Advances in Sample Preparation for better LC/MS Analysis of Vitamin D Metabolites in Plasma

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Vitamin D

- Central biological function:
  - Calcium and phosphate metabolism
  - Bone metabolism
- Additionally:
  - Cell differentiation
  - Insulin metabolism
  - Immune defence

Bischoff-Ferrari HA et al. (2006) Estimation of optimal serum concentrations of 15-hydroxy-vitamin D for multiple health outcomes. Am J Clin Nutr **84**: 18-28
Holick MF. (2007) Vitamin D defienceny. N Engl J Med **357**: 266-281
Zittermann A. (2003) Vitamin D in preventive medicine. Are we ignoring the evidence? Br J Nutr **89**: 552-572

Vitamin D

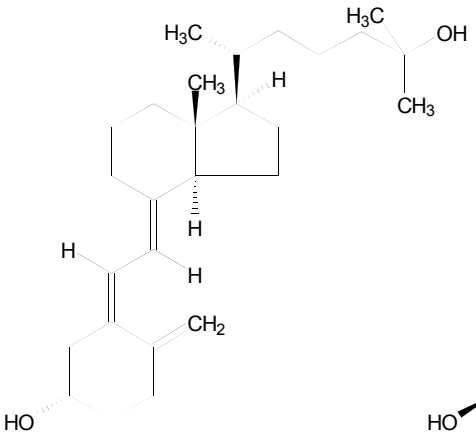
- Group of seco-steroids with biological activity of Vitamin D
- Lipid-soluble
- Structure similar to cholesterol
  - Cholecalciferol (Vitamin D_3)
  - Calcidiol (25-Hydroxyvitamin D_3)
  - Calcitriol (1,25-Dihydroxyvitamin D_3)
  - Ergocalciferol (Vitamin D_2)
- Can be metabolized by the body from cholesterol

Vitamin D

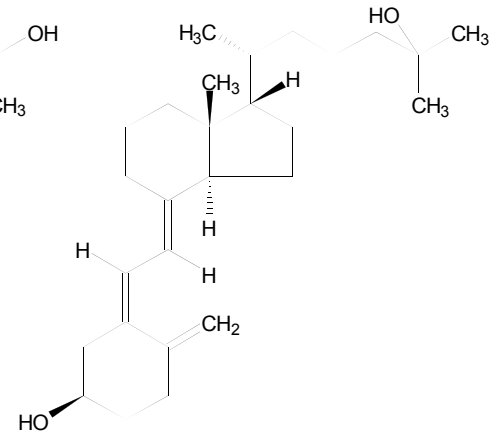
- Sources & metabolism:
  - Vitamin D3 is produced in the skin after exposure to ultraviolet light by conversion of 7-dehydrocholesterol
  - Vitamin D3 intake from animal sources
  - Vitamin D2 intake from plant sources
  - Vitamin D2 and D3 do have different biological activity [1]
    - Should not be analyzed as sum parameter
    - ELISAs can not distinguish between these
  - Biologically inactive 3-epi analogons of 25-OH D_2 and 25-OH D_3 have been reported, especially in young children [2,3]
    - Requirement on LC/MS method for chromatographic resolution of the isobaric compounds

[1] Holick MF. (2007) Vitamin D defienceny. N Engl J Med **357**: 266-281
[2] Higashi, T.; Shimada, K.; Toyo'oka, T. Journal of Chromatography B (2010), **878**, 1654-1661
[3] Singh, R.J. et al., J Clin Endocrinol Metab (2006), **91**, 305-3061

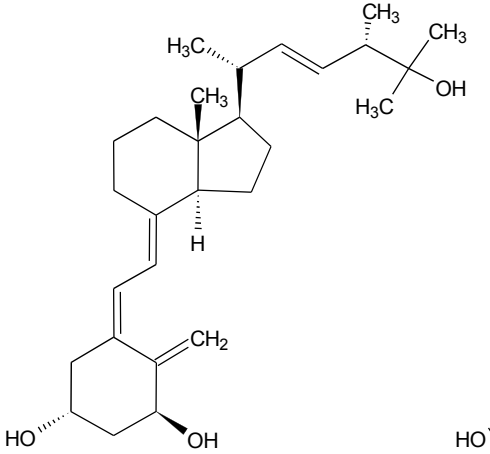
Vitamin D Metabolites with their monoisotopic masses



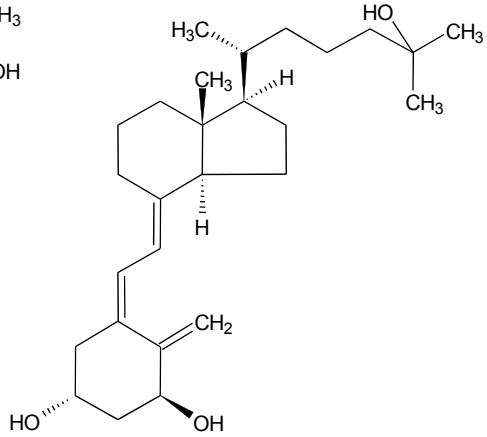
25-Hydroxyvitamin D3
M = 400.334131 Da



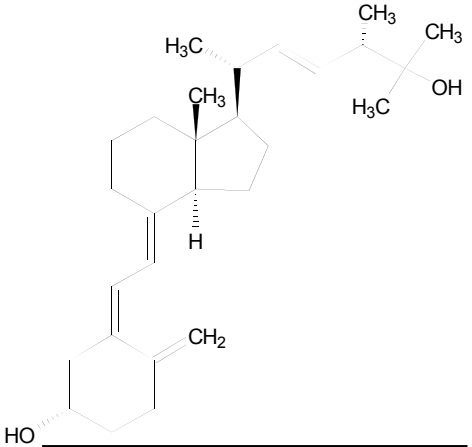
3-epi-25-Hydroxyvitamin D3
M = 400.334131 Da



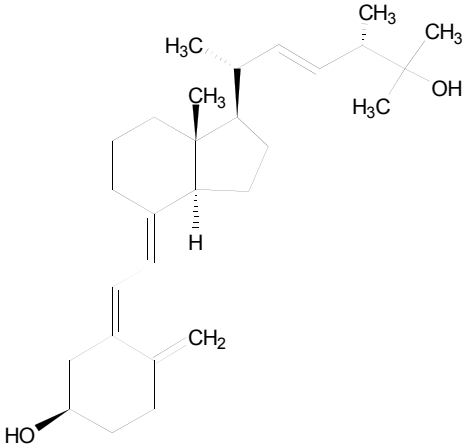
25-dihydroxyvitamin D2
M = 428.329045 Da



