Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

A high-throughput LC-MS/MS method suitable for population surveys measures five serum folate vitamers and one oxidation product

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Table S1 Instrument settings for the tandem mass spectrometer for each folate form[a]

Analyte[b]	Transition (*m/z*)	T_r[c] (min)	DP[d] (V)	CE (V)	CXP (V)	EP (V)
5-MethylTHF	460.2 → 313.2	2.28	120	25	10	10
5-MethylTHF ($^{13}C_5$)	465.2 → 313.2	2.28	120	27	10	10
FA	442.2 → 295.2	3.00	100	21	10	10
FA ($^{13}C_5$)	447.2 → 295.2	3.01	100	21	10	10
THF	446.2 → 299.2	2.24	95	30	10	10
THF ($^{13}C_5$)	451.1 → 299.1	2.24	95	30	10	10
5-FormylTHF	474.4 → 299.2	2.99	110	45	10	10
5-FormylTHF ($^{13}C_5$)	479.4 → 299.2	2.99	120	44	10	10
5,10-MethenylTHF	456.1 → 412.2	2.20	160	43	10	10
5,10-MethenylTHF ($^{13}C_5$)	461.1 → 416.2	2.19	160	44	10	10
MeFox	474.4 → 284.2	3.04	110	50	10	10
MeFox ($^{13}C_5$)	479.4 → 284.2	3.03	110	50	10	10

[a] These settings apply to all three methods (method 1: routine 8-probe SPE; method 2: scaled down 8-probe SPE; and method 3: scaled down 96-probe SPE)

[b] 5-MethyTHF, 5-methyltetrahydrofolate; FA, folic acid; THF, tetrahydrofolate; 5-formylTHF, 5-formyltetrahydrofolate; 5,10-methenylTHF, 5,10-methenyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF

[c] T_r, chromatographic retention time

[d] DP, declustering potential; CE, collision energy; CXP, collision cell exit potential; EP, entrance potential

Table S2 Analytical performance of method 2 (scaled down 8-probe SPE LC-MS/MS method)

Analyte[a]	Calibration[b]			Imprecision[c]				Accuracy[d]			Sensitivity[e]	
	Level (nmol/L)	Accuracy (%)	CV (%)	QC pool (nmol/L)	Total CV (%)	Within-run CV (%)	Between-run CV (%)	Spike (nmol/L)	Spike recovery (%)	CV (%)	LOD (nmol/L)	LLOQ (nmol/L)
5-MethylTHF	1	112	11	18.3	3.1	3.1	2.1	2	86	33	0.31	1.00
	2	103	4.7	32.9	2.4	2.2	1.8	4	93	15		
	4	101	2.7	48.2	3.5	2.4	3.0	20	94	2.8		
	20	98	2.1					100	95	2.4		
	100	99	2.0									
FA	0.5	107	9.4	0.67	11.3	3.9	11.0	1	96	7.2	0.14	0.47
	1	101	6.4	5.51	7.7	3.4	7.3	2	100	4.7		
	2	100	3.1	10.7	7.7	4.3	7.1	10	100	3.7		
	10	98	2.2					50	100	4.7		
	50	100	1.6									
THF	0.5	101	19	1.24	9.0	4.5	8.4	1	98	30	0.37	1.20
	1	101	12	4.18	6.7	5.8	5.3	2	92	7.9		
	2	102	5.2					10	95	1.5		
	10	100	3.4					50	95	5.6		
	50	101	2.6									
5-FormylTHF	0.5	99	14	0.60	13.4	5.2	12.9	1	81	14	0.30	1.00
	1	97	7.7	2.33	7.1	3.8	6.6	2	85	16		
	2	99	3.9					10	90	14		
	10	100	3.0					50	91	15		
	50	100	2.8									
5,10-MethenylTHF	0.5	100	17	1.46	5.7	2.9	5.3	1	99	7.0	0.34	1.14
	1	100	9.8	4.53	5.9	4.4	5.0	2	106	3.8		
	2	100	3.6					10	109	11		
	10	99	2.9					50	108	7.8		
	50	101	2.5									
MeFox	0.5	94	18	1.34	9.0	4.8	8.3	1	80	9.5	0.34	1.14
	1	97	7.6	1.42	9.1	4.6	8.5	2	97	6.4		
	2	99	2.0	2.81	6.7	2.4	6.5	10	98	3.2		
	10	101	2.3					50	96	2.7		
	50	101	2.9									

[a] 5-MethyTHF, 5-methyltetrahydrofolate; FA, folic acid; THF, tetrahydrofolate; 5-formylTHF, 5-formyltetrahydrofolate; 5,10-methenylTHF, 5,10-methenyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF

[b] Calibration was performed over 10 runs and calibrator accuracy was calculated as the mean percent difference between the measured and target value

