Enhanced LC-MS Sensitivity of Vitamin D Assay by Selection of Appropriate Mobile Phase

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Abstract

Liquid chromatography mass spectrometry is the best choice for the accurate measurement of vitamin D in clinical samples. Indeed, LC-MS/MS has been coined the "gold standard method" for vitamin D analysis. The major metabolite of vitamin D is 25-hydroxy vitamin D that circulates in blood plasma with extended half-life. In this report, we studied the increase in detection sensitivity of two metabolites: 25-hydroxy vitamin D_2 (m/z 413) and 25-hydroxy vitamin D_3 (m/z 401) utilizing selected ion monitoring (SIM) in positive mode ionization.

LC-MS methods often encounter ion suppression and reduced sensitivity of the target compound. Accordingly, we performed the LC-MS analysis of 25-hydroxy vitamin D using various formulations of the mobile phase produced using additives such as volatile organic acids, low salt or even mobile phase without additives. Small variations in pH and ionic strength introduced substantial changes in the SIM response for 25-hydroxy vitamin D_2 and D_3. Response comparisons were performed using both electro spray ionization (ESI) and jet stream ionization. The jet stream ionization showed a significant increase in sensitivity compared to ESI when mobile phase was used without any additive. A superior response was observed for low pH mobile phase in ESI. Trace amounts of vitamin D could be detected by fine tuning the mobile phase composition along with the instrument parameters using low pH mobile phases. To our knowledge, this is the first report of a comparative study of mobile phase effects on vitamin D detection sensitivity in LC-MS.

Introduction

- Vitamin D regulates calcium metabolism. Vitamin D deficiency leads to rickets and osteomalacia and is also associated with breast cancers, multiple sclerosis, dementia, rheumatoid arthritis, diabetes, Parkinson's and Alzheimer's diseases[1,2]. Vitamin D exists in two forms, namely vitamin D_2 and vitamin D_3, and is metabolized to 25-hydroxy vitamin D_2 and 25-hydroxy vitamin D_3. The half-life and concentration of 25-hydroxy vitamin D (25-OH-D) in blood is the longest and highest among vitamin D metabolites because 25-OH-D predominantly binds vitamin D binding protein[3,4,5].
- The long half-life of 25-OH-D makes its measurements ideal for assessing vitamin D level in the patient's sample. Therefore, an analytical method that can accurately quantify both forms of 25-OH-D is necessary to effectively diagnose and monitor patients who have vitamin D disorders.
- In this report, the detection response of 25-hydroxy vitamin D_2 and D_3 by single ion monitoring is evaluated with subtle change of ionic strength and pH of the mobile phase. The parent ions were monitored under the same analysis conditions to detect trace levels of analyte by LC-MS using standard compounds from NIST.
- Commercially available vitamin D_2 drop and vitamin D_3 soft gel capsules were purchased separately and the materials tested for further evaluation of the assay.

Materials and Methods

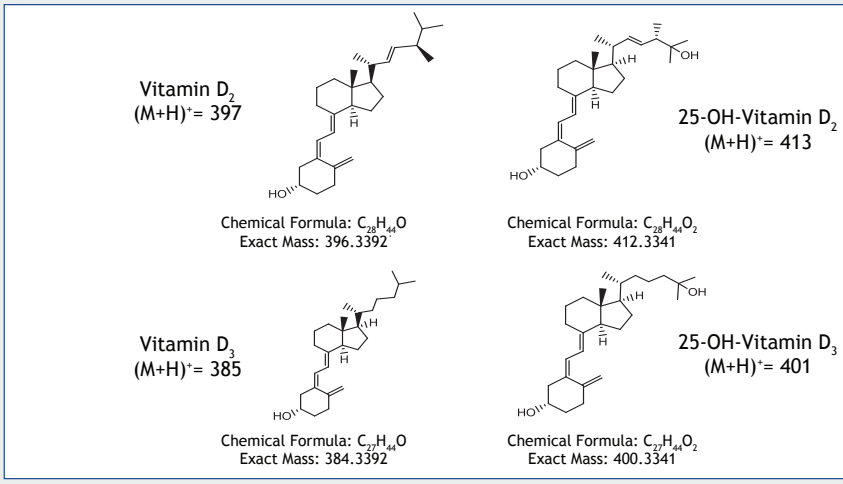
- HPLC-MS: Agilent 1100 HPLC equipped with auto-sampler and diode array detector (DAD) attached to Agilent MSD single quadrupole mass spectrometer. Agilent 1260 RRLC equipped with auto-sampler and diode array detector (DAD) attached to Agilent 6150 MSD single quadrupole mass spectrometer operated by jet stream technology.
- Column: Agilent Zorbax XDB C-18 (150 x 3 mm, 5 micron), Catalog No: 993967-302 and Poroshell 120 EC-C18 (50 x 4.6 mm, 2.7 micron), Catalog No: 69975-902

