Supplemental Material for Laha, et al., 2012

METHODS

Patient samples Use of patient samples was according to approved guidelines of the University of Washington Human Subjects Division.

24,25-dihydroxyvitamin D_3 assay using liquid-liquid extraction The sample extraction for 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] was a modification of a previous described method (1) and differs in important ways from another method recently described elsewhere.(2) Briefly 200 µL of calibrators, controls or patient serum were alkalinized with 200 µL of 1 N sodium hydroxide in a 2 mL 96-deep well plate (Greiner Bio-One, Monroe, NC) which was then covered and vortexed for 15 s. The plate was then incubated at room temperature for 15 min after which 200 µL of internal standard containing 50 ng/mL of 25$(OH)D_2$-d6, 25$(OH)D_3$-d6 & 24,25$(OH)_2D_3$-d6 was added to each well and the plate was covered and vortexed in a multitube vortexer (VWR, Randor, PA) for 15 seconds. Samples were then extracted with 1 mL of 50%:50% (v:v) n-heptane:methyl-tert-butyl ether (VWR) with vortexing for 5 min. The plate was then centrifuged at 1100x*g* for 4 min at ambient temperature. A 96-well plate transfer gasket was placed on top of the extraction plate followed by another clean 96-deep well plate. The sealed plates were then placed in a dry-ice acetone bath for 50 min to freeze the lower aqueous layer. The upper organic layer was then transferred to the new plate by inverting and gently tapping the assembly on the benchtop. The extraction plate was then removed and discarded. The extracts were dried under nitrogen at 35ºC in a Turbovap (Biotage, Charlotte, NC) and the residue was reconstituted in 100 µL of acetonitrile containing 0.5 mg/mL of 4-phenyl-1,2,4-triazoline-3,5-dione (Sigma-Aldrich, St. Louis, MO). This solution was allowed to react for 15 min at ambient temperature after which 10µL of the resulting solution was injected onto a Waters Acquity UPLC HSS T3, 2.1 x 50 mm, 1.7 micron analytical column fitted with a Waters Acquity UPLC BEH C18 2.1x5 mm 1.7 micron guard column. The sample was developed by a linear gradient starting at 31% mobile phase A [Opitima grade water (Fisher, Pittsburg, PA)/0.1% formic acid (VWR, Randor, PA)] and 69% mobile phase B [Optima grade methanol (Fisher)/0.1% formic acid], ending in 1 minute at 18% mobile phase A and 82% mobile phase B. The ending gradient condition was held for an additional 1 minute after which time the column was re-equilibrated to the initial start conditions. Mass spectrometer parameters were set as follows: capillary 4.00kV, desolvation temperature 500 °C, desolvation gas 1200 L/h, cone gas 30 L/h, collision gas flow 0.15 mL/min. Analyte specific transitions and cone/collision energies were: 25$(OH)D_3$, 558.34>298.12, 25/16; 25$(OH)D_3$-d6, 564.34>298.12, 25/16; 25$(OH)D_2$, 570.34>298.12, 25/16; 25$(OH)D_2$-d3, 573.34>301.12, 25/16; 24,25$(OH)_2D_3$, 574.34>298.12, 32/20; 24,25$(OH)_2D_3$-d6, 580.46>298.14, 28/22.

The assay correlated well with a previously described method for the determination of 25-hydroxyvitamin D by liquid-liquid extraction with Pearson correlation coefficients (r^2) of 0.9418 and 0.9883 for 25$(OH)D_3$ and 25$(OH)D_2$, respectively. The assay had lower limits of quantitation (20% CV) of <2.0 ng/mL, 5.0 ng/mL and 0.48 ng/mL for 25$(OH)D_3$, 25$(OH)D_2$ and 24,25$(OH)_2D_3$ respectively. Imprecision for each analyte was <19% near the lower limits of quantification. Mean recovery was 100.3%, 97.2% and 104.5% for 25$(OH)D_3$, 25$(OH)D_2$ and 24,25$(OH)_2D_3$ respectively (N=10). The calibration for the measurement of 25(OH)D was verified using SRM 972 from NIST [accuracy 91-95% for

25(OH)D_3 and 100-116% for 25(OH)D_2]. Descriptive data for the method evaluation can be found in **Supplemental Table 3**.

