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 • • • Additives: detection sensitivity of two metabolites: 25-hydroxy vitamin y, (m/z 413) and 25-hydroxy vitamin D, (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 D₂ and D₃. Response comparisons were 🕴 • Vitamin D₂ drop (0.2 mL) was diluted to 1 mL using a 10% methylene 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 . • Gradient of a comparative study of mobile phase effects on vitamin D detection sensitivity in LC-MS.

NTRODUCTION

- 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 D₃, and is metabolized to 25-hydroxy vitamin D₂ and 25-hydroxy vitamin D₂. 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 D₂ and D₂ 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, drop and vitamin D, soft gel capsules were purchased separately and the materials tested for further evaluation of the assay.

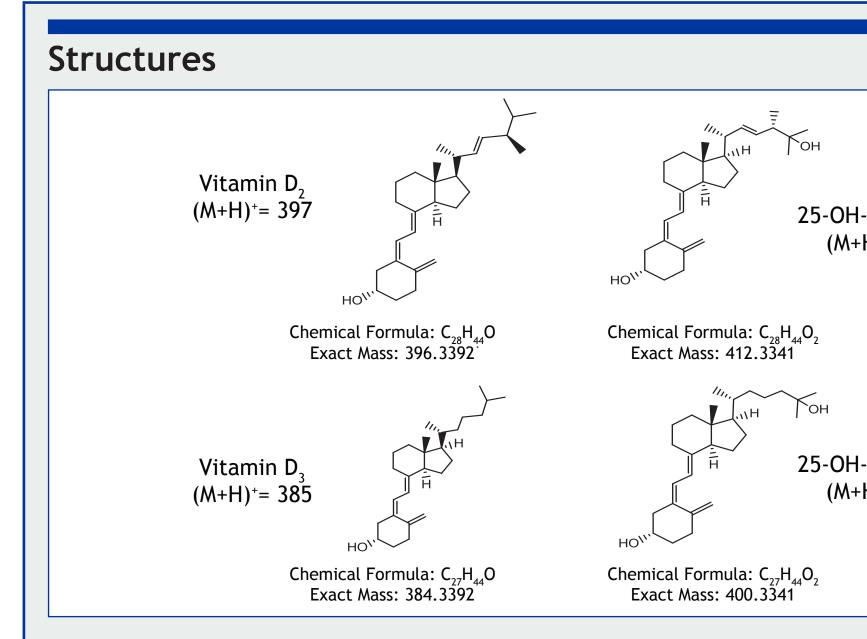
MATERIALS AND METHODS

- 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
- 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 methano
- 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, and vitamin D, were purchased from NIST (Cat #
- Vitamin D, drop (1000 IU per drop) and vitamin D, soft gel capsules (5000 IU per capsule) were purchased from Walgreens
- 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 D₂ 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.

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

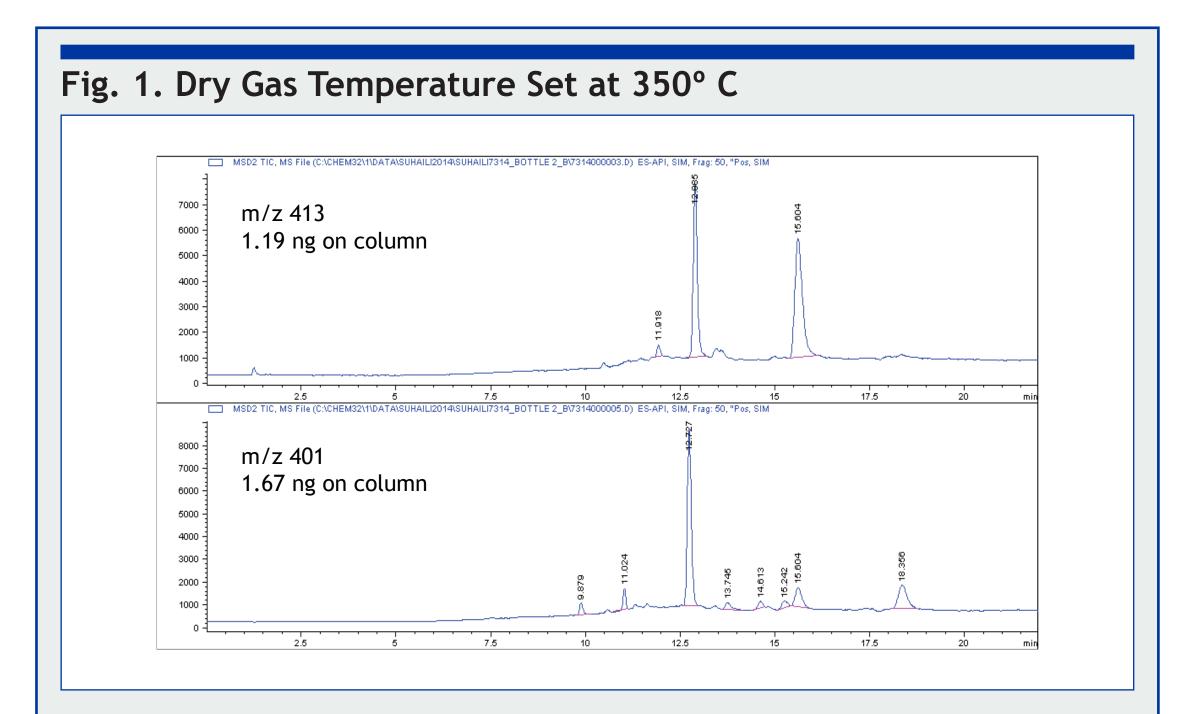
- Mass spectrometry conditions: positive mode, electro spray ionization (ESI) and thermal focusing jet stream electro spray ionization (TFJS-ESI) Fragmentor: 70V
- Gas Temperature: 250° C
- Drying gas: 11.0 l/min
- Gas flow: 5.0 l/min
- Neb gas: 30 psig
- Capillary: 3500 V
- 25-Hydroxy vitamin D₂ (m/z 413) and 25-hydroxy vitamin D₃ (m/z 401), vitamin D₂ (m/z 397) and vitamin D₂ (m/z 385) ions are monitored by selected ion monitoring (SIM)





