Supporting Information for:

UHPLC-ESI-MS/MS quantification of tryptophan metabolites and markers of gut health in serum and plasma – Application to clinical and epidemiology cohorts

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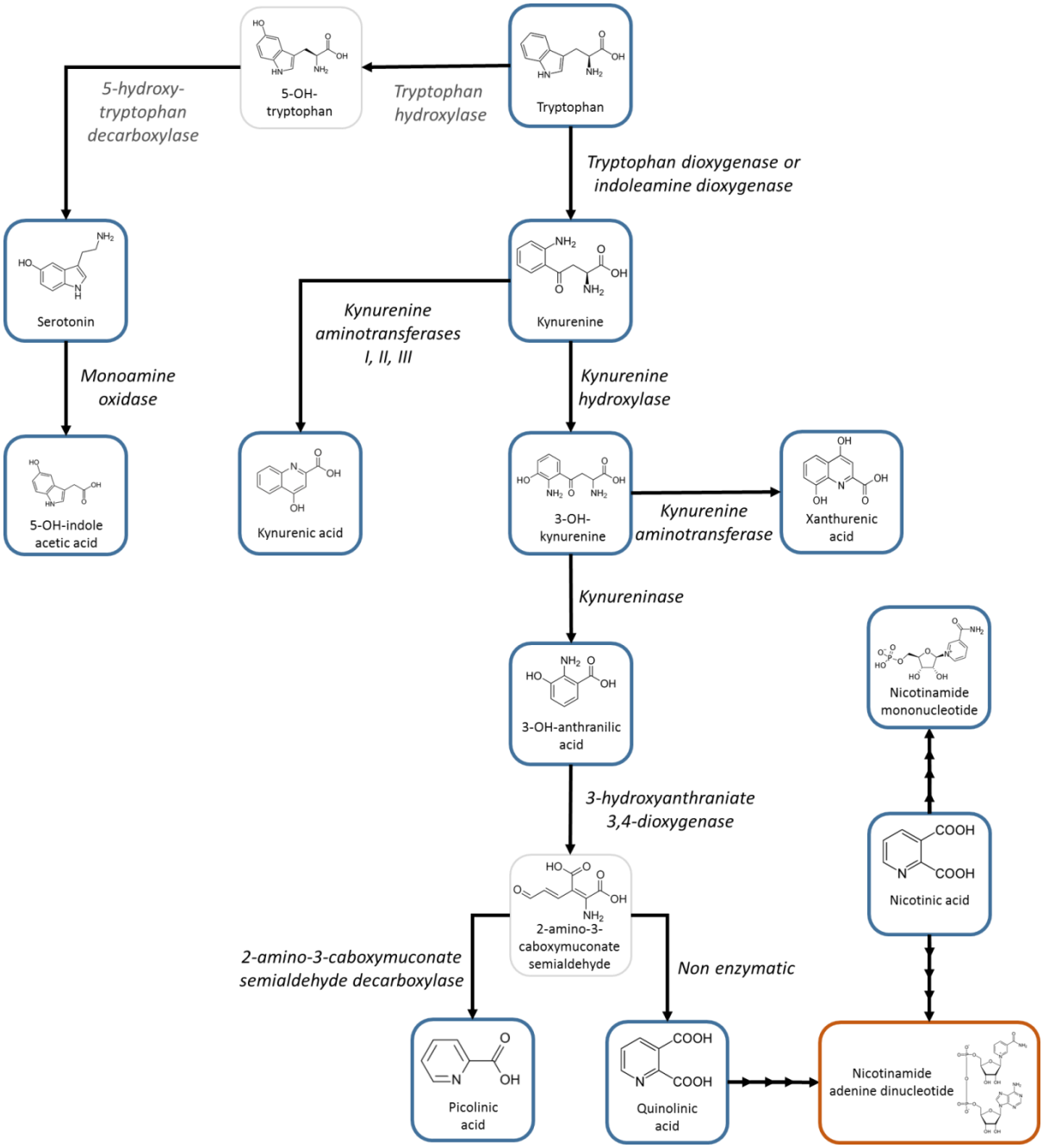
This supporting information document contains three figures: **Figure S1** shows tryptophan metabolism via the serotonin and kynurenine pathways; **Figure S2** depicts the 96 well plate map layout that was used in the assay; **Figure S3** illustrates the observed effect of detuning the tryptophan multiple reaction monitoring (MRM) transition in negative ionization MS detection mode.

This document also contains 12 tables: **Table S1** provides an overview describing the preparation of an upper limit of quantification stock; **Table S2** details the dilution preparation of working calibration and QC dilutions; **Table S3** provides final concentrations of each level of the calibration series and QC series used in the assay; **Table S4** details the preparation of the internal standard working solution used in the assay; **Table S5** details individual metabolite inter run calibration linearity; **Table S6** details the intra-day and inter-day accuracy and precision of each metabolite; **Table S7** presents results obtained from stability testing of the individual analytes; **Table S8** presents percentage recovery data for each metabolite from plasma; **Table S9** presents the percentage recovery data for each metabolite from serum; **Table S10** provides matrix effect data for each metabolite during analysis; **Table S11** presents mean concentrations, standard deviation and % coefficient of variation values derived from the quantification of analytes from repeat injections of a pool of serum; **Table S12** presents the mean concentration values obtained for repeat biological replicates.



Supporting Figure S1:

Tryptophan metabolism via the serotonin and kynurenine pathways. Figure adapted from [64]. Metabolites highlighted in blue were validated for quantitative analysis. Metabolites circled in grey were intermediate metabolites and did not undergo analysis. The mass spectrometry transition for nicotinamide adenine dinucleotide (NAD^+, highlighted in red) was included for monitoring only and was not quantitative.

a.

