

# High Throughput Reliable Quantitation of 25-hydroxyvitamin D in Serum by Offline Sample Preparation and a LC-MS/MS Instrument

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## INTRODUCTION

Vitamin D is a group of fat-soluble hormones, which have the two major forms: D2 (ergocalciferol) and D3 (cholecalciferol). The metabolites of vitamin D have a critical physiological function to maintain calcium and phosphate homeostasis. Vitamin D deficiency can be best diagnosed using 25(OH) vitamin D versus the other vitamin D metabolites because 25(OH) vitamin D levels in serum reflect the body's storage levels of vitamin D and correlate with the clinical symptoms of vitamin D deficiencies.[1,2] A simple and fast offline sample preparation coupled to a sensitive LC-MS/MS tandem mass spectrometer has been developed to simultaneously measure 25(OH) vitamin D3 and 25(OH) vitamin D2 over a commercial level I to IV analytical concentration range in human serum.

## METHOD

### Chemicals and Solvents

25(OH) vitamin D was purchased from Sigma (Milwaukee, WI) and vitamin D free human serum was purchased from Golden Western Biologicals (Temecula, CA). Serum level I to IV and Recipe 25(OH) vitamin D quality controls were purchased from IRIS (Olathe, KS). All of the chemicals were stored in the freezer. No IS was used.

### Sample Extraction

Sample preparation was carried out with the Orochem (Naperville, IL) PURITY Phospholipid Depletion Kit 96-well plate. The eluting step was performed with an Orochem Ezpress™ positive pressure manifold. Refer to **Table 1** for the steps used.

**Table 1: Steps & Procedure**

Step	Procedure
Load 1	300 µL of Vitamin D commercial precipitation reagent
Load 2	100 µL of serum sample, wait for 5 minutes,
Elution	apply a few pressure pulse until all solution passes through

### Mass Spectrometry Conditions

The LC-MS/MS analysis was performed using IONICS 3Q 220 triple quadrupole mass spectrometer. **Table 2** outlines the MS instrumental source parameter settings. The optimized MRM transition parameters for 25(OH) vitamin D are shown in **Table 3**.

**Table 2: MS Conditions**

ESI Voltage (V)	5050
HSID Temp (°C)	175
Nebulizer Gas Setting	450
Drying Gas Setting	120
Source Temp (°C)	350

**Table 3: Optimized MRM Parameters**

Compound Name	Precursor (m/z)	Fragment (m/z)	CCL	CE
25(OH) VD3	401.3	257.2	-51	23
	401.3	383.2	-60	13
25(OH) VD2	413.3	355.2	-55	16
	413.3	395.2	-60	13

### LC Conditions

Shimadzu UFLCxr system was used with a Imtakt Cadenza C18 –HT (2.1X 50mm) 3 µm particle size column. The LC was run with a gradient flow with a run time of 5min and the following conditions:

Mobile Phase: A (H<sub>2</sub>O, 0.1% Formic Acid, 5mM NH<sub>4</sub>OAc)  
 B (MeOH, 0.1% Formic Acid, 5mM NH<sub>4</sub>OAc)  
 Flow Rate: 0.6 mL/min  
 Injection Volume: 10 µL  
 Column Temperature: 32 °C

