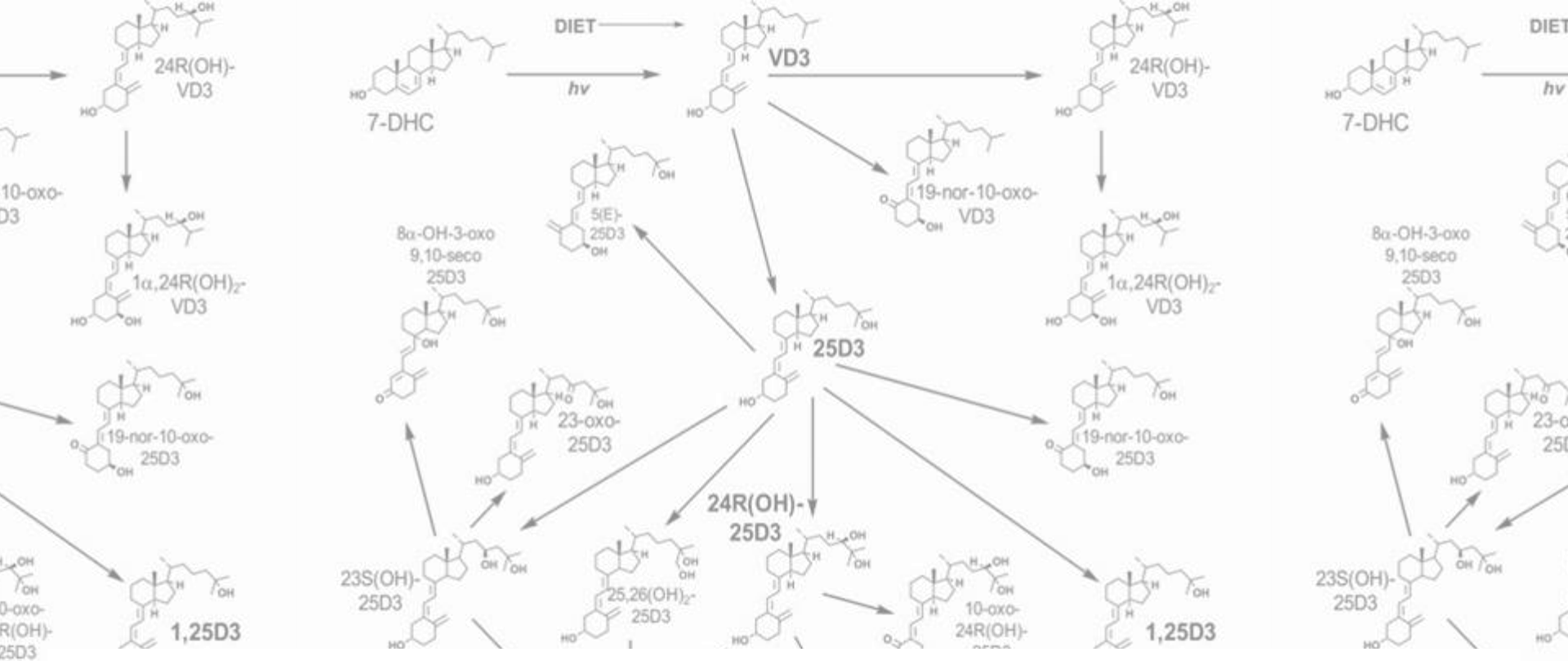


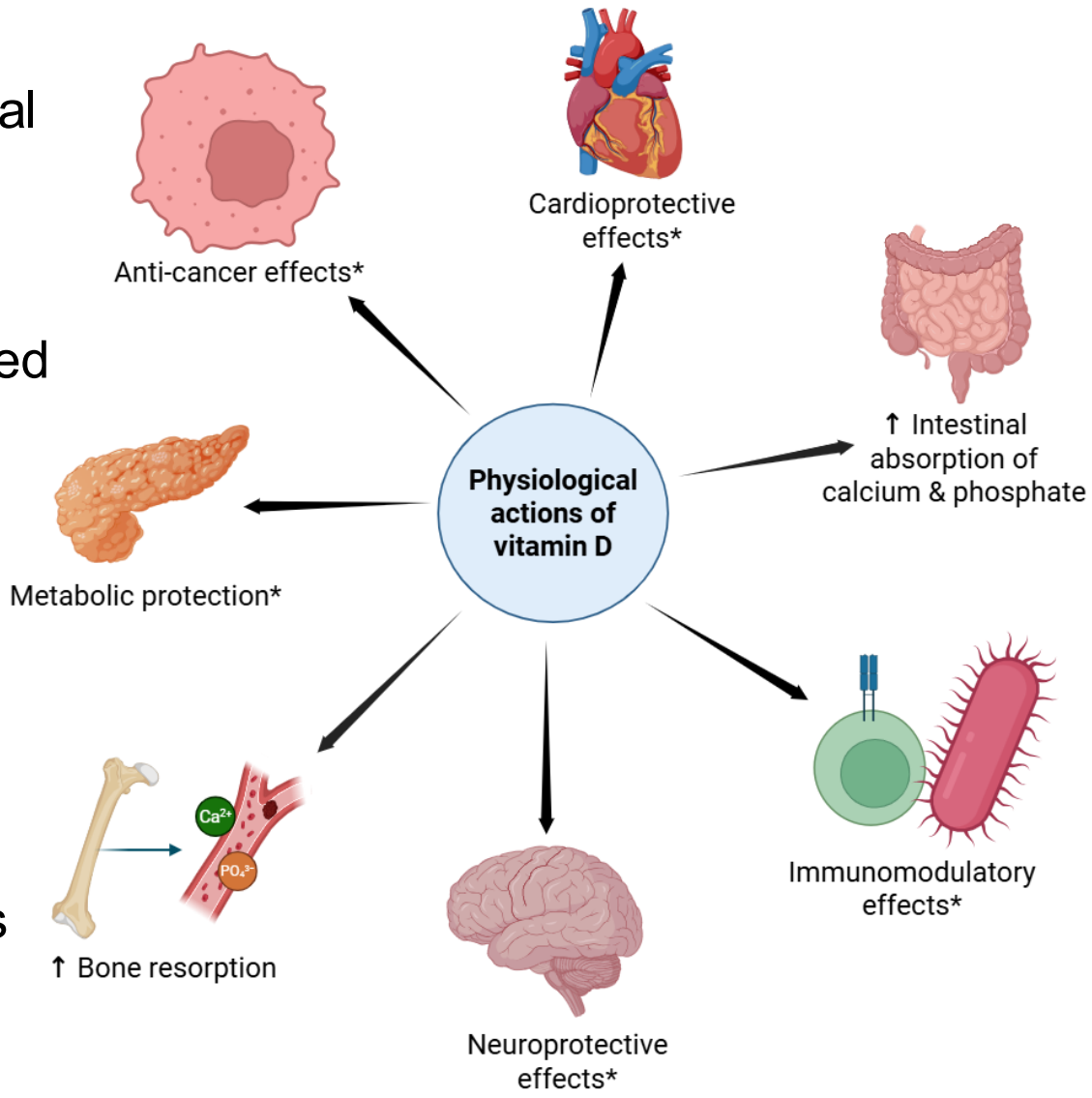
Challenges in the development of an LC-MS/MS method for the simultaneous measurement of vitamin D metabolites