Results

- SIM of parent ions of 25-hydroxy vitamin D₂ and D₃ was evaluated by:
- . 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).
- 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 D₂ and D₃ 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 D₂ (~100%); for 25-OH vitamin D₃ a recovery of 77 - 110% was observed in 2 to 300 ng/mL range.
- Analysis of commercially available vitamin D₂ drop and vitamin D₃ soft gel capsules is shown in the top panel of Fig. 11 with overlaid chromatograms standard 25-OH vitamin D_2 (blue trace) and 25-OH vitamin D_2 (red trace). Bottom panels showed the SIM (m/z 397) chromatograms of 100- and 1000-fold diluted vitamin D₂ from commercially available vitamin D_{2} drop.
- Fig. 12 provides the SIM response of vitamin D₂ drop and vitamin D₃ soft gel capsules.



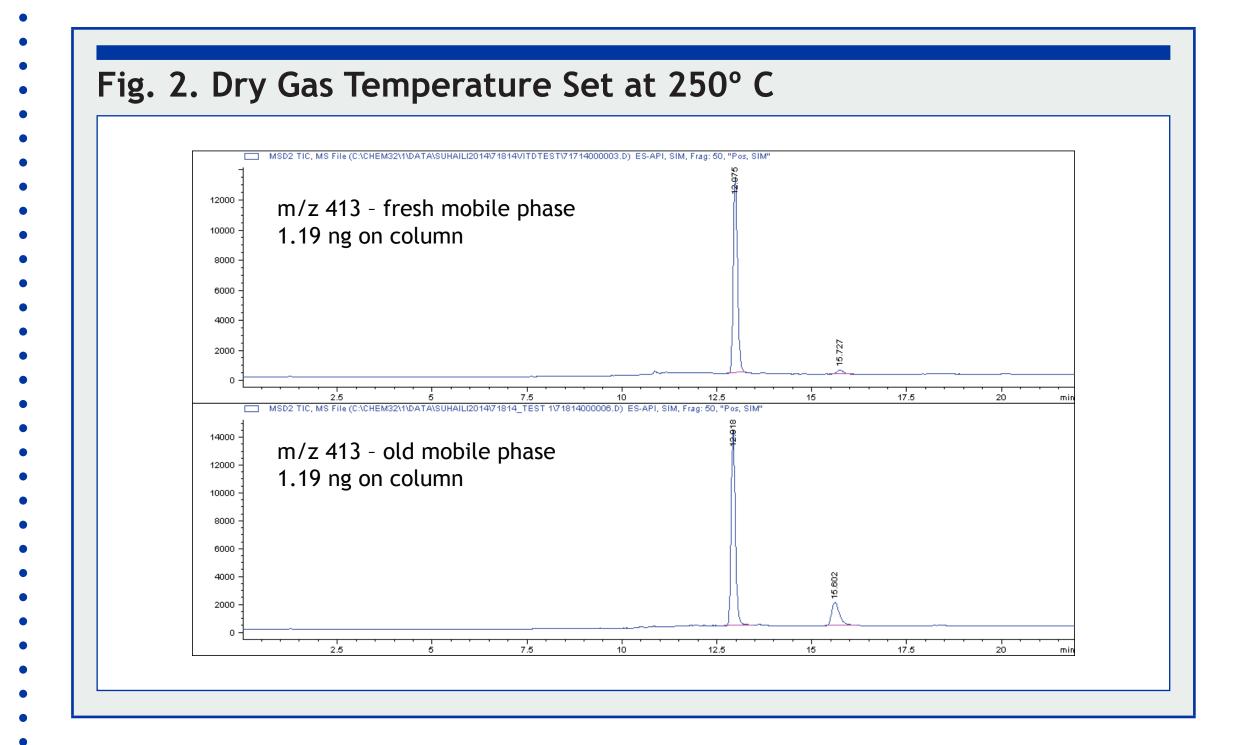


Fig. 3. Dr	ry Gas Temperature Set a
