



**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

# Vitamin D3 Determination - Automated, Streamlined, Robust and Reliable

Mohammed Abrar Unilabs York Bioanalytical Solutions





**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

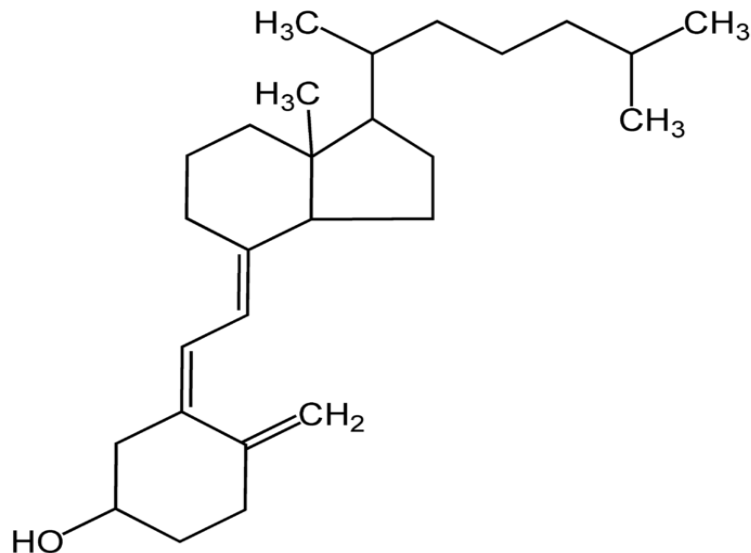
## Outline

- **Background**
- **Introduction**
- **Objectives**
- **Method challenges**
- **Novel solutions**
- **Results**
- **Summary**
- **Conclusions**



## Background

- Vitamin D3 is a calcium, phosphate and zinc regulator in blood
- It has 3 main metabolites 25-OH, 1,25-(OH)<sub>2</sub> and 24,25-(OH)<sub>2</sub>
- Vitamin D3 25-OH concentrations represent vitamin D3 status in individuals
- Vitamin D3 1,25-(OH)<sub>2</sub> is the biologically active metabolite
- Recent studies have indicated vitamin D3 deficiencies may play a major role in causing diseases such as osteoporosis, cancer, diabetes, hypertension and cardiovascular diseases.
- For these reasons Vitamin D3 metabolites have become very important biomarkers for PD purposes

