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Supplemental Information: Interrogation of the mammalian gut-brain-axis using LC-MS/MS-based targeted metabolomics using *in vitro* bacterial and organoid cultures and *in vivo* gnotobiotic mouse models

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Supplemental Methods

Modified Growth Medium for Quality Control (QC) Preparations

A bacterial minimal media containing amino acids (30 mg/L Glycine, 84 mg/L L-Arginine, 63 mg/L L-Cystine, 42 mg/L L-Histidine, 105 mg/L L-Isoleucine, 105 mg/L L-Leucine, 146 mg/L L-Lysine, 30 mg/L L-Methionine, 66 mg/L L-Phenylalanine, 42 mg/L L-Serine, 95 mg/L L-Threonine, and 94 mg/L L-Valine), vitamins (4 mg/L Choline chloride, 4 mg/L D-Calcium pantothenate, 4 mg/L Folic Acid, 4 mg/L Niacinamide, 4 mg/L Pyridoxine hydrochloride, 0.4 mg/L Riboflavin, 4 mg/L Thiamine hydrochloride, and 7.2 mg/L i-Inositol), inorganic salts (0.2 g/L Calcium Chloride, 97.67 mg/L Magnesium Sulfate, 0.4 g/L Potassium Chloride, 3.7g/L Sodium Bicarbonate, 6.4 g/L Sodium Chloride, and 125 mg/L Sodium Phosphate monobasic) and 4.55 g/L glucose was used as a surrogate matrix. The minimal medium was used to prepare QC standards for the Tryptophan and Tyrosine Pathways, Glutamate Cycle, and the short-chain fatty acid (SCFA) methods.

QC Preparations for the Tryptophan Pathway, Tyrosine Pathway, and Glutamate Cycle Methods

For the Tryptophan and Glutamate Cycle methods, a Combined Intermediate solution was prepared at a concentration of 100 µg/mL for all metabolites in the minimal medium. The Combined Intermediate solution for the Tyrosine Pathway method was prepared at a concentration of 100 µg/mL for all metabolites in a minimal medium that had been pH adjusted to 5.5 (0.850 mL of 2 M HCl into 40 mL of minimal medium). QC standards were prepared at the appropriate levels for each method using the scheme described in **Table S1** using either minimal medium (Tryptophan Pathway and Glutamate Cycle methods) or minimal medium pH adjusted to 5.5 (Tyrosine Pathway method). All growth medium-based Intermediate solutions and QC standards were prepared and stored in glass vials and were stored at -80°C while not in use. Glass autosampler vials were used for this study.

All QC standards were prepared at 10x the concentration of their intended concentration targets so that they can be used to assess the precision and accuracy of each assay at the intended concentration level after the standard 10x dilution of the growth medium.

Table S1: Quality Control Standard preparation scheme for the Tryptophan, Tyrosine, and Glutamate Cycle Methods

QC Standard	QC Level Targets	Undiluted [metabolite] (ng/mL)	Diluted 10x with ISS-A* [metabolite] (ng/mL)
QC - High	**0.8 * HCS**	**8,000**	**800**
QC - Medium	**QC-High and QC-Low geomean**	**484**	**48.4**
QC - Low	**3 x LCS**	**29.3**	**2.93**
QC - LCS	**LCS**	**9.77**	**0.977**

Abbreviations: QC, Quality Control Standard; ISS-A, internal standard solution A; HCS, high calibration standard (1,000 ng/mL for each metabolite); LCS, lowest calibration standard (0.977 ng/mL for each metabolite).

**The Tryptophan Pathway and Glutamate Cycle methods use ISS-A as the diluent. The Tyrosine Pathway Method uses an ISS-A solution that has been acidified with 1% formic acid.*

QC Preparations for the SCFA Method

For the SCFA methods, a Combined Intermediate solution was prepared at a concentration of 100 µM for the SCFA derivatives in minimal medium. QC standards were prepared at the appropriate levels for the SCFA method using the scheme described in **Table S2** using minimal medium. All growth medium-based Intermediate solutions and QC standards for the SCFA method were prepared and stored in polypropylene vials and were stored at -80°C while not in use. Polypropylene autosampler vials were used for this study.

The SCFA QC standards were prepared at 10x the concentration of their intended concentration targets so that they can be used to assess the precision and accuracy of each assay at the intended concentration level after the standard 10x dilution of the growth medium.