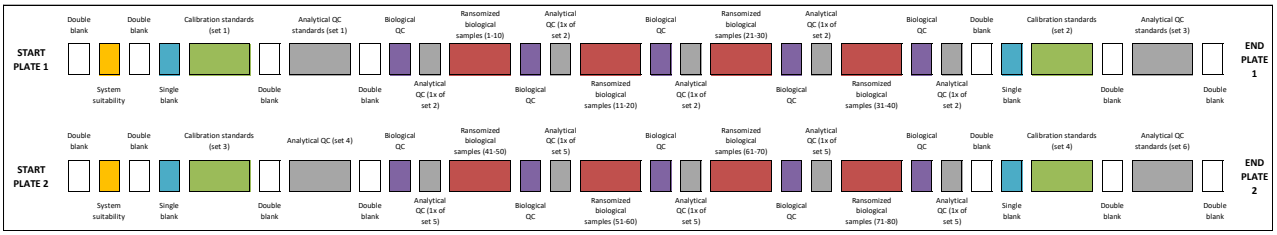
Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 Cal 1 (Set 1)	2 Cal 2 (Set 1)	3 Cal 3 (Set 1)	4 Cal 4 (Set 1)	5 Cal 5 (Set 1)	6 Cal 6 (Set 1)	7 Cal 7 (Set 1)	8 Cal 8 (Set 1)	9 QC4 (SS)	10 Single Blank	11 Single Blank	12 Double Blank
B	13 Cal 1 (Set 2)	14 Cal 2 (Set 2)	15 Cal 3 (Set 2)	16 Cal 4 (Set 2)	17 Cal 5 (Set 2)	18 Cal 6 (Set 2)	19 Cal 7 (Set 2)	20 Cal 8 (Set 2)	21 SPARE	22 SPARE	23 SPARE	24 Double Blank
C	25 QC 1 (Set 1)	26 QC 2 (Set 1)	27 QC3 (Set 1)	28 QC 4 (Set 1)	29 QC 5 (Set 1)	30 QC 6 (Set 1)	31 QC 7 (Set 1)	32 QC 1 (Set 2)	33 QC 2 (Set 2)	34 QC 3 (Set 2)	35 QC 4 (Set 2)	36 Double Blank
D	37 QC 5 (Set 2)	38 QC 6 (Set 2)	39 QC 7 (Set 2)	40 QC 1 (Set 3)	41 QC 2 (Set 3)	42 QC3 (Set 3)	43 QC 4 (Set 3)	44 QC 5 (Set 3)	45 QC 6 (Set 3)	46 QC 7 (Set 3)	47 Biological QC	48 Double Blank
E	49 Sample 1	50 Sample 2	51 Sample 3	52 Sample 4	53 Sample 5	54 Sample 6	55 Sample 7	56 Sample 8	57 Sample 9	58 Sample 10	59 Biological QC	60 Double Blank
F	61 Sample 11	62 Sample 12	63 Sample 13	64 Sample 14	65 Sample 15	66 Sample 16	67 Sample 17	68 Sample 18	69 Sample 19	70 Sample 20	71 Biological QC	72 Double Blank
G	73 Sample 21	74 Sample 22	75 Sample 23	76 Sample 24	77 Sample 25	78 Sample 26	79 Sample 27	80 Sample 28	81 Sample 29	82 Sample 30	83 Biological QC	84 Double Blank
H	85 Sample 31	86 Sample 32	87 Sample 33	88 Sample 34	89 Sample 35	90 Sample 36	91 Sample 37	92 Sample 38	93 Sample 39	94 Sample 40	95 Biological QC	96 Double Blank

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 Cal 1 (Set 3)	2 Cal 2 (Set 3)	3 Cal 3 (Set 3)	4 Cal 4 (Set 3)	5 Cal 5 (Set 3)	6 Cal 6 (Set 3)	7 Cal 7 (Set 3)	8 Cal 8 (Set 3)	9 QC4 (SS)	10 Single Blank	11 Single Blank	12 Double Blank
B	13 Cal 1 (Set 4)	14 Cal 2 (Set 4)	15 Cal 3 (Set 4)	16 Cal 4 (Set 4)	17 Cal 5 (Set 4)	18 Cal 6 (Set 4)	19 Cal 7 (Set 4)	20 Cal 8 (Set 4)	21 SPARE	22 SPARE	23 SPARE	24 Double Blank
C	25 QC 1 (Set 4)	26 QC 2 (Set 4)	27 QC3 (Set 4)	28 QC 4 (Set 4)	29 QC 5 (Set 4)	30 QC 6 (Set 4)	31 QC 7 (Set 4)	32 QC 1 (Set 5)	33 QC 2 (Set 5)	34 QC 3 (Set 5)	35 QC 4 (Set 5)	36 Double Blank
D	37 QC 5 (Set 5)	38 QC 6 (Set 5)	39 QC 7 (Set 5)	40 QC 1 (Set 6)	41 QC 2 (Set 6)	42 QC3 (Set 6)	43 QC 4 (Set 6)	44 QC 5 (Set 6)	45 QC 6 (Set 6)	46 QC 7 (Set 6)	47 Biological QC	48 Double Blank
E	49 Sample 41	50 Sample 42	51 Sample 43	52 Sample 44	53 Sample 45	54 Sample 46	55 Sample 47	56 Sample 48	57 Sample 49	58 Sample 50	59 Biological QC	60 Double Blank
F	61 Sample 51	62 Sample 52	63 Sample 53	64 Sample 54	65 Sample 55	66 Sample 56	67 Sample 57	68 Sample 58	69 Sample 59	70 Sample 60	71 Biological QC	72 Double Blank
G	73 Sample 61	74 Sample 62	75 Sample 63	76 Sample 64	77 Sample 65	78 Sample 66	79 Sample 67	80 Sample 68	81 Sample 69	82 Sample 70	83 Biological QC	84 Double Blank
H	85 Sample 71	86 Sample 72	87 Sample 73	88 Sample 74	89 Sample 75	90 Sample 76	91 Sample 77	92 Sample 78	93 Sample 79	94 Sample 80	95 Biological QC	96 Double Blank