- Flow: 0.5 mL/min
- Column temperature: 30° C
- Injection volume: 5 µL
- Solvents for mobile phase were Optima® LC/MS grade (A456 Methanol and W6 Water)
- Mobile phase A: Water and other additives in water
- Mobile phase B: Methanol and other additives in methanol
- Additives:
  - 10 mM Ammonium Formate + 0.05% Formic Acid (MPS1)
  - 5 mM Ammonium Formate + 0.05% Formic Acid in Water, 0.05% Formic Acid in Methanol (MPS2)
  - 0.05% Formic Acid (MPS3)
  - 0.1% Formic Acid (MPS4)
- 25-Hydroxy vitamin D_2 and vitamin D_3 were purchased from NIST (Cat # SRM2972)
- Vitamin D_2 drop (1000 IU per drop) and vitamin D_3 soft gel capsules (5000 IU per capsule) were purchased from Walgreens
- Vitamin D_2 drop (0.2 mL) was diluted to 1 mL using a 10% methylene chloride (DCM) in methanol mixture. Serial dilution of the stock was made in methanol to 10, 100, 1000 and 10,000-fold for LC-MS analysis
- Vitamin D_3 soft gel capsule (1 capsule) was punctured with a needle and the oil was transferred to a 1 mL volumetric flask. A mixture of 10% methylene chloride in methanol was used to dissolve the material. The solution was further diluted to 10, 100, 1000 and 10,000-fold in methanol from the stock for LC-MS analysis.
- Gradient

Time (min)	%MPA	%MPB
0	60	40
1	60	40
2	25	75
3	15	85
4	5	95
20	5	95

- Mass spectrometry conditions: positive mode, electro spray ionization (ESI) and thermal focusing jet stream electro spray ionization (TFJS-ESI)
  - Fragmentor: 70V
  - Gas Temperature: 250° C
  - Drying gas: 11.0 l/min
  - Gas flow: 5.0 l/min
  - Neb gas: 30 psig
  - Capillary: 3500 V
- 25-Hydroxy vitamin D_2 (m/z 413) and 25-hydroxy vitamin D_3 (m/z 401), vitamin D_2 (m/z 397) and vitamin D_3 (m/z 385) ions are monitored by selected ion monitoring (SIM)

Structures



Results

- SIM of parent ions of 25-hydroxy vitamin D_2 and D_3 was evaluated by:
  1. Effect of dry gas temperature on the response of analyte is shown in Figs. 1-3. A decrease in response was noted at higher dry gas temperature (350° C) compared to lower temperature (250° C).
  2. Both freshly prepared and 1 year old mobile phase containing 10 mM ammonium formate and 0.05% formic acid in water (MB123) and methanol (MB122) showed similar response at 250° C for 25-OH vitamin D_2 and D_3 by ESI (Figs. 2 and 3).
  3. Combination of different ionic strength and acid concentration in mobile phase showed varying response of analytes in five different mobile phases (Figs. 4-6) both by ESI and TFJS-ESI. Ionic strength of the buffer was reduced from 10 mM to 5 mM to 0 mM, keeping percent of acid (0.05%) constant in each. A mobile phase of 0.1% formic acid was also tested.
- Water/methanol mobile phase showed superior response in TFJS-ESI whereas regular ESI showed poor response with the same sample (injected from same vial) under identical analysis conditions (Figs. 5 and 6).
- Linearity of assay is shown in Figs. 7 and 8 using 25-OH vitamin D from 2 to 100 ng/mL concentration.
- Recovery of standard is shown in Figs. 9 and 10 using 2 to 300 ng/mL concentration of 25-OH vitamin D. Recovery of the standard was found better from 20 to 200 ng/mL for 25-OH vitamin D_2 (~100%); for 25-OH vitamin D_3 a recovery of 77 – 110% was observed in 2 to 300 ng/mL range.
- Analysis of commercially available vitamin D_2 drop and vitamin D_3 soft gel capsules is shown in the top panel of Fig. 11 with overlaid chromatograms of standard 25-OH vitamin D_2 (blue trace) and 25-OH vitamin D_3 (red trace). Bottom panels showed the SIM (m/z 397) chromatograms of 100- and 1000-fold diluted vitamin D_2 from commercially available vitamin D_2 drop.
- Fig. 12 provides the SIM response of vitamin D_2 drop and vitamin D_3 soft gel capsules.