Supplemental Table 1. Sources of vitamin D metabolites used in this study.

Compound	Commercial Source	Chemical Purity	Isotopic Purity
25(OH)D_3	Sigma Aldrich	≥98%	
25(OH)D_2	Sigma Aldrich	≥98%	
24(S),25(OH)$_2D_3$	Sigma Aldrich	≥99%	
24(R),25(OH)$_2D_3$	Enzo Life Sciences International	≥99%	
1α,25(OH)$_2D_3$	Sigma Aldrich	≥99%	
1α,25(OH)$_2D_2$	Medical Isotopes	≥97%	
23(S),25(OH)$_2D_3$	[Gift from Dr. Sakaki (Toyama Prefectural University, Japan)]	≥95%	
23(R),25(OH)$_2D_3$	Sigma Aldrich	≥99%	
25,26(OH)$_2D_3$	SAFC Pharma	≥95%	
3-epi-25(OH)D_3	IsoSciences	≥98%	
4β,25(OH)$_2D_3$	Enzymatic product purified by HPLC	≥98%	
3-epi-1α,25(OH)$_2D_3$	[Gift from Dr. Fujishima (Tokushima Bunri University, Japan)]	≥95%	
25(OH)D_2-d3	Sigma Aldrich	≥98%	≥97%
25(OH)D_3-d6	Medical Isotopes	≥97%	≥98%
1α,25(OH)$_2D_3$-d6	Medical Isotopes	≥97%	≥98%
1α,25(OH)$_2D_2$-d6	Medical Isotopes	≥97%	≥98%
24,25(OH)$_2D_3$-d6	Toronto Research Chemicals	≥98%	≥99%

Supplemental Table 2. Mass Spectrometer Parameters for the New Multi-analyte Method.

Parameter	Setting		
Capillary	2.75 kV		
Desolvation Temp	500 C		
Desolvation Gas	1000 L/Hr		
Cone Gas	30 L/Hr		
Collision Gas Flow	0.15 mL/Min		
Ionization Mode	Positive Ion ESI		
Compound	**Transition**	**Cone(V)**	**Collision(V)**
1α,25 dihydroxyvitamin D_3	623.45 > 314.18	22	22
1α,25 dihydroxyvitamin D_3-d6	629.54 > 314.19	22	22
1α,25 dihydroxyvitamin D_2	635.54 > 314.20	22	23
1α,25 dihydroxyvitamin D_2-d6	641.54 > 314.21	22	22
25 hydroxyvitamin D_3	607.56 > 298.20	21	22
25 hydroxyvitamin D_3-d6	613.56 > 298.21	22	22
25 hydroxyvitamin D_2	619.56 > 298.21	24	22
25 hydroxyvitamin D_2-d3	622.50 > 301.20	23	21
24,25 dihydroxyvitamin D_3	623.50 > 298.20	23	22
24,25 dihydroxyvitamin D_3-d6	629.56 > 298.21	22	24

Supplemental Table 3. Method evaluation data for the simultaneous measurement of 24,25-dihydroxyvitamin D, 25-hydroxyvitamin D_2, and 25-hydroxyvitamin D_3 by liquid-liquid extraction. A liquid-liquid extraction method for the simultaneous quantification of $24,25(OH)_2D_3$, $25(OH)D_2$, and $25(OH)D_3$ was developed and evaluated. Method comparison was performed against a previously described method for the quantification of $25(OH)D_2$ and $25(OH)D_3$ by liquid-liquid extraction (1). The method is described above on page 1 of supplemental material. The method evaluation data are presented here because they have not been published elsewhere, but the method is used as the comparator for $24,25(OH)_2D_3$ quantification in the new assay.