[c] Method imprecision was assessed by analyzing three QC pools (two for THF, 5-formylTHF, and 5,10-methenylTHF) over 10 runs (two replicates per run) and by calculating the total, within- and between-run coefficient of variation (CV)

[d] Method accuracy was assessed through spike recovery; the low serum QC pool was amended with a calibrator mixture containing each folate form at four levels (two replicates per level, three runs) and also measured unspiked (two replicates per run, three runs) for endogenous folate concentrations; the spike recovery was calculated as the measured concentration difference between the spiked and unspiked sample divided by the nominal concentration of the spike

[e] Method sensitivity was estimated as the limit of detection (LOD) for each analyte by serially diluting the medium serum QC pool with 0.1% ascorbic acid and calculating the standard deviation at a concentration of zero (σ_0) from an extrapolation of repeat analyte measurements (three replicates per dilution, three runs) made near the detection limit in these dilutions; the LOD was defined as 3 σ_0; the lower limit of quantitation (LLOQ) was defined as 10 σ_0; when using 4% albumin as a diluent to simulate protein matrix, we obtained similar LOD values (nmol/L): 5-methylTHF 0.19, FA 0.12, THF 0.36, 5-formylTHF 0.16, 5,10-methenylTHF 0.37, MeFox 0.10

Table S3 Comparison of folate results in serum and matched plasma samples[a]

Matrix[b]	Folate form[c]	Mean ± SD (nmol/L)	Pearson correlation coefficient (95% CI)[d]	Bland-Altman bias (95% CI) (%)[e]	Wilcoxon sign rank test *P*-value[f]
Serum	5-MethylTHF	30.4 ± 19.1	n/a	n/a	n/a
	FA	1.15 ± 0.40	n/a	n/a	n/a
	MeFox	2.76 ± 3.09	n/a	n/a	n/a
	tFOL	35.0 ± 22.0	n/a	n/a	n/a
Serum separator	5-MethylTHF	31.1 ± 20.6	1.00 (0.99 to 1.00)	1.2 (-1.7 to 4.1)	0.5186
	FA	1.11 ± 0.40	0.99 (0.95 to 1.00)	-3.6 (-7.3 to 0.1)	0.0923
	MeFox	2.36 ± 2.21	0.99 (0.97 to 1.00)	-9.2 (-21.2 to 2.9)	0.0771
	tFOL	35.2 ± 22.8	1.00 (0.99 to 1.00)	0.20 (-1.91 to 2.4)	0.9697
Plasma (K_2 EDTA)	5-MethylTHF	19.5 ± 12.9	1.00 (0.98 to 1.00)	-45 (-49 to -41)	0.0005
	FA	1.09 ± 0.40	0.98 (0.94 to 1.00)	-5.4 (-9.3 to -1.5)	0.0269
	MeFox	8.90 ± 5.55	0.78 (0.37 to 0.93)	108 (85 to 132)	0.0005
	tFOL	30.1 ± 18.6	1.00 (0.99 to 1.00)	-15 (-18 to -13)	0.0005
Plasma (Na heparin)	5-MethylTHF	30.9 ± 19.9	1.00 (0.99 to 1.00)	1.4 (-0.8 to 3.7)	0.2661
	FA	1.07 ± 0.40	0.97 (0.90 to 0.99)	-7.3 (-12.3 to -2.4)	0.0122
	MeFox	1.80 ± 1.58	0.98 (0.94 to 1.00)	-35.4 (-46.4 to -24.3)	0.0005
	tFOL	34.6 ± 21.5	1.00 (1.00 to 1.00)	-1.0 (-2.73 to 0.75)	0.2036
Plasma (Na citrate)	5-MethylTHF	30.8 ± 19.8	1.00 (0.99 to 1.00)	0.9 (-1.6 to 3.5)	0.5186
	FA	1.14 ± 0.42	0.99 (0.95 to 1.00)	-1.5 (-6.0 to 3.0)	0.7334
	MeFox	1.96 ± 1.66	0.98 (0.94 to 1.00)	-26 (-37 to -15)	0.0005
	tFOL	34.5 ± 21.4	1.00 (1.00 to 1.00)	-1.06 (-2.62 to 0.49)	0.1763

[a] Twelve matched serum and plasma samples were analyzed by method 3 (scaled down 96-probe SPE method)

[b] K_2 EDTA and Na heparin were spray dried anticoagulants, while Na citrate was a liquid (0.5 mL/5-mL vacutainer tube); folate results were multiplied by 1.1 to correct for this dilution

[c] 5-MethyTHF, 5-methyltetrahydrofolate; FA, folic acid; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; tFOL, total folate (sum of all folate forms including MeFox)

