Electronic Supplementary Material (ESI) for Analyst.

Supplementary Material

Determination of vitamin D_3 conjugated metabolites: a complementary view to hydroxylated metabolites

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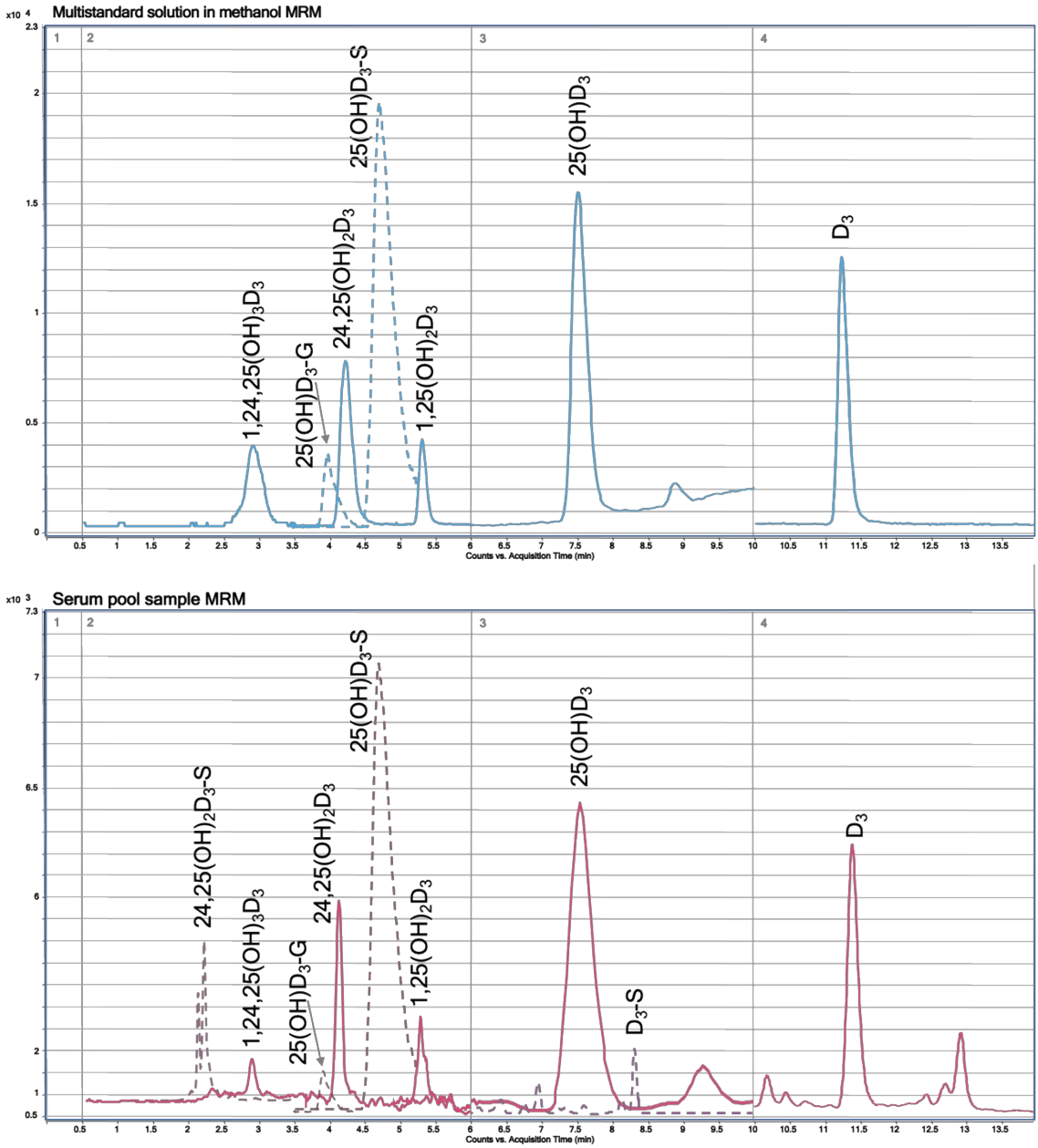
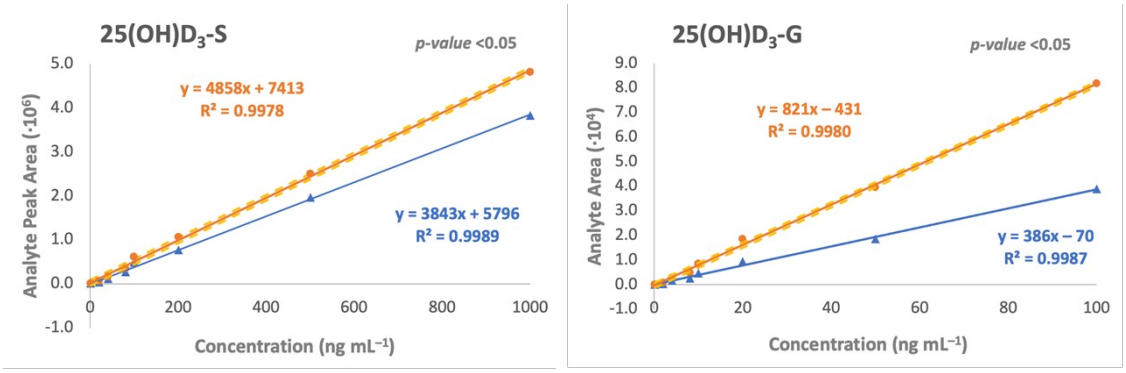
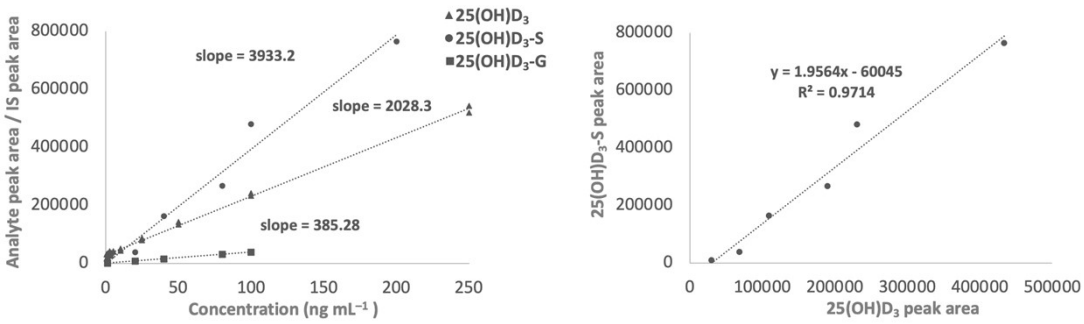