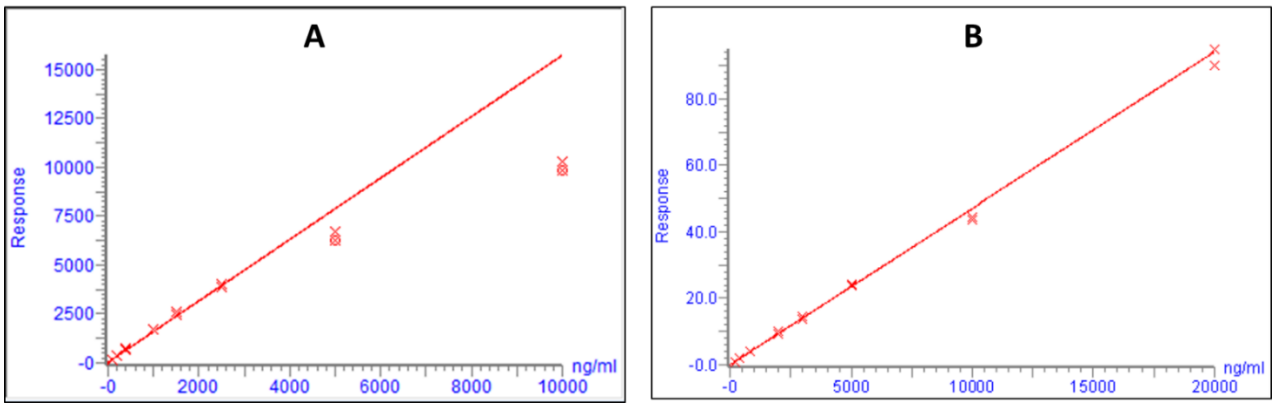
b.



Supporting Figure S2.

a. 96 well plate map layout used in the assay. The plate map enables two brackets of calibration standards and quality control standards per plate. Using a double plate layout allows for the analysis of 80 biological samples in 24 hours. The inclusion of biological QCs (pool of mixed study sample) allows for the comparison of stability of measurements for each metabolite in a biological matrix across plates in a batch. An extra QC was prepared and run prior to sample analysis and to act as a system suitability (SS) injection to ensure that the chromatography and mass spectrometry detection was performing correctly.

b. Sequence of analysis for metabolite quantification of randomized samples that are bracketed by calibration standards and interspersed with both Analytical QC and Biological QC injections.



Supporting Figure S3. Effect of detuning the tryptophan transition in negative ionization MS detection mode.

A. A calibration curve for tryptophan in positive ESI, tuned for sensitivity in the range 100-10,000 ng/mL. Non-linearity occurred at > 2500 ng/mL. This was hypothesized to be an effect of in-source saturation.
B. By polarity switching and detuning the MS capillary voltage (0.25 kV), linearity was achieved across a concentration range suitable for serum and plasma (200 -20,000 ng/mL).

Supporting Table S1. Overview describing the preparation of an upper limit of quantification stock C prepared in duplicate for calibration standards and quality control (QC) standards.

Calibration and QC mixed stock A (prepared in duplicate)		
Metabolites	**Source stock**	**Volume (µL)**
Neopterin	Parent 1 mg/mL stock	2.5
Kynurenic acid, xanthurenic acid, picolinic acid, nicotinic acid	Parent 1 mg/mL stock	5
Dopamine	Parent 1 mg/mL stock	10
Nicotinamide riboside	Parent 1 mg/mL stock	12.5
5-HIAA, NAD+, serotonin, quinolinic acid, 3-HK, 3-HAA	Parent 1 mg/mL stock	25
Kynurenine, βNM,	Parent 1 mg/mL stock	50
NA	Diluent-D2	705
Total volume	1000 µL	
Calibration and QC mixed stock B (prepared in duplicate)		
Metabolites	**Source stock**	**Volume (µL)**
I-3-AA	Parent 1 mg/mL stock	50
Citrulline	Parent 1 mg/mL stock	250
Tryptophan	Parent 1 mg/mL stock	500
Stock A	Stock A	200
Total volume	1000 µL	
Calibration and QC mixed stock C – ULOQ - (prepared in duplicate)		
Metabolites	**Source stock**	**Volume (µL)**
Stock B	Stock B	1000
NA	Diluent-D2	24000
Total volume	25000 µL	

Supporting Table S2. Preparation of working calibration and QC dilutions. The total volumes will require alteration depending on analytical batch size. These volumes are suitable for 2x 96 well plate preparation, consisting of 40 unknown samples per plate (see plate layout presented in **Figure S2**).

Calibration working solution preparation	Solution to dilute	Volume of solution (µL)	Volume of Diluent D2 (µL)
Calibration level 8 (ULOQ)	Stock C (Calibration)	NA	NA
Calibration level 7	Calibration 8 (ULOQ)	400	400
Calibration level 6	Calibration 7	400	400
Calibration level 5	Calibration 6	480	320
Calibration level 4	Calibration 5	384	192
Calibration level 3	Calibration 4	224	336
Calibration level 2	Calibration 3	240	240
Calibration level 1	Calibration 2	160	160
QC working solution preparation	**Solution to dilute (ng/mL)**	**Volume of solution (µL)**	**Volume of Diluent D2 (µL)**
QC level 7 (ULOQ)	Stock C (QC)	NA	NA
QC level 6	QC (ULOQ)	800	200
QC level 5	QC 6	400	400
QC level 4	QC 5	200	600
QC level 3	QC4	200	300
QC level 2	QC3	200	200
QC level 1 (LLOQ)	QC2	200	200

Supporting Table S3. Final concentrations of each level of the calibration series and QC series used in the assay