Megan Souness

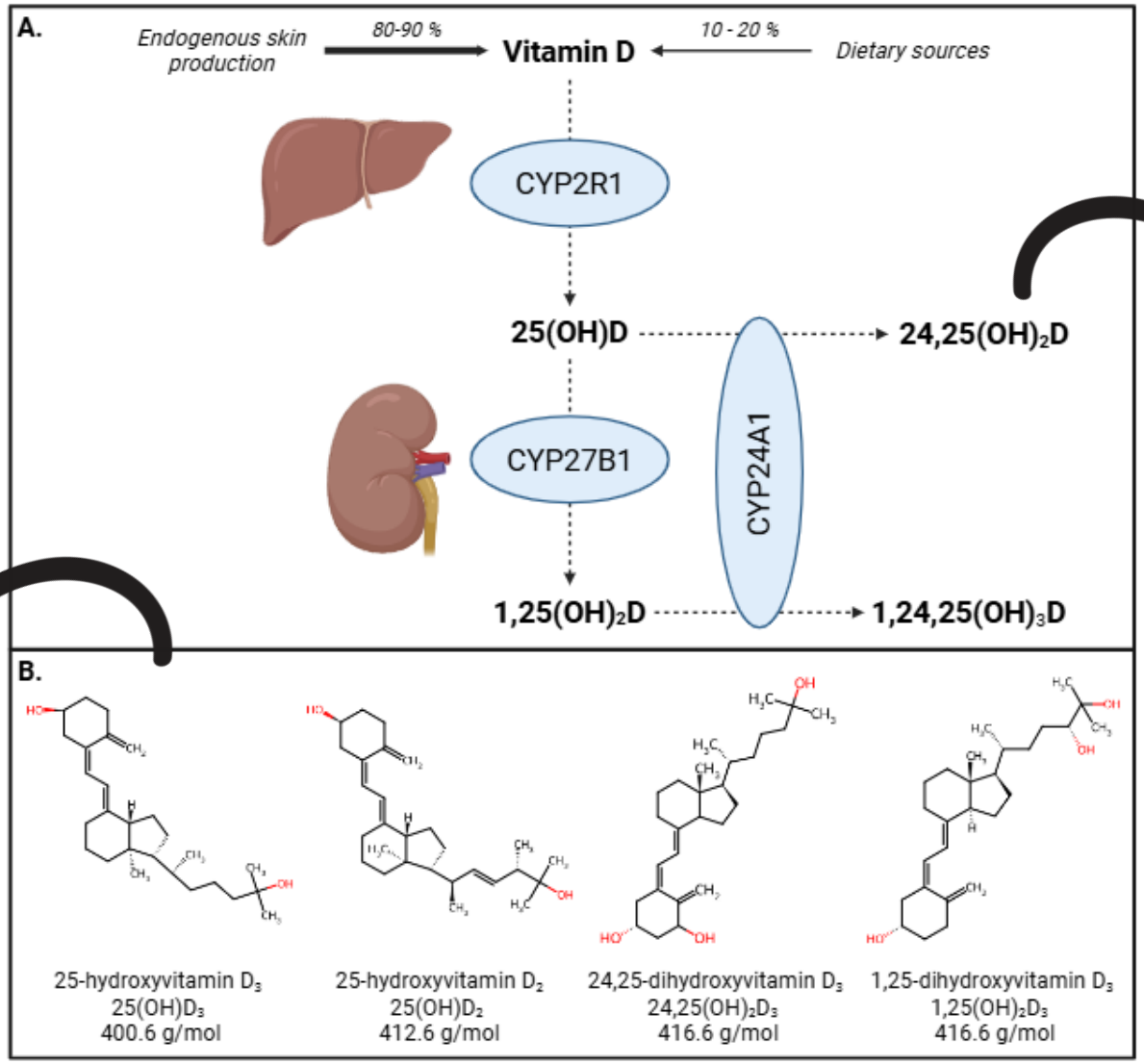
Imperial College Healthcare NHS Trust

Project Background

- Vitamin D's major physiological role is calcium-phosphate homeostasis & bone health.
- There are many other proposed non-classical roles of Vit D*.
- Vitamin D deficiency is a global pandemic.
- Vitamin D guidelines lack consensus in clinical cut-offs.
- Vitamin D measurement lacks standardization.



Project Background



The major catabolic product of vitamin D

Substantial variability in vitamin D assays

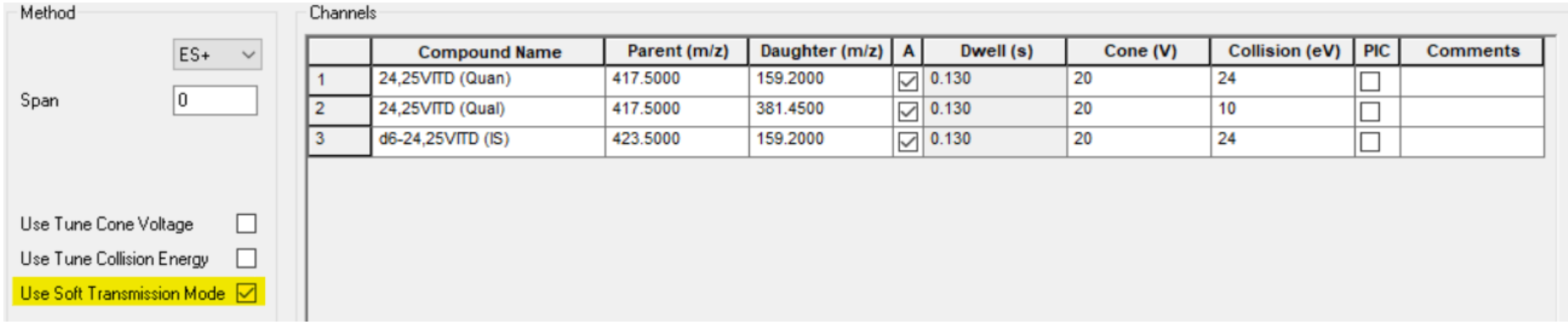
Project Aims & Objectives

To validate an LC-MS/MS method for the measurement of vitamin D metabolites, $24,25(OH)_2D_3$, $25(OH)D_3$, $25(OH)D_2$, that can:

- Provide clinical utility in the assessment of vitamin D deficiency & specific disease states e.g. Idiopathic infantile hypercalcaemia (IIH) & CKD.
- Allow us to better understand interference in routine vitamin D measurement.
- Contribute to a NIST & DEQAS collaborative intercomparison study on the assessment of $24,25(OH)_2D_3$ assay commutability.

Sensitivity - Ionization efficiency

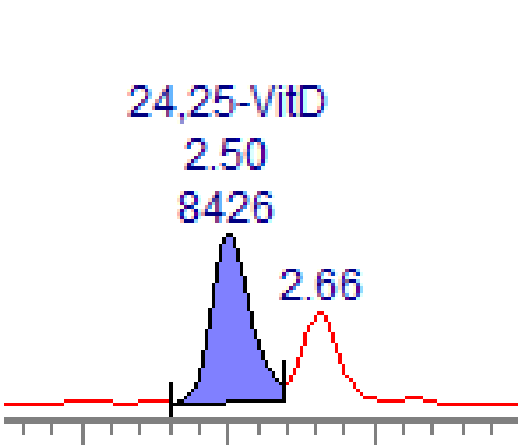
- Ionization efficiency describes how effectively compounds become ionized
- Ionization efficiency is affected by **in-source fragmentation**
- Water-loss $[M + H - H_2O]^+$ is a big problem for Vit D metabolites
- Can be overcome by using the water loss precursor ion mass
  - *i.e., m/z 417.5 becomes 399.5 (-18)*
- Or, using **Soft Transmission Mode (STM)**
  - *Selected for unstable ion species prone to in-source fragmentation*

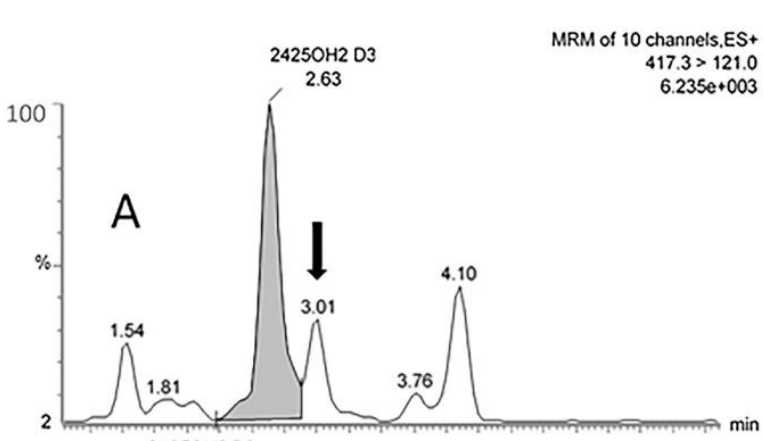


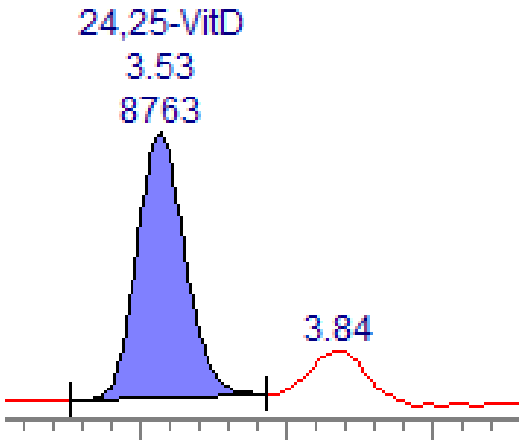
	Compound Name	Parent (m/z)	Daughter (m/z)	A	Dwell (s)	Cone (V)	Collision (eV)	PIC	Comments
1	24,25VITD (Quan)	417.5000	159.2000	☑	0.130	20	24	☐	
2	24,25VITD (Qual)	417.5000	381.4500	☑	0.130	20	10	☐	
3	d6-24,25VITD (IS)	423.5000	159.2000	☑	0.130	20	24	☐	