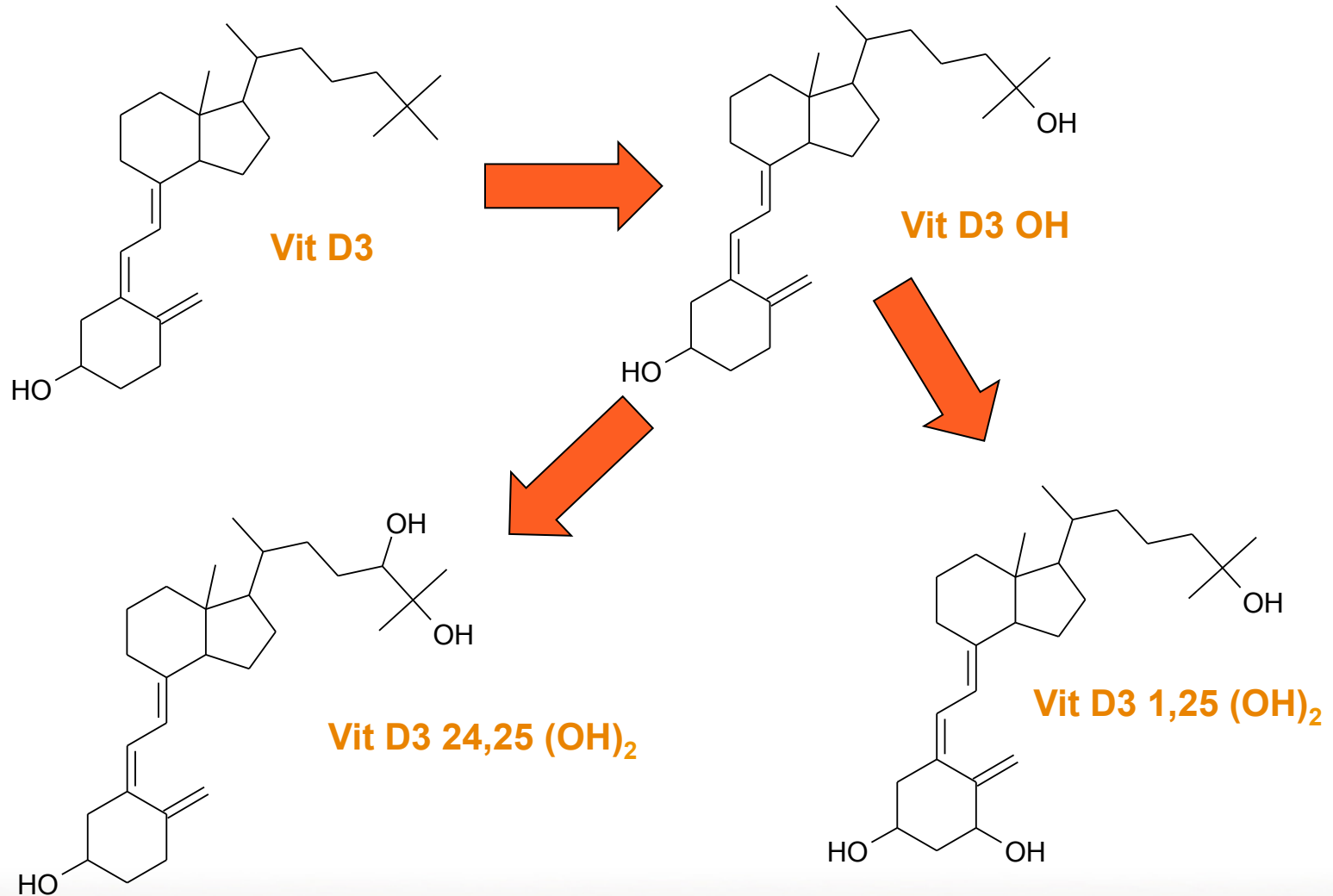


# Introduction



**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen



# Objectives



**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

- To develop LC-MS/MS methods for all 3 metabolites
  - Sensitive method 10pg mL for 1,25-(OH)<sub>2</sub>
  - Robust and Reliable
  - High throughput
  - 96 well format
  - Enable fast TATs for sample analysis





## Method Challenges

- Poor MS ionisation and fragmentation due to lack of suitable moiety on the compounds
- Strong protein binding to vitamin D specific plasma protein
- Difficult to achieve method selectivity due to very similar isobaric endogenous compounds
- Very high sensitivity requirements eg 10pg mL for 1,25-(OH)<sub>2</sub>
- Difficult to achieve high throughput 96 well plate format



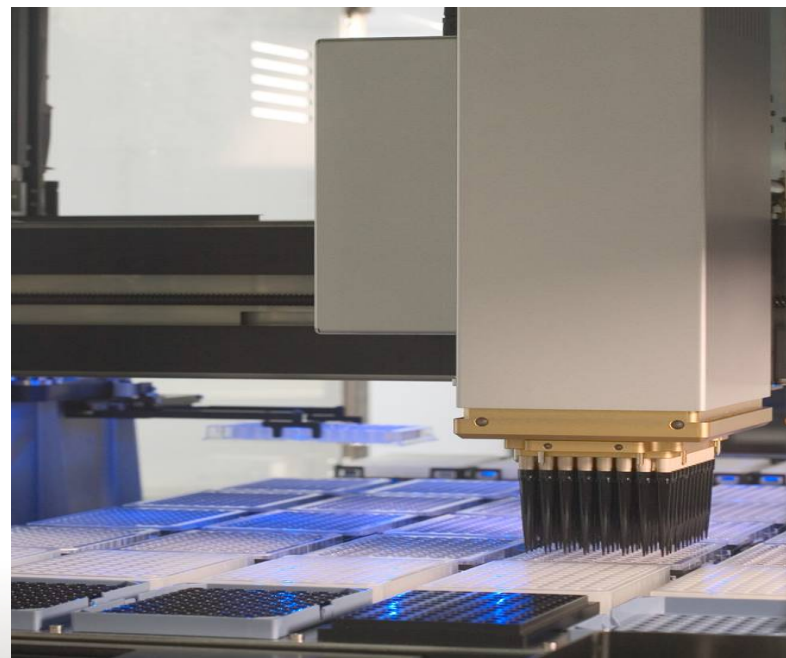


**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

## Novel Solutions

- Protein Binding Disruption
  - Plasma samples precipitated with a mixture of Chaotropic agent zinc sulphate and acetonitrile
  - Fully automated on Hamilton and in 96 well plate format
- Extraction
  - Samples taken from robot and directly applied to non-encapped C18 SPE
  - Analyte retention by polar interactions with silanols
  - Elution with pure organic hexane/IPA