Calibration series preparation - metabolite concentration (ng/mL)									
	Neopterin	Xanthurenic acid, kynurenic acid, picolinic acid, nicotinic acid	Dopamine	Nicotinamide riboside	5-HIAA, NAD+, serotonin, quinolinic acid, 3-HK, 3-HAA	Kynurenine, βNM	Indole-3-AA	Citrulline	Tryptophan
Calibration level 8 (ULOQ)	20	40	80	100	200	400	2000	10000	20000
Calibration level 7	10	20	40	50	100	200	1000	5000	10000
Calibration level 6	5	10	20	25	50	100	500	2500	5000
Calibration level 5	3	6	12	15	30	60	300	1500	3000
Calibration level 4	2	4	8	10	20	40	200	1000	2000
Calibration level 3	0.8	1.6	3.2	4	8	16	80	400	800
Calibration level 2	0.4	0.8	1.6	2	4	8	40	200	400
Calibration level 1	0.2	0.4	0.8	1	2	4	20	100	200
QC series preparation - metabolite concentration (ng/mL)									
	Neopterin	Xanthurenic acid, kynurenic acid, picolinic acid, nicotinic acid	Dopamine	Nicotinamide riboside	5-HIAA, NAD+, serotonin, quinolinic acid, 3-HK, 3-HAA	Kynurenine, βNM	Indole-3-AA	Citrulline	Tryptophan
QC level 7 (ULOQ)	20	40	80	100	200	400	2000	10000	20000
QC level 6	16	32	64	80	160	320	1600	8000	16000
QC level 5	8	16	32	40	80	160	800	4000	8000
QC level 4	2	4	8	10	20	40	200	1000	2000
QC level 3	0.8	1.6	3.2	4	8	16	80	400	800
QC level 2	0.4	0.8	1.6	2	4	8	40	200	400
QC level 1 (LLOQ)	0.2	0.4	0.8	1	2	4	20	100	200

Supporting Table S4. Preparation of the internal standard working solution used in the assay

Internal standard (IS) mixed stock A		
Metabolites	**Source stock**	**Volume (µL)**
I-3-AA-D_4, kynurenic acid-D_5	Parent 1 mg/mL stock	30
Neopterin-$^{13}C_5$, xanthurenic acid-D_4, 5-HIAA-D_5, citrulline-D_4, nicotinamide riboside-D_3	Parent 1 mg/mL stock	150
NA	Diluent-D2	190
Total volume	1000 µL	
Internal standard (IS) mixed stock B		
Metabolites	**Source stock**	**Volume (µL)**
Nicotinic acid-D_4, quinolinic acid-D_3, dopamine-D_4, 3-HK-$^{13}C_2$-^{15}N, kynurenine-D_4, 3-HAA-D_3, picolinic acid-D_3	Parent 1 mg/mL stock	75
Tryptophan-D_5	Parent 1 mg/mL stock	150
IS stock A	IS stock A	100
NA	Diluent-D2	225
Total volume	**1000 µL**	
Internal standard (IS) working solution		
IS stock B	IS stock B	500
NA	Diluent-D2	24500
Total volume	25000 µL	

Supporting Table S5. All metabolites that utilized an internal standard or a surrogate internal standard returned an r^2 value of > 0.990. MRM transitions for NAD+ were included for monitoring only, and therefore was not assessed for validation.

	Calibration range (ng/mL)	Linearity (r^2)		
		Calibration - repeat 1	Calibration - repeat 2	Calibration - repeat 3
3-HAA	2-200	0.991	0.994	0.992
3-HK	2-200	0.997	0.997	0.999
5-HIAA	2-200	0.994	0.997	0.995
βNM	4-400	0.998	0.998	0.998
Citrulline	100-10,000	0.999	0.999	0.998
Dopamine	0.8-80	0.991	0.997	0.998
I-3-AA	20-2000	0.999	0.998	0.996
Kynurenic acid	0.4-40	0.999	0.998	0.997
Kynurenine	8-400	0.992	0.992	0.997
NAD+	*NA (monitored MRM transition only)*			
Neopterin	0.2-200	0.996	0.999	0.993
Nicotinic acid	0.4-40	0.990	0.993	0.993
Nicotinamide riboside	1-100	0.997	0.997	0.997
Picolinic Acid	0.4-40	0.996	0.996	0.994
Quinolinic acid	1-100	0.994	0.995	0.995
Serotonin	2-200	0.995	0.996	0.995
Tryptophan	200-20,000	0.994	0.997	0.993
Xanthurenic acid	0.4-40	0.993	0.997	0.996