14000 12000 10000 8000 4000 2000 0 14000 12000 12000 12000 8000 10000 8000	MSD2 TIC, MS FIle (C:CHEM3211DATA\SUHAILI2014\71814\71814\71714000004.D) E m/z 401 - fresh mobile phase 1.67 ng on column MSD2 TIC, MS FIle (C:CHEM3211DATA\SUHAILI2014\71814_TEST 1\71814000010.D) ES m/z 401 - old mobile phase 1.67 ng on column
6000 - 4000 - 2000 - 0 -	88 80 8
	2.5 5 7.5 10

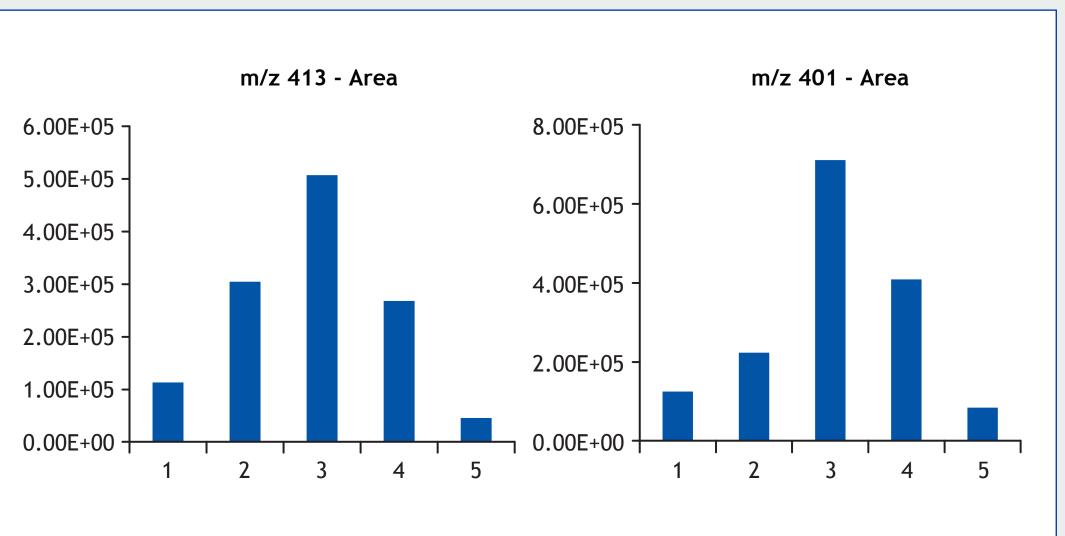
Fig. 4. Respo Phase Using F				-	
Mobile phase	MPS1	MPS2	MPS3	MPS4	MPS5
Plot number	1	2	3	4	5
m/z 413 - Area	1.13E+05	3.04E+05	5.07E+05	2.68E+05	4.60E+04
m/z 413 - Height	1.46E+04	3.91E+04	6.57E+04	3.58E+04	6.36E+03
m/z 401 - Area	1.25E+05	2.23E+05	7.11E+05	4.09E+05	8.40E+04
m/z 401 - Height	1.64E+04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

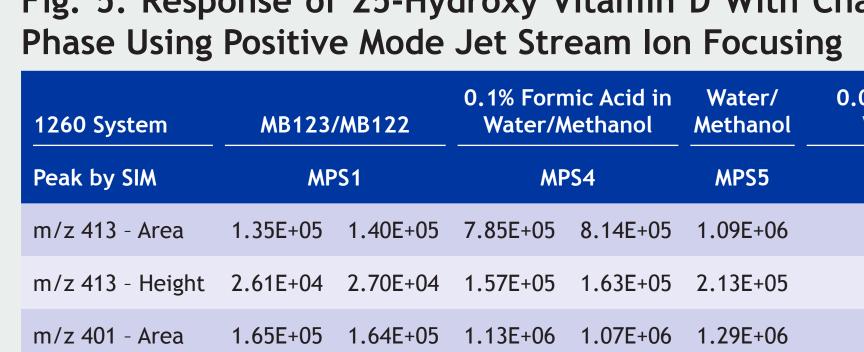
2.5 mM ammonium formate, 0.05% formic acid in water, 0.05% formic

