A fast and simple method for simultaneous measurements of 25(OH)D, 24,25$(OH)_2$D and the Vitamin D Metabolite Ratio (VMR) in serum samples by LC-MS/MS

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Why should we be interested in $24,25(OH)_2D$ and VMR?

- $24,25(OH)_2D$ is the major product of 25(OH)D catabolism
- Levels of both 25(OH)D and $24,25(OH)_2D$ are strongly correlated in healthy persons
- Screening for 24-hydroxylase (CYP24A1) deficiency for cases of PTH-independent hypercalcemia, (idiopathic infantile hypercalcemia)
- Can only be determined by LC-MS/MS

Herrmann et al., CCLM 2016

Why should we be interested in $24,25(OH)_2D$ and VMR?

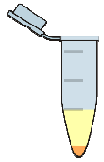
Vitamin D Metabolite Ratio (VMR)

$$VMR(\%) = \frac{Conc\ \ 24,25(OH)_2D_3(\mu g/L)}{Conc\ \ 25(OH)D_3(\mu g/L)} \cdot 100$$

- New candidate for vitamin D status
- VMR does not differ significantly between races
- VMR tends to be disproportionately decreased in patients with low 25OHD concentrations and in patients who have functional vitamin D deficiency because of CKD

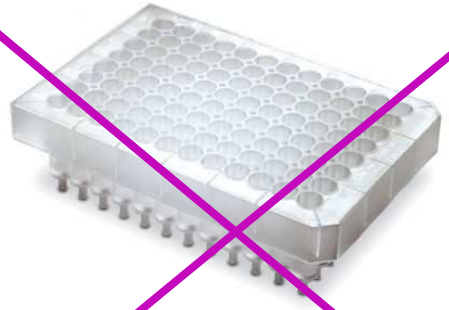
Berg, Clin Chem 2015

State of the Art



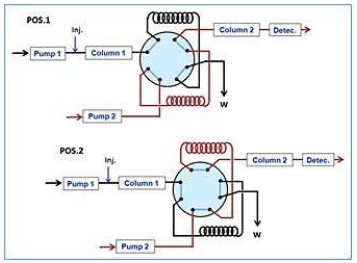
Protein precipitation

+



SPE, SLE, LLE, etc.

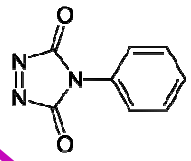
Two-dimension LC



One-dimension LC

25(OH)D,
epi-25(OH)D &
24,25(OH)$_2$D
by LC-MS

Amplifex®



PTAD

Derivatization

ESI

APCI

Ionization Source

Method development: *Instrumentation*

Liquid Chromatography (LC)

- Nexera X2 UPLC (Shimadzu) equipped with three binary pumps (A:LC30-AD, B:LC30-AD, C:LC20-AB)

<u>Column</u>

- Kinetex®PFP 100Å (100 x 2,1mm, 2,6µm)

  Flow : 0.4 mL/min

  Injection volume: 30 µL.

<u>Mobile phase</u>

A: Water B: Methanol

(both with 0.1% HCOOH)