Supporting Table S6. Intra-day and inter-day accuracy and precision

		QC concentration in ng/mL (Coefficients of Variation (%))						
Compound	**Run**	**QC 1 (LLOQ)**	**QC 2**	**QC 3**	**QC 4**	**QC 5**	**QC 6**	**QC 7 (ULOQ)**
Target concentrations (ng/mL)		**200**	**400**	**800**	**2000**	**8000**	**16,000**	**20,000**
Tryptophan	Intra-run 1	198.9 (5.4)	409.2 (6.7)	804.7 (7.1)	2078.4 (8.6)	7839.0 (4.3)	15895.2 (5.6)	19750.9 (4.2)
	Intra-run 2	194.4 (6.2)	412.4 (3.9)	843.0 (4.1)	2136.9 (5.9)	8158.5 (5.7)	15759.8 (4.0)	20065.9 (3.9)
	Intra-run 3	204.3 (9.3)	420.0 (4.6)	829.4 (6.3)	2022.2 (5.0)	7882.3 (4.0)	15173.1 (7.2)	18472.7 (4.4)
	Inter-run	199.2 (7.1)	413.9 (5.0)	825.7 (5.9)	2079.2 (6.7)	7960.0 (4.8)	15609.4 (5.7)	19429.8 (5.3)
Target concentrations (ng/mL)		**100**	**200**	**400**	**1000**	**4000**	**8000**	**10,000**
Citrulline	Intra-run 1	99.5 (3.7)	201.1 (3.4)	400.0 (2.8)	1012.9 (2.1)	3983.4 (2.3)	8062.5 (2.2)	10144.5 (1.3)
	Intra-run 2	95.8 (4.6)	198.3 (2.8)	414.2 (2.5)	1015.6 (4.3)	4034.2 (2.1)	8022.9 (1.0)	9783.2 (4.0)
	Intra-run 3	101.9 (6.6)	203.2 (3.3)	407.6 (3.9)	1005.2 (4.0)	4018.4 (1.9)	7988.5 (4.3)	9638.4 (2.8)
	Inter-run	99.2 (5.5)	200.9 (3.2)	407.3 (3.3)	1011.2 (3.4)	4012.0 (2.1)	8024.6 (2.7)	9855.4 (3.5)
Target concentrations (ng/mL)		**20**	**40**	**80**	**200**	**800**	**1600**	**2000**
I-3-AA	Intra-run 1	20.5 (3.5)	42.1 (1.0)	83.3 (3.3)	207.2 (2.2)	795.2 (1.0)	1547.9 (3.6)	1900.2 (2.4)
	Intra-run 2	21.2 (4.0)	42.8 (3.9)	83.8 (1.4)	209.1 (2.4)	824.1 (3.1)	1555.9 (2.9)	1877.8 (2.8)
	Intra-run 3	20.3 (2.3)	41.6 (3.3)	85.3 (2.7)	208.6 (4.7)	804.7 (2.4)	1544.3 (4.1)	1832.8 (3.3)
	Inter-run	20.7 (3.6)	42.2 (3.1)	84.1 (2.6)	208.3 (3.1)	808.0 (2.7)	1549.4 (3.4)	1870.2 (3.1)
Target concentrations (ng/mL)		**4**	**8**	**16**	**40**	**160**	**320**	**400**
Kynurenine	Intra-run 1	-	7.0 (6.7)	14.6 (3.8)	39.9 (4.3)	140.0 (2.5)	280.4 (2.1)	349.5 (1.8)
	Intra-run 2	-	7.0 (5.0)	16.8 (3.7)	40.9 (6.5)	144.9 (3.6)	278.3 (2.0)	345.8 (1.8)
	Intra-run 3	-	7.6 (4.9)	16.1 (5.9)	42.5 (3.1)	159.9 (3.6)	299.4 (5.3)	373.7 (4.6)
	Inter-run	-	7.2 (6.6)	15.8 (7.3)	41.1 (5.3)	148.2 (6.6)	286.0 (4.8)	356.3 (4.6)
βNM	Intra-run 1	4.1 (2.1)	8.1 (2.4)	15.9 (3.7)	39.2 (2.4)	154.8 (2.1)	311.2 (3.6)	397.3 (2.8)
	Intra-run 2	3.8 (7.4)	8.1 (4.7)	16.2 (8.7)	40.3 (5.5)	161.4 (3.1)	337.4 (3.6)	421.3 (4.7)
	Intra-run 3	4.2 (4.7)	8.0 (3.1)	16.5 (2.8)	40.0 (4.1)	160.7 (3.4)	324.1 (5.3)	403.9 (2.6)
	Inter-run	4.0 (6.2)	8.1 (3.3)	16.2 (5.3)	39.8 (4.2)	158.8 (3.4)	323.5 (5.2)	406.7 (4.1)
Target concentrations (ng/mL)		**2**	**4**	**8**	**20**	**80**	**160**	**200**
3-HAA	Intra-run 1	2.1 (13.2)	4.1 (8.8)	8.5 (7.6)	21.4 (2.7)	89.7 (3.2)	178.0 (2.4)	221.5 (1.8)
	Intra-run 2	2.1 (12.2)	4.0 (6.4)	8.3 (6.3)	22.0 (2.4)	86.4 (4.4)	176.2 (2.1)	220.2 (2.8)
	Intra-run 3	2.2 (6.4)	4.2 (8.2)	8.6 (5.7)	21.3 (3.8)	88.9 (2.5)	175.6 (2.7)	212.6 (2.9)
	Inter-run	2.1 (10.4)	4.1 (7.7)	8.4 (6.3)	21.5 (3.2)	88.3 (3.7)	176.6 (2.4)	218.4 (3.0)
Serotonin	Intra-run 1	2.2 (12.6)	4.1 (4.8)	8.1 (6.0)	20.2 (5.9)	80.6 (4.4)	168.3 (4.6)	211.8 (4.8)
	Intra-run 2	2.3 (3.6)	4.2 (3.0)	8.1 (3.0)	20.4 (6.7)	82.8 (2.4)	172.5 (3.9)	213.7 (3.8)
	Intra-run 3	2.0 (5.7)	3.9 (6.0)	8.1 (6.4)	20.5 (5.9)	82.1 (7.9)	165.3 (6.0)	190.7 (4.0)
	Inter-run	2.2 (12.6)	4.1 (4.8)	8.1 (6.0)	20.2 (5.9)	80.6 (4.4)	168.3 (4.6)	211.8 (4.8)