Time(min)	0.1	0.5	2.8	3.1	3.2	5
B%	10	70	100	100	10	10

## RESULTS

### Sample Extraction Results

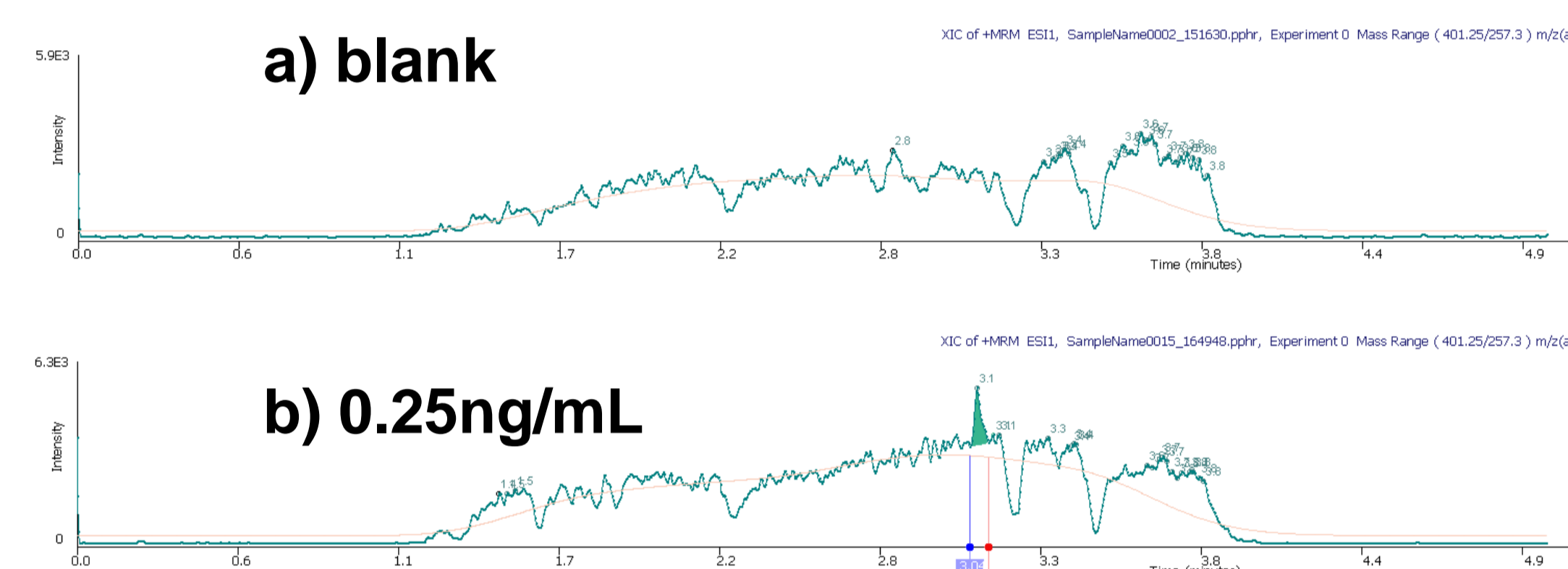
The extraction recovery rate on samples using PURITY Phospholipid Depletion Kit 96-well plate are about 60 and 65% for 25(OH) VD3 and 25(OH) VD2, respectively. Overall the extraction efficiency is about 50% for serum samples. Summary of the extraction performance is shown in **Table 4**:

**Table 4: Sample Extraction Performance.**

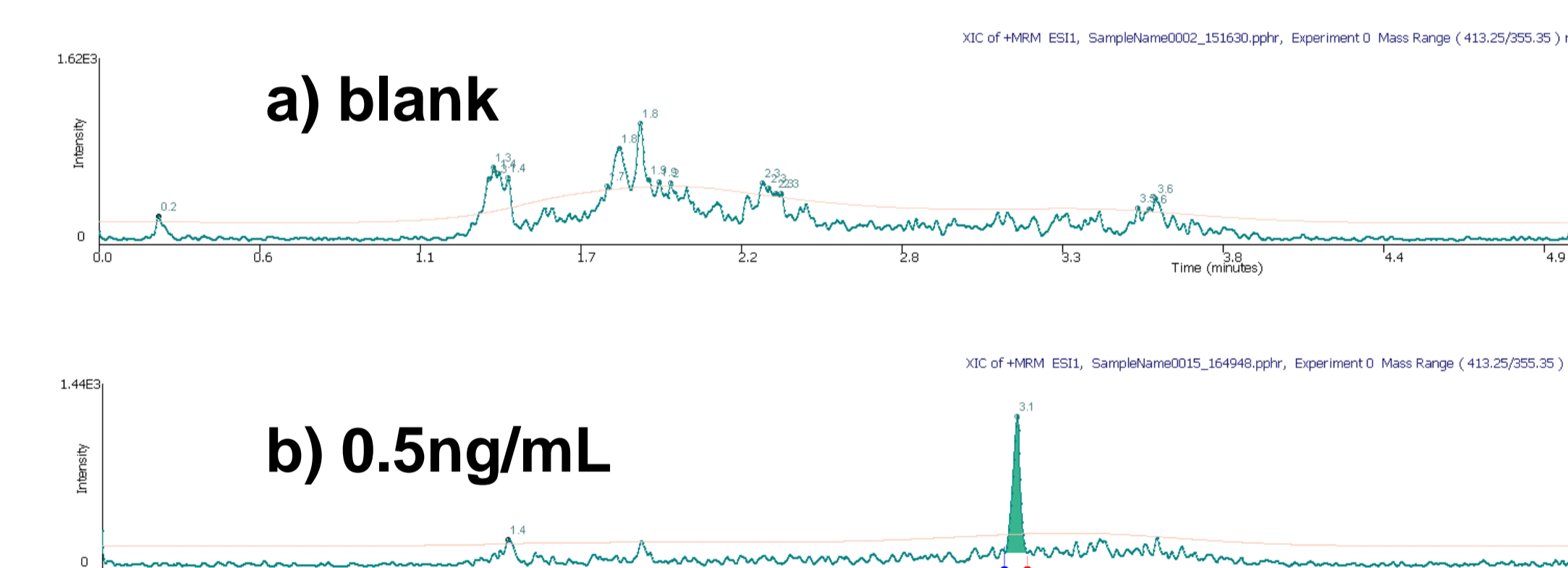
%	25-(OH)-VD <sub>3</sub>	25-(OH)-VD <sub>2</sub>
Recovery rate	57.9	64.6
Matrix effect	87.5	74.9
Process efficiency	50.6	48.4

### Extracted Ion Chromatograms (EICs)

EIC Chromatogram in serum blank and spiked one with 0.25 and 0.5 ng/mL 25 -Hydroxyvitamin D<sub>3</sub> and D<sub>2</sub> is shown in **Figure 1a-b & 2a-b**.



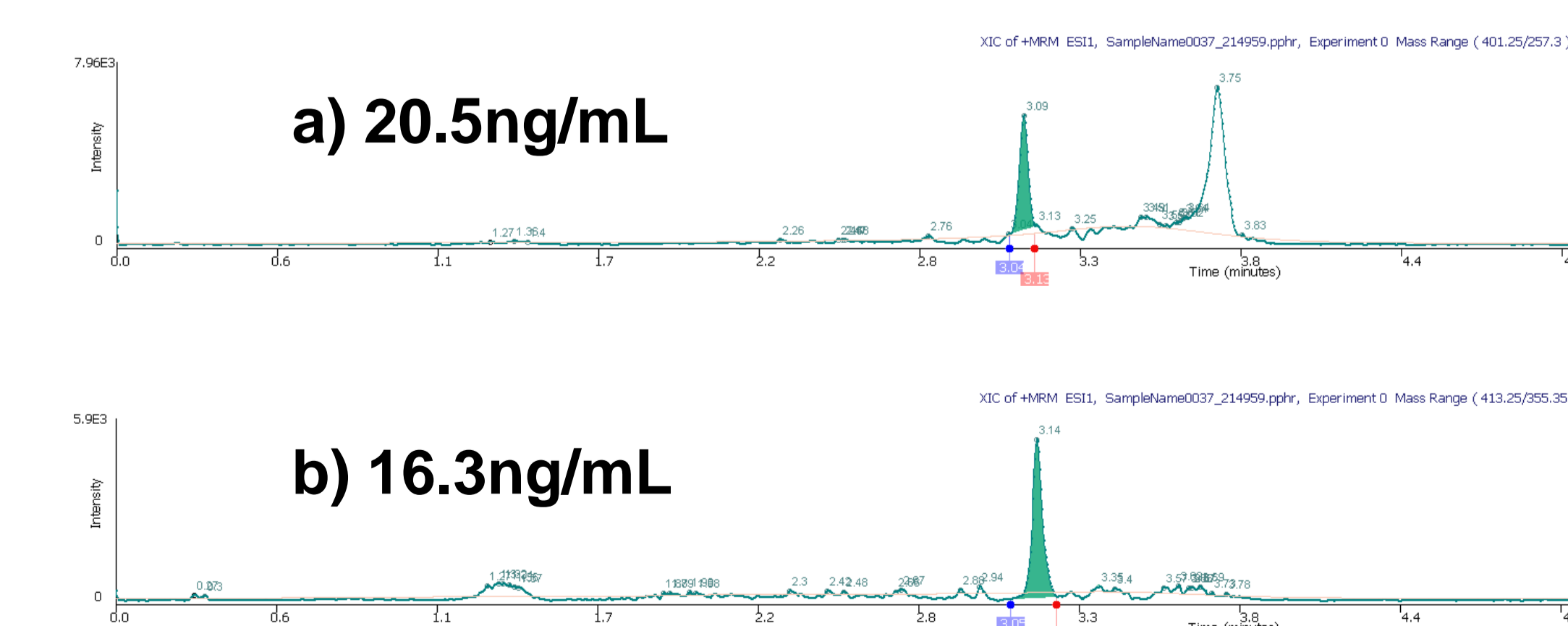
**Fig. 1. Chromatograms of 25 -Hydroxyvitamin D<sub>3</sub> for blank and 0.25ng/mL.**



