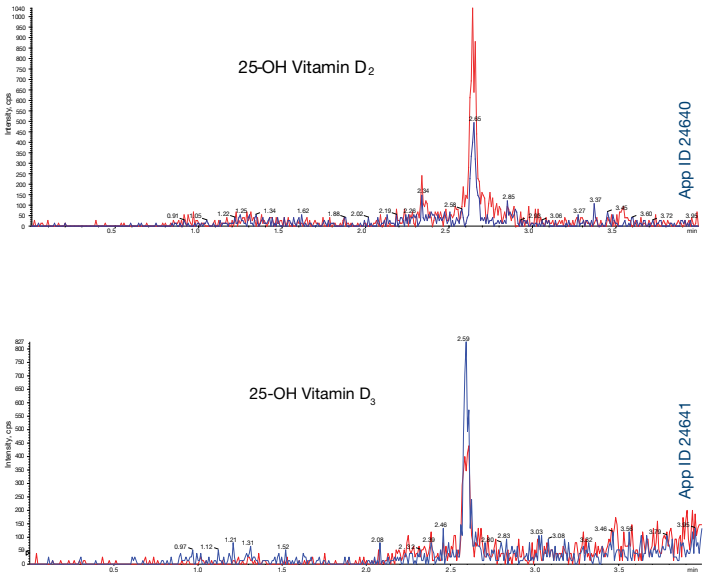

Figure 3.
Representative of chromatogram of ULOQ at 100 ng/mL in human serum

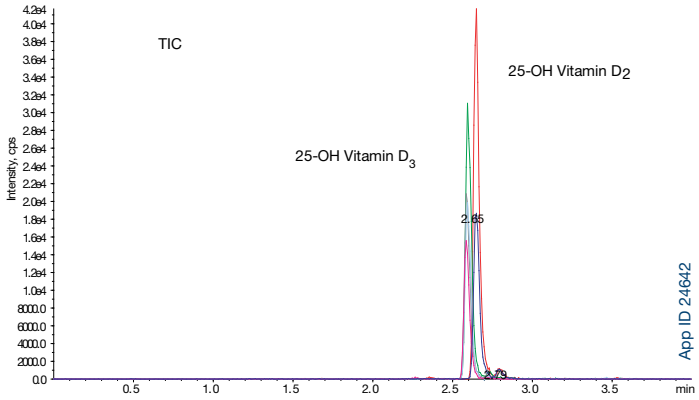

Figure 4.
Representative chromatograms of blank human serum matrix

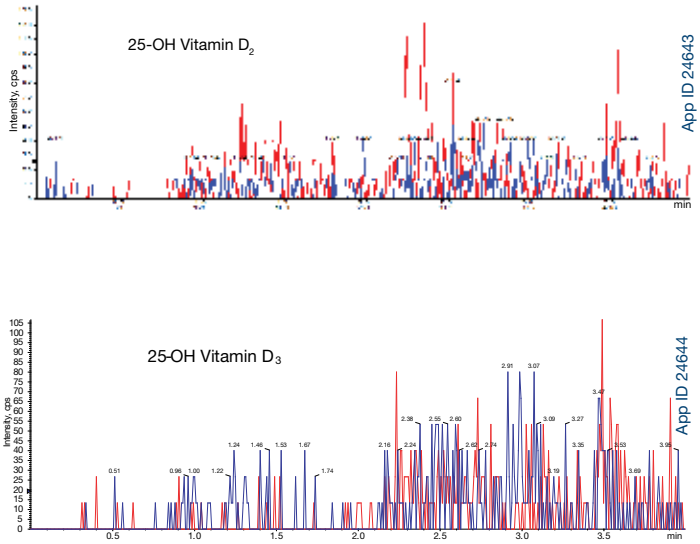

Figure 5.
Representative of the linearity of the curve (n=2)

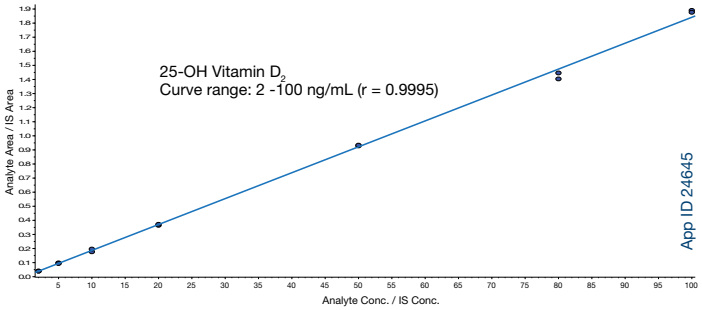

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Table 5.
Accuracy and Precision