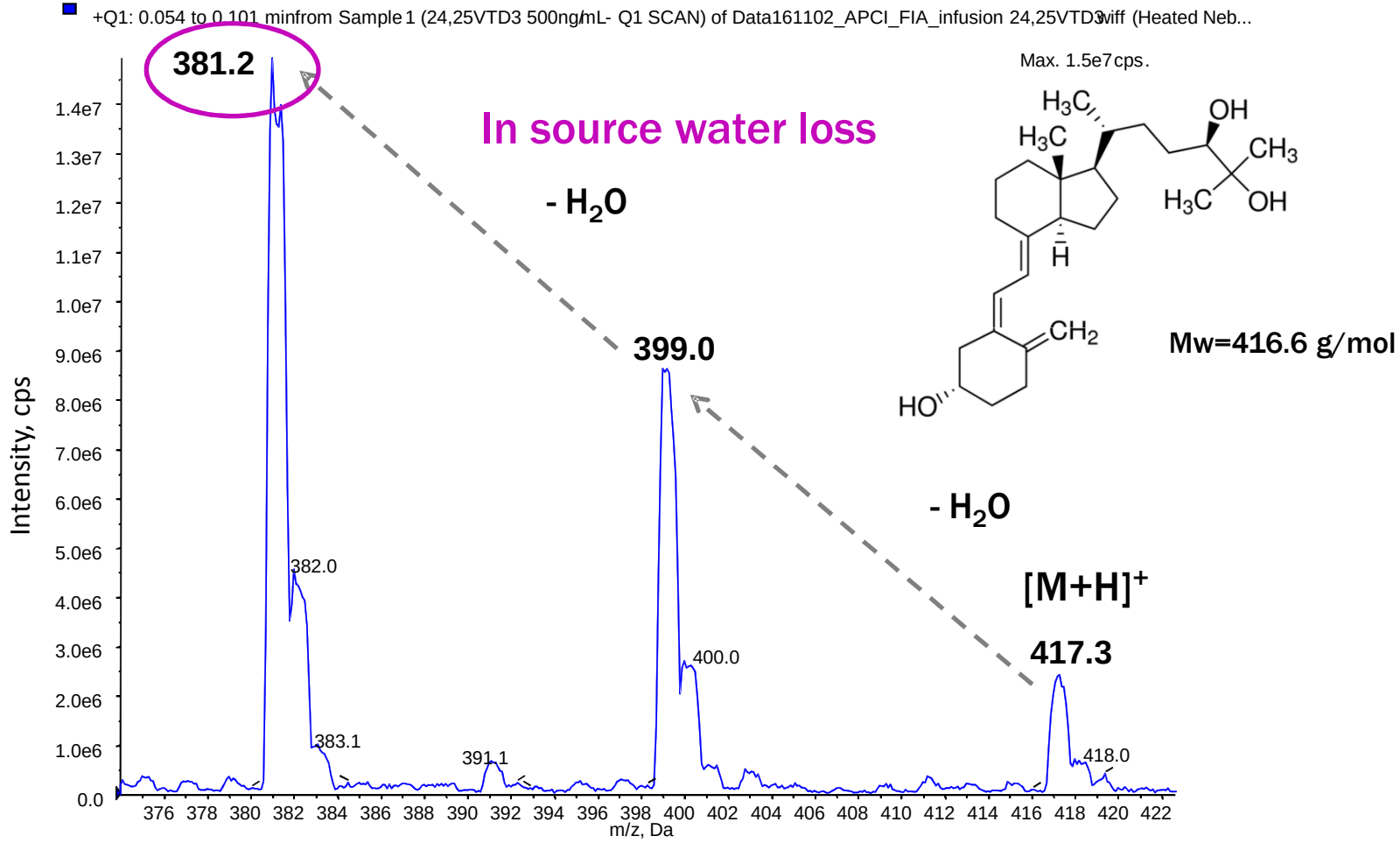
Gradient conditions

MS (Mass Spectrometry)