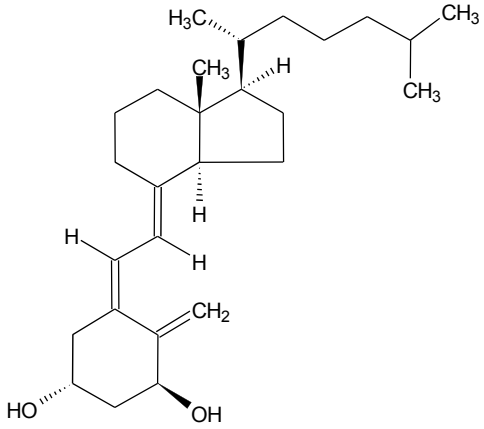
25-dihydroxyvitamin D3
M = 416.329045 Da



25-Hydroxyvitamin D2
M = 412.334131 Da



epi-25-Hydroxyvitamin D2
M = 412.334131 Da



1-α-Hydroxyvitamin D3
M = 400.334131 Da

HPLC/MS Method Development

Three Factors Control HPLC and UHPLC Resolution [4]

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{k}{k+1} \cdot \frac{\alpha-1}{\alpha}$$

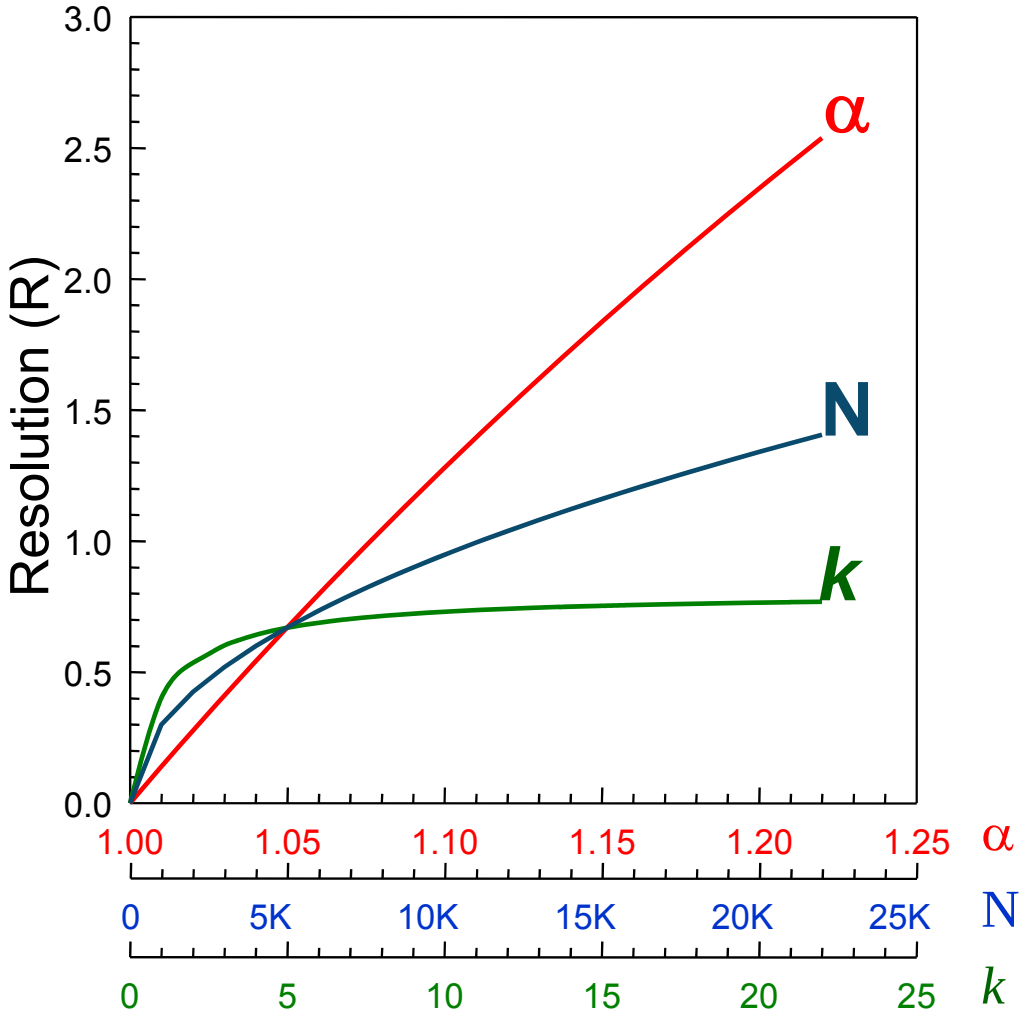
$N = 16\,(t_R/w)^2$ or

$N = 5.5\,(t_R/\delta)^2$

$k = (t_R - t_0)/t_0$

$\alpha = k_2/k_1$

All factors are important, but selectivity is considered the most powerful term.



[4] Yun Mao, PhD Dissertation, University of Minnesota, 2001.

Selectivity Variables in Reversed-Phase *

Continuous variables (solvent):

- type (organic, water)
- pH (especially ionizable solutes)
- additives (type and concentration)
- temperature
- solvent strength

More predictable

(modeling software available)

Some analysts may spend too much time here "force feeding" C18 columns.

Discontinuous variable (column):

- type (phase and substrate)

Less predictable

(screening required and beneficial)

* Excerpted with permission from John Dolan, 2009 Minnesota Chromatography Forum Spring Symposium; adapted by R. Henry.

Analytical assessment of Vitamin D levels

- Usually done by HPLC on C18 phases
  - Co-elution of the epimers with the parent compounds
- Alternative method by Phinney et al. [5] on cyano phase
  - Run time of more than 40 min
- Method developent by systematic screening of different stationary and mobile phases
  - Ascentis® Express F5
  - Ascentis® Express Phenyl-Hexyl
  - Ascentis® Express ES-Cyano
  - Acetonitrile
  - Ethanol
  - Methanol

[5] Tai, S. S.-C.; Bedner, M.; Phinney, K. W. Analytical Chemistry (2010), **82**, 1942-1948

Classification by Possible Chemical Interactions[a]

Bonded Phase	Hydrophobic	H-Bonding	Dipolar	π-π	Steric	Ionic
C18	Very Strong	Weak	No	No	No	Moderate
C8	Strong	Weak	No	No	No	Weak
RP-Amide	Strong	Strong Acceptor	Mode-rate	No	Weak	Very weak
Phenyl-Hexyl	Strong	Weak Acceptor	Weak	Strong Donor	Strong (Rigid)	Weak
F5 or PFP	Moderate	Moderate Acceptor	Strong	Strong Acceptor	Strong (Rigid)	Moderate
Cyano	Light to Moderate	Weak Acceptor	Strong	Weak	No	Moderate

a. Using Euerby variation of Snyder-Dolan-Carr Hydrophobic Subtraction Model [6,7].