Co-eluting interferences

- Optimized SLE to finally provide the desired sensitivity…
- Co-eluting peak for 24,25(OH)$_2$D$_3$





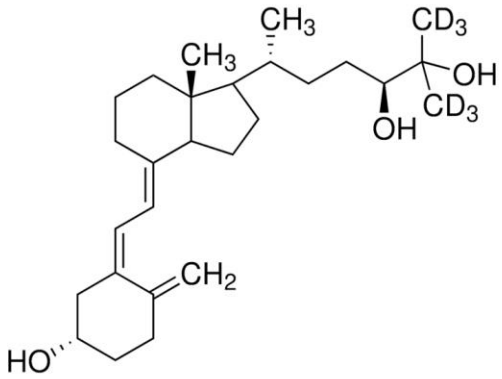


24R,25(OH)$_2$D$_3$. A modified gradient method (as described in Section 2.5) was able to separate this peak, as shown in Fig. 2C. The mean retention time difference between 24R,25(OH)$_2$D$_3$ and the unknown was 0.60 ± 0.03 min (10 runs over two days).

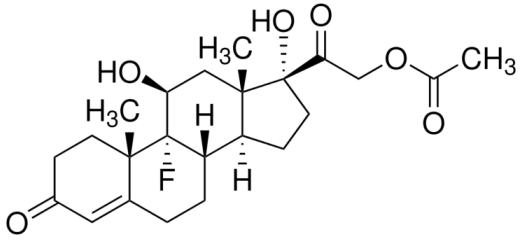
J. Chromatogr. A 1497 (2017) 102–109

Isobaric interferences & Matrix Effects

- Isobaric compounds are those with the same m/z.
- Identify isobaric compounds with computer searches (MassBank) and through literature review of existing methods.
- We must ensure these do not co-elute with our target compounds.



m/z = 423.5



d6-24,25(OH)$_2$D3 (IS)

Fludrocortisone

- Sample matrix components can also co-elute with target compounds.
- Phospholipids are a well-known cause of ion suppression.

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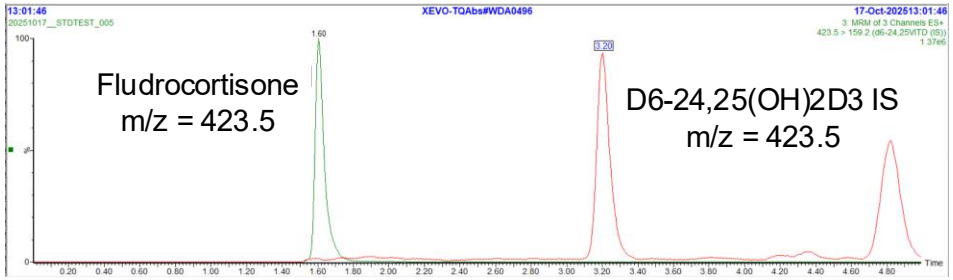
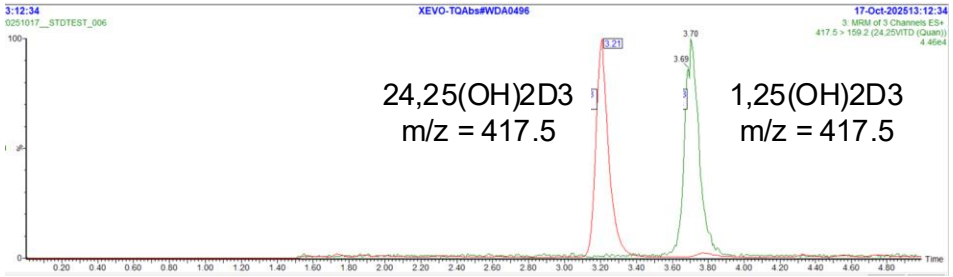
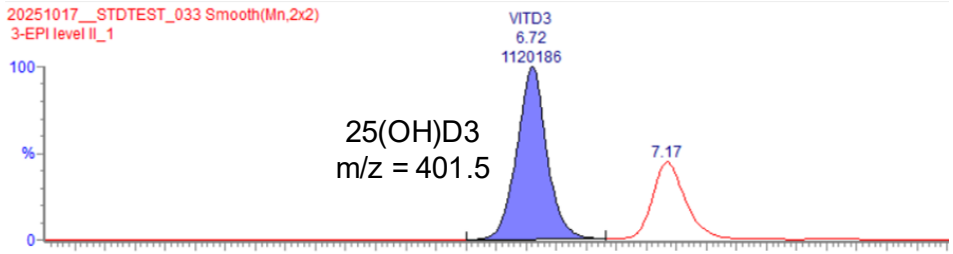
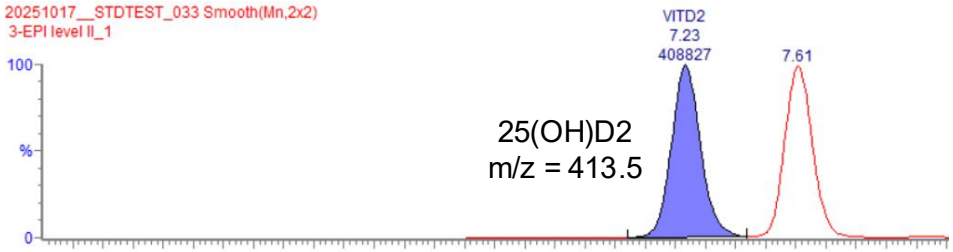
Isobaric interferences & Matrix Effects



