



Methods Development and Standard Reference Materials for 25(OH)D

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Development of SRM 972

- Discussions regarding design of SRM and procurement specifications began in 2005
- Panel of experts convened by NIST and NIH:
 - Neal Binkley (University of Wisconsin)
 - Bruce Hollis (MUSC)
 - Reinhold Vieth (University of Toronto/Mount Sinai Hospital)
 - Robert Heaney (Creighton University)
 - Donald Wiebe* (University of Wisconsin)

* First round only



Development of SRM 972

- Original design was to have “high” and “low” serum pools for $25(\text{OH})\text{D}_2$ and $25(\text{OH})\text{D}_3$
- Difficulty in obtaining these materials, particularly sera with predominantly $25(\text{OH})\text{D}_2$



Development of SRM 972

Level 1

65 ± 15 nmol/L 25-hydroxyvitamin D₃ (“normal”)

Level 2

Blend of “normal” serum and horse serum to obtain approximately half the level of 25-hydroxyvitamin D₃ in the “normal” pool (35 ± 5 nmol/L)

Level 3

“Normal” serum spiked with an equivalent amount of 25-hydroxyvitamin D₂

Level 4

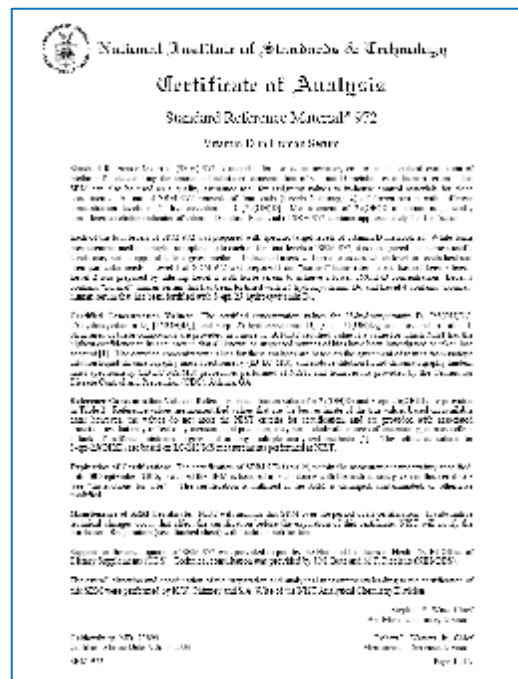
“Normal” serum spiked with 3-epi-25-hydroxyvitamin D₃