[6] M. R. Euerby, et. al., "Classification of Phenyl Columns", J Chrom A, **1154** (2007), 138-151.
[7] L.R. Snyder, J.W. Dolan and P.W. Carr, "Hydrophobic Subtraction Model for Classification of Reversed-Phase Columns", J Chrom A, **1060** (2004), 77.

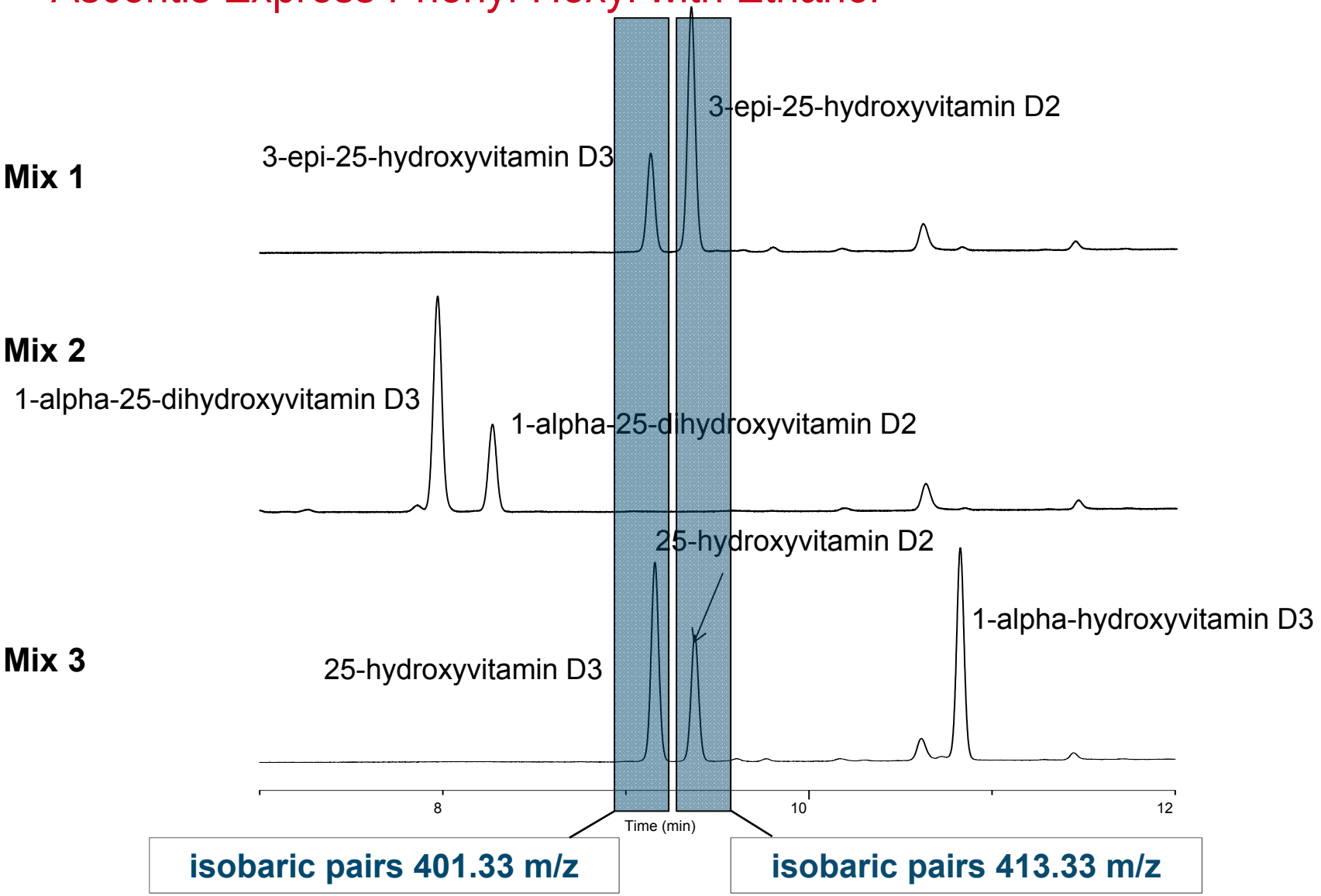
Screening Samples

- Minimization of likelihood of coelution of target analytes
- Isobaric pairs highlighted in red and blue:
- Vitamin D Mix 1
  - 3-epi-25-hydroxyvitamin D2 (412.33 Da)
  - 3-epi-25-hydroxyvitamin D3 (400.33 Da)
- Vitamin D Mix 2
  - 1-α-25-dihydroxyvitamin D2 (428.33 Da)
  - 1-α-25-dihydroxyvitamin D3 (416.33 Da)
- Vitamin D Mix 3
  - 25-hydroxyvitamin D2 (412.33 Da)
  - 25-hydroxyvitamin D3 (400.33 Da)
  - 1-α-hydroxyvitamin D3 (400.33 Da)

Experimental Conditions

column:	Ascentis Express 100 x 3.0 mm, 2.7 µm
mobile phase:	(A) 1% formic acid; (B) water; (C) as listed below;
gradient methanol:	10% A constant; 40% B, 50% C held for 1 min; to 0% B, 90% C, in 8 min;0% B, 90% C, held for 3 min
gradient ethanol:	10% A constant; 50% B, 40% C held for 1 min; to 0% B, 90% C, in 10 min;0% B, 90% C, held for 3 min
gradient acetonitrile:	10% A constant; 50% B, 40% C held for 1 min; to 0% B, 90% C, in 10 min;0% B, 90% C, held for 3 min
flow rate:	0.6 mL/min
column temp.:	35 ° C
injection:	5 µL
sample 1:	vitamin D epi mix, 5 µg/mL in methanol
sample 2:	vitamin D alpha di mix, 5 µg/mL in methanol
sample 3:	vitamin D alpha hydroxy + hydroxy mix, 5 µg/mL in methanol