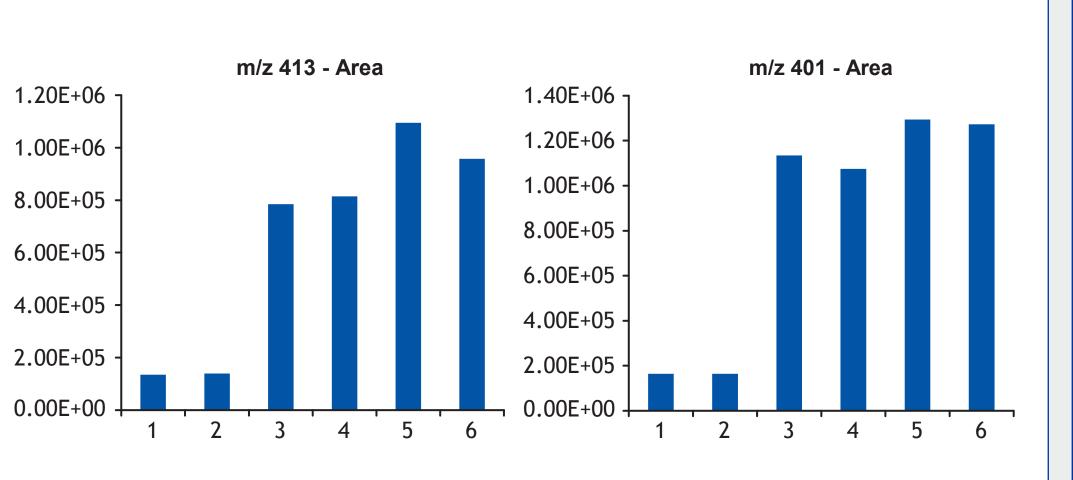
acid in methanol - MPS2

3. 0.05% formic acid in water and methanol - MPS3

4.0.1% formic acid in water/methanol - MPS4 5. Water/methanol - MPS5







25-OH-Vitamin D (M+H)⁺= 413

25-OH-Vitamin D₃ (M+H)⁺= 401

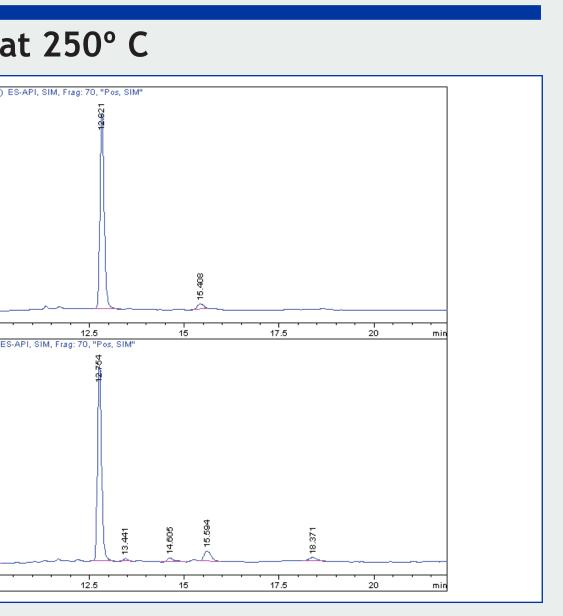


Fig. 5. Response of 25-Hydroxy Vitamin D With Change of Mobile

1260 System	MB123/	/MB122		nic Acid in Aethanol	Water/ Methanol	0.05% Formic Acid in Water/Methanol
Peak by SIM	MP	S1	MP	PS4	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