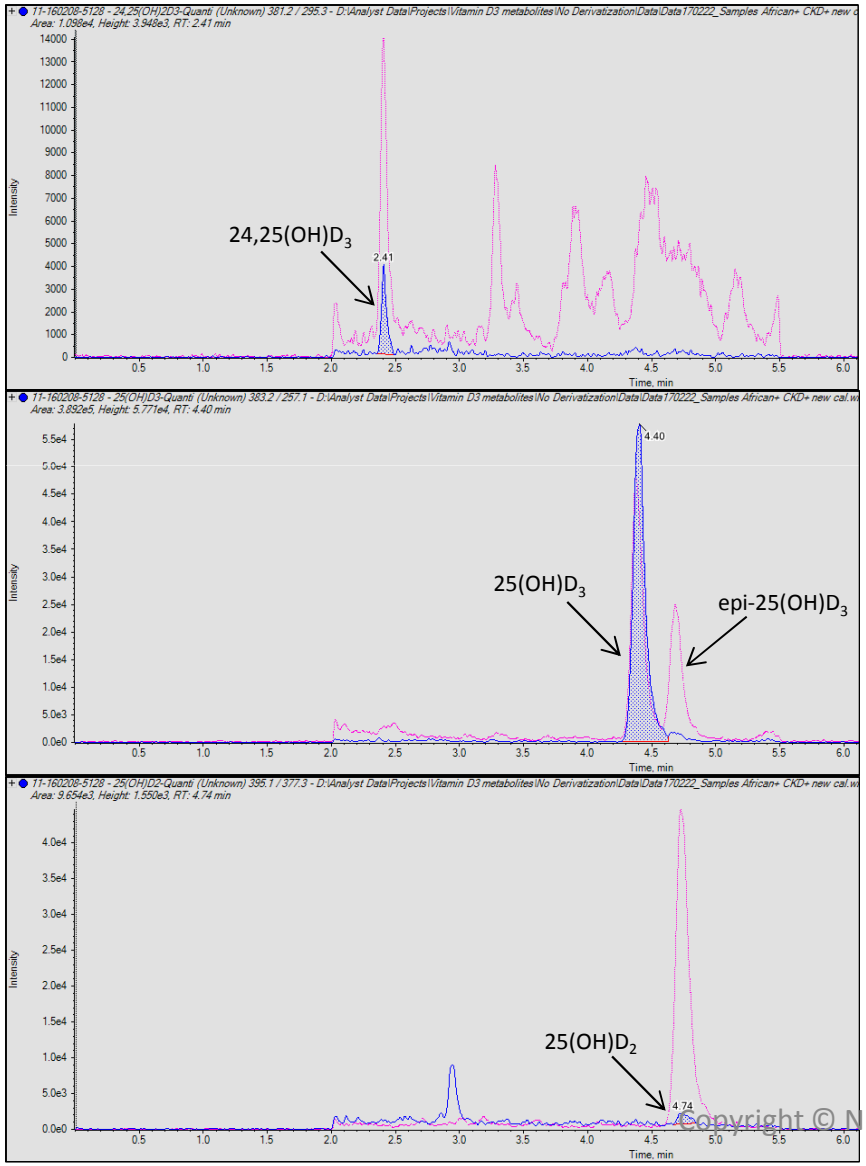
- Sciex QTRAP 6500 Quadrupole-linear ion trap
- Atmospheric Pressure Chemical Ionization (APCI+).
- Multiple Reaction Monitoring (MRM)
- Source/Gas Parameters optimized for $24,25(OH)_2D_3$