Ascentis Express Phenyl-Hexyl with Ethanol



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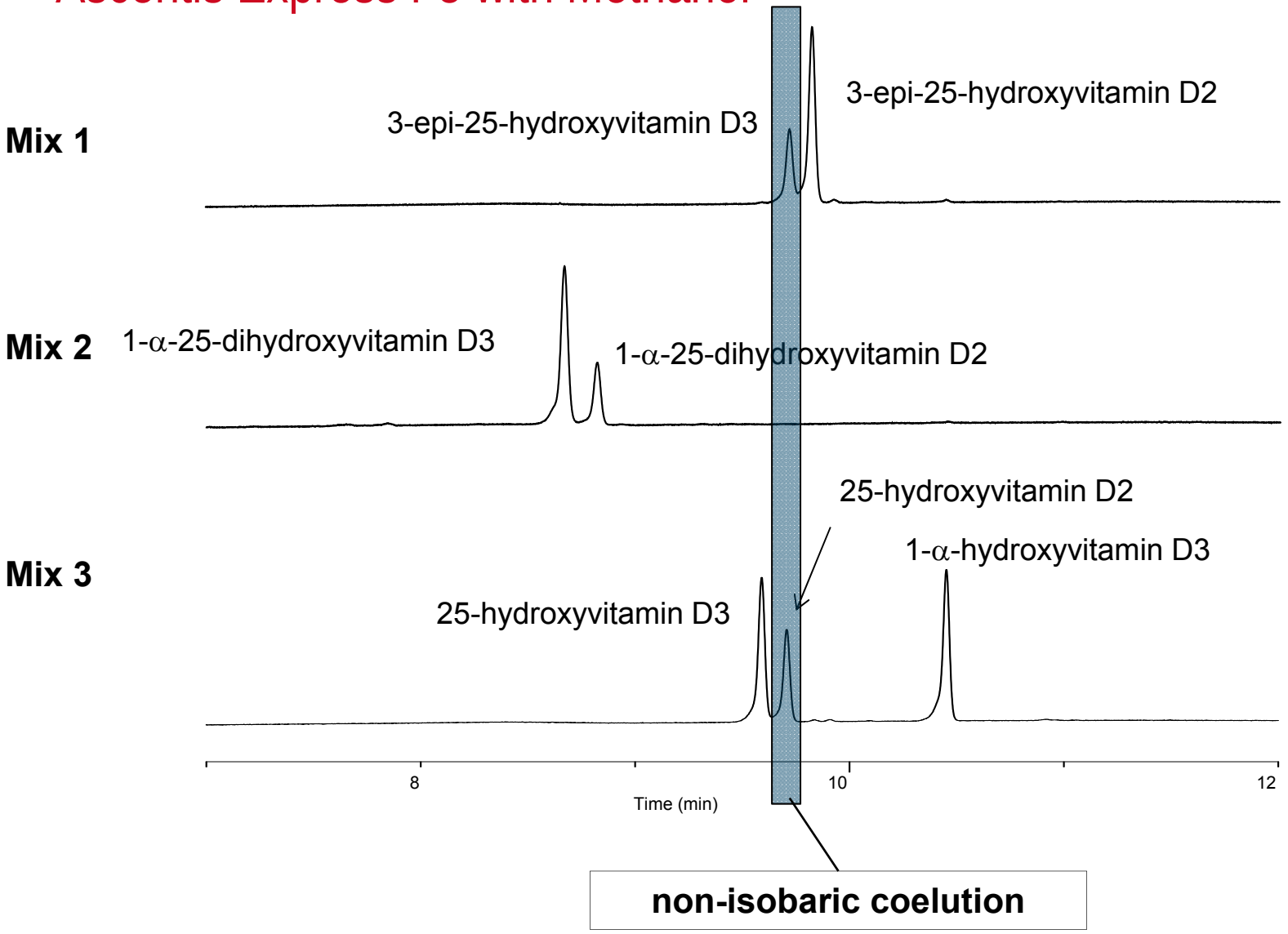
Ascentis Express F5 with Methanol



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Summary of Minimum Isobaric Resolution by stationary and mobile Phase

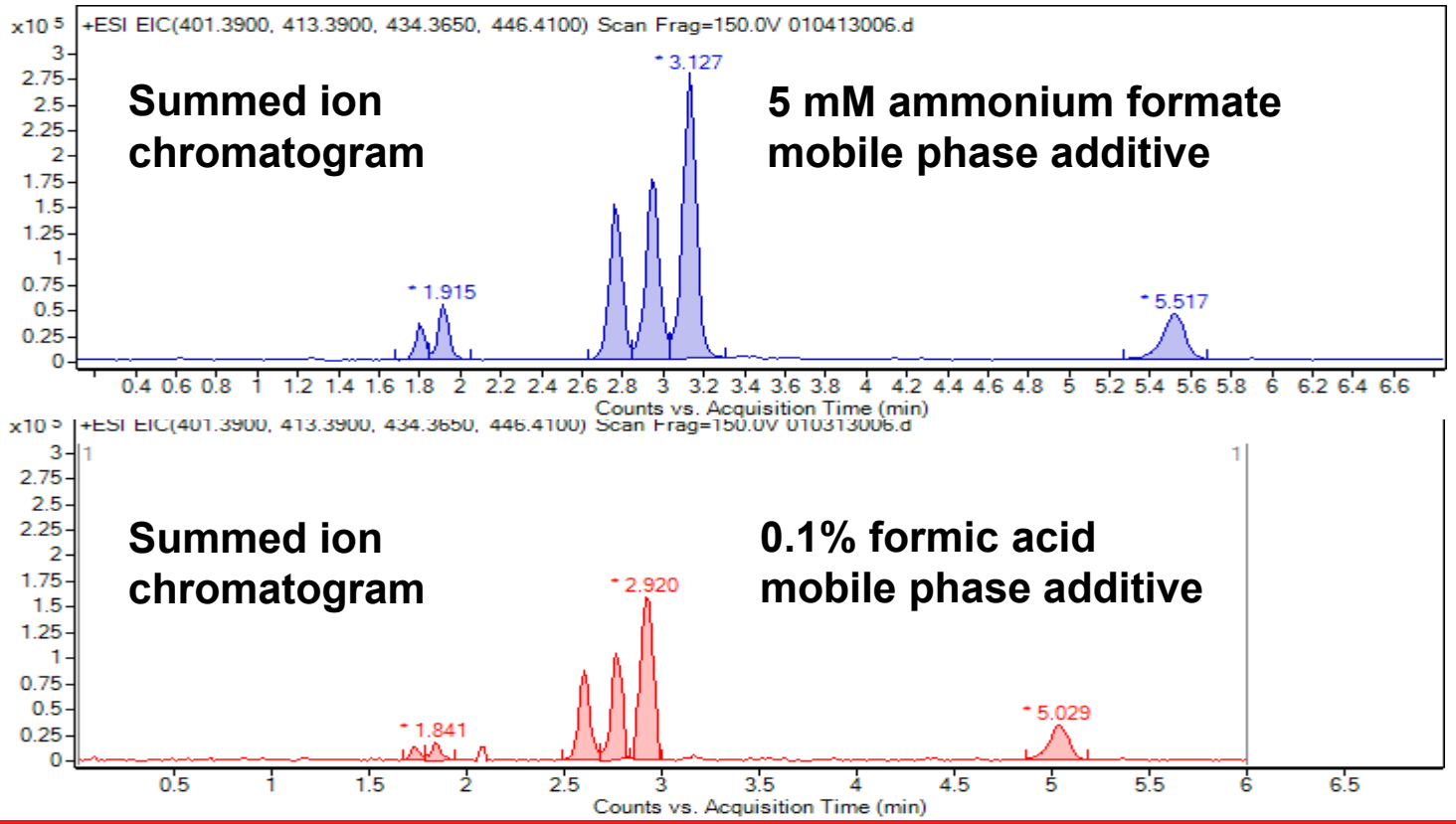
Column	Organic Modifier	Minimum Resolution Between Isobars
Ascentis Express F5	methanol	2
Ascentis Express F5	acetonitrile	1.2
Ascentis Express F5	ethanol	1.1
Ascentis Express ES-CN	methanol	1.2
Ascentis Express ES-CN	acetonitrile	0.8
Ascentis Express ES-CN	ethanol	0.5
Ascentis Express Phenyl-Hexyl	methanol	0
Ascentis Express Phenyl-Hexyl	acetonitrile	0
Ascentis Express Phenyl-Hexyl	ethanol	0.3