	$24,25(OH)_2D_3$	$25(OH)D_2$	$25(OH)D_3$
Mean % Recovery[a]	104.5%	97.2%	100.3%
r^2	NA[b]	0.988	0.942
Regression equations (new method = slope * original +intercept)	NA[b]	y = 1.05 * x – 1.04	y = 1.05 * x + 0.34
Intra-assay %CV (concentration analyzed)[c]	12.7 (2.2 ng/mL)	12.6 (19.8 ng/mL)	10.3 (10.3 ng/mL)
Inter-assay %CV (concentration analyzed)[d]	16.6 (4.2 ng/mL)	8.6 (24.4 ng/mL)	9.2 (27.4 ng/mL)
LLOQ (ng/mL)[e]	0.48	5.0	2.0

[a] Recovery was assessed by addition of each metabolite into 10 separate human serum samples with total protein values ranging from 8.2–10.4 g/dL; triglyceride values in the range of 323–429 mg/dL and total cholesterol ranging from 139–241 mg/dL. The 10 recovery samples were assayed in parallel with sample blanks to which a volume of methanol equal to the volume of the standard addition was added to a separate aliquot of each sample. The percent recovery was calculated as the concentration of the spiked sample minus the concentration of the sample blank divided by the expected concentration of the spike addition.

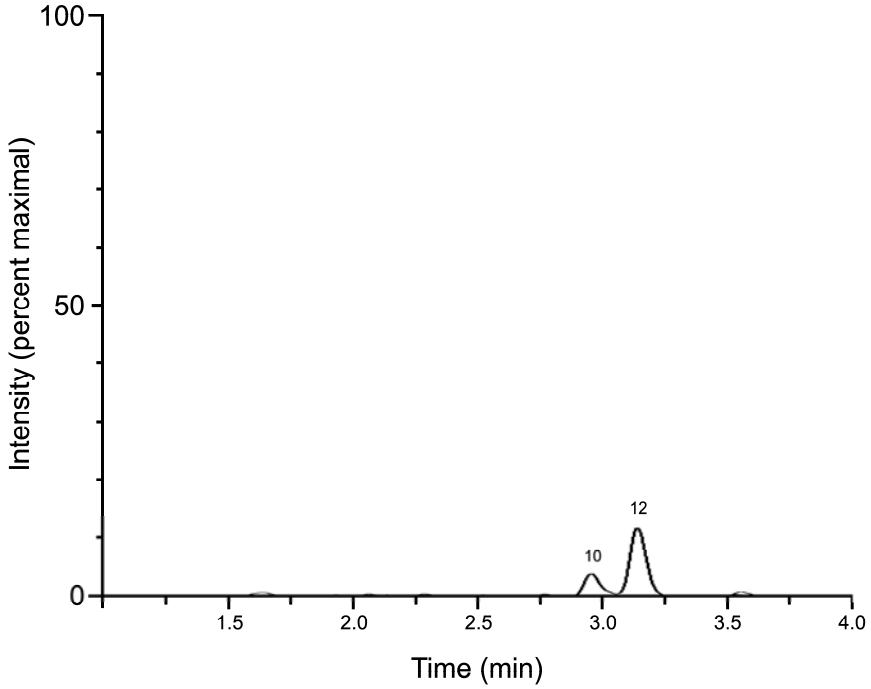
[b] NA = not applicable [$24,25(OH)_2D_3$ is not measured in the original $25(OH)D_2$ and $25(OH)D_3$ assay].

