Application Note 587

An Automated Liquid Handling and Sample Preparation Workflow for LC-MS/MS Analysis of Vitamin D Metabolites, Testosterone, and Cortisol

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Key Words
Versette, ControlMate, Prelude SPLC, SRM, bioanalytical validation

Goal

To develop an automated research workflow that eliminates manual pipetting from the analysis of vitamin D metabolites, testosterone, and cortisol; and to evaluate the accuracy and precision of data acquired using the method.

Introduction

The use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the analysis of vitamin D metabolites, testosterone, and cortisol is well established. This note presents a method for analysis of these compounds that incorporates automated liquid handling and eliminates manual pipetting through the use of a Thermo Scientific™ Versette™ automated liquid handler (Figure 1) for sample preparation. The accuracy and precision of the method were tested using the analysis of 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, testosterone, and cortisol. For validation, the data generated was required to fall within a set of parameters outlined by Thermo Scientific standard operating procedures (SOPs). These parameters include, but are not limited to:

- The lower limit of quantitation (LLOQ) and low quality control (LQC) must be ±20% of the expected concentration.
- All of the remaining calibrators and controls must be ±15%.
- To determine both inter- and intraday accuracy and precision, these requirements must be met for three consecutive days.

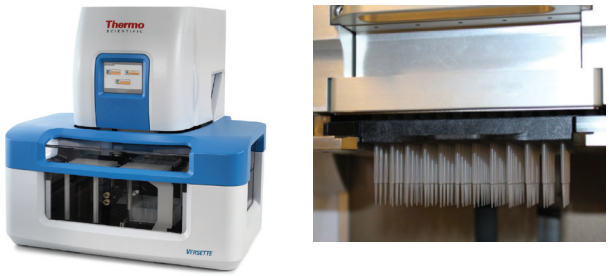

Figure 1. The Versette automated liquid handler (left) and 96-channel head with Thermo Scientific™ D.A.R.T.'S™ tips (right) used for the preparation of vitamin D, testosterone, and cortisol

Experimental

The Versette automated liquid handler was used to aliquot samples, internal standards, and protein precipitation reagents. A Thermo Scientific™ Prelude SPLC™ system was equipped with Thermo Scientific™ TurboFlow™ Cyclone-P™ column (50 x 0.5 mm) and a Thermo Scientific™ Accucore™ PFP analytical column (50 x 2.1 mm, 2.6 µm particle size). The detector for the system was a Thermo Scientific™ TSQ Vantage™ triple-stage quadrupole mass spectrometer equipped with a HESI-II ion source.

Sample Preparation

Samples of 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, and testosterone were prepared in human serum. The cortisol sample was prepared in urine. The optimum sample preparation protocol for each method was created and designed using the Thermo Scientific™ Versette™ ControlMate™ software (shown in Figure 2).

A glass or plastic sample-containing plate was placed onto the Versette stage. Then, 100 µL of each sample vial was aliquoted into a 2 mL, 96-well plate. To each sample, 200 µL of crashing reagent containing internal standard was added. The samples were vortexed for 30 s and centrifuged at 4500 rpm for 3 min. Then, 200 µL of supernatant was transferred into a clean plate for injection.

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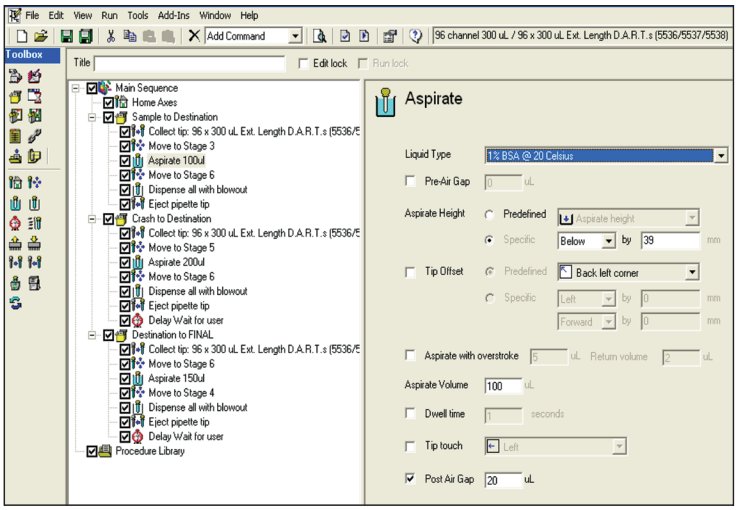