**Fig. 2. Chromatograms of 25 -Hydroxyvitamin D<sub>2</sub> for blank and 0.5ng/mL.**

### Extracted Ion Chromatograms for QC samples

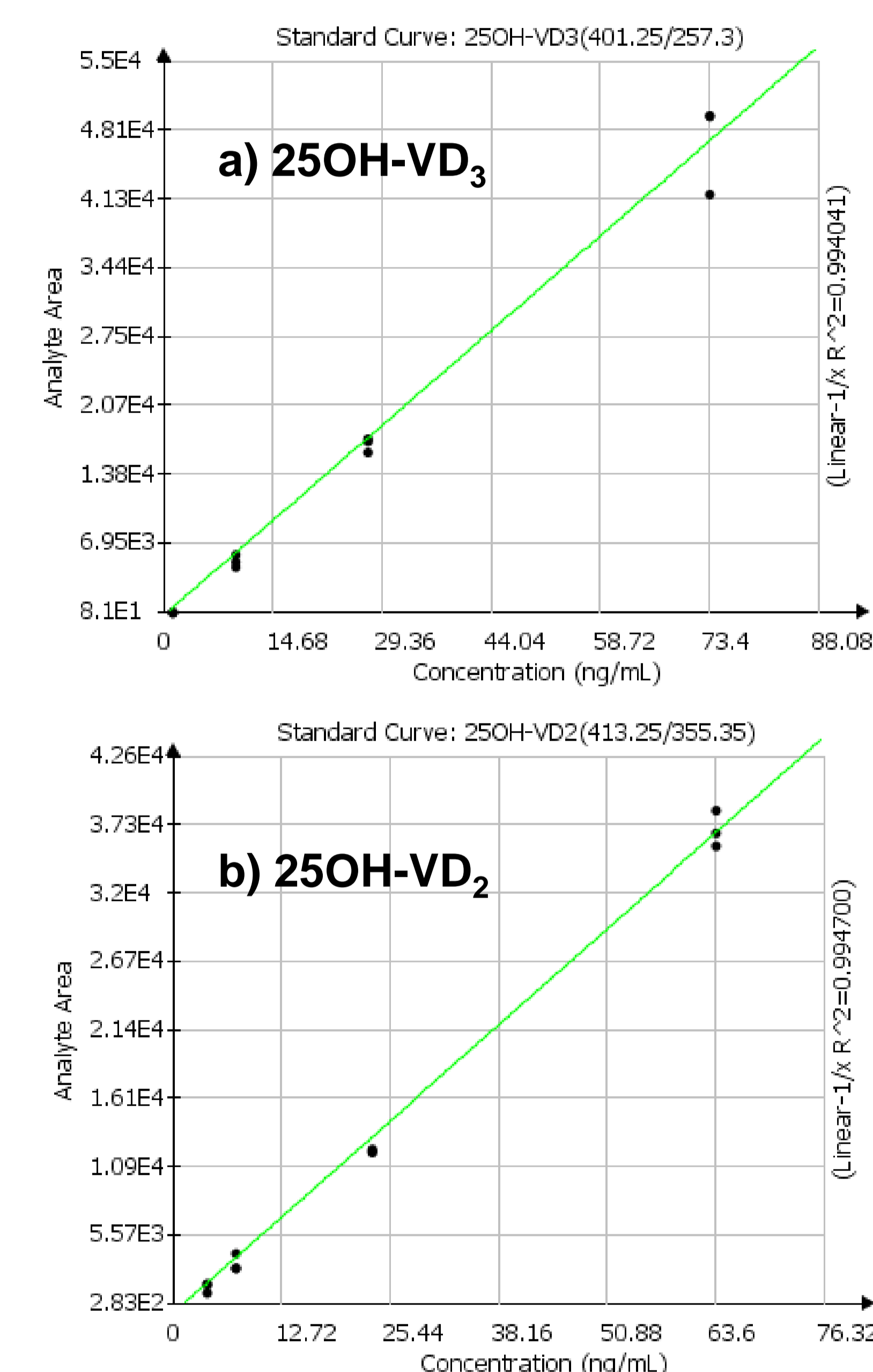
Representative chromatograms of 25 -Hydroxyvitamin D<sub>3</sub> and D<sub>2</sub> for a Recipe level I serum control (20.5 and 16.3 ng/mL, respectively) in this study, are shown in **Figure 3a-b**.