Goal was to have serum pools that presented different analytical challenges

SRM 972 Vitamin D in Human Serum

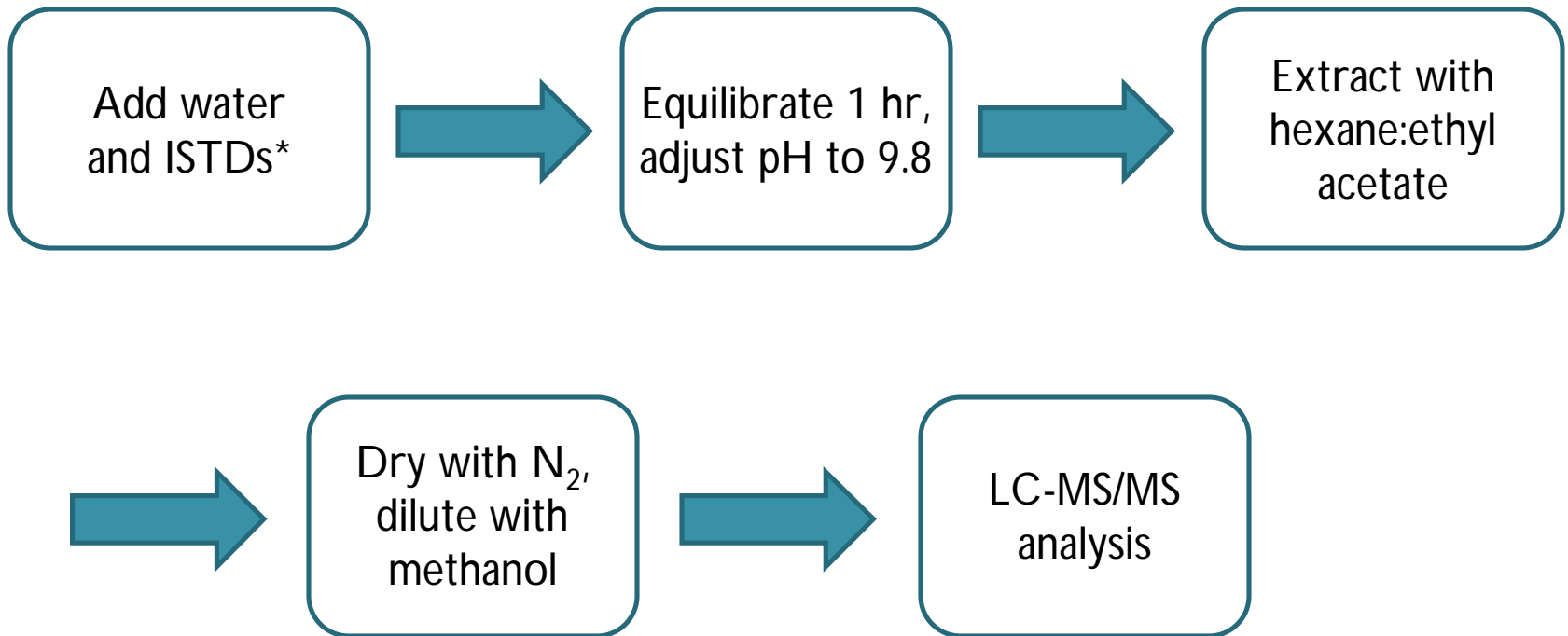


- Four levels, each containing 1.0 mL serum
- Certified and reference values for 25(OH)D₂, 25(OH)D₃, and 3-epi-25(OH)D₃
- Value assignment by isotope-dilution LC-MS and LC-MS/MS using data from NIST and CDC



- Metabolite concentrations reported in ng/g, ng/mL, and nmol/L
- COA does not provide data from other analytical techniques

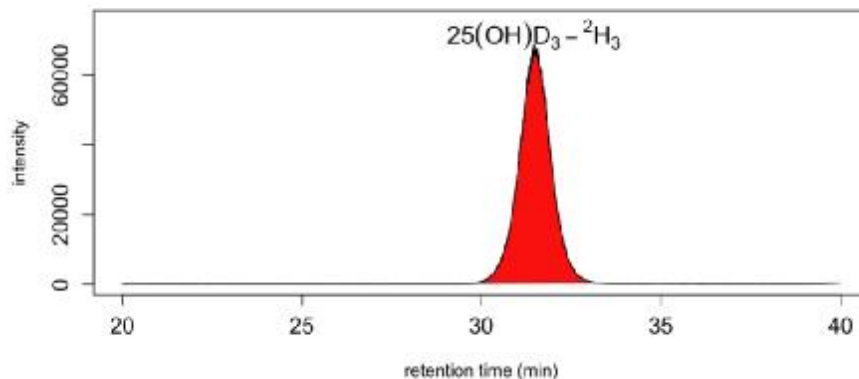
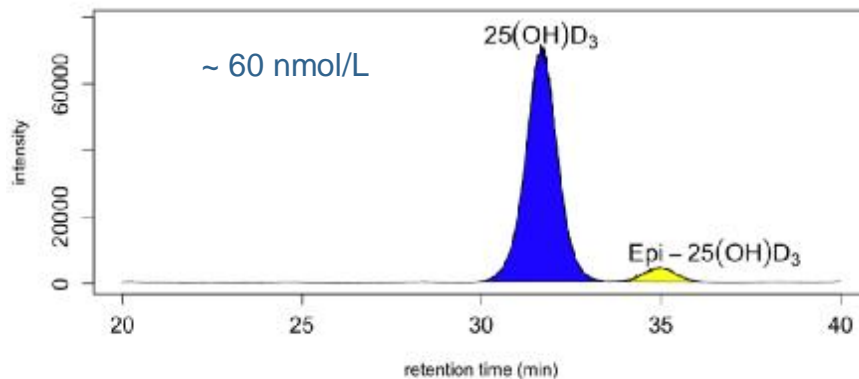
NIST LC-MS/MS Methodology for 25(OH)D