Table S2: Quality Control Standard preparation scheme for the Short-Chain Fatty Acid (SCFA) Method

QC Standard	QC Level Targets	Undiluted [metabolite] (nM)	Diluted 10x with ISS-A [metabolite] (nM)
QC - High	**0.8 * HCS**	**80,000**	**8,000**
QC - Medium	**QC-High and QC-Low geomean**	**4,840**	**484**
QC - Low	**3 x LCS**	**293**	**29.3**
QC - LCS	**LCS**	**97.7**	**9.77**

Abbreviations: QC, Quality Control Standard; ISS-A, internal standard solution A; HCS, high calibration standard (10,000 nM for each metabolite); LCS, lowest calibration standard (9.77 nM for each metabolite).

Limit-of-Detection (LOD) and Limit-of-Quantitation (LOQ) Determinations

A total of three calibration curves were used to perform the LOD and LOQ assessments for the metabolites contained in each method. Calibration curves were constructed for each metabolite by plotting the instrument response (IR = $Area_{analyte}$ / A_{IS}) factor of each Calibrator against their respective nominal concentration. From this plot, a least-squares, linear regression with weighting (1/x) was used to calculate the line of best fit for each metabolite. LOD and LOQ estimates were calculated using the standard deviation of the y-intercepts and the mean slope of the three calibration curves as described previously [1]. The slope and Y-intercept for each calibration curve, and the estimated LODs and LOQs for each metabolite measured in the Glutamate Cycle method (**Table 3**), SCFA method (**Table S5**), Tryptophan (**Table S7**) and Tyrosine (**Table S9**) Pathway Methods are tabulated below.

QC Accuracy (%Bias) and Precision (coefficient of variance; %CV) Assessments and Acceptance Criteria

For each method, analytical accuracy of the method was assessed for 6 individual QC standards assayed at each QC level by computing the Percent Bias (%Bias) of the measured metabolite concentration against the nominal concentrations of the QC preparation at each level. The pooled analytical accuracy of the method was assessed at each QC level by computing the %Bias between the mean of the measured metabolite concentration of the 6 QCs in the pool against the nominal concentrations of the QC preparation at each level. Acceptance criteria for individual and pooled accuracy determinations was specified as ± 20% of the nominal concentration at the QC-LCS level, and ± 15% of the nominal concentration at the QC-Low, QC-Medium, and QC-High levels. For each method, the pooled analytical precision was assessed by computing the Coefficient of Variation (%CV) of the metabolite concentrations for each QC standard at each QC level. Acceptance criteria for pooled precision determination was specified as ± 20% of the nominal concentration at the QC-LCS level, and ± 15% of the nominal concentration at the QC-Low, QC-Medium, and QC-High levels.

The experimental data for the individual and pooled accuracy, and pooled precision assessments described directly above for the Glutamate Cycle Method (**Table S4**), SCFA method (**Table S6**), Tryptophan (**Table S8**) and Tyrosine (**Table S10**) Pathway Methods are tabulated below.

References

1. ICH harmonized tripartite guideline: validation of analytical procedures: text and methodology Q2(R1), International Conference of harmonization of technical requirements for registration of pharmaceuticals for human use (**2005)**.

Glutamate Cycle Method Benchmarking

Table S3: Limit-of-detection (LOD) and limit-of-quantitation (LOQ) assessments for the metabolites measured in the Glutamate Cycle Method.

GABA (linear 1/x model)				Glutamate (linear 1/x model)				Glutamine (linear 1/x model)			
	Slope (m)	Y-Int. (b)	r		Slope (m)	Y-Int. (b)	r		Slope (m)	Y-Int. (b)	r
Curve 1	0.00470	0.00135	0.99954	Curve 1	0.00343	0.00424	0.99995	Curve 1	0.00581	0.00218	0.99987
Curve 2	0.00454	0.00135	0.99988	Curve 2	0.00336	0.00399	0.99995	Curve 2	0.00581	0.00177	0.99976
Curve 3	0.00480	0.00104	0.99997	Curve 3	0.00348	0.00394	0.99998	Curve 3	0.00588	0.00214	0.99994
Mean	0.00468	0.00125	0.99980	Mean	0.00342	0.00406	0.99996	Mean	0.00583	0.00203	0.99986
Std. Dev.	1.31E-04	1.79E-04	2.27E-04	Std. Dev.	6.03E-05	0.00016	1.73E-05	Std. Dev.	4.04E-05	2.26E-04	9.07E-05
LOD	0.126 ng/mL			LOD	0.155 ng/mL			LOD	0.128 ng/mL		
LOQ	0.382 ng/mL			LOQ	0.470 ng/mL			LOQ	0.388 ng/mL		

Abbreviations: Std Dev., standard deviation; LOD, limit-of-detection; LOQ, limit-of-quantitation.