		QC concentration in ng/mL (Coefficients of Variation (%))						
Compound	**Run**	**QC 1 (LLOQ)**	**QC 2**	**QC 3**	**QC 4**	**QC 5**	**QC 6**	**QC 7 (ULOQ)**
Quinolinic acid	Intra-run 1	2.1 (9.1)	4.2 (5.3)	8.2 (4.6)	19.9 (6.4)	79.7 (2.9)	154.9 (5.7)	194.6 (2.8)
	Intra-run 2	2.1 (8.4)	4.2 (5.8)	8.5 (2.9)	20.3 (3.5)	80.6 (2.9)	158.3 (3.5)	201.9 (3.4)
	Intra-run 3	1.9 (12.9)	4.1 (8.0)	8.3 (4.6)	20.3 (3.0)	80.6 (6.3)	158.4 (4.3)	197.5 (3.6)
	Inter-run	2.0 (10.9)	4.1 (6.2)	8.3 (4.2)	20.2 (4.4)	80.3 (4.1)	157.2 (4.4)	198.0 (3.5)
5-HIAA	Intra-run 1	1.9 (1.8)	4.1 (2.8)	8.5 (1.4)	20.8 (5.0)	85.3 (4.8)	170.9 (4.9)	207.6 (4.0)
	Intra-run 2	2.2 (5.9)	4.3 (5.2)	8.5 (3.7)	21.5 (6.1)	81.6 (8.4)	172.1 (2.7)	198.2 (2.7)
	Intra-run 3	2.0 (1.2)	4.2 (5.4)	8.5 (4.1)	20.2 (4.6)	85.1 (4.5)	163.3 (4.3)	202.5 (3.6)
	Inter-run	2.1 (6.8)	4.2 (4.8)	8.5 (3.1)	20.8 (5.6)	84.0 (6.1)	168.7 (4.5)	202.8 (3.8)
3-HK	Intra-run 1	2.2 (12.8)	4.5 (3.1)	8.7 (3.3)	21.9 (1.6)	87.1 (2.6)	176.3 (2.7)	225.6 (0.8)
	Intra-run 2	2.0 (6.3)	4.0 (5.0)	8.5 (2.2)	21.8 (2.1)	89.0 (1.7)	179.2 (1.1)	225.0 (1.5)
	Intra-run 3	2.1 (3.8)	4.3 (3.9)	8.5 (5.5)	21.4 (2.6)	87.2 (3.0)	168.9 (3.5)	216.1 (1.5)
	Inter-run	2.1 (9.3)	4.3 (6.3)	8.6 (3.7)	21.7 (2.2)	87.8 (2.6)	174.8 (3.5)	222.2 (2.3)
NAD+ (monitored metabolite only)	Intra-run 1	*NA (monitored MRM transition only)*						
	Intra-run 2							
	Intra-run 3							
	Inter-run							
Target concentrations (ng/mL)		**1**	**2**	**4**	**10**	**40**	**80**	**100**
Nicotinamide riboside	Intra-run 1	1.0 (1.7)	2.0 (4.5)	4.0 (4.1)	10.0 (3.4)	39.4 (4.0)	80.8 (4.2)	100.8 (3.0)
	Intra-run 2	1.0 (8.7)	2.0 (4.1)	4.1 (5.2)	10.5 (3.4)	40.9 (5.1)	82.6 (3.2)	102.6 (4.0)
	Intra-run 3	1.0 (6.6)	2.0 (5.0)	4.1 (3.6)	9.9 (3.4)	40.4 (3.0)	80.1 (3.7)	100.0 (3.1)
	Inter-run	1.0 (6.1)	2.0 (4.4)	4.1 (4.4)	10.1 (4.0)	40.3 (4.2)	81.2 (3.7)	101.1 (3.4)
Target concentrations (ng/mL)		**0.8**	**1.6**	**3.2**	**8**	**32**	**64**	**80**
Dopamine	Intra-run 1	0.9 (8.8)	1.7 (5.5)	3.4 (5.6)	8.2 (3.3)	31.6 (2.2)	61.9 (3.8)	80.8 (5.2)
	Intra-run 2	0.9 (4.5)	1.8 (3.1)	3.4 (5.3)	8.2 (2.9)	33.7 (4.0)	64.0 (2.6)	82.2 (2.3)
	Intra-run 3	0.9 (3.8)	1.6 (6.2)	3.3 (4.9)	8.0 (2.4)	32.9 (3.9)	64.5 (5.2)	80.3 (1.8)
	Inter-run	0.9 (6.0)	1.7 (5.8)	3.4 (5.0)	8.1 (2.9)	32.8 (4.3)	63.5 (4.2)	81.1 (3.4)
Target concentrations (ng/mL)		**0.4**	**0.8**	**1.6**	**4**	**16**	**32**	**40**
Picolinic acid	Intra-run 1	0.4 (6.7)	0.8 (6.5)	1.6 (11.2)	4.1 (9.7)	16.6 (5.4)	32.8 (3.0)	41.6 (1.3)
	Intra-run 2	0.4 (11.4)	0.8 (7.5)	1.6 (8.9)	4.4 (4.6)	16.4 (3.2)	33.0 (3.1)	41.4 (4.6)
	Intra-run 3	0.4 (15.4)	0.9 (6.5)	1.7 (4.3)	4.1 (8.1)	16.2 (4.2)	33.1 (4.1)	39.9 (1.5)
	Inter-run	0.4 (11.6)	0.8 (7.7)	1.6 (9.0)	4.2 (7.8)	16.4 (4.3)	33.0 (3.3)	41.0 (3.4)
Nicotinic acid	Intra-run 1	0.4 (9.8)	0.8 (10.5)	1.6 (8.7)	4.1 (7.1)	15.9 (4.2)	31.4 (3.8)	38.5 (3.1)
	Intra-run 2	0.4 (8.0)	0.8 (8.3)	1.6 (1.9)	4.0 (4.7)	16.0 (5.1)	32.4 (4.9)	37.6 (4.5)
	Intra-run 3	0.4 (8.4)	0.8 (7.1)	1.6 (9.3)	4.1 (3.7)	16.4 (5.3)	32.4 (5.4)	38.1 (3.7)
	Inter-run	0.4 (8.7)	0.8 (9.1)	1.6 (7.3)	4.1 (5.1)	16.1 (4.8)	32.1 (4.7)	38.1 (3.7)

		QC concentration in ng/mL (Coefficients of Variation (%))						
Compound	**Run**	**QC 1 (LLOQ)**	**QC 2**	**QC 3**	**QC 4**	**QC 5**	**QC 6**	**QC 7 (ULOQ)**
Kynurenic acid	Intra-run 1	0.4 (7.0)	0.8 (4.8)	1.6 (1.1)	4.1 (2.8)	16.0 (2.7)	32.1 (2.4)	40.3 (2.7)
	Intra-run 2	0.4 (1.8)	0.8 (5.9)	1.7 (4.6)	4.2 (3.8)	16.4 (1.1)	32.9 (3.4)	40.2 (1.3)
	Intra-run 3	0.4 (5.4)	0.9 (2.6)	1.7 (4.4)	4.1 (3.9)	16.2 (2.4)	32.5 (4.8)	39.2 (4.1)
	Inter-run	0.4 (6.8)	0.8 (4.9)	1.7 (3.6)	4.1 (3.8)	16.2 (2.3)	32.5 (3.6)	40.0 (3.0)
Xanthurenic acid	Intra-run 1	0.4 (8.2)	0.8 (8.6)	1.6 (5.4)	4.0 (3.1)	15.6 (1.8)	31.6 (5.5)	40.1 (3.0)
	Intra-run 2	0.4 (8.0)	0.8 (7.2)	1.7 (4.5)	4.1 (3.3)	16.2 (2.8)	32.9 (4.4)	40.5 (2.3)
	Intra-run 3	0.4 (8.8)	0.8 (6.2)	1.6 (2.8)	3.9 (3.5)	16.5 (4.1)	32.5 (3.6)	40.7 (4.7)
	Inter-run	0.4 (9.0)	0.8 (7.3)	1.6 (4.5)	4.0 (4.4)	16.1 (3.7)	32.3 (4.6)	40.4 (3.3)
Target concentrations (ng/mL)		**0.2**	**0.4**	**0.8**	**2**	**8**	**16**	**20**
Neopterin	Intra-run 1	0.2 (5.1)	0.4 (5.6)	0.8 (3.4)	2.0 (3.4)	7.9 (3.4)	15.9 (3.8)	20.3 (3.8)
	Intra-run 2	0.2 (12.5)	0.4 (4.9)	0.9 (4.6)	2.1 (4.1)	8.3 (2.0)	16.8 (2.1)	21.0 (3.3)
	Intra-run 3	0.2 (8.5)	0.4 (5.9)	0.9 (4.4)	2.0 (6.7)	8.4 (3.0)	16.7 (3.1)	20.4 (1.4)
	Inter-run	0.2 (9.1)	0.4 (5.3)	0.8 (4.5)	2.0 (4.8)	8.2 (3.9)	16.5 (3.9)	20.6 (3.2)