[d] Correlation was assessed relative to serum

[e] Relative Bland-Altman bias was used to assess the magnitude of the difference between a certain specimen type and serum because of increasing SD over the range of folate concentrations

[f] Because the distribution of differences was not normal, we used the non-parametric Wilcoxon sign rank test to assess significant ($P < 0.05$) differences between a certain specimen type and serum

Table S4 Comparison of total folate results in serum samples obtained by different LC-MS/MS methods and microbiologic assay[a]

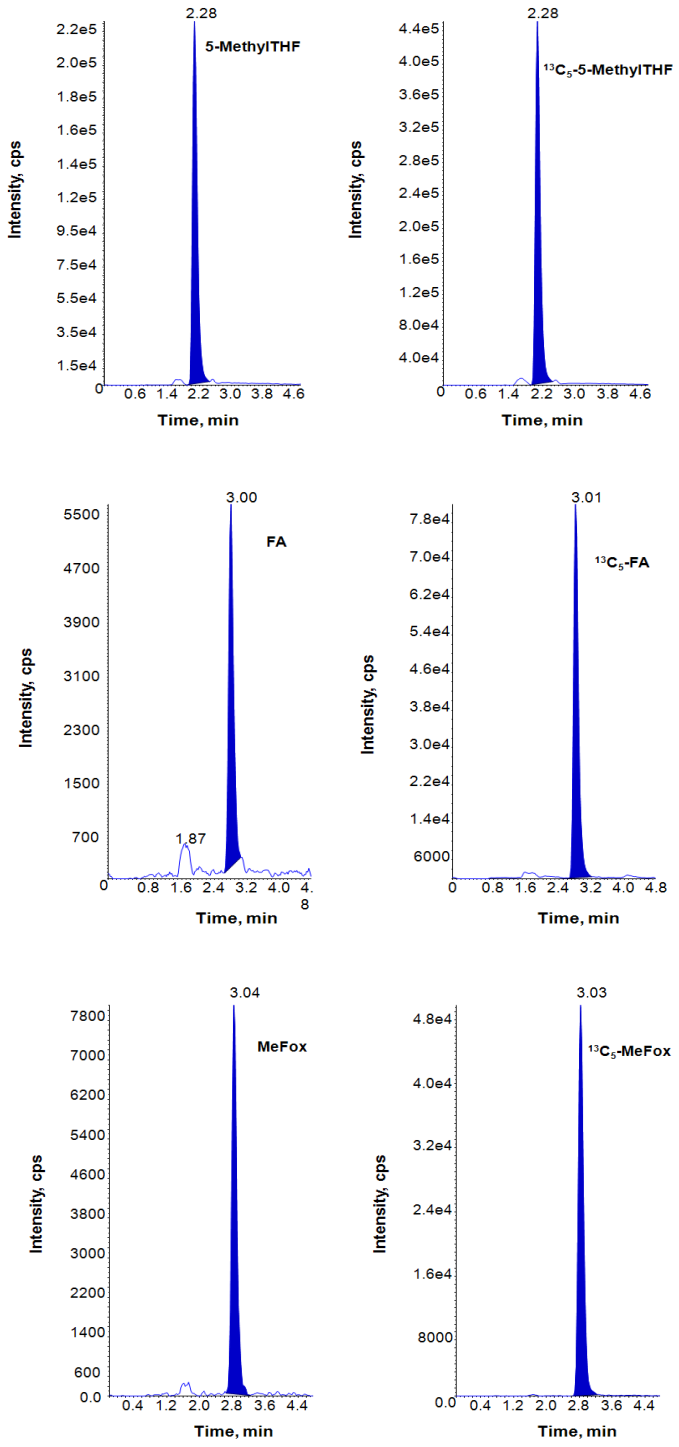
Method pair (y vs. x)	Pearson correlation coefficient (95% CI)	Deming slope (95% CI) (nmol/L)[b]	Deming intercept (95% CI) (nmol/L) [b]	Bland-Altman bias (95% CI) (%)[c]
LC-MS/MS 1 vs. MA	0.97 (0.96 to 0.98)	0.99 (0.95 to 1.03)	1.96 (0.98 to 2.94)	5.8 (3.6 to 7.9)
LC-MS/MS 2 vs. MA	0.97 (0.96 to 0.98)	0.99 (0.95 to 1.04)	2.05 (0.97 to 3.12)	6.2 (4.2 to 8.2)
LC-MS/MS 3 vs. MA	0.97 (0.96 to 0.98)	1.07 (1.03 to 1.11)	1.50 (0.53 to 2.48)	11.4 (9.5 to 13.3)
MA vs. LC-MS/MS 1	0.97 (0.96 to 0.98)	1.01 (0.97 to 1.05)	-1.97 (-3.03 to -0.92)	-5.8 (-7.9 to -3.6)
MA vs. LC-MS/MS 2	0.97 (0.96 to 0.98)	1.01 (0.96 to 1.05)	-2.06 (-3.22 to -0.90)	-6.2 (-8.2 to -4.2)
MA vs. LC-MS/MS 3	0.97 (0.96 to 0.98)	0.94 (0.90 to 0.97)	-1.41 (-2.37 to -0.45)	-11.4 (-13.3 to -9.5)