Table S4: Accuracy (%Bias) and Precision (coefficient of variance; %CV) assessments for the Glutamate Cycle Method.

	GABA				Glutamate				Glutamine			
QC	LCS	Low	Medium	High	LCS	Low	Medium	High	LCS	Low	Medium	High
Conc. (ng/mL)	**9.77**	**29.3**	**484**	**8,000**	**9.77**	**29.3**	**484**	**8,000**	**9.77**	**29.3**	**484**	**8,000**
QC 1	8.22	28.97	473	7,673	9.06	27.91	447	7,901	9.02	30.35	474	7,879
QC 2	9.45	26.73	457	7,576	8.47	25.20	470	7,610	9.56	26.44	477	7,581
QC 3	8.81	29.86	537	8,062	9.38	28.52	527	7,529	9.10	30.26	519	7,939
QC 4	9.56	28.78	467	7,713	8.20	28.83	442	7,771	10.30	30.77	476	7,632
QC 5	10.5	30.41	489	7,886	10.52	28.66	470	7,590	11.56	31.45	479	7,780
QC 6	9.85	29.58	485	8,230	8.26	25.04	443	8,184	8.46	27.75	478	8,380
Passing Incidence	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
Mean Conc. (ng/mL)	9.40	29.05	485	7,857	8.98	27.36	467	7,764	9.67	29.50	484	7,865
Std. Dev. (ng/mL)	0.80	1.28	28.22	251.25	0.89	1.76	32.40	246.61	1.12	1.96	17.33	287.65
Accuracy (% Bias)	96.2%	99.2%	100.2%	98.2%	91.9%	93.4%	96.4%	97.1%	98.9%	100.7%	100.0%	98.3%
Precision (% CV)	8.5%	4.4%	5.8%	3.2%	9.9%	6.4%	6.9%	3.2%	11.5%	6.6%	3.6%	3.7%

Note: Bolded and underlined metabolite concentration values fail acceptance criteria.

Abbreviations: QC, quality control standard; LCS, lowest calibration standard; Conc., concentration; Mean Conc., mean concentration; Std Dev., standard deviation.

Derivatized Short-Chain Fatty Acid (SCFA) Method Benchmarking

Table S5: Limit-of-detection (LOD) and limit-of-quantitation (LOQ) assessments for the metabolites measured in the SCFA method.

Acetic Acid (linear 1/x model)			
	Slope (m)	Y-Int. (b)	r
Curve 1	0.00403	0.00556	0.99991
Curve 2	0.00404	0.00459	0.99993
Curve 3	0.00401	0.00549	0.99990
Mean	0.00403	0.00521	0.99991
Std. Dev.	1.53E-05	5.41E-04	1.53E-05
LOD	0.443 nM		
LOQ	1.34 nM		

Propionic Acid (linear 1/x model)			
	Slope (m)	Y-Int. (b)	r
Curve 1	0.00413	0.00436	0.99997
Curve 2	0.00415	0.00442	0.99996
Curve 3	0.00413	0.00396	0.99997
Mean	0.00414	0.00425	0.99997
Std. Dev.	1.15E-05	2.50E-04	5.77E-06
LOD	0.199 nM		
LOQ	0.605 nM		

Isobutyric Acid (linear 1/x model)			
	Slope (m)	Y-Int. (b)	r
Curve 1	0.00402	0.00409	0.99996
Curve 2	0.00406	0.00383	0.99997
Curve 3	0.00409	0.00337	0.99991
Mean	0.00406	0.00376	0.99995
Std. Dev.	3.51E-05	0.00036	3.21E-05
LOD	0.297 nM		
LOQ	0.899 nM		

Butyric Acid (linear 1/x model)[A]			
	Slope (m)	Y-Int. (b)	r
Curve 1	0.00425	0.00324	0.99977
Curve 2	0.00431	0.00266	0.99997
Curve 3	0.00441	5.64E-04	0.99964
Mean	0.00432	0.00215	0.99979
Std. Dev.	8.08E-05	1.41E-03	1.66E-04
LOD	1.07 nM		
LOQ	3.26 nM		