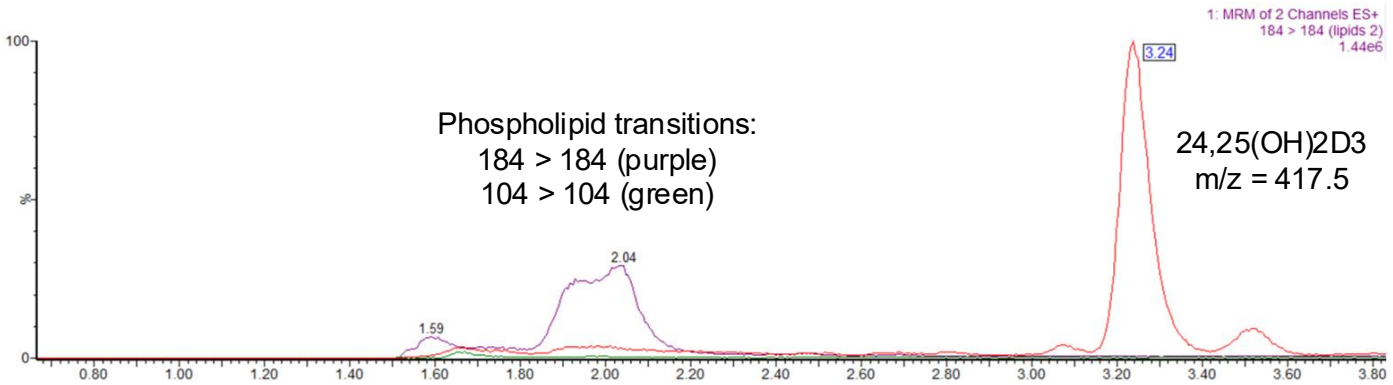




3-epi-25(OH)D3
m/z = 401.5



3-epi-25(OH)D2
m/z = 413.5



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NIST & DEQAS collaboration

NIST - National Institute of Standards and Technology (USA)

DEQAS - The Vitamin D External Quality Assessment Scheme

- We participated in a NIST 24,25$(OH)_2D_3$ interlaboratory comparison study.
- We tested 50 unknown samples in duplicate with inter-batch NIST SRMs.
- Results were compared to the NIST RMP and 5 other participating labs.