Figure 2. Versette ControlMate software

Liquid Chromatography

LC system	Prelude SPLC system powered by TurboFlow technology
Mobile phases	A: aqueous phase, 10 mM ammonium formate, 0.01% formic acid in water B: organic phase, 10 mM ammonium formate, 0.01% formic acid in methanol C: column wash, 45% isopropyl alcohol, 45% acetonitrile and 10% acetone *All mobile phase solvents were Fisher Chemical brand.*
Needle washes	Aqueous: 60% water, 40% methanol, and 0.5% formic acid Organic: 45% isopropyl alcohol, 45% acetonitrile, and 10% acetone
Columns	TurboFlow Cyclone-P column (50 x 0.5 mm) Accucore PFP analytical column (50 x 2.1 mm, 2.6 µm particle size) encased in a 70 °C column heater

Mass Spectrometry

The mass spectrometer was a TSQ Vantage triple-stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. It was operated in positive ion, selected-reaction monitoring (SRM) mode.

Results and Discussion

Vitamin D

The analytical measurement range for 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 was 2.0–100 ng/mL. The MRM chromatograms at the LLOQ and the calibration curves for 25-hydroxyvitamin D_2 and D_3 are shown in Figures 3 and 4, respectively. The accuracy and precision data for the quality controls for the three days of validation for 25-hydroxyvitamin D_2 and D_3 are listed in Tables 1 and 2, respectively. The coefficient of variability (R^2) for all three days for both analytes ranged from 0.9918 to 0.9980.

Testosterone

The analytical measurement range for testosterone was 0.020–10 ng/mL The SRM chromatogram at the LLOQ and the calibration curve for testosterone are shown in Figure 5. The accuracy and precision data for the quality controls for the three days of validation for testosterone are listed in Table 3. The R^2 values for all three days ranged from 0.9978 to 0.9985.

Cortisol

The analytical measurement range for cortisol was 3.62–362 ng/mL. The SRM chromatogram at the LLOQ and the calibration curve for cortisol are shown in Figure 6. The accuracy and precision data for the quality controls for the three days of validation for cortisol are listed in Table 4. The R^2 values for all three days ranged from 0.9971 to 0.9988.