2-Methylbutyric Acid (linear 1/x model)[B]			
	Slope (m)	Y-Int. (b)	r
Curve 1	0.00396	0.00257	0.99986
Curve 2	0.00400	0.00253	0.99939
Curve 3	0.00383	0.00460	0.99985
Mean	0.0039	0.00323	0.99970
Std. Dev.	8.89E-05	1.18E-03	2.69E-04
LOD	0.994 nM		
LOQ	3.01 nM		

Isovaleric Acid (linear 1/x model)			
	Slope (m)	Y-Int. (b)	r
Curve 1	0.00441	-0.00044	0.99521
Curve 2	0.00421	0.00148	0.99847
Curve 3	0.00452	-0.00233	0.99896
Mean	0.00438	-0.00043	0.99755
Std. Dev.	1.57E-04	1.91E-03	2.04E-03
LOD	1.46 nM		
LOQ	4.35 nM		

Valeric Acid (linear 1/x model)			
	Slope (m)	Y-Int. (b)	r
Curve 1	0.00496	-0.00607	0.99519
Curve 2	0.00546	-0.01603	0.98801

Curve 3	0.00490	-0.00597	0.99668
Mean	0.00511	-0.00936	0.99329
Std. Dev.	3.07E-04	5.78E-03	0.00464
LOD	3.74 nM		
LOQ	11.3 nM		

Abbreviations: Std Dev., standard deviation; LOD, limit-of-detection; LOQ, limit-of-quantitation.

Table S6: Accuracy (%Bias) and Precision (%CV) assessments for the SCFA Method.

	Acetic Acid				Propionic Acid				Isobutyric Acid			
QC	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**
Conc. (nM)	**97.7**	**293**	**4,840**	**80,000**	**97.7**	**293**	**4,840**	**80,000**	**97.7**	**293**	**4,840**	**80,000**
QC 1	83.8	278	4,510	74,297	84.4	269	4,630	74,633	89.3	287	4,702	74,878
QC 2	85.5	276	4,517	75,189	84.2	278	4,680	76,646	87.2	290	4,755	77,668
QC 3	85.1	278	4,612	76,191	85.9	285	4,664	77,091	87.9	290	4,861	77,581
QC 4	91.0	281	4,626	73,855	87.9	273	4,449	75,301	87.5	284	4,701	74,369
QC 5	82.1	280	4,610	77,025	82.9	282	4,532	76,773	86.4	291	4,513	77,026
QC 6	81.5	268	4,616	76,041	84.8	266	4,565	75,358	91.0	276	4,607	77,400
Pass Incidence	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
Mean Conc. (nM)	84.8	277	4,582	75,433	85.0	275	4,587	75,967	88.2	286	4,690	76,487
Std. Dev. (nM)	3.41	4.53	53.39	1209.99	1.70	7.34	88.29	996.78	1.68	5.55	120.16	1469.02
Accuracy (% Bias)	86.8%	94.4%	94.7%	94.3%	87.0%	94.0%	94.8%	95.0%	90.3%	97.7%	96.9%	95.6%
Precision (% CV)	4.0%	1.6%	1.2%	1.6%	2.0%	2.7%	1.9%	1.3%	1.9%	1.9%	2.6%	1.9%
	Butyric Acid				**2-Methylbutyric Acid**				**Isovaleric Acid**			
QC	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**
Conc. (nM)	**97.7**	**293**	**4,840**	**80,000**	**97.7**	**293**	**4,840**	**80,000**	**97.7**	**293**	**4,840**	**80,000**
QC 1	89.2	299	4,762	78,160	90.0	273	4,514	75,477	97.6	300	**3,614**	**57,670**
QC 2	83.7	282	4,722	80,191	92.8	280	4,810	75,334	85.9	265	4,185	71,590
QC 3	88.9	288	4,735	78,753	88.8	268	4,475	78,936	90.5	269	**3,952**	**66,519**
QC 4	93.6	290	4,572	77,983	101.0	283	4,460	78,304	91.2	274	4,671	78,447