[c] N= 14

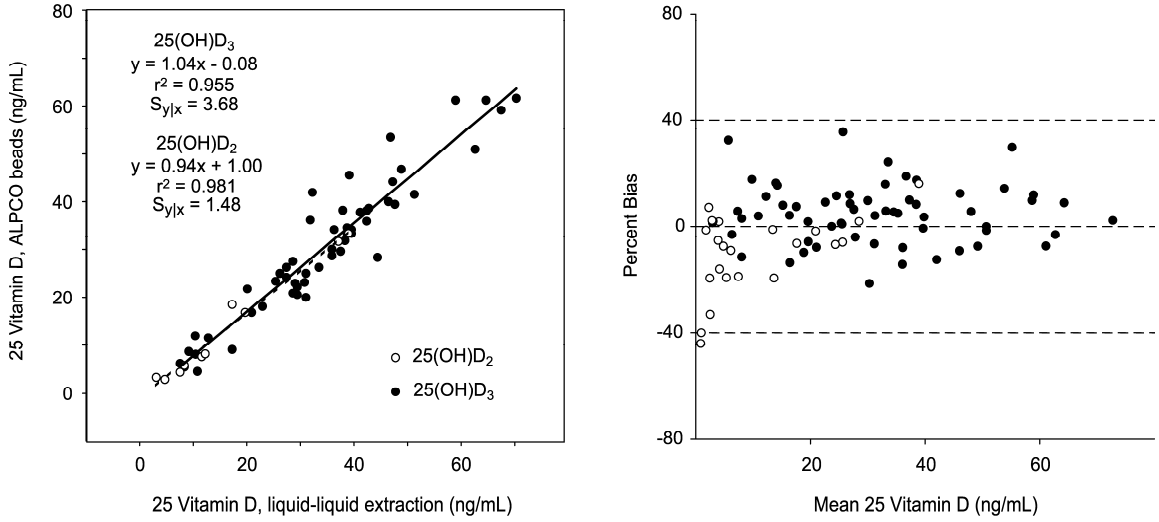
[d] N= 102

[e] Commercial serum controls were diluted serially and 10 aliquots of each level were assayed to determine imprecision at each level. The LLOQ was defined as the concentration where the %CV approached 20%.

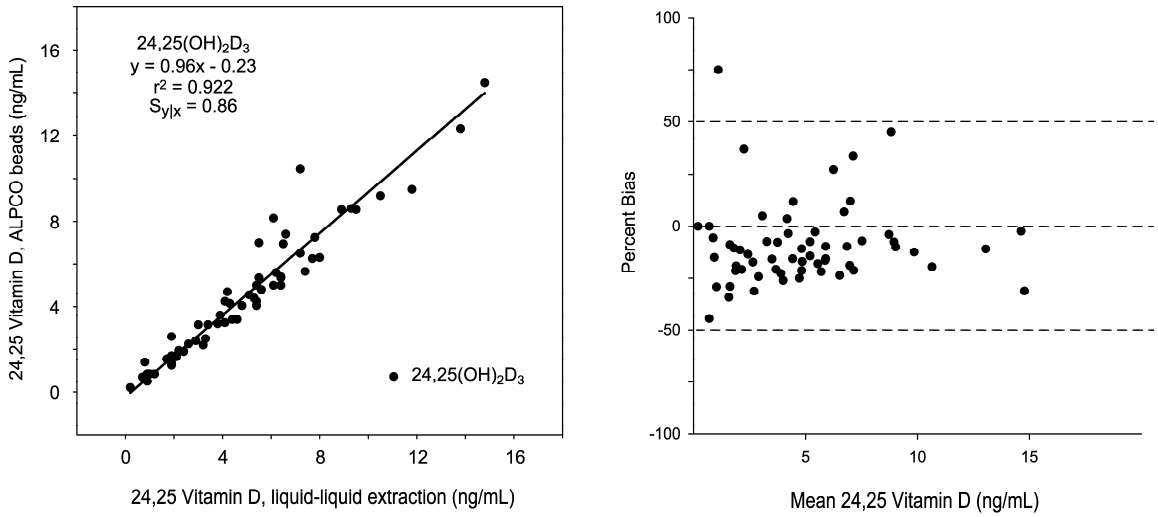
Supplemental Figure 1. Representative chromatogram of a stripped serum sample. As a supplement to Figure 1 in the main text, MSG-4000 was analyzed using the new method and is shown on a scale approximating that in Figure 1. Peak 10: Minor 25(OH)D_3 PTAD isomer and peak 12: 25(OH)D_3