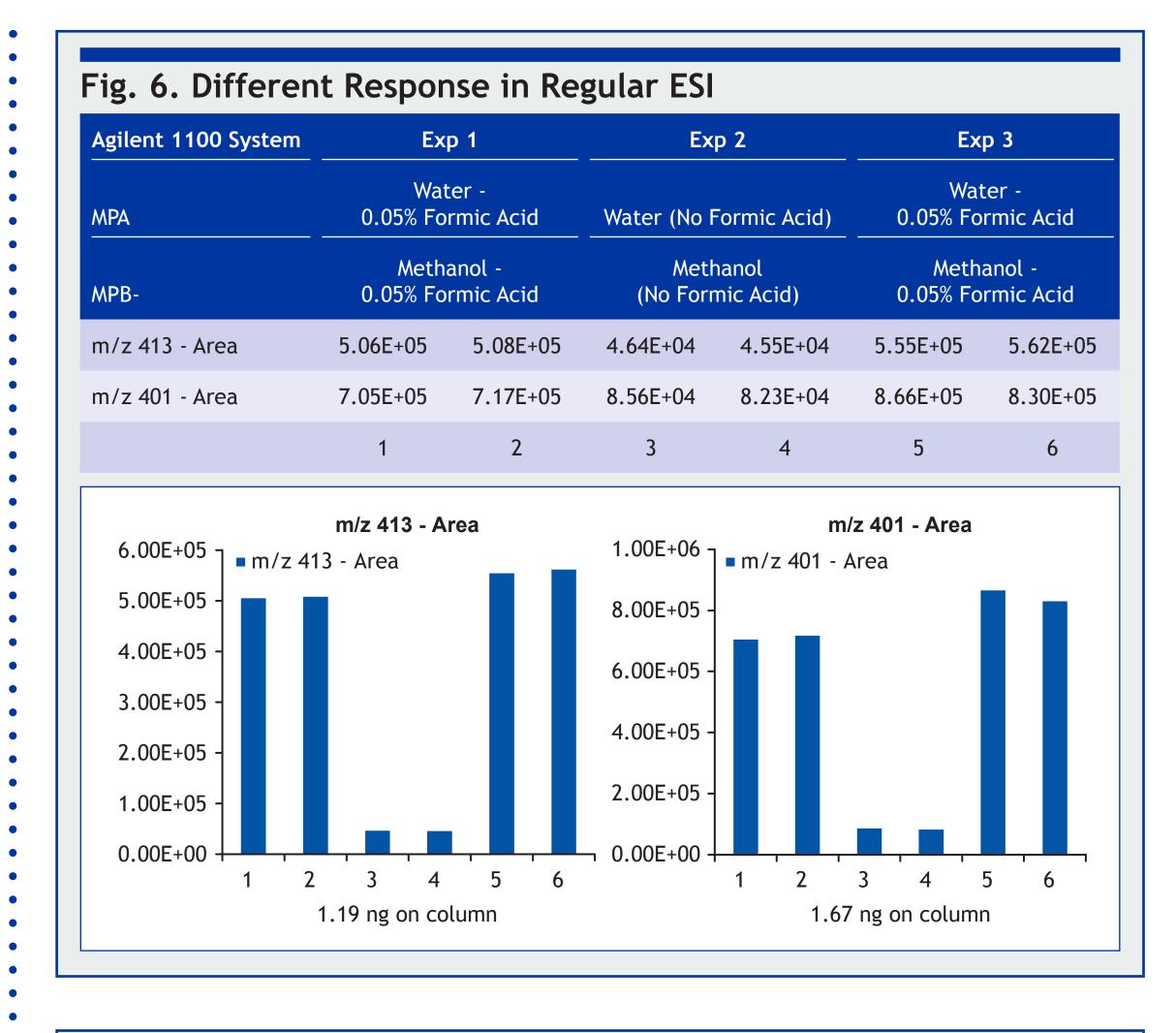


Fig. 7. Linearity Assay m/z 413

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

votune 5 pe		
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

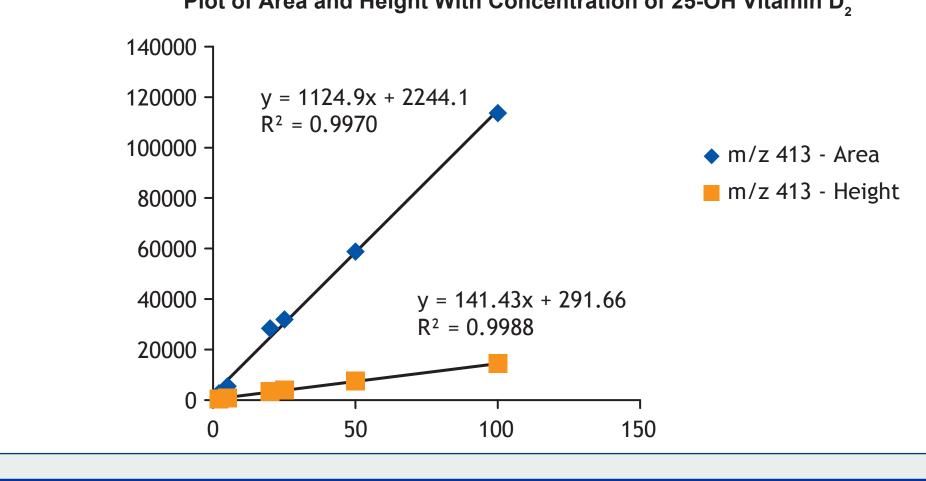


Fig. 8. Linearity Assay m/z 401				
Conc (ng/mL)	m/z 401 - Area	m/z 401 - Height		
2	2254.82	338.75		
5	4254.84	629.8		
10	8392.73	1207.07		
25	29401.3	3933.2		
50	53360.6	7130.2		
100	126071	16789.9		