Fig. 1. Dry Gas Temperature Set at 350º C

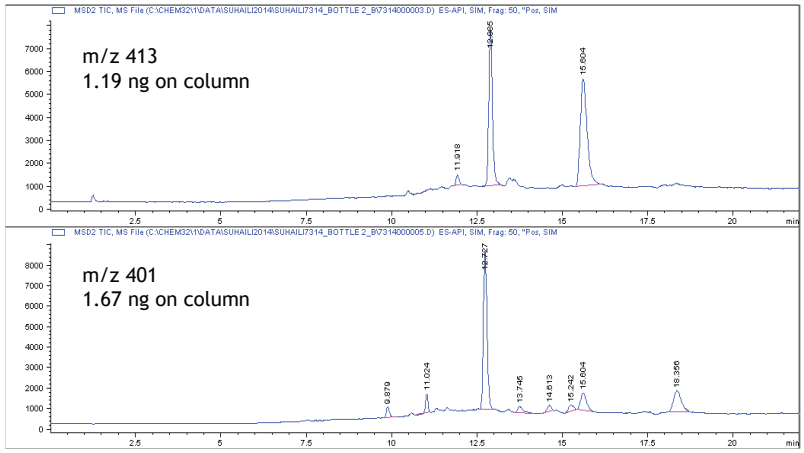


Fig. 2. Dry Gas Temperature Set at 250º C

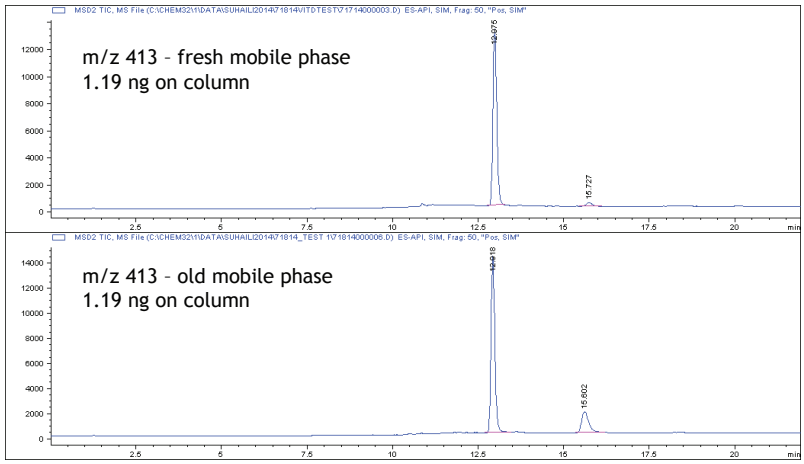


Fig. 3. Dry Gas Temperature Set at 250° C

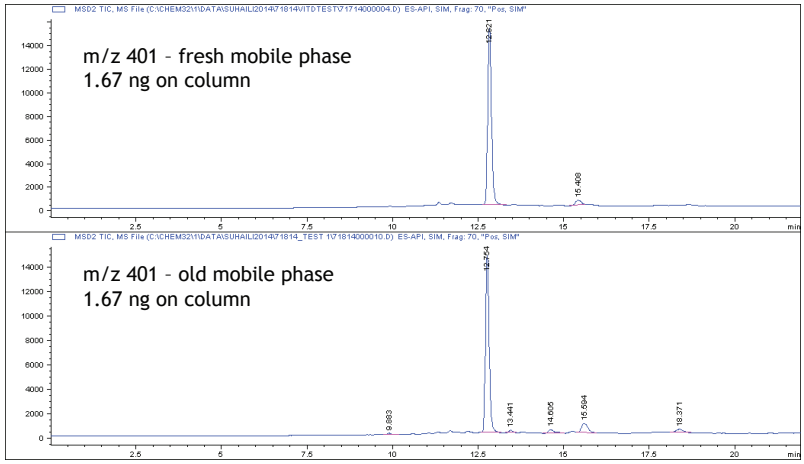


Fig. 4. Response of 25-Hydroxy Vitamin D With Change of Mobile Phase Using Positive Mode Electro Spray Ionization