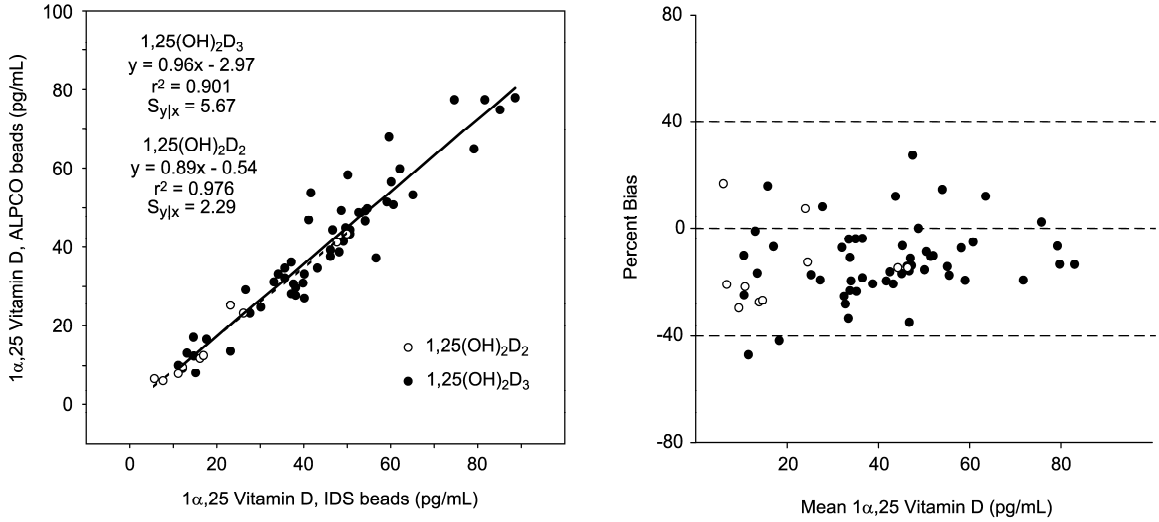
Supplemental Figure 2. Method comparison with a liquid-liquid extraction for the quantification of 25-hydroxyvitamin D. Samples were extracted with heptane as previously described (1) or immunoextracted with ALPCO beads and analyzed with LC-MS/MS (N=61). Prohormone 25(OH)D_2 was detectable by both methods in 24 patients, one outlier was removed from the 25(OH)D_2 method comparison based on Cook's Distance >1.0. **Left.** Regression equations are presented, along with Pearson correlation coefficients and standard errors of residuals ($S_{y|x}$) for each analyte. **Right.** Bias plot is presented as percent bias vs. the mean concentration determined by both methods.



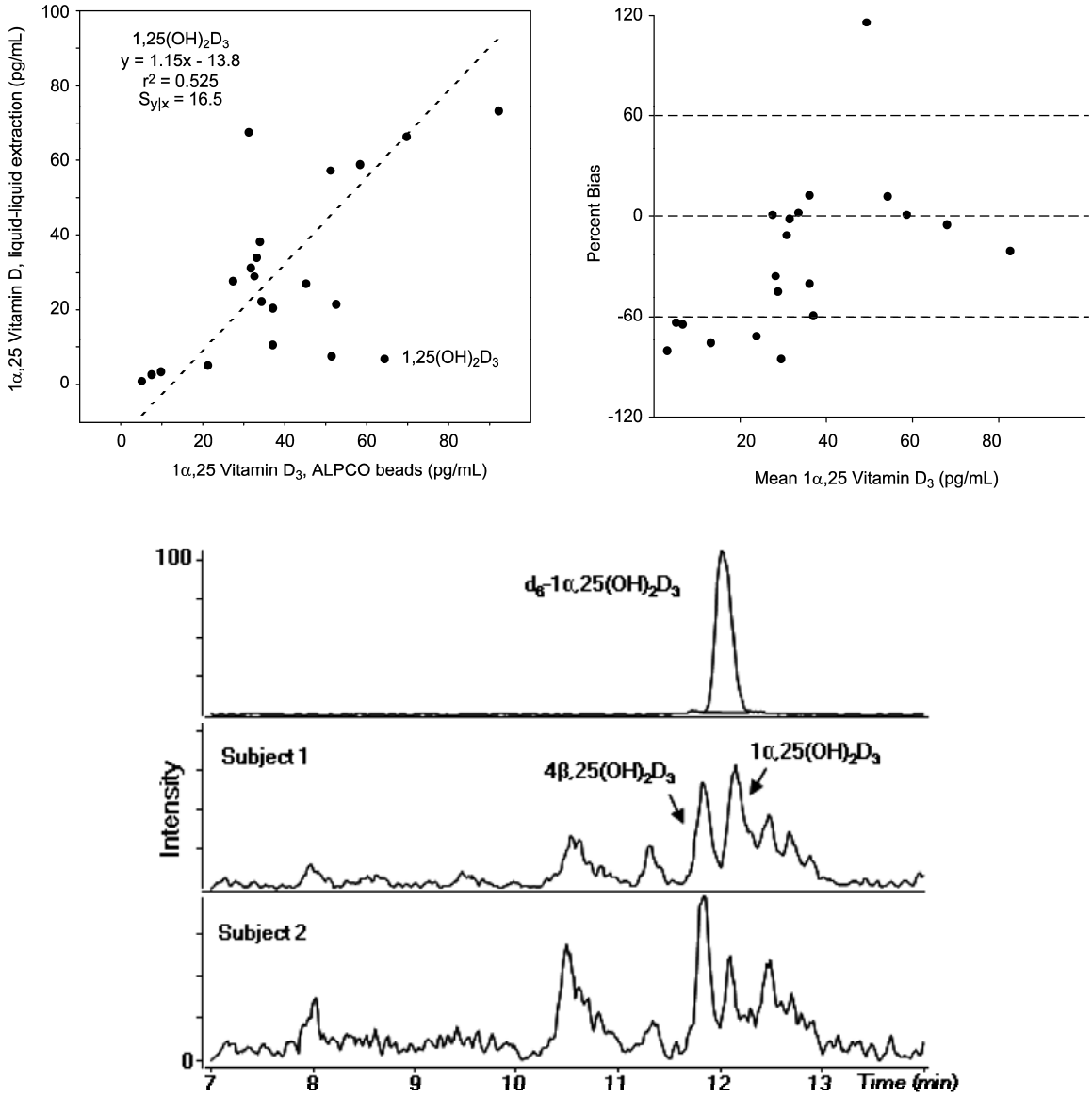
Supplemental Figure 3. Method comparison with a liquid-liquid extraction for the quantification of 24,25-dihydroxyvitamin D_3. Samples were extracted with heptane:MTBE as described above or immunoextracted with ALPCO beads and analyzed with LC-MS/MS (N=59). Two outliers were removed from the method comparison based on Cook's Distance >1.0. **Left.** Regression equations are presented, along with Pearson correlation coefficients and standard errors of residuals ($S_{y|x}$) for each analyte. **Right.** Bias plot is presented as percent bias vs. the mean concentration determined by both methods.