[a] Method comparison consisted of two separate aliquots of 150 pristine serum samples, one aliquot analyzed by microbiologic assay (MA) for $tFOL_{MA}$ and the second aliquot analyzed by three different LC-MS/MS methods (method 1: routine 8-probe SPE; method 2: scaled down 8-probe SPE; method 3: scaled down 96-probe SPE) for folate forms that were summed up for $tFOL_{without\ MeFox}$; MeFox was not included in the summation because the MA does not respond to the biologically inactive MeFox

[b] Weighted Deming regression was used because the SD increased over the range of folate concentrations

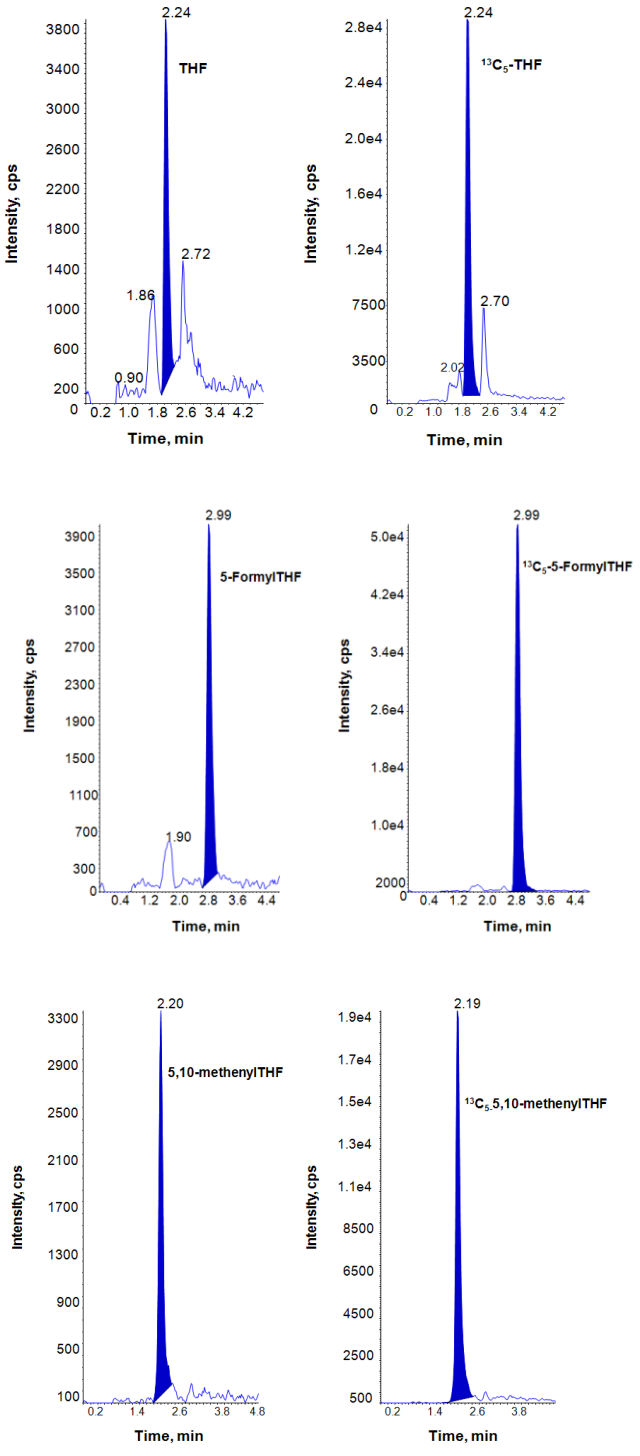
[e] Relative Bland-Altman bias was used to assess the magnitude of the difference between two methods because of increasing SD over the range of folate concentrations

Figure S1 Typical tandem MRM profiles for folate forms for the extracted low-concentration serum QC pool[a]



[a] Applies to method 3 (scaled down 96-probe SPE method); serum concentration of 5-methylTHF (19.5 nmol/L), FA (0.72 nmol/L) and MeFox (1.44 nmol/L)

Figure S2 Typical tandem MRM profiles for folate forms for the extracted medium-concentration serum QC pool[a]



[a] Applies to method 3 (scaled down 96-probe SPE method); serum concentration of THF (1.33 nmol/L), 5-formylTHF (0.68 nmol/L) and 5,10-methenylTHF (1.56 nmol/L)