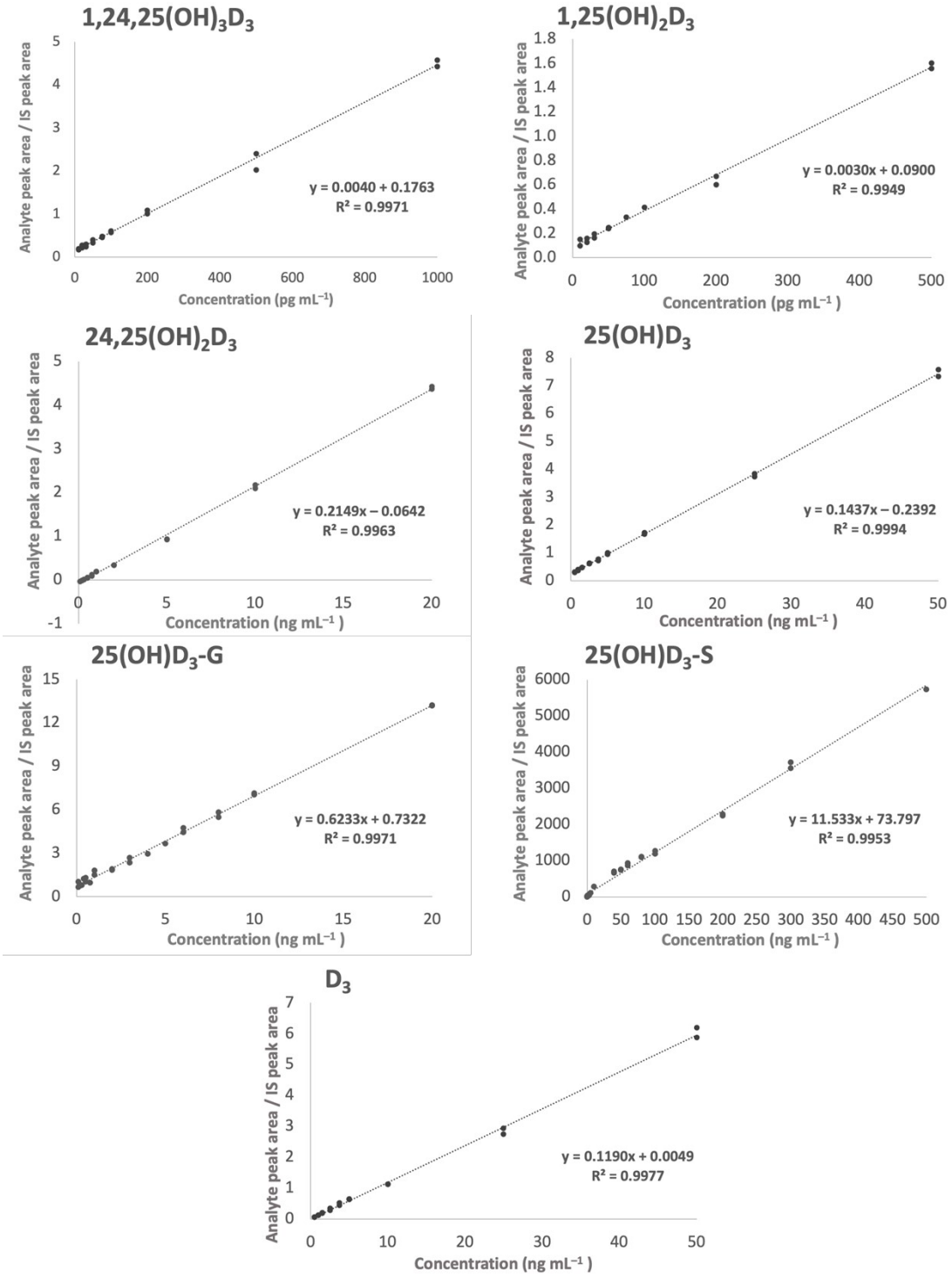
Supplementary Fig. 1. Chromatograms obtained from MRM analyses of a multistandard solution in methanol (blue) and a serum pool sample (pink).