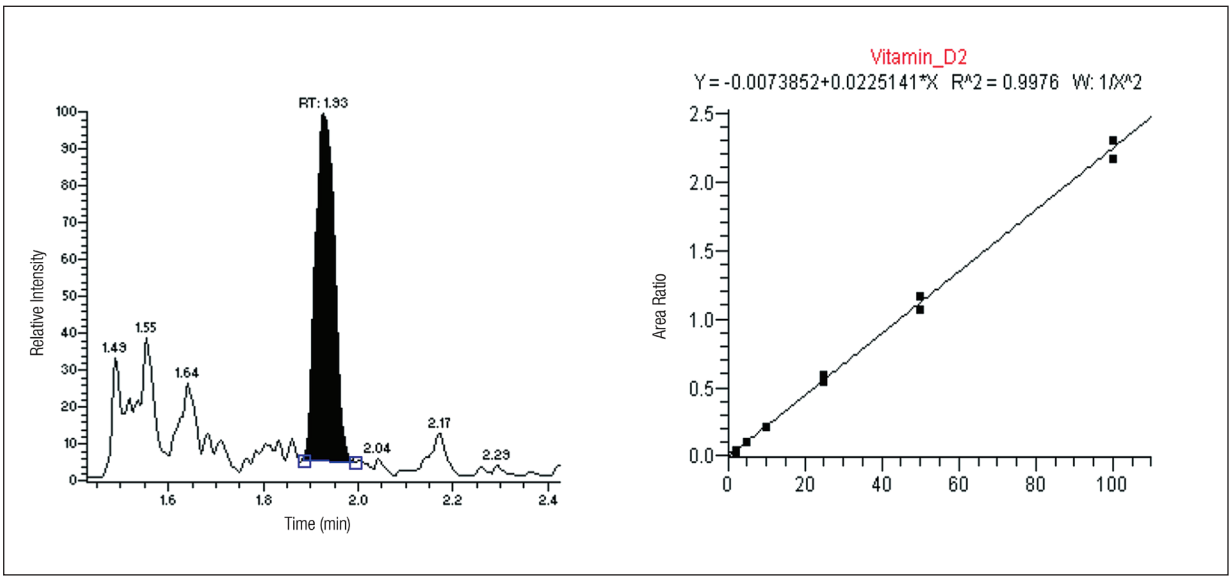

Figure 3. SRM chromatogram of 25-hydroxyvitamin D_2 at the LLOQ and an example calibration curve

Table 1. Accuracy and precision data for 25-hydroxyvitamin D_2

Low QC	Day 1		Day 2		Day 3	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
6.0	6.26	4.3	4.93	-17.8	6.79	13.2
6.0	6.73	12.1	5.46	-9.1	5.18	-13.6
6.0	5.50	-8.4	6.01	0.1	5.30	-11.7
6.0	5.46	-9.1	6.77	12.9	6.12	2.0
6.0	6.00	-0.1	6.43	7.2	5.03	-16.2
Average	5.99	-0.2	5.92	-1.4	5.68	-5.3
SD	0.53		0.74		0.75	
%RSD	8.9		12.5		13.2	
Mid QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
40.0	34.9	-12.8	38.7	-3.4	37.0	-7.5
40.0	38.7	-3.2	41.2	3.1	37.0	-7.6
40.0	36.5	-8.7	39.4	-1.5	38.5	-3.7
40.0	35.0	-12.4	39.2	-2.0	37.9	-5.2
40.0	40.4	0.9	36.7	-8.2	39.6	-1.0
Average	37.1	-7.2	39.1	-2.4	38.0	-5.0
SD	2.38		1.62		1.11	
%RSD	6.4		4.1		2.9	
High QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
80.0	72.3	-9.6	85.1	6.4	80.3	0.4
80.0	75.6	-5.5	80.7	0.9	77.9	-2.7
80.0	74.7	-6.6	81.9	2.3	80.5	0.7
80.0	78.6	-1.8	81.7	2.1	85.6	7.0
80.0	82.5	3.1	84.0	5.0	80.8	1.0
Average	76.7	-4.1	82.7	3.3	81.0	1.3
SD	3.91		1.81		2.82	
%RSD	5.1		2.2		3.5	