Supporting Table S7. Results from stability testing of the analytes**.** Standards were prepared in diluent containing citric acid and were assessed for stability at 24 hours in a 4°C autosampler and both at 1 and 2 weeks at -20°C. This was repeated with a low and a high QC (QC 2 and 6). The QC samples that underwent quantification after storage reported acceptable stability of > 97% for the high QC for all metabolites, and greater than 90% in the low QC. The exception was picolinic acid where stability was 90.74% after 1 week, and 83.33% after 2 weeks.

	Low QC			High QC		
	% Stability 24-hour autosampler	% Stability 1 Week at -20 °C	% Stability 2 Weeks at -20 °C	% Stability 24-hour autosampler	% Stability 1 Week at -20 °C	% Stability 2 Weeks at -20 °C
Picolinic Acid	97.8	90.7	83.3	102.8	103.3	102.0
Nicotinic acid	95.6	95.9	100.0	98.2	105.0	102.9
3-HAA	100.0	98.8	97.3	100.1	101.6	101.0
Dopamine	103.1	100.9	96.2	103.3	97.5	98.6
Serotonin	102.6	99.2	95.4	102.6	101.1	98.1
Quinolinic acid	99.5	97.0	94.7	99.3	101.2	100.9
Citrulline	98.1	96.0	98.7	102.9	103.6	101.5
I-3-AA	101.2	102.6	96.1	104.4	104.1	101.5
Kynurenic acid	96.3	106.4	104.3	102.3	100.9	97.7
5-HIAA	99.6	103.1	98.8	103.2	111.7	105.4
Xanthurenic acid	106.2	98.0	95.9	102.5	99.9	97.7
Kynurenine	91.3	95.3	101.6	101.6	99.0	99.7
3-HK	101.9	98.0	97.2	102.9	105.2	105.3
Neopterin	102.8	100.0	100.0	102.9	103.5	99.8
Nicotinamide riboside	102.1	101.6	97.5	104.0	104.2	100.8
βNM	94.6	104.7	101.9	102.5	110.8	98.1
NAD+	117.3	96.2	105.8	106.6	99.4	105.1
Tryptophan	98.7	101.4	100.2	104.3	98.7	98.6

Supporting Table 8. Results of recovery study for plasma. Extraction recoveries were calculated by comparing SIL internal standards spiked into matrix after the protein precipitation/SPE extraction protocol, with SIL internal standards that were spiked into matrix prior to extraction. Plasma metabolite recoveries were typically greater than 90%, with only dopamine-D_4 (83 %), quinolinic acid-D_3 (80 %) and nicotinamide riboside-D_3 (53%) being the exceptions.

	Recovery % Plasma 1	Recovery % Plasma 2	Recovery % Plasma 3	Recovery % Plasma 4	Recovery % Plasma 5	Recovery % Plasma 6	Recovery % mean recovery
3-HAA-D_3	97.0	103.5	92.8	91.4	97.0	97.9	96.6
3-HK-$^{13}C_2$-^{15}N	97.3	97.7	93.3	96.6	97.7	91.3	95.6
5-HIAA-D_5	102.5	114.2	103.2	113.6	110.5	95.5	106.6
Citrulline-D_4	92.7	100.0	88.1	82.7	90.7	102.8	92.8
Dopamine-D_4	78.8	91.3	79.2	83.1	84.3	81.1	83.0
I-3-AA-D_4	83.5	97.8	95.5	96.6	97.5	102.3	95.5
Kynurenic acid-D_5	92.0	92.2	92.0	91.7	94.1	96.0	93.0
Kynurenine-D_4	94.0	95.1	93.5	97.3	97.2	92.8	95.0
Neopterin-$^{13}C_5$	90.9	92.9	92.3	90.6	92.7	93.6	92.2
Nicotinic acid-D_4	90.4	92.9	92.0	93.7	93.4	95.4	93.0
Nicotinamide riboside-D_3	52.9	53.1	52.5	54.8	53.2	50.3	52.8
Picolinic acid-D_3	92.0	94.5	90.5	83.1	93.1	90.6	90.6
Quinolinic acid-D_3	80.2	81.2	80.7	74.4	80.0	81.6	79.7
Tryptophan-D_5	90.4	86.4	94.0	78.8	105.6	93.9	91.5
Xanthurenic acid-D_4	93.0	91.3	91.7	91.0	92.5	96.0	92.6

Supporting Table S9. Results of recovery study for serum. Extraction recoveries were calculated by comparing SIL internal standards spiked into matrix after the protein precipitation/SPE extraction protocol, with SIL internal standards that were spiked into matrix prior to extraction. Serum metabolite recoveries were typically greater than 85%, with quinolinic acid-D_3 (79%) and nicotinamide riboside-D_3 (52%) being the exceptions.