Mobile phase	MPS1	MPS2	MPS3	MPS4	MPS5
Plot number	1	2	3	4	5
m/z 413 - Area	1.13E+05	3.04E+05	5.07E+05	2.68E+05	4.60E+04
m/z 413 - Height	1.46E+04	3.91E+04	6.57E+04	3.58E+04	6.36E+03
m/z 401 - Area	1.25E+05	2.23E+05	7.11E+05	4.09E+05	8.40E+04
m/z 401 - Height	1.64E+04	2.97E+04	9.43E+04	5.46E+04	1.17E+04

1. 10 mM ammonium formate, 0.05% formic acid in water/methanol – MPS1
2. 5 mM ammonium formate, 0.05% formic acid in water, 0.05% formic acid in methanol – MPS2
3. 0.05% formic acid in water and methanol – MPS3
4. 0.1% formic acid in water/methanol – MPS4
5. Water/methanol – MPS5

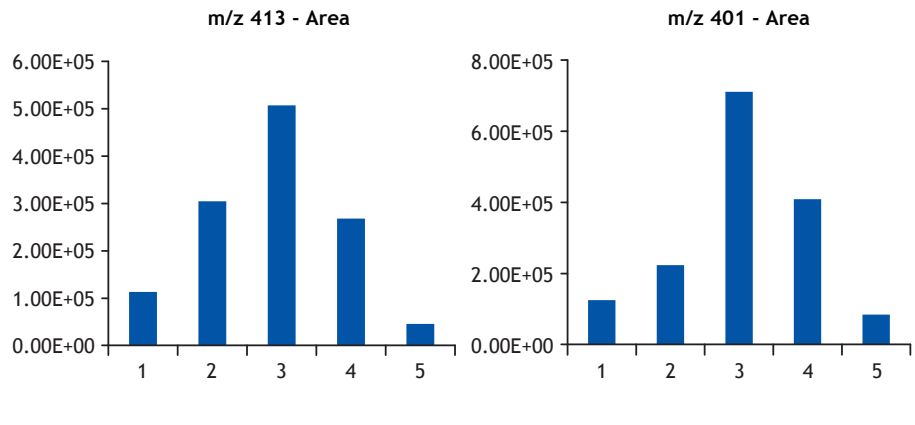


Fig. 5. Response of 25-Hydroxy Vitamin D With Change of Mobile Phase Using Positive Mode Jet Stream Ion Focusing

1260 System	MB123/MB122		0.1% Formic Acid in Water/Methanol		Water/ Methanol	0.05% Formic Acid in Water/Methanol
Peak by SIM	MPS1		MPS4		MPS5	MPS3
m/z 413 - Area	1.35E+05	1.40E+05	7.85E+05	8.14E+05	1.09E+06	9.58E+05
m/z 413 - Height	2.61E+04	2.70E+04	1.57E+05	1.63E+05	2.13E+05	1.91E+05
m/z 401 - Area	1.65E+05	1.64E+05	1.13E+06	1.07E+06	1.29E+06	1.27E+06
m/z 401 - Height	3.26E+04	3.20E+04	2.26E+05	2.15E+05	2.56E+05	2.52E+05
Plot Number	1	2	3	4	5	6