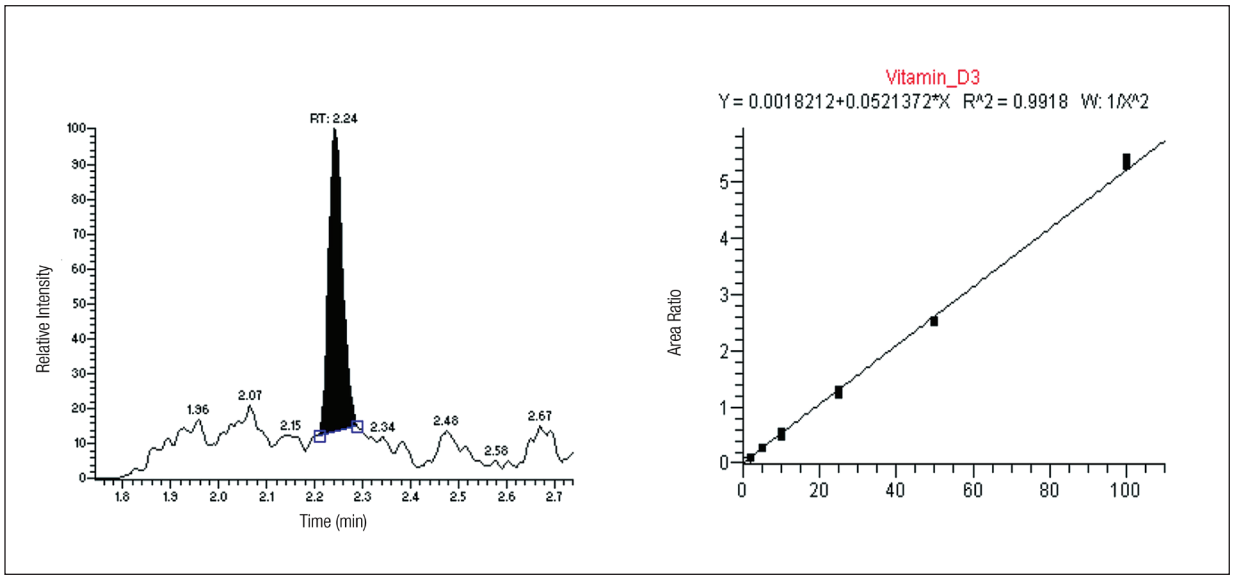

Figure 4. SRM chromatogram of 25-hydroxyvitamin D_3 at the LLOQ and an example calibration curve

Table 2. Accuracy and precision data for 25-hydroxyvitamin D_3

Low QC	Day 1		Day 2		Day 3	
Theoretical Concentration (ng/mL)	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**
6.0	6.24	4.1	6.30	4.9	6.09	1.4
6.0	6.00	-0.1	5.83	-2.8	6.16	2.7
6.0	5.49	-8.5	5.81	-3.2	6.65	10.9
6.0	5.98	-0.4	6.77	12.9	6.65	10.8
6.0	5.35	-10.9	5.63	-6.3	6.49	8.1
Average	5.81	-3.1	6.07	1.1	6.41	6.8
SD	0.38		0.47		0.27	
%RSD	6.54		7.74		4.21	
Mid QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**
40.0	35.6	-11.00	41.9	4.81	39.2	-2.04
40.0	38.6	-3.462	40.3	0.7	37.4	-6.50
40.0	39.0	-2.607	37.7	-5.65	39.0	-2.57
40.0	36.0	-9.97	38.8	-3.08	40.8	1.97
40.0	41.2	2.92	38.7	-3.14	38.1	-4.79
Average	38.1	-4.8	39.5	-1.3	38.9	-2.8
SD	2.29		1.64		1.28	
%RSD	6.01		4.15		3.29	
High QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**
80.0	78.5	-1.8	81.2	1.5	81.7	2.1
80.0	83.5	4.4	79.2	-1.0	81.4	1.7
80.0	79.2	-1.0	79.3	-0.9	77.7	-2.9
80.0	82.0	2.5	77.9	-2.7	80.9	1.1
80.0	80.3	0.4	78.2	-2.2	84.1	5.2
Average	80.7	0.9	79.2	-1.1	81.2	1.4
SD	2.04		1.28		2.32	
%RSD	2.5		1.62		2.86	