Method development: *Optimization of Mass spectrometry*

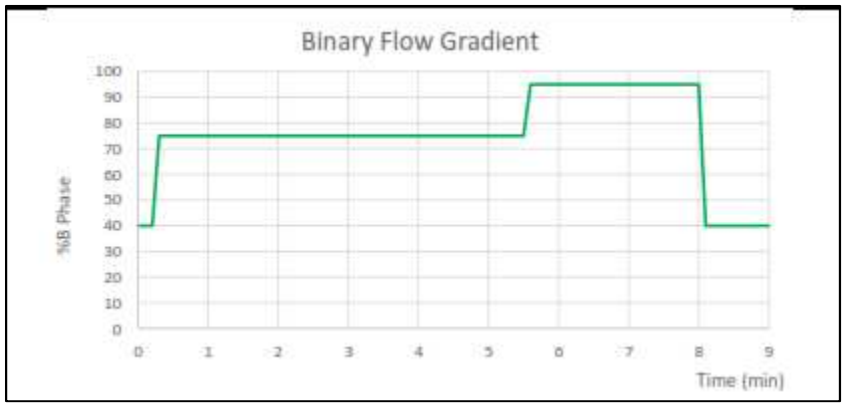
MS precursor ion scan



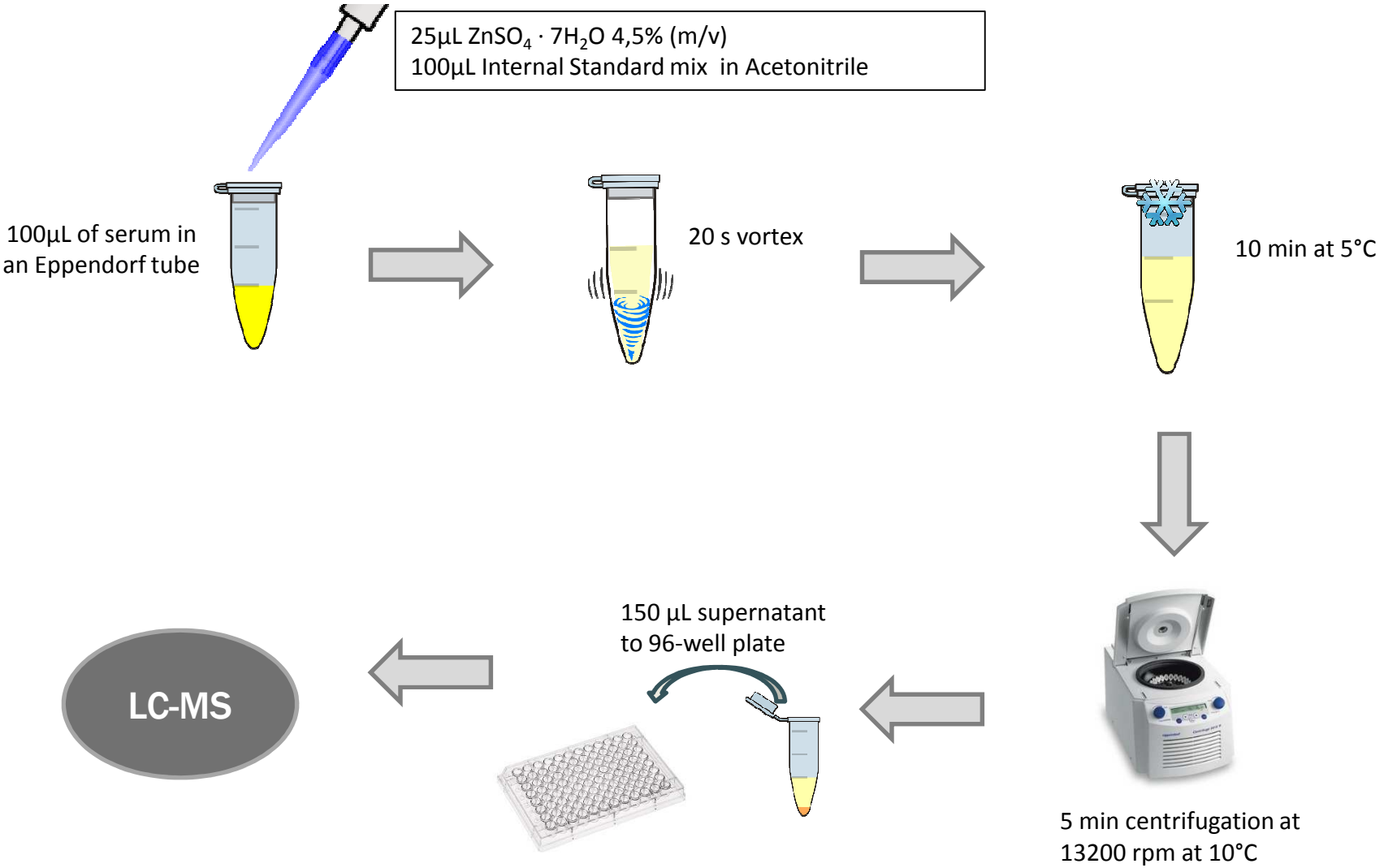
Method development: *Optimization of chromatography*



Chromatogram from a Chronic Kidney Disease (CKD) patient



Proposed procedure



Method validation

Analytical Validation

- Certified Reference Materials used:

  NIST 972a (n=4) and Labquality (n=6)

  (in triplicate during 3 days)

- Selectivity: separation from isobaric interferences $23,25(OH)_2D_3$ and $1\alpha(OH)D_3$.