Minimum resolution defined as resolution between two sets of isobaric compounds:

	m/z
25-hydroxyvitamin D2 and epi-25-hydroxyvitamin D2	413.33
25-hydroxyvitamin D3 and epi-25-hydroxyvitamin D3	401.33

Method Optimization

- Isocratic method with short run time => MeOH:water 75:25
- Optimization of mobile phase additives for resolution/ionization



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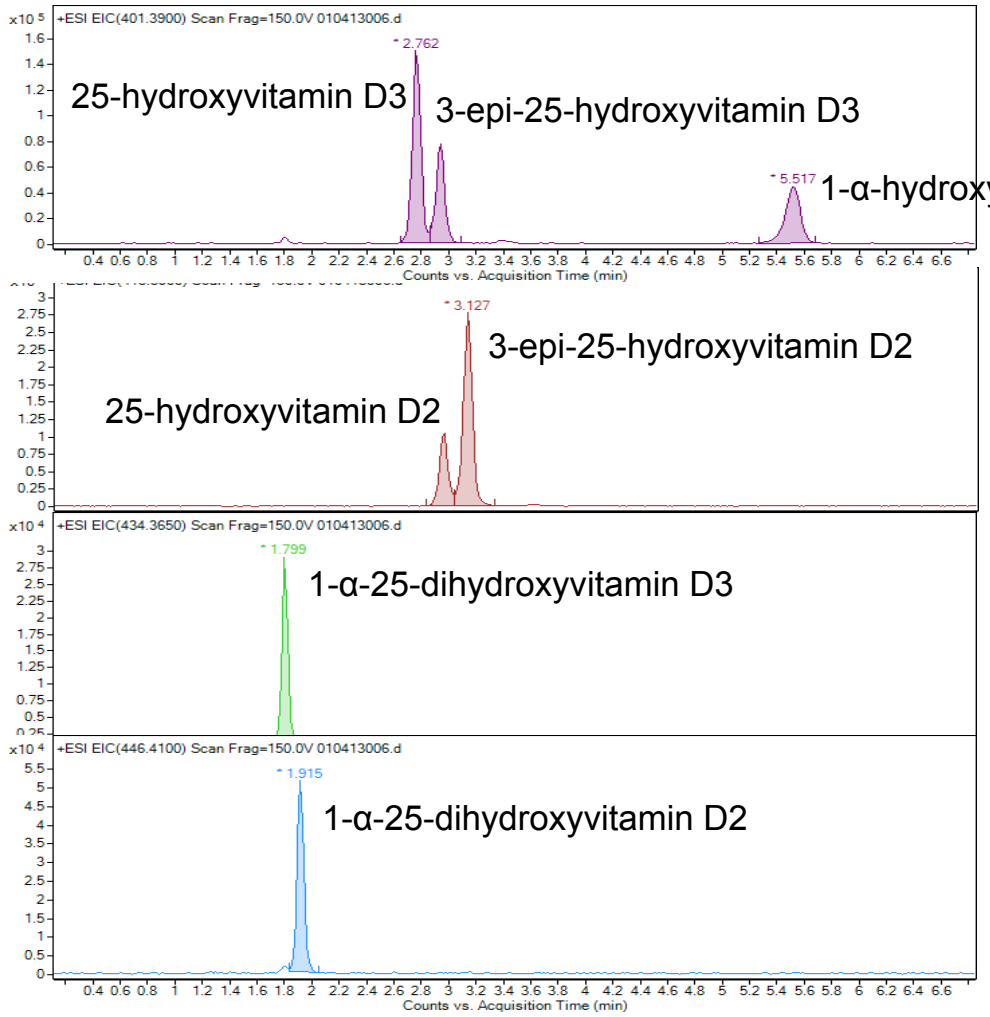
Separation of Vitamin D Metabolites on Ascentis Express F5

m/z 401.39

m/z 413.39

m/z 434.36

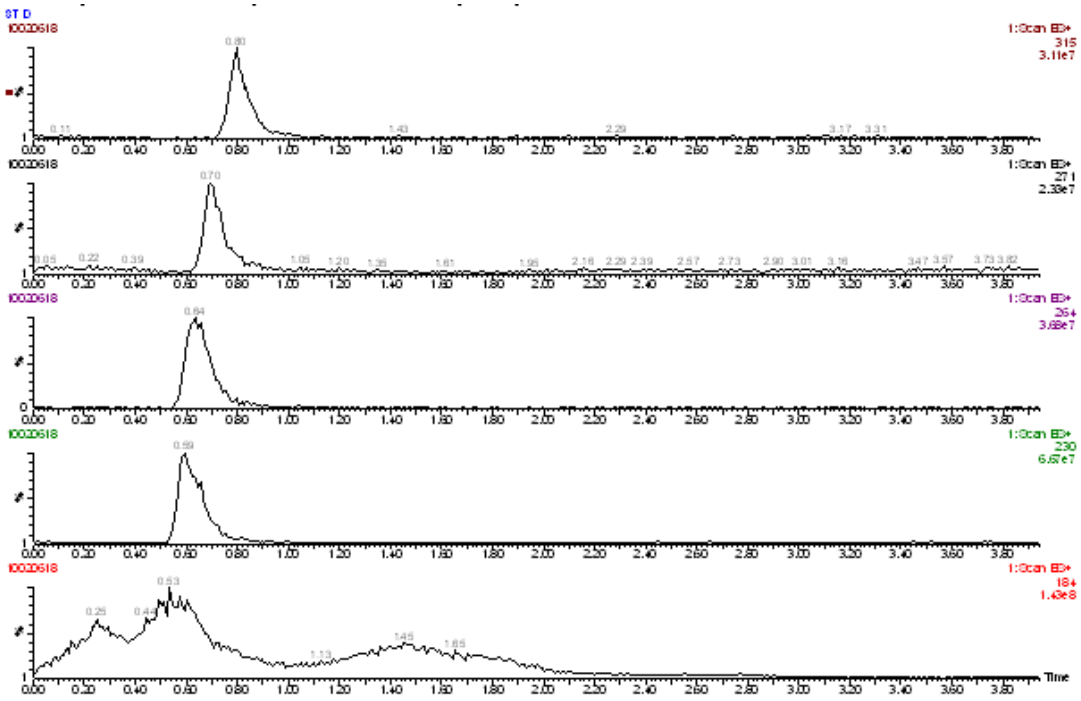
m/z 446.41



System: Agilent® 1290, 6210 TOF
Column: Ascentis Express F5, 150 x 2.1 mm, 2.7 µm
Mobile phase: 25:75, (A) 5 mM NH_4 formate; (B) MeOH
Flow rate: 0.4 mL/min
Temp.: 40 ºC
MS detector: ESI+, 100-1000m/z
Injection: 2 µL