	Recovery % Serum 1	Recovery % Serum 2	Recovery % Serum 3	Recovery % Serum 4	Recovery % Serum 5	Recovery % Serum 6	Recovery % mean recovery
3-HAA-D_3	107.1	99.9	103.0	98.3	101.2	93.1	100.4
3-HK-$^{13}C_2$-^{15}N	91.8	95.3	94.8	94.6	103.0	91.3	95.1
5-HIAA-D_5	102.7	105.8	110.0	107.5	107.9	110.7	107.4
Citrulline-D_4	86.7	95.8	92.1	100.9	93.9	82.6	92.0
Dopamine-D_4	89.9	83.5	86.7	87.4	89.6	85.9	87.2
I-3-AA-D_4	96.6	90.8	97.8	91.7	100.0	99.8	96.1
Kynurenic acid-D_5	93.7	90.9	94.3	92.7	97.4	91.5	93.4
Kynurenine-D_4	89.1	94.3	92.4	94.2	103.5	90.7	94.0
Neopterin-$^{13}C_5$	94.7	90.3	92.3	91.8	94.3	90.1	92.3
Nicotinic acid-D_4	90.7	89.6	93.4	94.3	98.2	93.4	93.3
Nicotinamide riboside-D_3	48.4	49.9	49.9	55.8	58.8	51.8	52.4
Picolinic acid-D_3	81.9	91.2	93.3	92.6	91.7	86.9	89.6
Quinolinic acid-D_3	79.0	79.1	80.9	81.0	81.0	74.8	79.3
Tryptophan-D_5	87.6	91.0	86.7	99.7	105.2	93.9	94.0
Xanthurenic acid-D_4	91.6	90.0	92.2	91.9	97.8	94.3	93.0

Supporting Table S10. Results of studies into matrix effects during analysis. Analytes were assessed for matrix effects by spiking SIL internal standard into both matrix and diluent blanks following extraction. In both plasma and serum matrix effects were minimal for most metabolites, with results suggesting a matrix area response of 77 %-113 % of metabolites spiked into blanks. The exception in both biofluids was citrulline-D_4 (plasma = 23 %, serum = 18 %). Whilst nicotinamide riboside experienced greater matrix effects in serum. To compensate for matrix effects a SIL internal standard was included in the assay.

	Plasma	Serum
3-HAA-D_3	102.0	103.7
3-HK-$^{13}C_2$-^{15}N	88.8	95.3
5-HIAA-D_5	89.0	94.7
Citrulline-D_4	23.5	18.4
Dopamine-D_4	88.7	87.3
I-3-AA-D_4	88.9	91.1
Kynurenic acid-D_5	103.9	103.6
Kynurenine-D_4	79.5	82.2
Neopterin-13C5	95.6	95.3
Nicotinic acid-D_4	84.8	85.8
Nicotinamide riboside-D_3	92.8	40.0
Picolinic acid-D_3	99.6	98.0
Quinolinic acid-D_3	105.2	94.6
Tryptophan-D_5	77.1	78.3
Xanthurenic acid-D_4	112.7	111.7

Supporting Table S11. Mean concentrations, standard deviation and % coefficient of variation values derived from the quantification of analytes from repeat injections of a pool of serum. The study was performed to assess multi-plate analytical performance and a pool of serum was analyzed at frequent intervals during an analytical run that consisted of eighteen plates analyzed across nine days. A total of 92 injections of the serum pool were analyzed across the eighteen plates. Note – the study pool was collected from different donors to the one used in **Table S12** and therefore reports different analyte concentrations.

	QC pool mean concentration	Standard deviation	%CV
3-HAA	*<LLOQ*	*<LLOQ*	*<LLOQ*
3-OH-kynurenine	1.9	0.2	10.2
5-OH-IAA	12.3	0.6	4.9
Beta-nicotinamide mononucleotide	*<LLOQ*	*<LLOQ*	*<LLOQ*
Citrulline	6068.2	247.1	4.1
Dopamine	*<LLOQ*	*<LLOQ*	*<LLOQ*
Indole-3-acetic-acid	357.4	9.9	2.8
Kynurenic acid	12.1	0.4	3.6
Kynurenine	266.1	39.6	14.9
NAD+	*NA (monitored MRM transition only)*		
Neopterin	0.8	0.05	5.9
Nicotinic acid	3.7	0.5	14.5
Nicotinamide riboside	80.5	16.0	20.0
Picolinic Acid	6.7	0.5	8.1
Quinolinic acid	67.3	3.0	4.5
Serotonin	23.4	2.5	10.7
Tryptophan	14828.7	771.9	5.2
Xanthurenic acid	3.4	0.1	4.2

Supporting Table S12. Mean concentration values for repeat biological replicates (n=8). Plasma samples were pooled and analyzed at periodic timepoints across the analytical run. % CV values were <10% for each metabolite. Note – the pool of plasma was collected from different donors to the one used in **Table S11** and therefore reports different analyte concentrations.

	Mean concentration (ng/mL)	Standard deviation	% CV
3-HAA	17.8	1.5	8.5
3-HK	26.8	0.9	3.4
5-HIAA	13.0	0.4	3.1
βNM	27.6	2.2	7.9
Citrulline	5428.5	158.3	2.9
Dopamine	*NA (<LLOQ)*	*NA (<LLOQ)*	*NA (<LLOQ)*
I-3-AA	291.5	8.7	3.0
Kynurenic acid	7.9	0.3	3.9
Kynurenine	121.1	5.9	4.9
NAD+	*NA (monitored MRM transition only)*		
Neopterin	2.6	0.2	8.1
Nicotinic acid	*NA (<LLOQ)*	*NA (<LLOQ)*	*NA (<LLOQ)*
Nicotinamide riboside	801.2 (*>ULOQ)*	71.9 (*>ULOQ)*	9.0 (*>ULOQ)*
Picolinic Acid	5.4	0.3	6.5
Quinolinic acid	69.0	4.1	6.0
Serotonin	43.0	2.5	5.8
Tryptophan	12681.0	1067.4	8.4
Xanthurenic acid	3.5	0.1	3.4