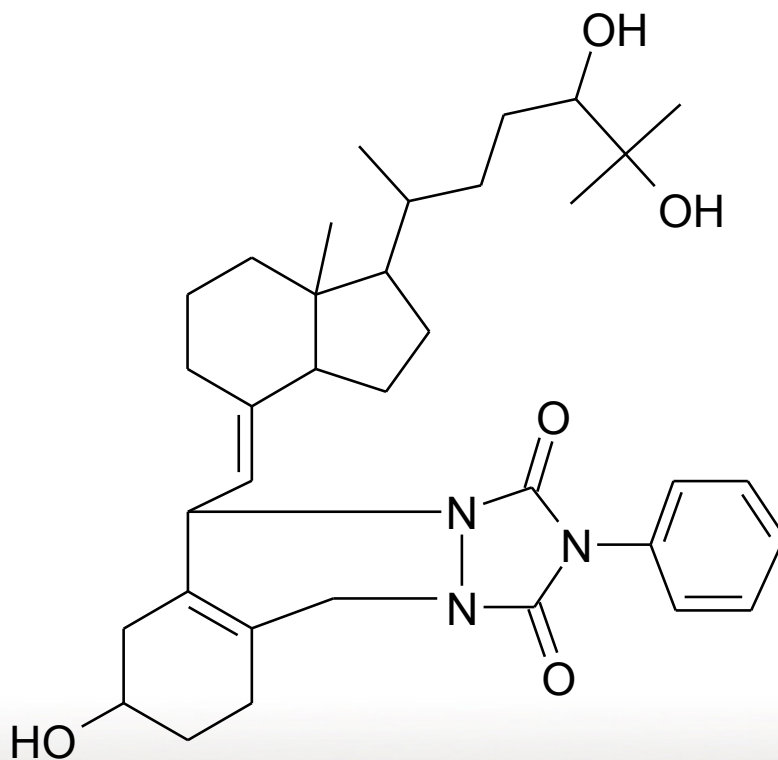
# Derivatisation with PTAD



**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

- PTAD (phenyl triazole dione)
  - Simple & fast derivatisation in 1 hour at room temperature
  - Enhances MS sensitivity and aids chromatographic separation
  - Diels-Alder reaction of conjugated dienes with dienophiles to produce cyclic product







## MS Detection

- **Vitamin D3 25 OH**
  - MRM [M-H<sub>2</sub>O+H] 383 > 257,211
  - SIL internal std d<sup>6</sup> [M-H<sub>2</sub>O+H] 389 > 211
- **Vitamin D3 1,25 (OH)<sub>2</sub>**
  - MRM [M+PTAD-H<sub>2</sub>O+H] 574 > 314
  - SIL internal std d<sup>6</sup> [M+PTAD-H<sub>2</sub>O+H] 580 > 314
- **Vitamin D3 24,25 (OH)<sub>2</sub>**
  - MRM [M+PTAD-H<sub>2</sub>O+H] 574 > 298
  - SIL internal std d<sup>6</sup> [M+PTAD-H<sub>2</sub>O+H] 580 > 298