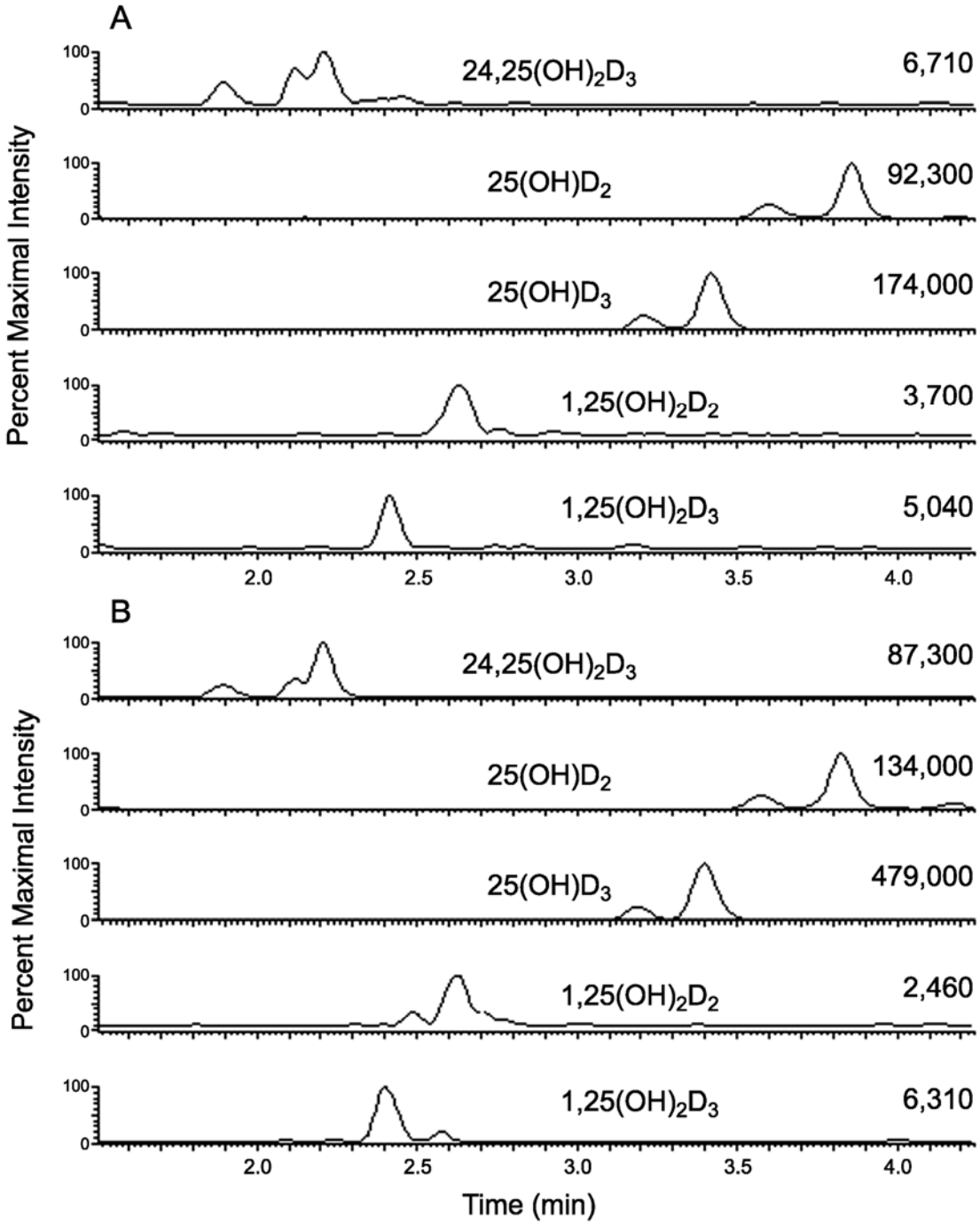
Supplemental Figure 4. Method comparison with IDS immunoaffinity purification after protein precipitation for the quantification of 1α,25-dihydroxyvitamin D. Samples were immunoextracted with IDS beads after protein precipitation as previously described (3) or immunoextracted with ALPCO beads and analyzed with LC-MS/MS (N=52). 1α,25(OH)D_2 was detectable by both methods in 10 patients. **Left.** Regression equations are presented, along with Pearson correlation coefficients and standard errors of residuals ($S_{y|x}$) for each analyte. **Right.** Bias plot is presented as percent bias vs. the mean concentration determined by both methods.