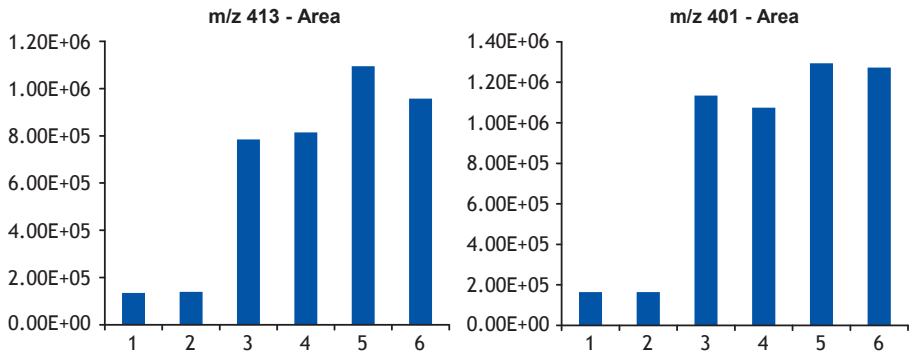


Fig. 6. Different Response in Regular ESI

Agilent 1100 System	Exp 1		Exp 2		Exp 3	
MPA	Water - 0.05% Formic Acid		Water (No Formic Acid)		Water - 0.05% Formic Acid	
MPB-	Methanol - 0.05% Formic Acid		Methanol (No Formic Acid)		Methanol - 0.05% Formic Acid	
m/z 413 - Area	5.06E+05	5.08E+05	4.64E+04	4.55E+04	5.55E+05	5.62E+05
m/z 401 - Area	7.05E+05	7.17E+05	8.56E+04	8.23E+04	8.66E+05	8.30E+05
	1	2	3	4	5	6

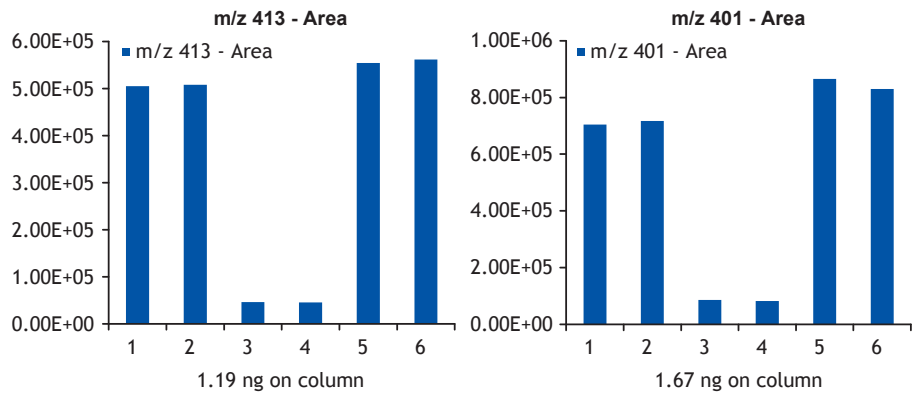


Fig. 7. Linearity Assay m/z 413

Linearity assay using 0.05% formic acid in water/methanol (ESI) - injection volume 5 µL

Conc (ng/mL)	m/z 413 - Area	m/z 413 - Height
2	2574.76	412.03
5	5433.33	836.37
20	28316.5	3424.83
25	31940.8	3897.87
50	58793.4	7396.83
100	113630	14350.2

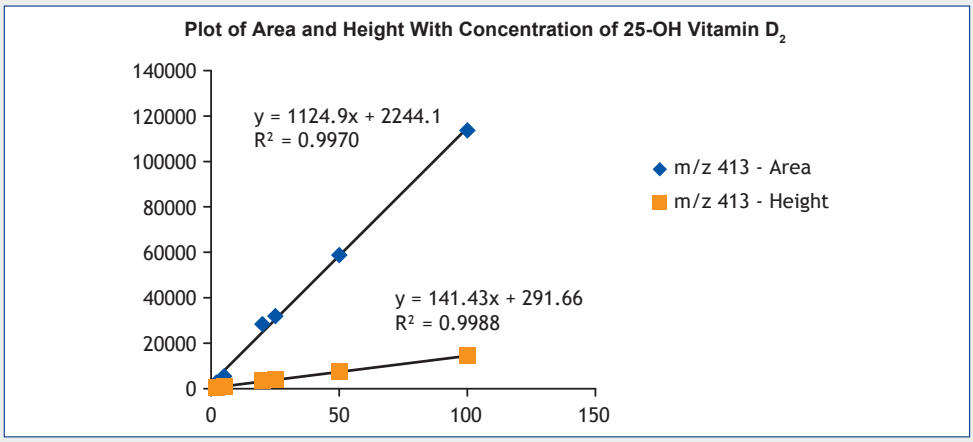


Fig. 8. Linearity Assay m/z 401

Conc (ng/mL)	m/z 401 - Area	m/z 401 - Height
2	2254.82	338.75
5	4254.84	629.8
10	8392.73	1207.07
25	29401.3	3933.2
50	53360.6	7130.2
100	126071	16789.9