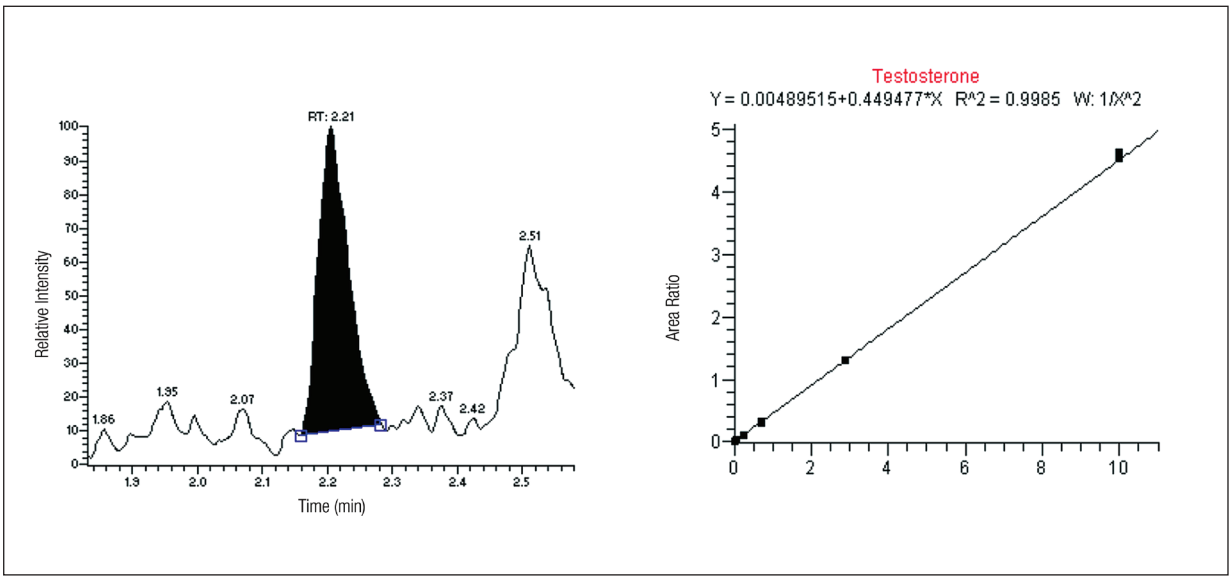

Figure 5. SRM chromatogram of testosterone at the LLOQ and an example calibration curve

Table 3. Accuracy and precision data for testosterone

Low QC	Day 1		Day 2		Day 3	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
0.06	0.064	6.18	0.054	-10.06	0.054	-10.42
0.06	0.058	-3.44	0.063	5.50	0.061	1.13
0.06	0.061	1.84	0.058	-3.95	0.058	-2.52
0.06	0.063	4.91	0.064	5.99	0.057	-5.31
0.06	0.059	-1.18	0.059	-1.30	0.062	3.40
Average	0.061	1.66	0.059	-2.45	0.059	-2.74
SD	0.002		0.004		0.003	
%RSD	3.28		6.72		5.09	
Mid QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
0.45	0.438	-2.58	0.434	-3.53	0.413	-8.20
0.45	0.450	0.05	0.422	-6.23	0.435	-3.32
0.45	0.468	4.05	0.456	1.33	0.422	-6.23
0.45	0.461	2.54	0.439	-2.34	0.436	-3.06
0.45	0.435	-3.36	0.451	0.23	0.415	-7.87
Average	38.1	0.14	0.44	-2.11	0.42	-5.74
SD	2.29		0.014		0.011	
%RSD	6.01		3.18		2.59	
High QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
8	8.04	0.54	7.89	-1.35	7.85	-1.82
8	7.88	-1.49	7.84	-2.02	8.18	2.24
8	7.89	-1.41	8.07	0.91	8.12	1.44
8	7.95	-0.68	8.04	0.55	8.18	2.25
8	7.84	-2.00	7.96	-0.46	7.98	-0.28
Average	7.92	-1.01	7.96	-0.47	8.06	0.77
SD	0.079		0.099		0.142	
%RSD	1.00		1.24		1.76	