QC 5	85.8	288	4,599	78,768	86.1	295	4,518	84,732	88.8	251	4,224	75,044
QC 6	88.1	282	4,526	80,526	93.9	281	4,597	70,209	78.4	276	4,395	79,111
Pass Incidence	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	4/6	4/6
Mean Conc. (nM)	88.2	288	4,653	79,064	92.1	280	4,562	77,165	88.7	273	4,174	71,397
Std. Dev. (nM)	3.36	6.36	98.99	1056.24	5.18	9.36	130.14	4822.50	6.38	16.09	363.51	8182.40
Accuracy (% Bias)	90.3%	98.3%	96.1%	98.8%	94.3%	95.6%	94.3%	96.5%	90.8%	93.1%	86.2%	89.2%
Precision (% CV)	3.8%	2.2%	2.1%	1.3%	5.6%	3.3%	2.9%	6.2%	7.2%	5.9%	8.7%	11.5%

	Valeric Acid			
QC	**LCS**	**Low**	**Medium**	**High**
Conc. (nM)	**97.7**	**293**	**4,840**	**80,000**
QC 1	87.6	265	4,388	79,829
QC 2	94.2	258	4,160	68,849
QC 3	94.4	279	**4,033**	75,940
QC 4	103.9	274	4,386	76,885
QC 5	88.0	**221**	**3,942**	69,157
QC 6	96.6	299	4,704	71,216
Pass Incidence	6/6	5/6	4/6	6/6
Mean Conc. (nM)	94.1	266	4,269	73,646
Std. Dev. (nM)	6.05	26.34	279.74	4539.76
Accuracy (% Bias)	96.4%	90.8%	88.2%	92.1%
Precision (% CV)	6.4%	9.9%	6.6%	6.2%

Note: Bolded and underlined metabolite concentration values fail acceptance criteria.
Abbreviations: QC, quality control standard; LCS, lowest calibration standard; Conc., concentration; Mean Conc., mean concentration; Std Dev., standard deviation.

Tryptophan Pathway Method Benchmarking

Table S7: Limit-of-detection (LOD) and limit-of-quantitation (LOQ) assessments for the metabolites measured in the Tryptophan Method

Tryptophan (linear 1/x model)				Serotonin (linear 1/x model)				Melatonin (linear 1/x model)			
	Slope (m)	Y-Int. (b)	r		Slope (m)	Y-Int. (b)	r		Slope (m)	Y-Int. (b)	r
Curve 1	0.00387	0.00429	0.99998	Curve 1	0.00331	5.13E-04	0.99992	Curve 1	0.00360	1.92E-04	0.99997
Curve 2	0.00374	0.00360	0.99990	Curve 2	0.00315	6.45E-04	0.99956	Curve 2	0.00340	2.46E-04	0.99992
Curve 3	0.00378	0.00369	0.99990	Curve 3	0.00250	5.89E-04	0.99921	Curve 3	0.00397	1.07E-04	0.99950
Mean	0.00380	0.00386	0.99993	Mean	0.00299	5.82E-04	0.99956	Mean	0.00366	1.82E-04	0.99980
Std. Dev.	6.66E-05	3.75E-04	4.62E-05	Std. Dev.	4.29E-04	6.63E-05	3.55E-04	Std. Dev.	2.89E-04	6.99E-05	2.58E-04
LOD	0.326 ng/mL			LOD	0.073 ng/mL			LOD	0.063 ng/mL		
LOQ	0.988 ng/mL			LOQ	0.222 ng/mL			LOQ	0.191 ng/mL		
5-HIAA (linear 1/x model)				5-Hydroxytryptophan[A] (linear 1/x model)				N-Acetylserotonin[B] (linear 1/x model)			
	Slope (m)	Y-Int. (b)	r		Slope (m)	Y-Int. (b)	r		Slope (m)	Y-Int. (b)	r
Curve 1	0.00270	0.00132	0.99946	Curve 1	8.82E-04	4.10E-05	0.99912	Curve 1	0.00547	0.00140	0.99990
Curve 2	0.00264	9.89E-04	0.99986	Curve 2	7.07E-04	-4.23E-05	0.99943	Curve 2	0.00427	2.56E-04	0.99986
Curve 3	0.00288	3.79E-04	0.99957	Curve 3	7.15E-04	-4.36E-05	0.99957	Curve 3	0.00379	1.96E-04	0.99956
Mean	0.00274	8.96E-04	0.99963	Mean	7.68E-04	-1.50E-05	0.99937	Mean	0.00451	6.17E-04	0.999773
Std. Dev.	1.25E-04	4.77E-04	2.07E-04	Std. Dev.	9.8797E-05	4.85E-05	2.30E-04	Std. Dev.	8.65E-04	6.78E-04	1.86E-04
LOD	0.575 ng/mL			LOD	0.208 ng/mL			LOD	0.496 ng/mL		
LOQ	1.74 ng/mL			LOQ	0.631 ng/mL			LOQ	1.50 ng/mL		