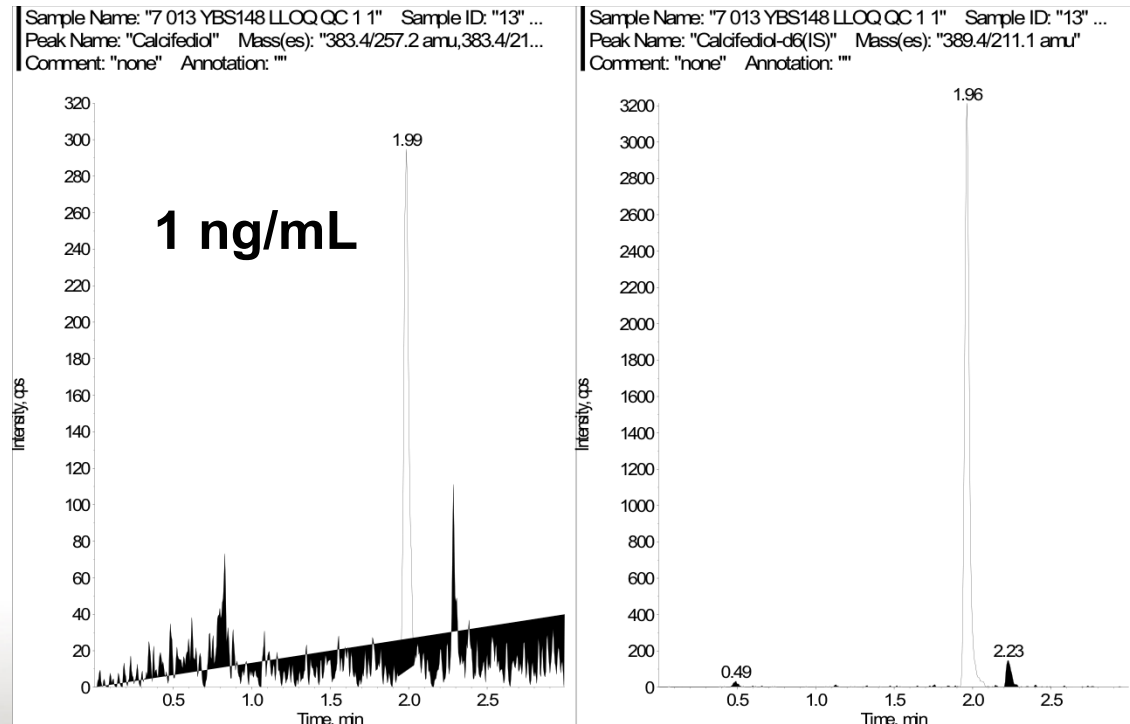
# Results (Methods)



**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

- **Method 1** Vitamin D3 25 (OH)
- Assay range 1 to 200ng/mL
- Surrogate matrix 5% BSA in PBS
- Assay volume 200 µL
- Extraction protein precipitation followed by C18 SPE (Generic)
- Chromatography on sub 2 µm phenyl column, cycle time 4 min with APCI+ MS detection
- No derivatisation



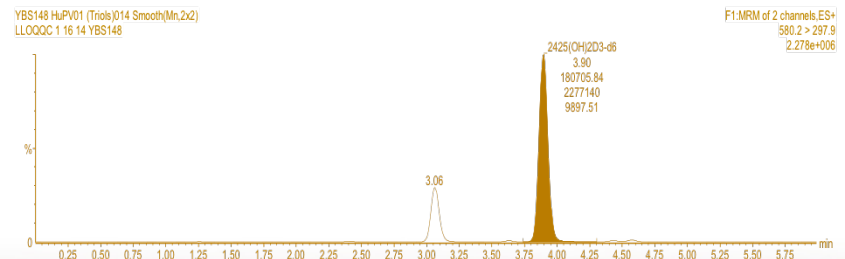
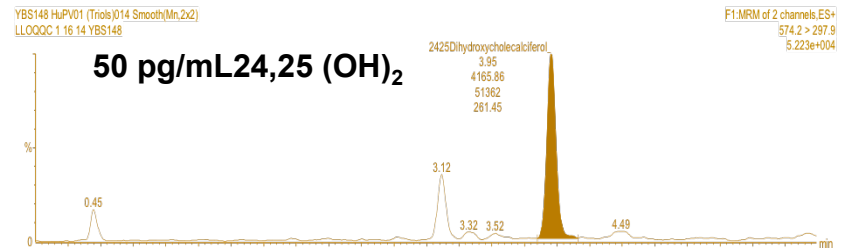
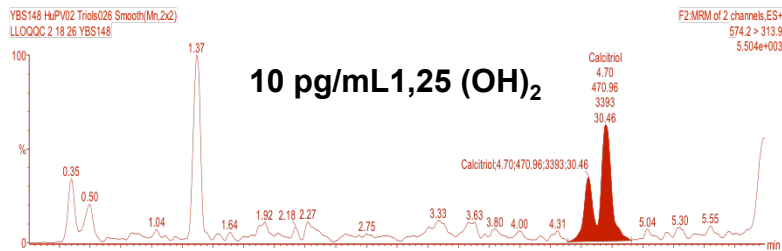
# Results (Methods)