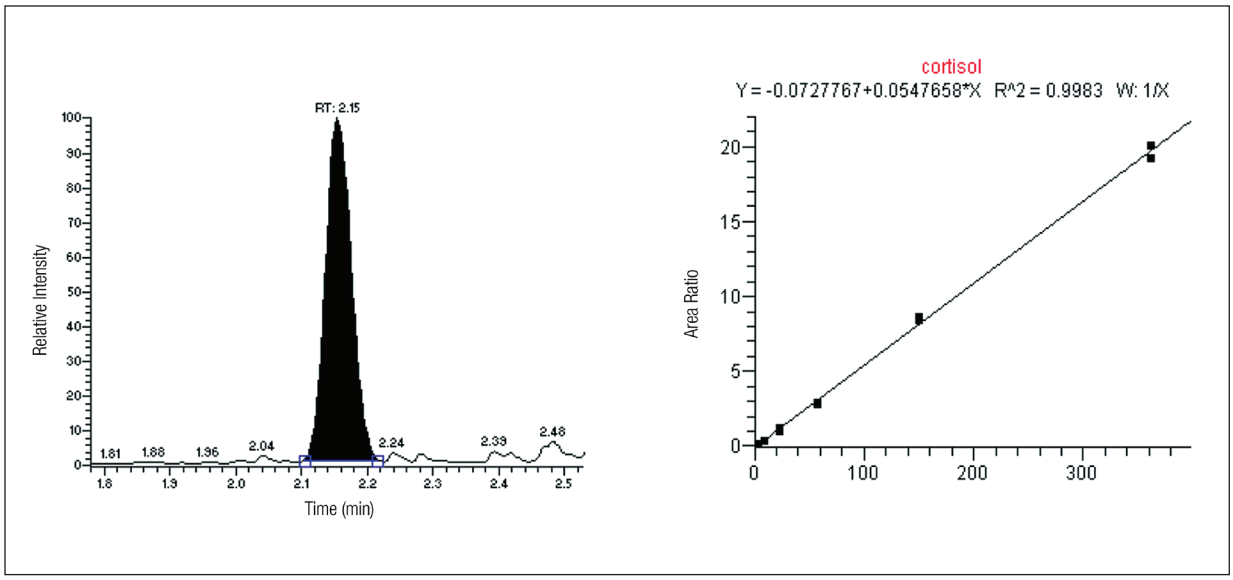

Figure 6. SRM chromatogram of cortisol at the LLOQ and an example calibration curve

Table 4. Accuracy and precision data for cortisol

Low QC	Day 1		Day 2		Day 3	
Theoretical Concentration (ng/mL)	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**
10.0	10.9	9.3	9.3	-7.1	10.4	3.5
10.0	10.2	2.3	9.5	-5.5	9.8	-1.6
10.0	9.5	-4.7	9.8	-1.7	10.6	6.2
10.0	9.7	-3.4	9.6	-3.6	11.0	9.6
10.0	n/a	n/a	10.7	7.2	10.6	6.1
Average	10.09	0.9	9.79	-2.1	10.48	4.8
SD	0.64		0.56		0.42	
%RSD	6.34		5.72		4.01	
Mid QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**
145.0	136	-6.52	132	-8.67	141	-2.93
145.0	138	-4.70	128	-12.0	144	-0.64
145.0	130	-10.25	138	-4.94	148	2.25
145.0	137	-5.50	141	-2.98	140	-3.43
145.0	134	-7.70	132	-9.14	144	-0.76
Average	134.9	-6.9	134.1	-7.5	143.4	-1.1
SD	3.14		5.17		3.27	
%RSD	2.33		3.86		2.28	
High QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**	**Measured Concentration (ng/mL)**	**%Difference**
290.0	278	-4.1	286	-1.4	313	7.9
290.0	298	2.7	NA	36.4	318	9.5
290.0	317	9.5	275	-5.3	334	15.1
290.0	311	7.3	291	0.3	315	8.7
290.0	310	6.8	300	3.5	317	9.3
Average	302.8	4.4	288.0	6.7	319.3	10.1
SD	15.54		10.40		8.34	
%RSD	5.10		0.04		2.61	

Conclusion

- The Versette automated liquid handler provided accuracy and precision with high efficiency. All methods were validated, with the R^2 values ranging from 0.9918 to 0.9988.
- The Versette automated liquid handler demonstrated 90% time savings over manual pipetting and passed all acceptance criteria for the compounds tested. Approximate sample preparation time for one full 96 well plate for these methods, when performed manually, ranges from 2 to 3 hours; whereas the Versette automated liquid handler sample preparation time was 10–15 minutes. This minimized technician sample handling and saved time, money, and resources.
- The Versette system, in combination with the Prelude SPLC system and the TSQ Vantage mass spectrometer, allowed for highly automated preparation of samples for LC-MS/MS research.

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