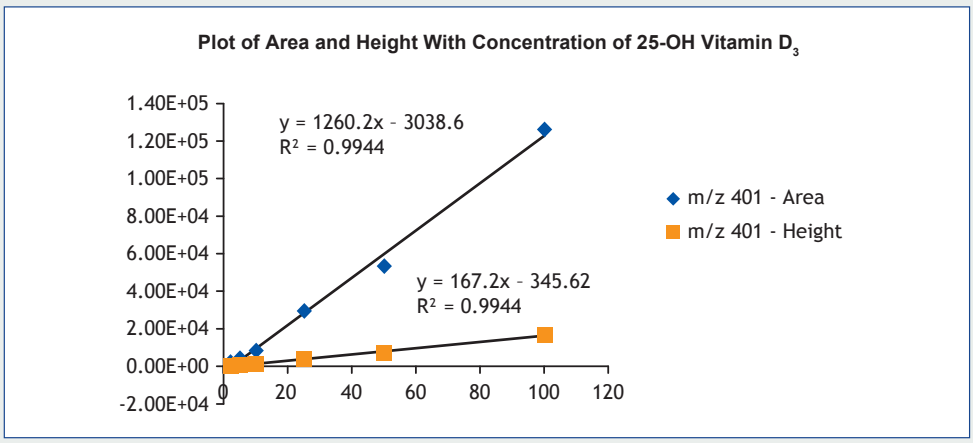


Fig. 9. Recovery of 25-OH Vitamin D_2

Conc. (ng/mL)	Retention Time (min)	Area D2	Recovery From Area (ng/mL)	% Recovery
5	12.88	1.65E+04	2.63	52.6
10	12.90	2.56E+04	6.52	65.2
20	12.91	5.71E+04	20.03	100.1
25	12.90	6.90E+04	25.13	100.5
50	12.91	1.31E+05	51.75	103.5
100	12.92	2.61E+05	107.14	107.1
238	12.92	5.59E+05	234.82	98.66

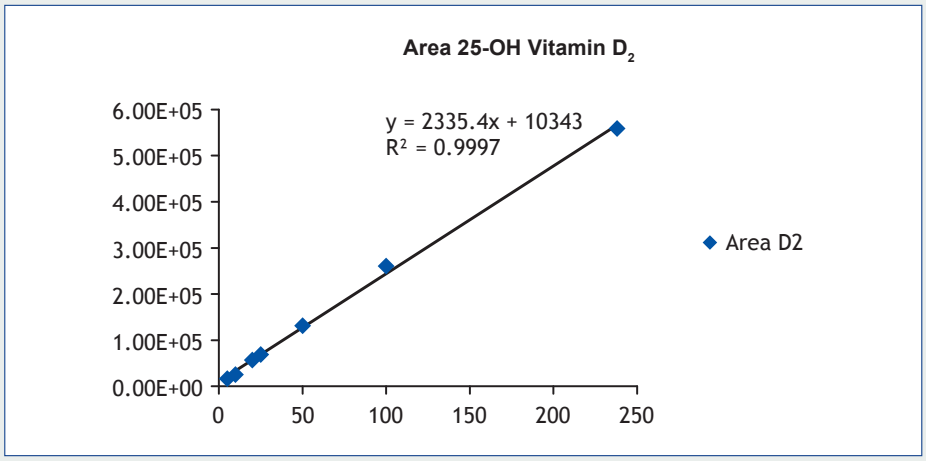


Fig. 10. Recovery of 25-OH Vitamin D_3

Conc. (ng/mL)	Retention Time (min)	Area D3	Recovery From Area (ng/mL)	% Recovery
2	12.76	5057.95	1.55	77.5
5	12.76	9662.88	3.7	73.8
10	12.77	1.68E+04	7.04	70.4
25	12.76	5.86E+04	26.55	106.2
50	12.77	1.17E+05	53.72	107.4
334	12.76	7.15E+05	333.44	99.8