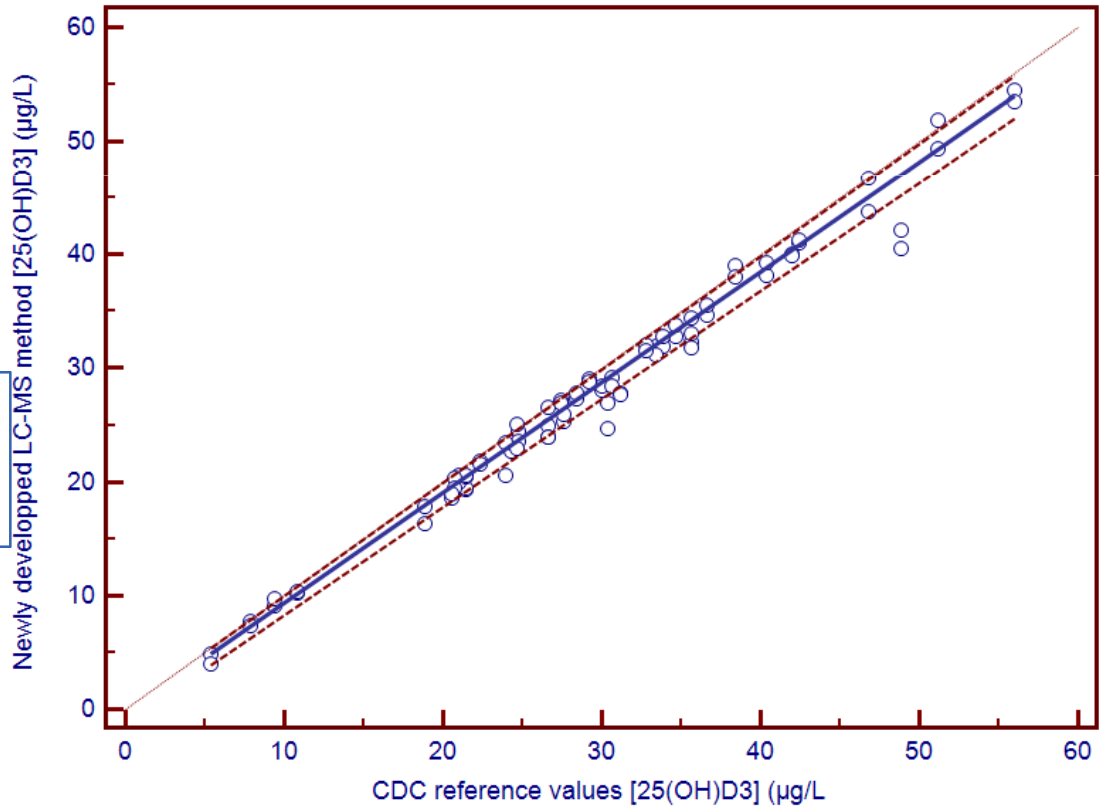
	LOQ (µg/L)	Matrix effect (%)	Recovery (%)	CV (%) Intra-assay	CV (%) inter-assay
$24,25(OH)_2D_3$	0.5	-6	99-102	2.5-5.5	2.5-5.5
$25(OH)D_3$	1.1	+5	95-104	1.3-4.8	2.6-4.8
$25(OH)D_2$	1.7	-17	101-105	2.9-5.1	3.6-5.5
epi-$25(OH)D_3$	1.1	-3	96-103	2.7-6.0	3.6-6.4

Method validation

Clinical Validation:

External control from the Center for Disease Control and Prevention (CDC), within the scope of the Vitamin D Standardization Program VDSP (n=80)