Supplemental Figure 5. Method comparison with liquid-liquid extraction for the quantification of 1α,25-dihydroxyvitamin D_3. Samples were liquid-liquid extracted and analyzed with LC-MS/MS as previously described (4) or immunoextracted with ALPCO beads and analyzed with LC-MS/MS (N=20). **Top left.** Regression equations are presented, along with Pearson correlation coefficients and standard errors of residuals ($S_{y|x}$) for each analyte. **Top right.** Bias plot is presented as percent bias vs. the mean concentration determined by both methods. **Bottom.** Representative chromatograms from two patients are shown for the liquid-liquid extraction method illustrating the complicated matrix observed without an immunoaffinity enrichment step.



Supplemental Figure 6. Comparison of IDS and ALPCO solid-phase reagents in a single patient. (A) A single patient sample was immunoaffinity extracted with IDS beads after protein precipitation and analyzed using LC-MS/MS. (B) The same sample was immunoaffinity extracted with ALPCO beads without protein precipitation and analyzed using LC-MS/MS. Each analyte monitored is labeled and the maximum intensity for each chromatogram is listed on the right hand side of each trace.



Supplemental References

1. Hoofnagle AN, Laha TJ, Donaldson TF. A rubber transfer gasket to improve the throughput of liquid-liquid extraction in 96-well plates: application to vitamin D testing. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2010;878:1639-42.
2. Wagner D, Hanwell HE, Schnabl K, Yazdanpanah M, Kimball S, Fu L, et al. The ratio of serum 24,25-dihydroxyvitamin D(3) to 25-hydroxyvitamin D(3) is predictive of 25-hydroxyvitamin D(3) response to vitamin D(3) supplementation. *J Steroid Biochem Mol Biol.* 2011;126:72-7.
3. Strathmann FG, Laha TJ, Hoofnagle AN. Quantification of 1alpha,25-dihydroxy vitamin D by immunoextraction and liquid chromatography-tandem mass spectrometry. *Clin Chem.* 2011;57:1279-85.
4. Wang Z, Senn T, Kalhorn T, Zheng XE, Zheng S, Davis CL, et al. Simultaneous measurement of plasma vitamin D(3) metabolites, including 4beta,25-dihydroxyvitamin D(3), using liquid chromatography-tandem mass spectrometry. *Anal Biochem.* 2011;418:126-33.