➢ We confirmed our robust assay precision

➢ We confirmed our positive bias of +30% - *similar to 1 other laboratory*

➢ Revealed impurity of our 24,25$(OH)_2D_3$ material - *contributing to bias*

NIST National Institute of Standards and Technology

D·E·Q·A·S Vitamin D External Quality Assessment Scheme

Positive bias troubleshooting

- *Ongoing troubleshooting experiments for our positive bias*

- *Possible causes…*

➢ Calibrator calculation errors

➢ Poor calibrator preparation

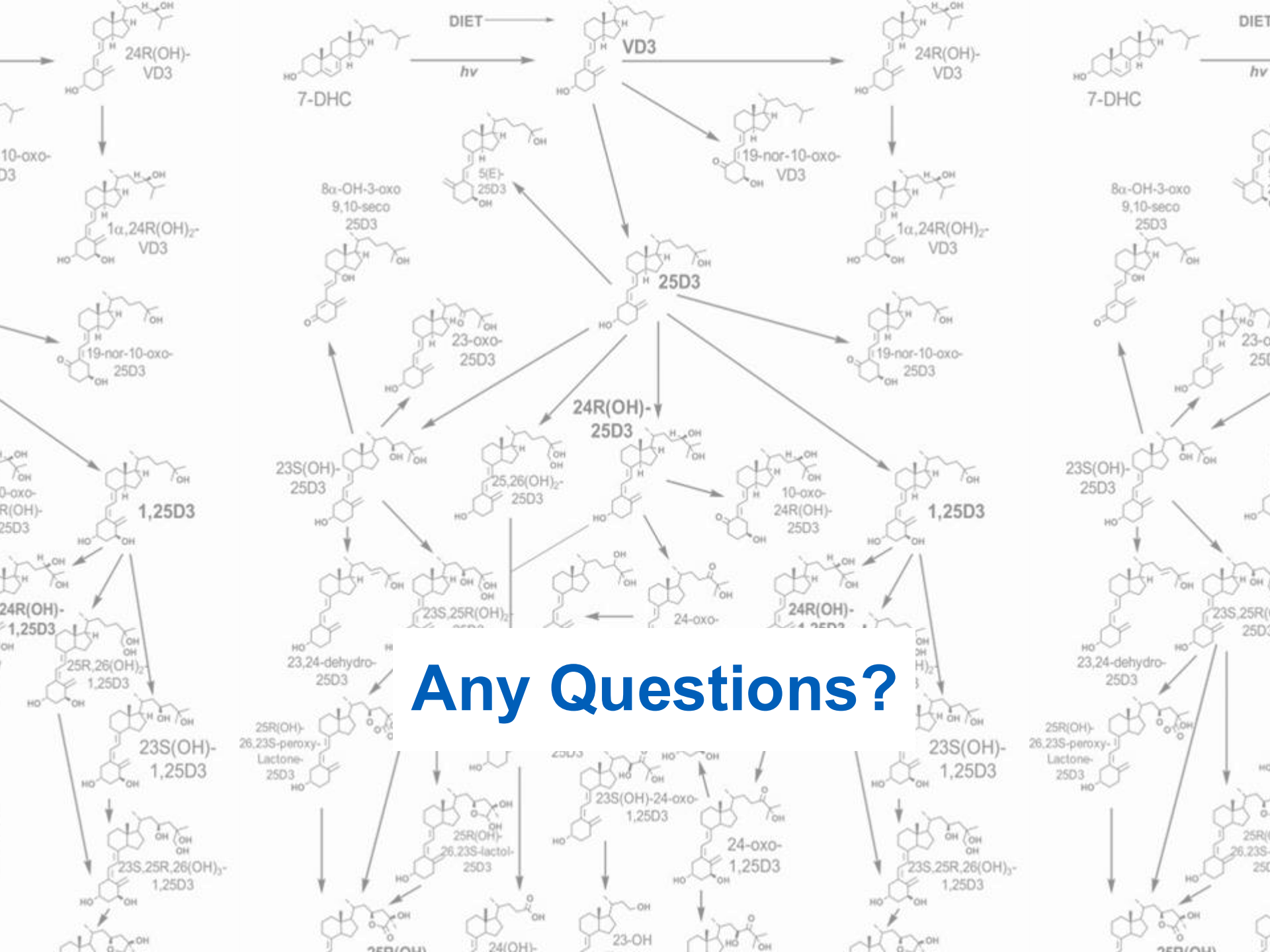
➢ Calibrator degradation

➢ Purity of primary materials

➢ Calibrator matrix commutability

Project Outcomes

Developed a high-sensitivity vitamin D metabolites LC-MS/MS method	• Tool for deficiency assessment • CYP24A1-mediated hypercalcaemia e.g. IIH • Bone metabolism, fracture risk, CKD
Partook in a NIST intercomparison study & contributed to the vitamin D standardization program	• Evidenced good intra & inter-assay precision • Confirmed issues with method accuracy
Highlighted the issue of primary stock impurity and calibrator matrix-matching	• Performed troubleshooting experiments • Positive bias remains an issue
Facilitated future DEQAS experimental studies on $24,25(OH)_2D$	• *Once the assay is fully validated!*



Any Questions?