**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

- **Method 2** Vitamin D3 1,25 (OH)<sub>2</sub> and 24,25 (OH)<sub>2</sub>
- Assay range 10 to 2000 pg/mL 1,25 (OH)<sub>2</sub>
- Assay range 50 to 10000 pg/mL 24,25 (OH)<sub>2</sub>
- Surrogate matrix 5% BSA in PBS
- Assay volume 300 µL
- Extraction protein precipitation followed by C18 SPE (Generic)
- Chromatography on sub 2 µm C18 column, cycle time 7 min with ESI+ MS detection
- PTAD derivatisation





## Results (Methods)

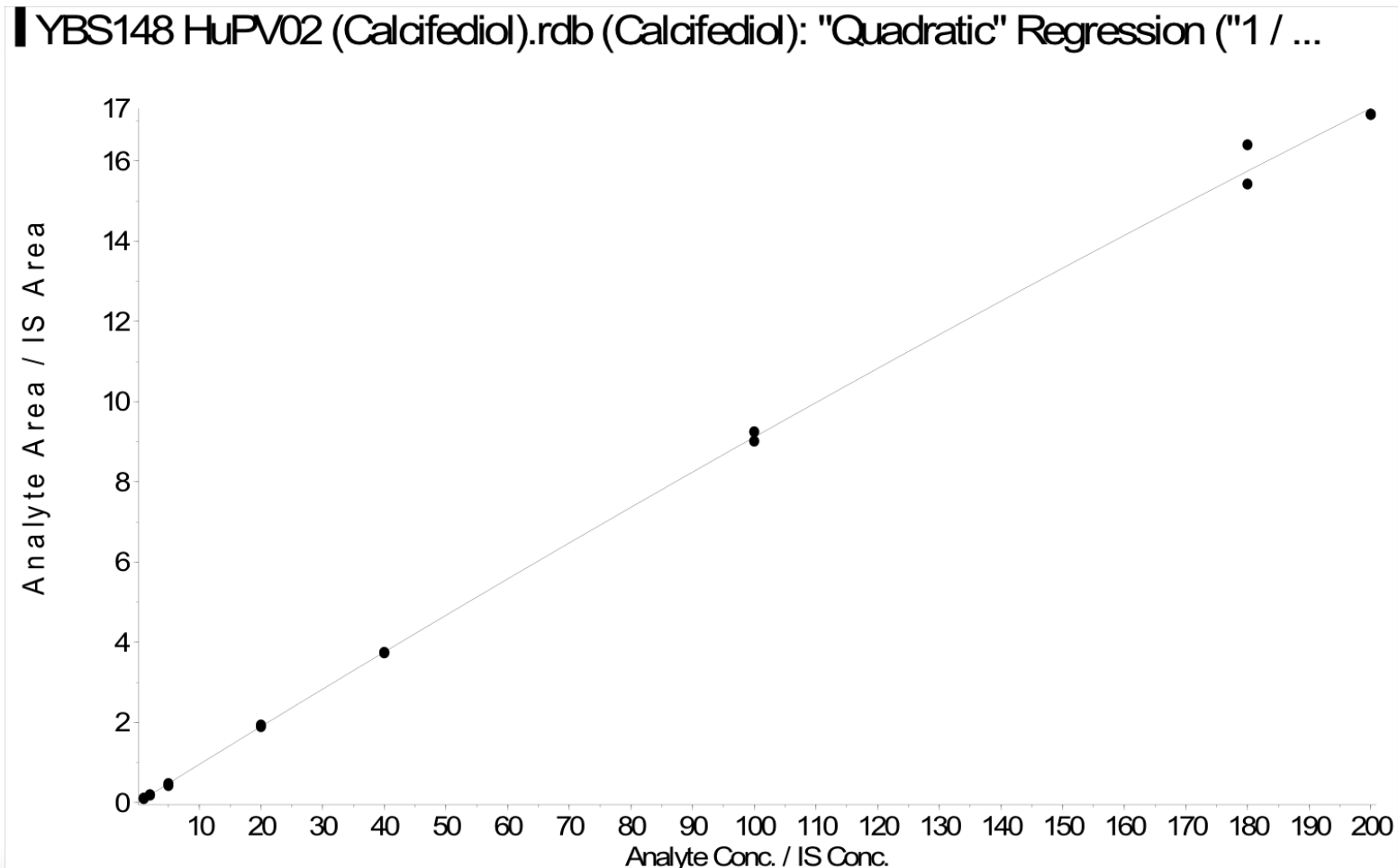
- Calibration curves:- LLOQQC and LoQC's prepared in surrogate in 5.0% BSA in PBS
- MeQCs pre-determined endogenous plasma concentrations (pre-quantified)
- HiQC, ULOQQC, DiQC prepared in (pre-quantified) endogenous plasma with std addition
- DiQC's diluted with surrogate