* The internal standards were ²H₃-25(OH)D₂ and ²H₃-25(OH)D₃

NIST LC-MS/MS Methodology – 25(OH)D₃

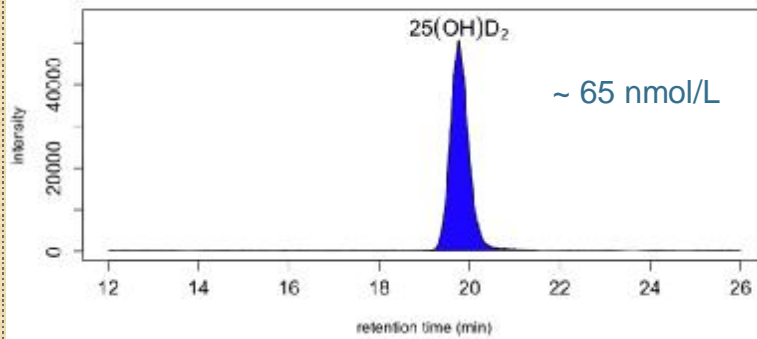
SRM 972 Level 1



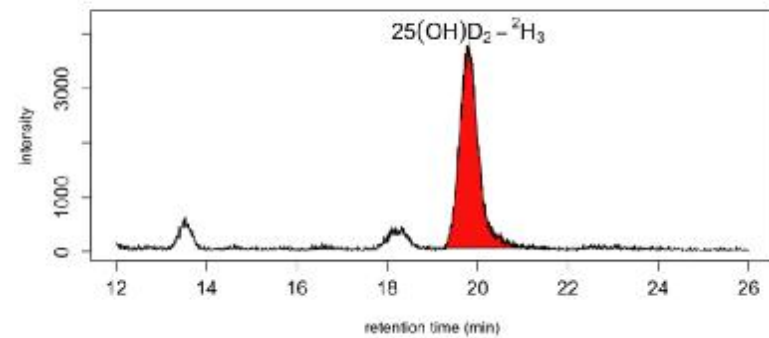
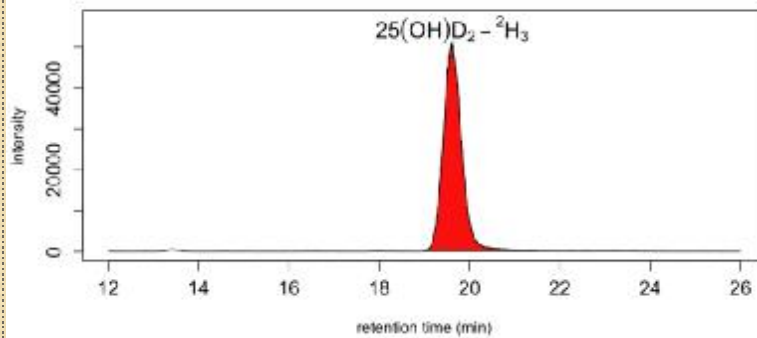
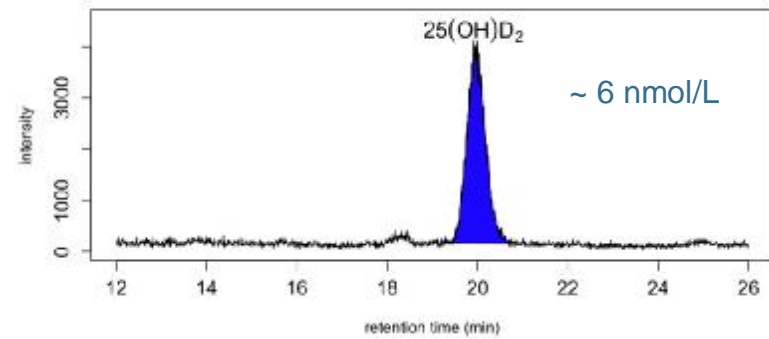
- Separation based on work of Lensmeyer et al.
- 3-epi-25(OH)D₃ fully resolved from 25(OH)D₃
- Same internal standard used for both

NIST LC-MS/MS Methodology – 25(OH)D₂

SRM 972 Level 3



SRM 972 Level 4





Recovery Study

- Stripped serum from DiaSorin used for 25OHD₃ recovery study
- Commercially available human serum used for 25OHD₂ study
- Samples spiked with 25(OH)D at approximately 20 ng/g
- Internal standard added either before or after extraction and intensity ratios compared

Recovery for 25(OH)D₂: 97% (0.4% CV, n = 5)

Recovery for 25(OH)D₃: 92% (0.9% CV, n = 5)

Labeled internal standard should correct for less than complete recovery if equilibrium with analyte is achieved

Presence of 3-epi-25(OH)D₃

| SRM 972 | 25(OH)D ₃ (ng/mL) | 3-epi-25(OH)D ₃ (ng/mL) |
|---------|---------------------------------|---------------------------------------|
| Level 1 | 23.9 | 1.39 |
| Level 2 | 12.3 | 0.76 |
| Level 3 | 18.5 | 1.06 |



Commutability

Commutability is defined as the equivalence of the mathematical relationships between the results of different measurement procedures for a reference material and for representative samples from healthy and diseased individuals.

Vesper et al., Clin. Biochem. Rev., 28 (2007) 139

Reasons a reference material may not be commutable:

- Material handling, processing (lyophilization, filtering, etc.)
- Supplementation with native or non-native analytes

Supplementation does not necessarily result in non-commutable materials
Not always possible to predict non-commutability



SRM 2972 25-Hydroxyvitamin D₂ and D₃ Calibration Solutions

- Ethanol-based solutions, one each for 25(OH)D₂ and 25(OH)D₃
- Concentrations approx. 10X serum levels, can be diluted to prepare calibration curves
- Certified values for 25(OH)D₂ and 25(OH)D₃
- Documentation currently under review