	LLOQ	QCL	QCM	QCH
Target Concentration (ng/mL)	2	6	50	80
25-OH Vitamin D_2				
Mean Concentration Found (ng/mL)	2.05	6.26	50.1	82.5
% CV (n=6)	8.8	3.42	3.63	3.5
% Accuracy (n=6)	103	104	100	103
25-OH Vitamin D_3				
Mean Concentration Found (ng/mL)	1.83	6.05	50	84.4
% CV (n=6)	9.78	3.56	3.11	3.84
% Accuracy (n=6)	92	101	100	106

Results and Discussion

Sample Preparation and Cohesive System Set-Up

The on-line extraction is supplemented with an off-line protein precipitation. The precipitating reagent, Methanol/Acetonitrile/Zinc sulfate (5:2:1), has been optimized for both efficient protein removal and acceptable analyte recovery. Protein precipitation is required to prevent 25-OH Vitamin D_2/D_3 from binding to proteins in solution which would otherwise significantly reduce overall method sensitivity.

Table 1 displays the experimental details for the on-line SPE column. Contrary to off-line solid phase extraction, Step 1 (Blue) serves as both the loading and washing steps in this method and as such, the sample is loaded onto the extraction column under 70 % organic, (i.e. 70 % Mobile Phase B). A flow rate of 0.75 mL/min provides a good solvent volume that thoroughly washes the extraction column while the bed length is suitable for analyte retention.

Step 3 (Blue) is the elution of analytes from the extraction column and subsequent transfer to the analytical column. The flow rate for the elution is reduced to 0.35 mL/min and the organic content is increased to 98 % Mobile Phase B to maximize recovery off the extraction column. For **Table 2**, in the same step (3 in Pink) , the elution solvent mixes with a "dilution solvent" of 30 % mobile phase A flowing at 0.35 mL/min prior to reaching the analytical column. This mixing effectively serves as a dilution to mitigate against strong solvent effect to analytical column, since the analytical method (Step 4 in Pink) starts at 90 % Mobile Phase B.

Because the Cohesive system is engineering with focus mode, the dilution solvent and elution solvent's flow rates are additive, so it is important to reduce their combined flow rates equal to or below the starting 0.75 mL/min flow rate for the analytical method in Step 4 (Pink).

Steps 4-8 (Blue) serve to simultaneously wash and re-equilibrate the Strata RP On-line SPE column, while the isocratic LC method runs to completion of the separation of the analytes (Pink).

Assay Performance

To assess the performance of the method, an assay recovery was evaluated in **Table 4**, which is calculated by the peak area ratio of analytes spiked into serum divided by the response for analytes in a neat solution that is passed through both the extraction and analytical columns (TX Mode). This data shows that for both 25-OH Vitamin D_2 and D_3 recovery is greater than or equal to 93.3 %, while the RSD is less than or equal to 1.28 %.

The linear dynamic range of this method was tested with seven calibrators (n=2) from 2-100 ng/mL and the linearity of curve is shown in **Figure 5**, which shows an r=0.9995. Chromatograms of LLOQ and ULOQ are shown in **Figure 2** and **Figure 3**, respectively. The chromatogram for the blank matrix, double charcoal-stripped serum, is shown **Figure 4**, indicating that there is no detectable levels of 25-OH Vitamin D_2/D_3, so it was selected for standards and QCs preparation during the assay evaluation.

This assay was subsequently evaluated using four different level QC's, including LLOQ at n=6 for each sample set. The accuracy and precision is shown in **Table 5**, and it meets GLP environment regulations, respectively.

Conclusion

The combined Strata® RP On-Line SPE and LC analytical method result in a method with a total runtime of less than six minutes. The speed of this method associated with its accuracy and precision make it ideal for the high-throughput research environment.

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Ordering Information
Kinetex® Core Shell LC Columns

2.6 µm MidBore™ Columns (mm)						SecurityGuard™ ULTRA Cartridges‡
Phases	30 x 3.0	50 x 3.0	75 x 3.0	100 x 3.0	150 x 3.0	3/pk
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775 for 3.0 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000.

Ordering Information
Strata® On-Line SPE

On-Line Extraction Columns (mm)			
Phase	50 x 0.5	50 x 1.0	20 x 2.1
Strata RP	00B-S326-AF	00B-S326-A0	00M-S326-AN

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