Development of Sample Preparation Method

- Challenges with plasma/serum samples in LC/MS:
  - Proteins and phospholipids
  - Just protein precipitation often insufficient:



Clomipramine (m/z 184)

Recovery: 110, 9%

Desmethyl-diazepam (m/z 315)

Recovery: 81,9%

Protryptiline (m/z 264)

Recovery: 44,7%

Clonidine (m/z 230)

Recovery: 54,5 %

Phospholipids (m/z 184)

Challenges in Phospholipid Removal

Protein Precipitation:

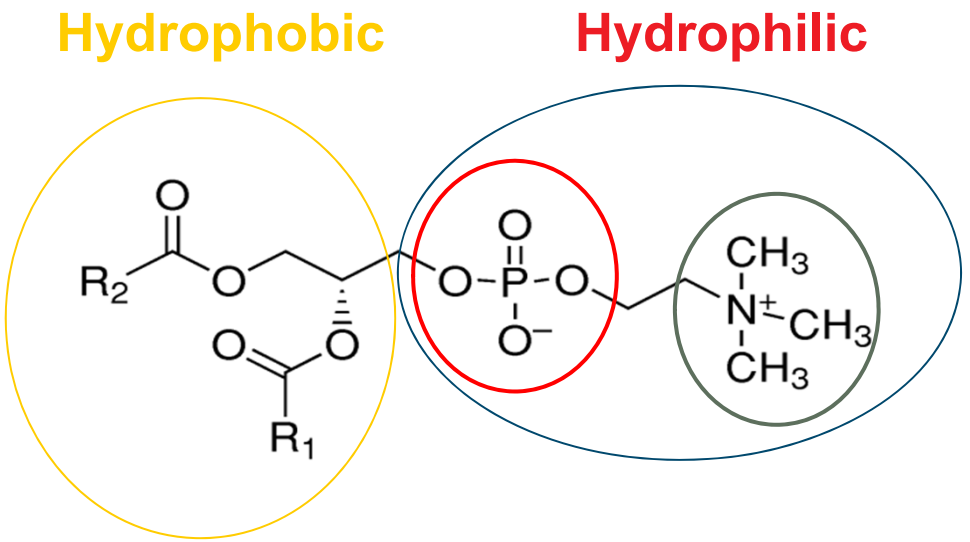
- Will only remove gross levels of protein (albumin)

Liquid Liquid Extraction:

- Hydrophobic tail allows for co-extraction with analytes of interest

Solid Phase Extraction:

- **Mixed-Mode & IEX –** often co-extract with basic or acidic analytes of interest
- **Reversed-Phase –** will result in co-extraction with analytes of interest
- **Multi Step clean up**

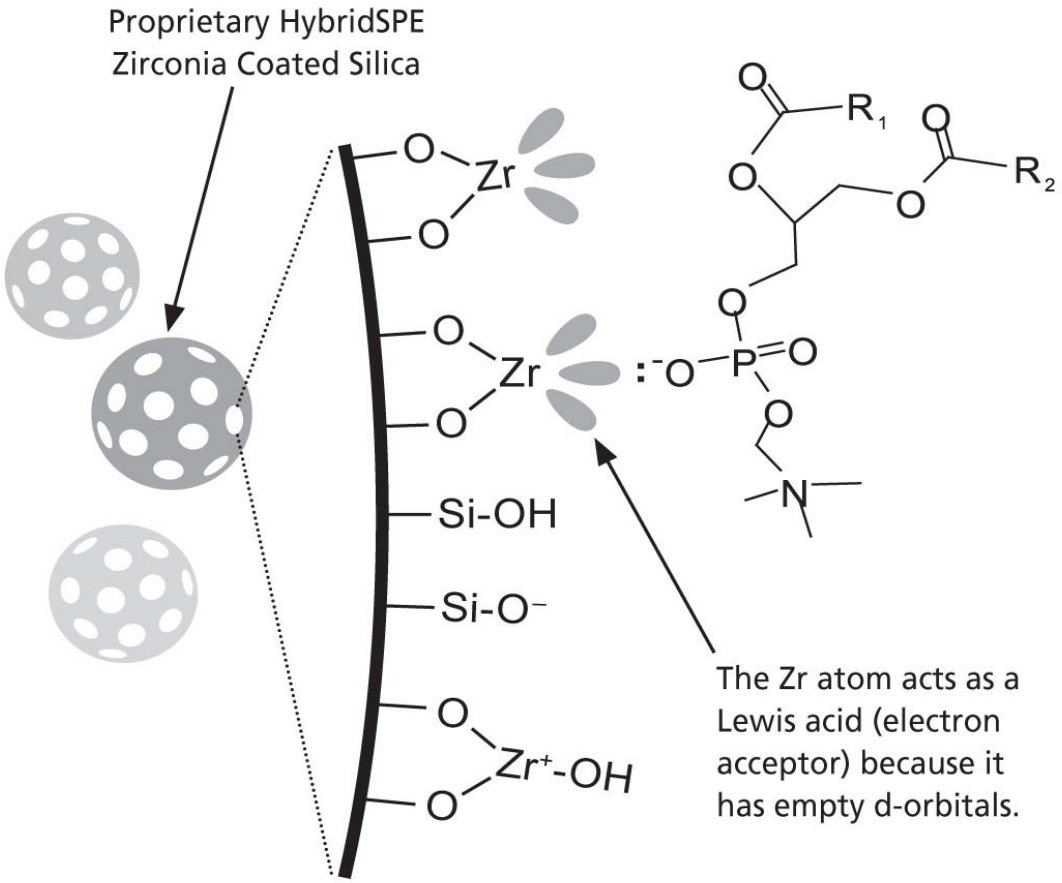


Polar head group – zwitterionic phosphonate (remains charged at from strong alkaline to strong acid)