**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

## Vitamin D3 25 (OH) Calibration line





**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

## Vitamin D3 1,25 (OH)<sub>2</sub> Calibration line:

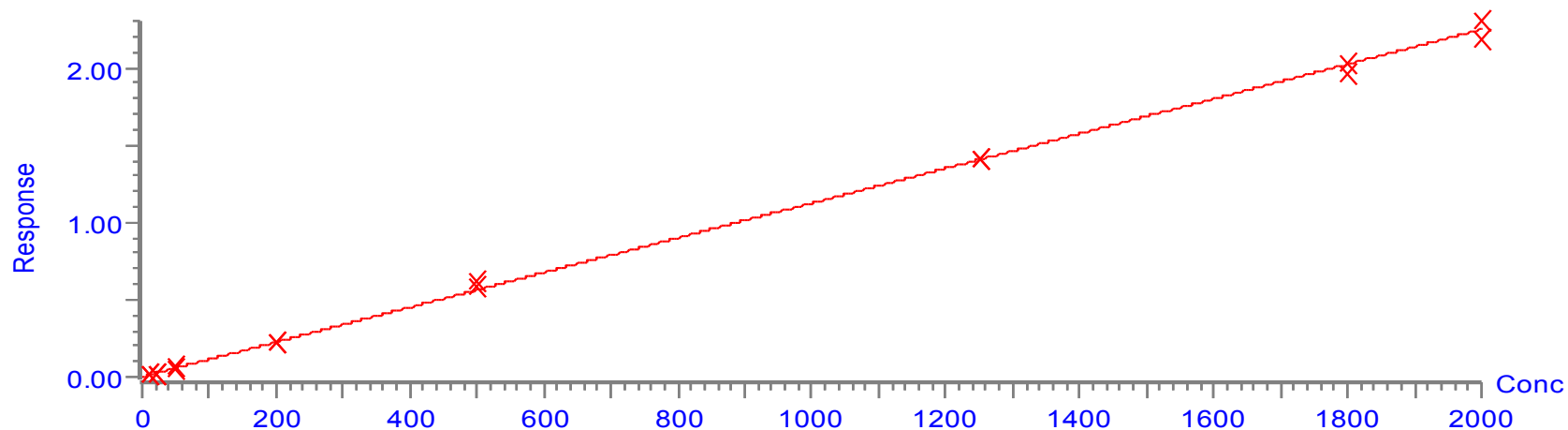
Compound name: Calcitriol

Correlation coefficient:  $r = 0.999504$ ,  $r^2 = 0.999008$

Calibration curve:  $0.00112319 * x + 0.00135242$

Response type: Internal Std ( Ref 4 ), Area \* ( IS Conc. / IS Area )

Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None





**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

## Vitamin D3 24,25 (OH)<sub>2</sub> Calibration line:

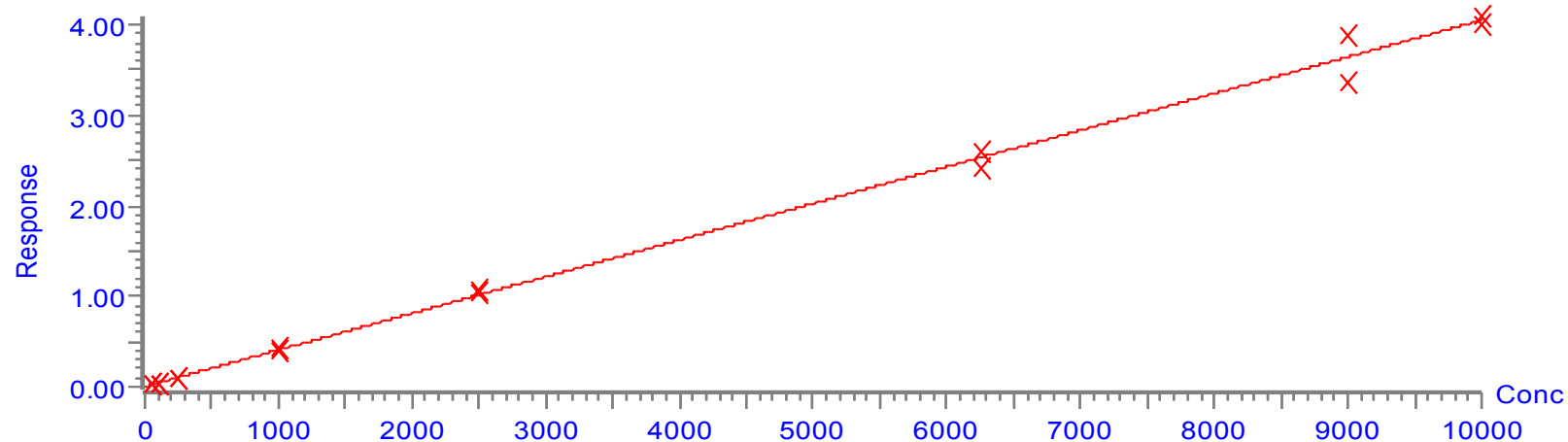
Compound name: 2425Dihydroxycholecalciferol

Correlation coefficient:  $r = 0.998958$ ,  $r^2 = 0.997917$

Calibration curve:  $0.000404505 * x + 0.00228599$

Response type: Internal Std ( Ref 2 ), Area \* ( IS Conc. / IS Area )

Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None





## The validation data for Vitamin D3 25 OH

Concentration (ng/mL)	Mean intra-run precision and bias		Inter-run precision and bias	
	Precision (CV%)	Bias (%)	Precision (CV%)	Bias (%)
1.00	7.2	8.7	7.8	9.0
3.00	4.4	-2.7	5.3	-2.7
19.2	3.4	-3.8	3.7	-3.6
169	2.8	4.9	4.1	4.7
Room temperature		Freeze-thaw cycles		
24 hours		3		
Whole blood on ice and at room temperature	Extracts at +4 °C		Solution in methanol -20°C	
2 hours	20 hours		122 days	





## The validation data for Vitamin D3 1,25 (OH)<sub>2</sub>

Concentration (pg/mL)	Mean intra-run precision and bias		Inter-run precision and bias	
	Precision (CV%)	Bias (%)	Precision (CV%)	Bias (%)
10.0	9.2	0.7	10.5	0.0
30.0	5.6	-4.1	9.3	-4.0
170	3.9	2.8	6.4	2.9
1520	5.1	2.9	5.7	2.6
Room temperature		Freeze-thaw cycles		
24 hours		3		
Whole blood on ice and at room temperature	Extracts at +4 °C		Solution in methanol -20°C	
2 hours	24 hours		171 days	



## The validation data for Vitamin D3 24,25 (OH)<sub>2</sub>

Concentration (pg/mL)	Mean intra-run precision and bias		Inter-run precision and bias	
	Precision (CV%)	Bias (%)	Precision (CV%)	Bias (%)
50.0	3.2	8.5	6.3	9.0
150	2.9	12.3	3.8	12.0
1390	2.8	1.0	3.4	0.7
7140	2.1	0.6	2.9	0.6
Room temperature		Freeze-thaw cycles		
24 hours		3		
Whole blood on ice and at room temperature	Extracts at +4 °C		Solution in methanol -20°C	
2 hours	24 hours		167 days	

# Summary



**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

- TATS:
- Samples received Monday afternoon
- Analysis Tuesday; 1 analyst 69 samples (one batch for 25 OH, one batch 1,25 & 24,25 (OH)<sub>2</sub> metabolites)
- 2 mass specs
- QC checked data to sponsor Wednesday midday
- 48 hour TAT



# Conclusions



**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

- Lots of labs run Vitamin D3 methods
- Use of automation and 96 well plate format enable high throughput
- Combination of good analytics ensures robust and reliable methods
- Biomarkers are becoming more important for early disease detection and prevention
- More biomarker assays now being converted from Elisa to MS
- Lots of new biomarker assays being developed small & large molecules
- They will require a combination of techniques to measure them eg Elisa, Quadrupole & High Res

*it ain't  
what you do, it's  
the way that  
you do it*

# Acknowledgements



**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

- YBS
  - Colleagues for their support on this project



**Unilabs**

Bioanalytical Solutions · York · Sandwich · Copenhagen