Passing-Bablok
APCI-PFP method = - 0.44+ 0.97CDC

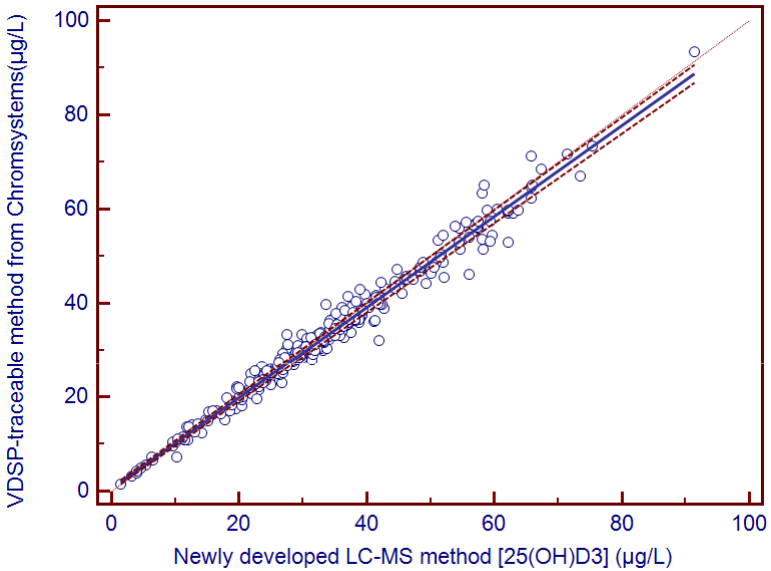


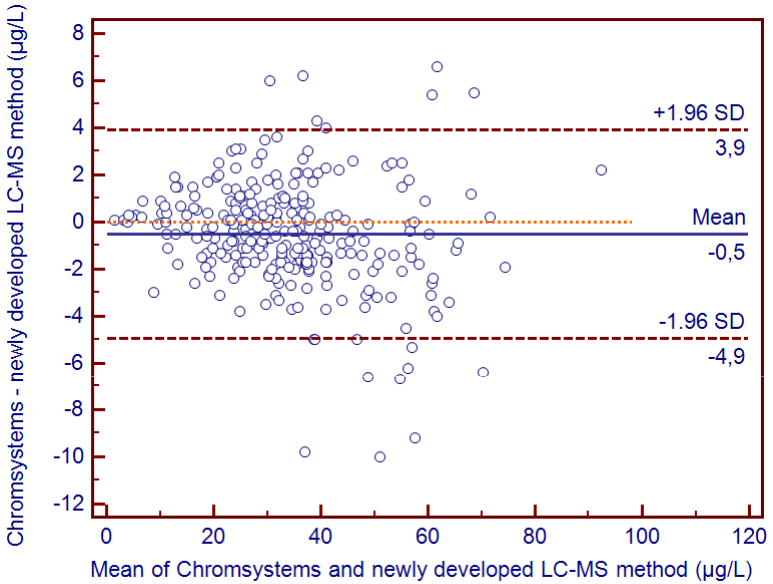
Method comparison

- Caucasian healthy subjects supplemented with vitamin D (n=50),
- African healthy subjects from Abidjan, Côte d'Ivoire (n=31).
- CKD patients with Glomerular Filtration Rate (GFR) <30 mL/min/1.73 m^2 (n=50)
- Stable hemodialyzed patients (n=50),
- Women referred to specialized osteoporosis clinic (n=50),
- 3rd trimester pregnant women (n=50),

The "true" value for 25(OH)D in these samples was determined with our VDSP-traceable LC-MS/MS method from Chromsystems® (MassChrom® 25-OH-Vitamin D3/D2 (LC-MS/MS), Chromsystems)