Hybrophobic tail – two fatty acyl groups that are hydrophobic

Zirconia-coated silica for targeted removal of phospholipids

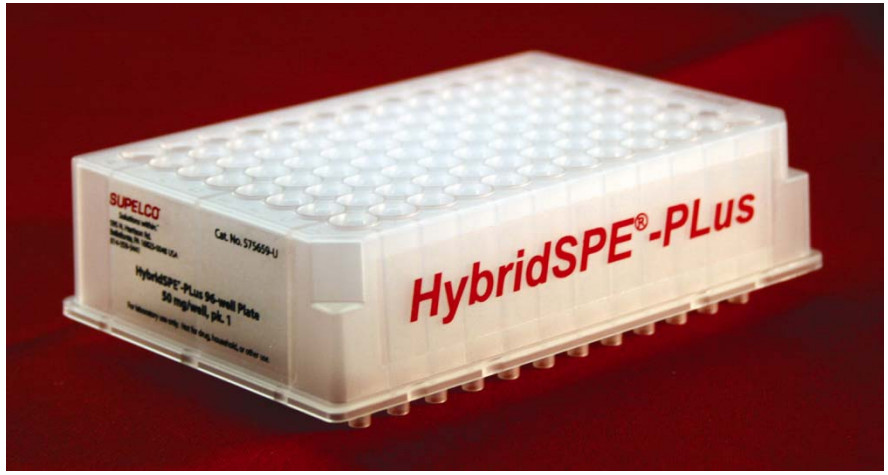
- Highly selective removal of phospholipids due to Lewis acid/base interaction
- Coating on zirconia on silica:
  - Acidity of zirconia is reduced: highly efficient removal of phospholipids not compromizing other compounds
  - Larger surface area for higher capacity

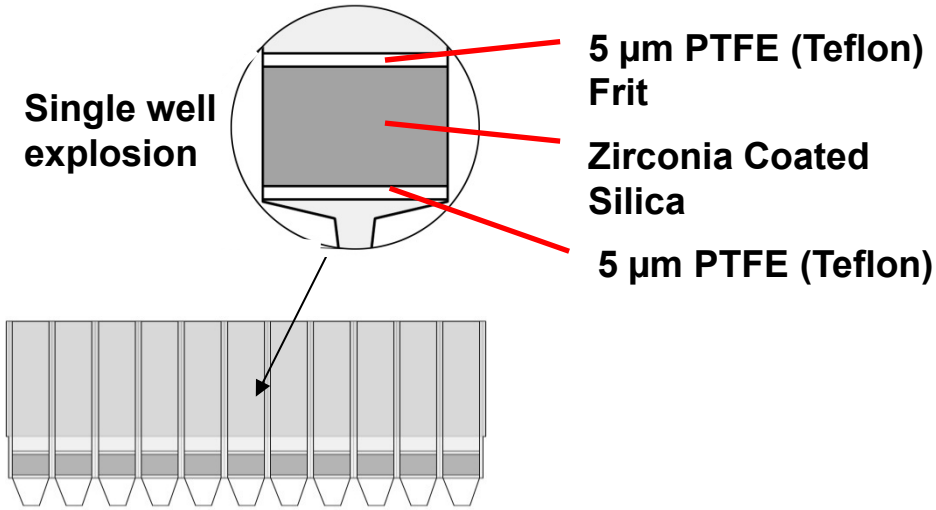


Development Sample Preparation Method

- Samples:
  - Human serum spiked at 25 ng/mL vitamin D metabolites
- Sample Prep Approaches:
  - Standard protein precipitation
    - 100 µL of sample into 2 mL centrifuge vial plus 300 µL of 1% formic acid in acetonitrile.
    - Samples were vortexed and centrifuged
    - Supernatant was collected and analyzed directly
  - HybridSPE®-PLus (zirconia-coated silica)

Sample Process for HybridSPE PLus 96-well Plate





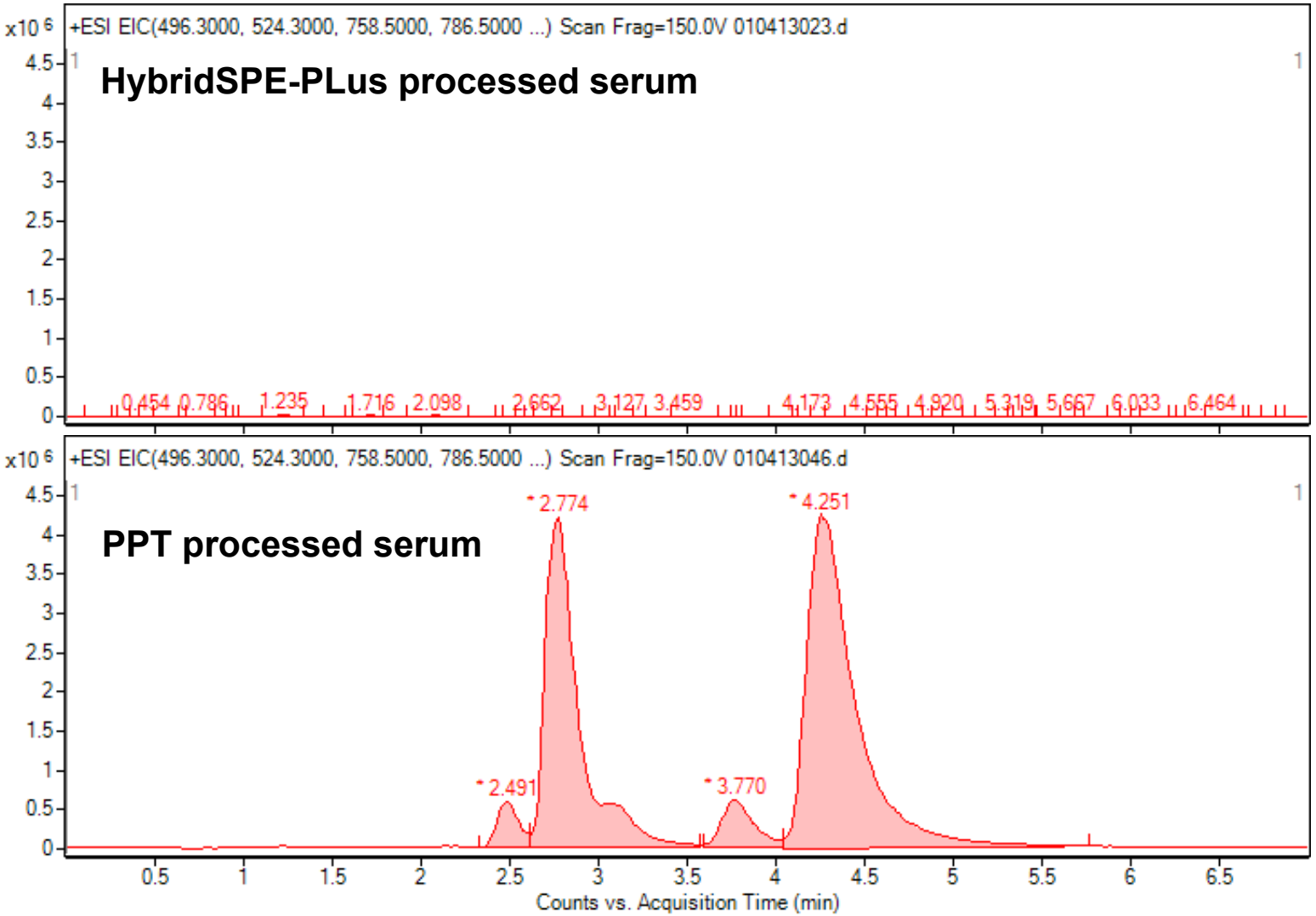
- HybridSPE-Plus
  - Offline Protein precipitation of 100 µL of sample plus 300 µL of 1% formic acid in acetonitrile
  - Mixing by five 300 µL draw/aspiration cycles using digital pipetter
  - After setting of precipitate transferring of 300 µL of supernatant into the HybridSPE-PLus 96-well plate
  - Passing through 96-well plate by applying 10” Hg vacuum for 4 min
  - HPLC/MS analysis