Notes: [A]D5-Tryptophan was used as a surrogate IS for 5-hydroxytryptophan; [B]D4-Serotonin was used as a surrogate IS for N-acetylserotonin.

Abbreviations: Std. Dev., standard deviation; LOD, limit-of-detection; LOQ, limit-of-quantitation.

Table S8: Accuracy (%Bias) and Precision (%CV) and assessments for the Tryptophan Method.

	Tryptophan				Serotonin				Melatonin			
QC	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**
Conc. (ng/mL)	**9.77**	**29.3**	**484**	**8,000**	**9.77**	**29.3**	**484**	**8,000**	**9.77**	**29.3**	**484**	**8,000**
QC 1	9.62	31.89	485	8,075	9.01	25.19	441	7,334	10.12	30.96	512	8,128
QC 2	9.53	28.81	484	7,741	8.21	27.62	449	7,094	10.25	29.92	490	7,849
QC 3	9.22	31.03	498	8,550	8.49	27.00	458	7,474	9.42	30.62	525	8,109
QC 4	8.82	29.00	489	7,739	**7.66**	26.20	442	7,234	9.76	29.54	499	7,733
QC 5[C]	**18.3**	29.66	510	8,144	**13.97**	25.48	449	7,273	**13.90**	28.53	505	7,884
QC 6	9.32	27.54	486	7,859	8.12	27.67	431	7,411	9.83	29.66	477	8,000
Passing Incidence	5/6	6/6	6/6	6/6	4/6	6/6	6/6	6/6	5/6	6/6	6/6	6/6
Mean Conc. (ng/mL)	9.30	29.65	492	8,018	8.30	26.53	445	7,304	9.88	29.87	501	7,951
Std. Dev. (ng/mL)	0.31	1.58	10.17	310.50	0.50	1.07	9.36	135.21	0.33	0.86	16.56	155.80
Accuracy (% Bias)	95.2%	101.2%	101.7%	100.2%	84.9%	90.5%	92.0%	91.3%	101.1%	102.0%	103.6%	99.4%
Precision (% CV)	3.4%	5.3%	2.1%	3.9%	6.0%	4.0%	2.1%	1.9%	3.3%	2.9%	3.3%	2.0%
	5-HIAA				**5-Hydroxytrytophan[A]**				**N-acetylserotonin[B]**			
QC	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**
Conc. (ng/mL)	**9.77**	**29.3**	**484**	**8,000**	**9.77**	**29.3**	**484**	**8,000**	**9.77**	**29.3**	**484**	**8,000**
QC 1	11.38	27.57	539	8,650	10.32	29.42	435	8,107	9.78	31.80	508	8,231
QC 2	10.36	29.24	544	8,404	9.10	28.03	438	8,051	9.55	30.19	555	8,351
QC 3	8.48	29.42	513	8,695	10.65	29.57	434	8,439	9.02	28.84	529	8,305
QC 4	9.49	28.45	509	8,322	9.09	28.66	448	7,814	9.11	30.35	521	8,097
QC 5[C]	11.57	31.04	536	8,950	**13.54**	29.21	458	8,172	**13.83**	29.45	**569**	8,248
QC 6	**6.28**	29.99	509	8,272	9.96	28.48	441	8,401	10.33	31.62	539	7,975
Passing Incidence	5/6	6/6	6/6	6/6	5/6	6/6	6/6	6/6	5/6	6/6	5/6	6/6
Mean Conc. (ng/mL)	9.20	29.28	525	8,549	9.82	28.90	442	8,164	9.56	30.38	537	8,201
Std. Dev. (ng/mL)	1.95	1.20	16.20	261.12	0.71	0.60	9.07	232.45	0.53	1.17	22.56	140.10
Accuracy (% Bias)	94.1%	99.9%	108.4%	106.9%	100.6%	98.6%	91.4%	102.1%	97.8%	103.7%	111.0%	102.5%
Precision (% CV)	21.2%	4.1%	3.1%	3.1%	7.2%	2.1%	2.1%	2.8%	5.6%	3.8%	4.2%	1.7%

Notes: [A]D5-Tryptophan was used as a surrogate IS for 5-Hydroxytryptophan; [B]D4-Serotonin was used as a surrogate IS for N-acetylserotonin; [C]QC data for the fifth QC-LCS sample was rejected from the QC pooled statistics because of a noted sample preparation error during batch preparation; Bolded and underlined metabolite concentration values fail acceptance criteria.