Method comparison



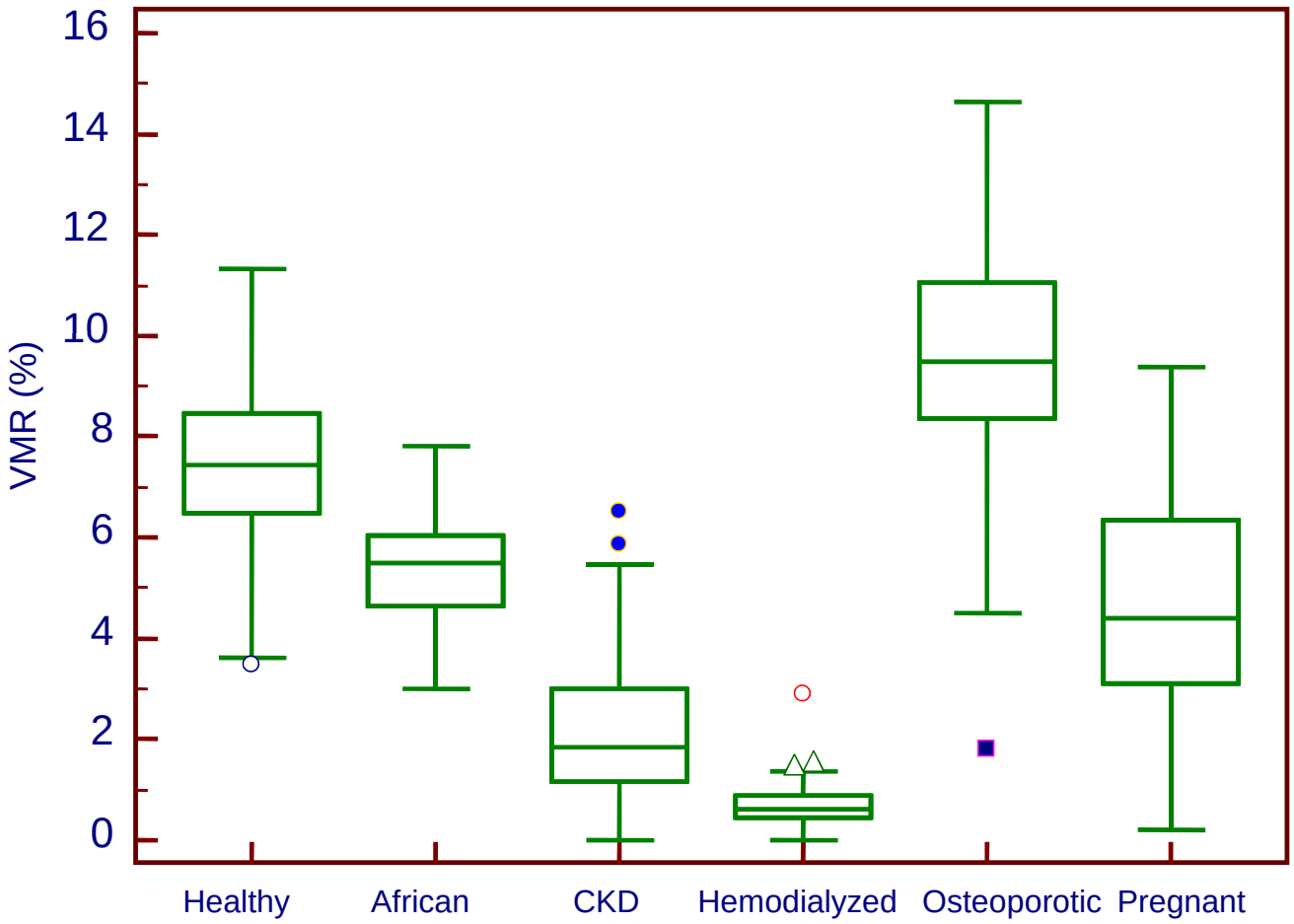


Passing-Bablok
VDSP-traceable method = 0,52 + 0,96 APCI-PFP

Concordance Lin's Correlation Coefficient (CCC) =0.99

Results

$$VMR(\%) = \frac{Conc\ 24{,}25(OH)_2D_3(\mu g/L)}{Conc\ 25(OH)D_3(\mu g/L)} \cdot 100$$



Conclusions

We have developed and validated a method for the measurement of the 25(OH)D, epi-25(OH)D & 24,25$(OH)_2D$ by LC-MS/MS in serum samples.

Simple, fast and easy sample preparation completely adequate for routine testing

LOQ of 0.5 µg/L for 24,25$(OH)_2D_3$ with 100 µL sample

Research article

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