Plot of Area and Height With Concentration of 25-OH Vitamin D

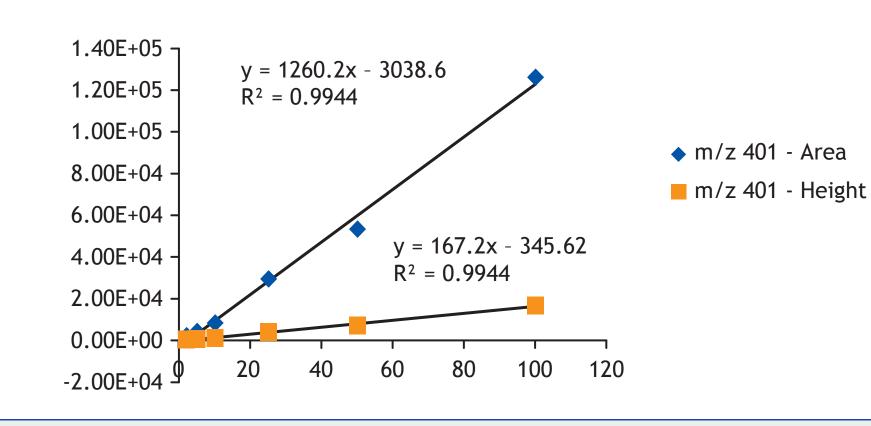
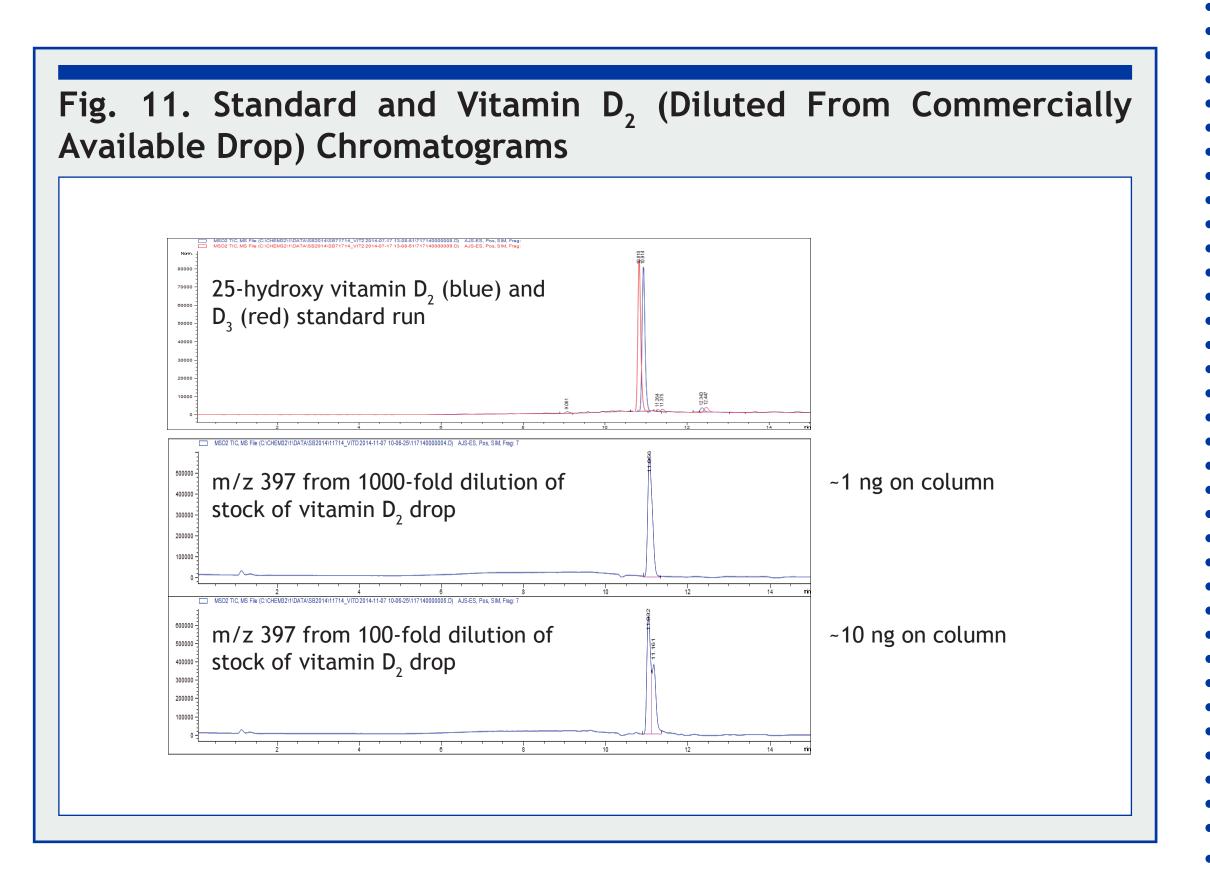
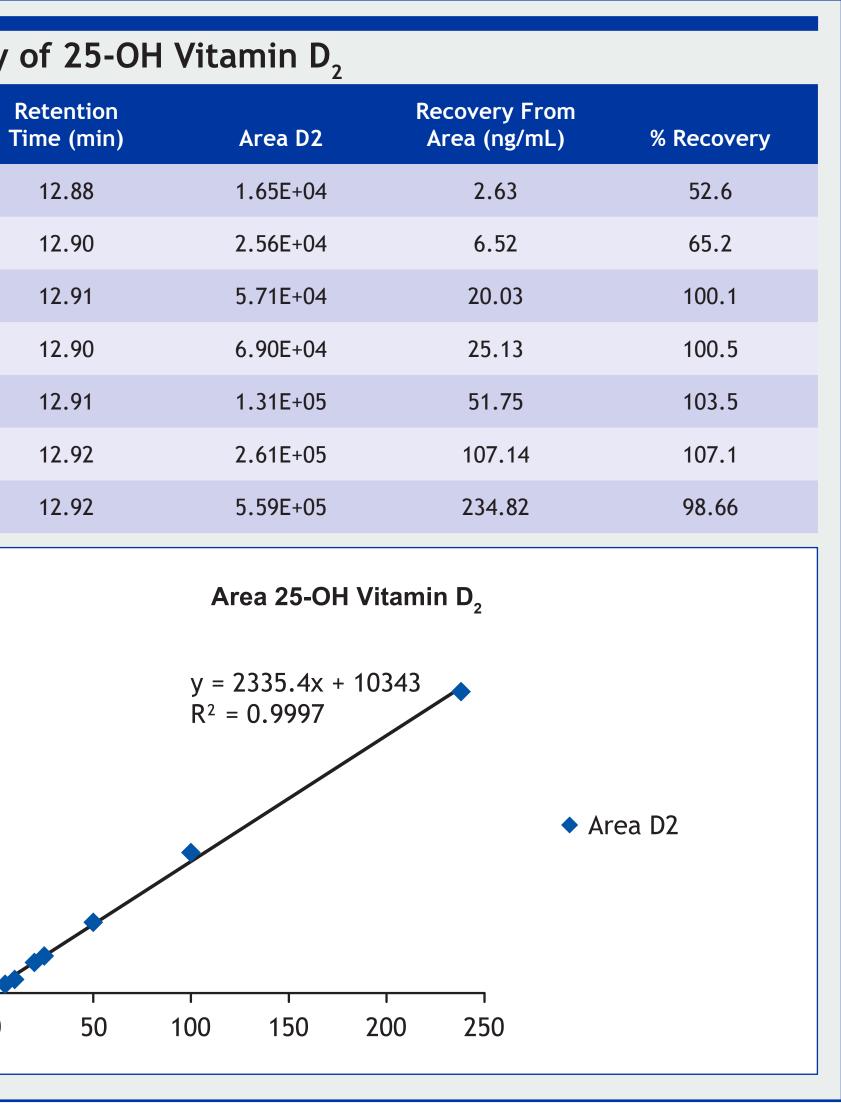