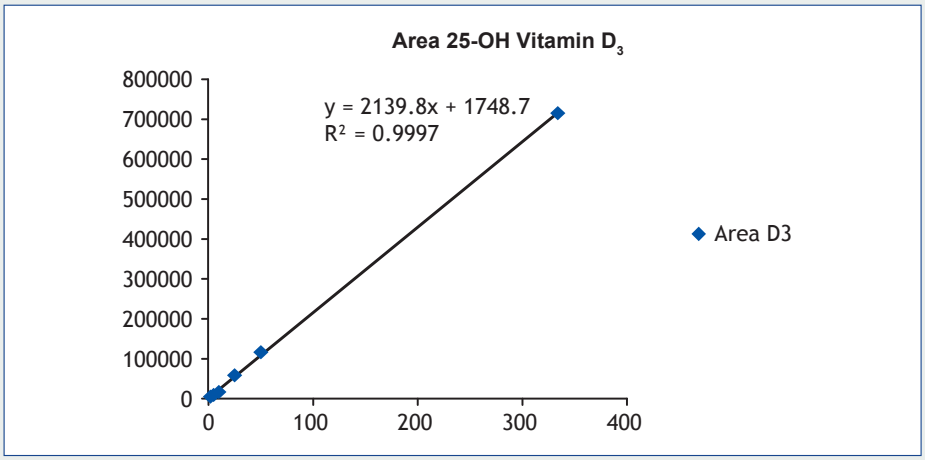


Fig. 11. Standard and Vitamin D_2 (Diluted From Commercially Available Drop) Chromatograms

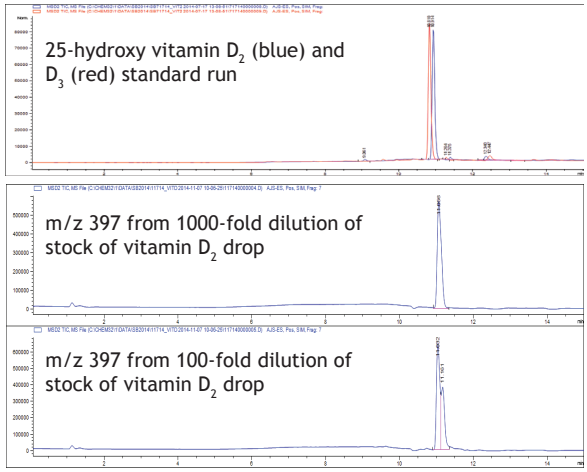
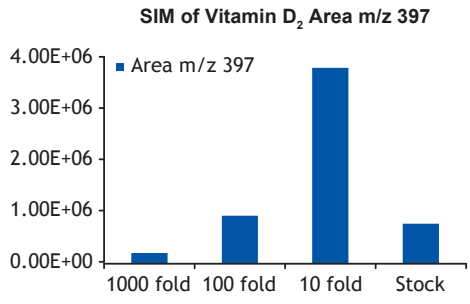
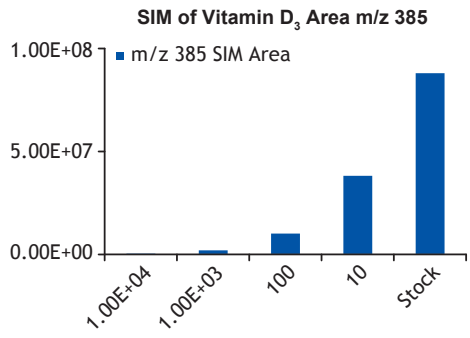


Fig. 12. Commercially Available Vitamin D_2 and D_3 LC-MS Response





Vitamin D_2 Initial Stock ~ 0.18 µg/mL

Vitamin D_2 Drop		
Dilution	Area m/z 397	Height m/z 397
1000 - fold	1.82E+05	9.39E+03
100 - fold	8.15E+05	5.64E+04
10 - fold	3.31E+06	1.49E+05
Stock	6.79E+05	2.71E+04

Vitamin D_3 Initial Stock ~ 0.125 µg/mL

Soft Gel Capsules	Vitamin D_3 SIM m/z 385	
Dilution	Area	Height
10,000 - fold	8.50E+04	6895.807
1000 - fold	1.29E+06	1.01E+05
100 - fold	6.89E+06	4.68E+05
10 - fold	2.66E+07	1.41E+06
Stock	6.14E+07	3.05E+06

Conclusions

- Better response of 25-OH-vitamin D_2 and D_3 was observed in decreased ionic strength mobile phase.
- In regular ESI, a difference in response was noted between 0.1% and 0.05% formic acid containing mobile phase. This variation may be due to subtle change in pH of the two solutions.
- A significant difference in response of 25-OH-vitamin D_2 and D_3 was noted when water/methanol mobile phase was run in jet stream ion focusing ESI compared to regular ESI. The results clearly indicate that ion focusing is a key factor for obtaining a better response of analyte in a jet stream operated instrument.
- The assay method was useable for both NIST standards and samples from a commercially available source.

References

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