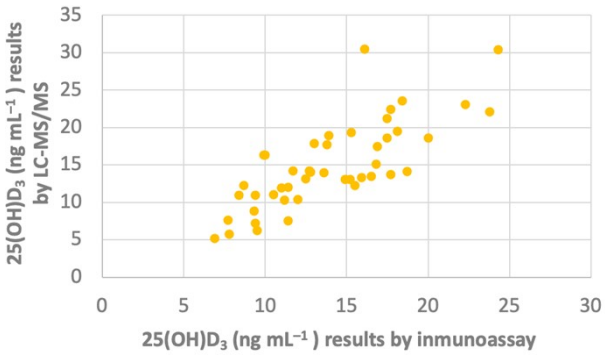
Supplementary Fig. 2. Estimated matrix effects for representative conjugated metabolites included in the proposed quantitative method by comparing calibration curves of standard solutions in methanol (orange circles) and spiked human serum aliquots (blue triangles).



Supplementary Fig. 3. Comparison between 25(OH)D_3, 25(OH)D_3-G and 25(OH)D_3-S calibration curves to check the suitability of the 25(OH)D_3-d_3 as internal standard for quantitation of both conjugated metabolites and correlation between 25(OH)D_3 and 25(OH)D_3-S calibration curves slopes.



Supplementary Fig. 4. Calibration curves for target metabolites analyzed with the proposed method.



Supplementary Fig. 5. Correlation of $25(OH)_2D_3$ results obtained by immunoassay and LC–MS/MS analysis of serum samples in the studied cohort.

Supplementary Table 1. Evaluation of accuracy and precision of the proposed method for quantitation of hydroxylated and conjugated metabolites of vitamin D_3.

Analyte	Concentration level	Matrix effect* (%) with not using IS	Matrix effect† (%) using IS
25(OH)D_3-3-G	2 ng mL^{-1}	32.3	17.2
	10 ng mL^{-1}	21.5	14.2
	20 ng mL^{-1}	23.0	15.0
25(OH)D_3-3-S	20 ng mL^{-1}	46.5	–5.7
	100 ng mL^{-1}	23.7	–14.9
	200 ng mL^{-1}	25.0	–14.2

*(Measured concentration in serum – Measured concentration in methanol)/Measured concentration in methanol, expressed as a percentage. Concentrations are estimated interpolating analytes peak areas in calibration curves represented by analytes peak areas *versus* concentration levels.

†(Measured concentration in serum – Measured concentration in methanol)/Measured concentration in methanol, expressed as a percentage. Concentrations are estimated interpolating ratios of analytes and ISs peak areas in calibration curve represented by ratios of analytes and ISs peak areas *versus* concentration levels.

Supplementary Table 2. Concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ obtained by analysis of DEQAS samples with the proposed method, and bias (expressed in %) from the corresponding reference values.

$25(OH)D_3$					
Samples	487	488	490	498	499
Proposed method (ng mL^{-1})	84.2	59.4	120.3	28.5	43.1
NIST (ng mL^{-1})	92.2	65.6	116.5	28.3	49.8
ALTM* (ng mL^{-1})	100.3	69	129.4	31.7	54.2
Bias from NIST (%)	8.7%	9.5%	-3.3%	-0.7%	13.5%
Bias from ALTM* (%)	16.1%	13.9%	7.0%	10.1%	20.5%
$1,25(OH)_2D_3$					
Sample code	343	344	345	355	356
Proposed method(pg mL^{-1})	105.1	83.8	94.5	127.5	135.6
LC–MS/MS (pg mL^{-1})	100.4	91.9	84.5	124.8	152.6
ALTM* (pg mL^{-1})	114.3	87.1	103.4	144.7	180.7
Bias from LC–MS/MS (%)	-4.7%	8.8%	-11.8%	-2.2%	11.1%
Bias from ALTM* (%)	8.0%	3.8%	8.6%	11.9%	25.0%

ALTM*; All-Laboratory Trimmed Mean, average of results from five different analytical techniques recognized by DEQAS program.