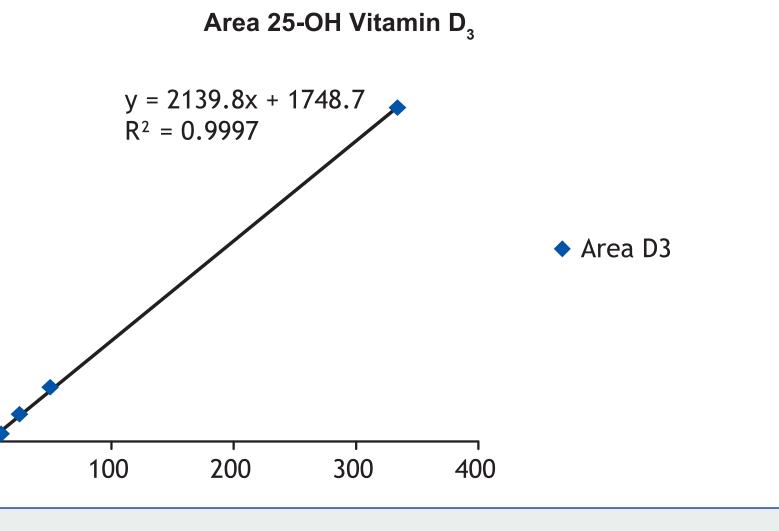
Fig. 9.	Recovery
Conc. (ng	g/mL)
5	
10	
20	
25	
50	
100	
238	
	ך 6.00E+05
	5.00E+05 -
	4.00E+05 -
	3.00E+05 -
	2.00E+05 -
	1.00E+05 -
	0.00E+00
	0