**Fig. 3. Chromatograms of 25 -Hydroxyvitamin D quality control level I.**

### Quantitation Results

The calibration curves generated for 25-hydroxyvitamin D<sub>2</sub> (413.2/355.2) and 25-hydroxyvitamin D<sub>3</sub> (401.3/257.2) show injections which covers a concentration range of nearly 2 orders of magnitude from 1.1 to 73.4 ng/mL for 25-hydroxyvitamin D<sub>3</sub> (413.2/355.2) and from 3.9 to 63.6 for 25-hydroxyvitamin D<sub>2</sub> (401.3/257.2) (**Figure 4a-b**, respectively). The linear regression has a weighting factor, 1/x. Good linearity (R<sup>2</sup>>0.994) was found for both analytes. Level I and II Recipe quality controls results with 3 injections were found to be excellent as shown in summary **Table 5**.



**Fig. 4. Calibration curves of 25 -Hydroxyvitamin D<sub>3</sub> and D<sub>2</sub> .**

**Table 5: Level I and II QCs quantification results (n=3) .**

25 -Hydroxyvitamin D <sub>3</sub>			25 -Hydroxyvitamin D <sub>2</sub>		
Conc. (ng/mL)	Avg. accuracy(%)	CV (%)	Conc. (ng/mL)	Avg. accuracy(%)	CV (%)
20.5	92.6	8.3	16.3	95.7	4.0
44.3	101.3	1.1	36.6	101.0	1.9

## CONCLUSION

A 5-min, sensitive, and reliable LC-MS/MS method was developed for quantitative determination of 25(OH) vitamin D in human serum. The LLOQ achieved by IONICS 3Q 220 triple quadrupole mass spectrometer for 25-OH-D<sub>3</sub> and 25-OH-D<sub>2</sub> in human serum are 0.25 and 0.5 ng/mL, respectively. The load, filter two-step simple method showed no signs of interferences. The results show a good linearity and selectivity over level I to IV Recipe calibrators. The offline sample preparation for this LC-MS/MS method is simple and well suited for routine clinical analysis of 25(OH) vitamin D.

## REFERENCES

- [1] Reinhold Vieth, Am J Clin Nutr 1999;69:842–56.
- [2] Robert P. Heaney, Clin J Am Soc Nephrol 3: 1535–1541, 2008.