Vitamin D Metabolite Recovery Comparison

Sample	HybridSPE-PLus 100 ng/mL Standard	HybridSPE-PLus Plasma 25 ng/mL	PPT Plasma 25 ng/mL
	Average n=16	Average n=16	Average n=8
1-α 25-dihydroxy vitamin D3	92.6	21.1	17.1
1-α 25-dihydroxy vitamin D2	95.9	19.7	19.0
25-hydroxy vitamin D3	93.8	24.3	15.5
3-epi-25-hydroxy vitamin D3	98.8	21.3	15.3
25-hydroxy vitamin D2	97.0	29.8	21.4
3-epi-25-hydroxy vitamin D2	72.7	24.5	23.0
1-α hydroxy vitamin D3	110.3	27.7	21.0

- Excellent recovery in serum of all analytes in both standard solutions using HybridSPE-Plus plate
- Protein precipitation results in significantly reduced recovery for 25-hydroxy vitamin D3 and 3-epi-25-hydroxy vitamin D3.

Phospholipid Matrix Monitoring



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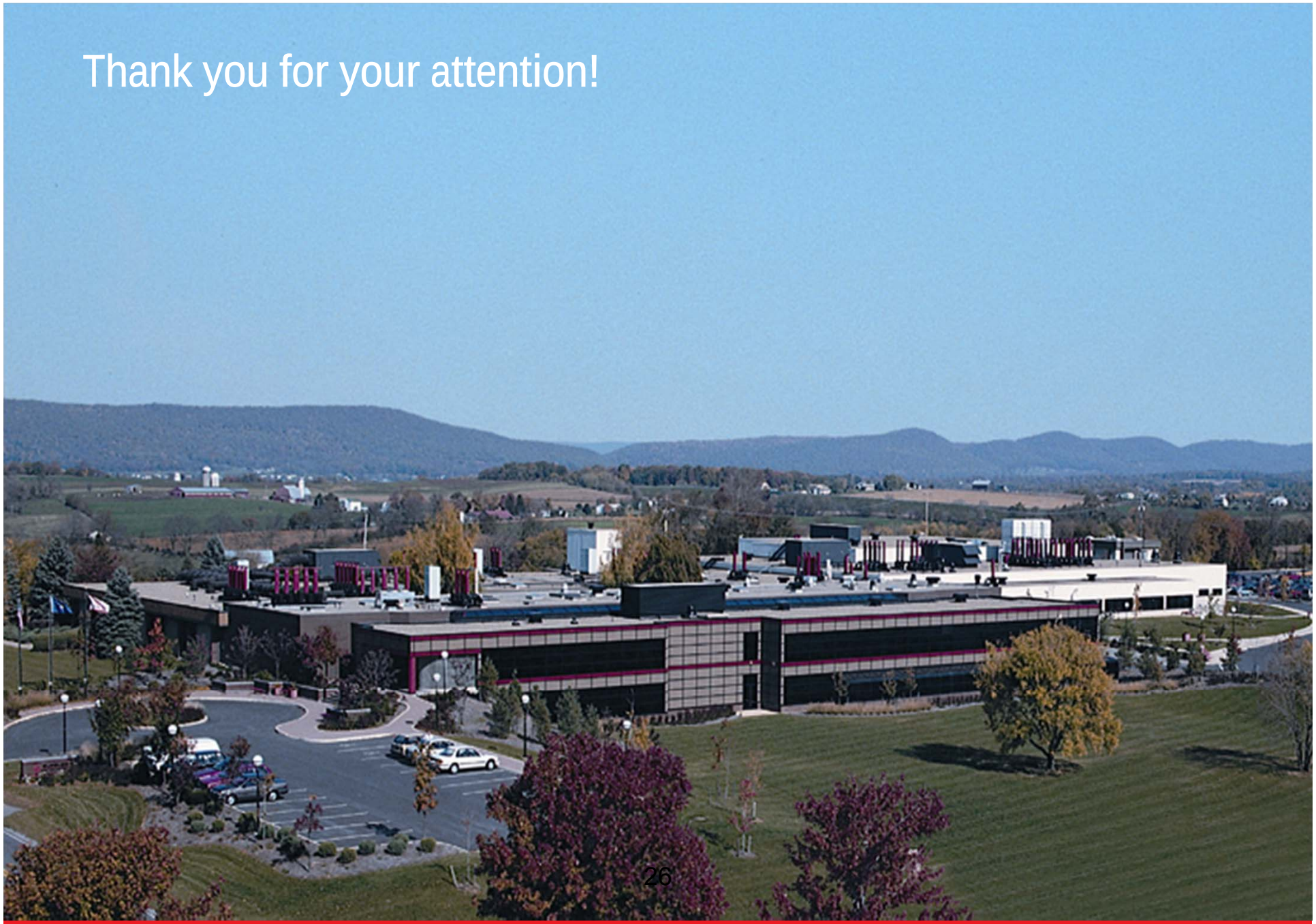
Summary

- Column screening of different stationary is the best approach for HPLC method development
  - Unique selectivity of Ascentis Express F5 enables a fast and efficient method for the analysis of vitamin D metabolites from serum samples.
- Samples processed using HybridSPE-PLus technique show no matrix interference resulting in lower limits of quantitation
  - Phospholipid matrix interference was observed using the standard protein precipitation technique resulting in a 40% reduction of response for three of the metabolites
  - The selective phospholipid depletion of the HybridSPE-PLus 96-well plate enables an efficient sample cleanup, increasing method reproducibility and accuracy
- This approach demonstrates how selectivity, in both sample preparation and chromatography, allows for efficient LC/MS analysis

Thank you for your attention!



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