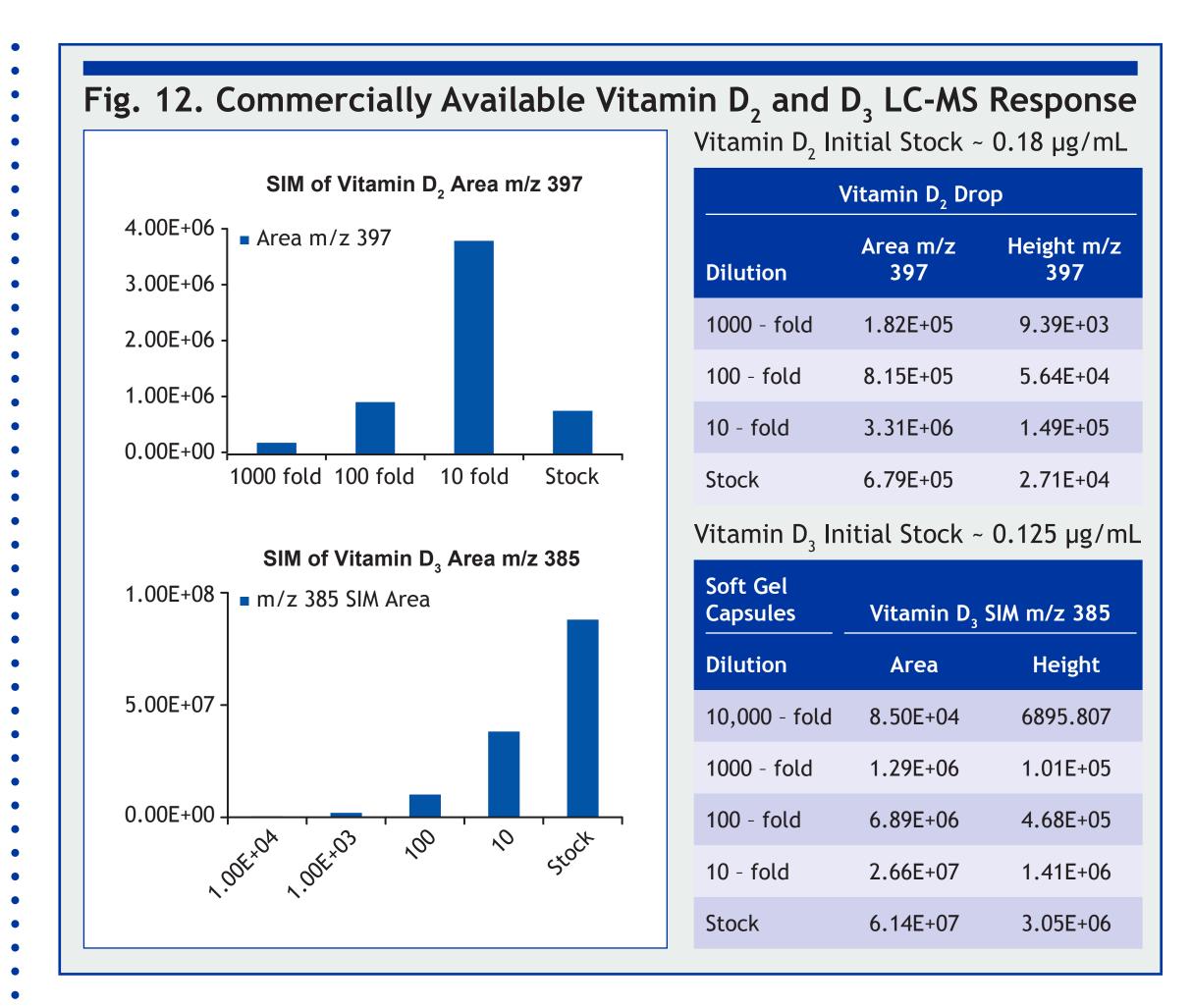
ig.	10.	Recov	/er
Conc	. (ng/n	nL)	
2			
5			
10			
25			
50			
334			
		800000 700000 600000 500000 400000 300000 200000 100000 0	





ry of 25-OH Vitamin D ₃				
Retention Time (min)	Area D3	Recovery From Area (ng/mL)	% Recovery	
12.76	5057.95	1.55	77.5	
12.76	9662.88	3.7	73.8	
12.77	1.68E+04	7.04	70.4	
12.76	5.86E+04	26.55	106.2	
12.77	1.17E+05	53.72	107.4	
12.76	7.15E+05	333.44	99.8	





CONCLUSIONS

- Better response of 25-OH-vitamin D₂ and D₃ 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 D₂ and D, was noted when water/methanol mobile phase was run n 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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