Abbreviations: QC, quality control standard; LCS, lowest calibration standard; Conc., concentration; Mean Conc., mean concentration; Std Dev., standard deviation.

Tyrosine Method Benchmarking

Table S9: Limit-of-detection (LOD) and limit-of-quantitation (LOQ) assessments for the metabolites measured in the Tyrosine Method.

Tyramine (linear 1/x model)				Dopamine (linear 1/x model)				L-DOPA (linear 1/x model)			
	Slope (m)	Y-Int. (b)	r		Slope (m)	Y-Int. (b)	r		Slope (m)	Y-Int. (b)	r
Curve 1	0.00488	1.33E-04	0.99966	Curve 1	0.00207	0.00059	0.99976	Curve 1	0.00889	0.00652	0.99984
Curve 2	0.00515	1.31E-04	0.99987	Curve 2	0.00197	6.32E-04	0.99986	Curve 2	0.00911	0.00264	0.99992
Curve 3	0.00482	6.95E-04	0.99965	Curve 3	0.00202	5.11E-04	0.99968	Curve 3	0.00948	0.00280	0.99993
Mean	0.00495	3.20E-04	0.99973	Mean	0.00202	5.77E-04	0.99977	Mean	0.00916	0.00399	0.99990
Std. Dev.	1.76E-04	3.25E-04	1.24E-04	Std. Dev.	5.00E-05	6.15E-05	9.02E-05	Std. Dev.	2.98E-04	0.00220	4.93E-05
LOD	0.217 ng/mL			LOD	0.101 ng/mL			LOD	0.791 ng/mL		
LOQ	0.656 ng/mL			LOQ	0.305 ng/mL			LOQ	2.40 ng/mL		
Tyrosine (linear 1/x model)[A]				Norepinephrine (linear 1/x model)[B]				Epinephrine (linear 1/x model)			
	Slope (m)	Y-Int. (b)	r		Slope (m)	Y-Int. (b)	r		Slope (m)	Y-Int. (b)	r
Curve 1	0.00136	0.00334	0.99813	Curve 1	0.0141	0.00255	0.99960	Curve 1	0.00861	0.00307	0.99932
Curve 2	0.00146	0.00305	0.99837	Curve 2	0.0141	0.00189	0.99976	Curve 2	0.00874	0.00213	0.99914
Curve 3	0.00137	0.00301	0.99950	Curve 3	0.0145	0.00157	0.99985	Curve 3	0.00885	0.00219	0.99974
Mean	0.00140	0.00313	0.99867	Mean	0.0143	0.00200	0.99974	Mean	0.00873	0.00246	0.99940
Std. Dev.	5.51E-05	1.80E-04	7.32E-04	Std. Dev.	2.34E-04	5.00E-04	1.27E-04	Std. Dev.	1.20E-04	5.26E-04	3.08E-04
LOD	0.426 ng/mL			LOD	0.116 ng/mL			LOD	0.199 ng/mL		

LOQ	1.29 ng/mL			**LOQ**	0.350 ng/mL			**LOQ**	0.603 ng/mL		
Anthranilic Acid (linear 1/x model)[C]											
	Slope (m)	**Y-Int. (b)**	**r**								
Curve 1	0.00420	0.00781	0.99771								
Curve 2	0.00488	0.00812	0.99540								
Curve 3	0.00472	0.00775	0.99907								
Mean	0.00460	0.00789	0.99739								
Std. Dev.	3.56E-04	1.99E-04	0.00186								
LOD	0.142 ng/mL										
LOQ	0.432 ng/mL										

Notes: [A]D4-Tyramine was used as a surrogate IS for Tyrosine; [B]D3-L-DOPA was used as a surrogate IS for Norepinephrine; [C]D4-Tyramine was used as a surrogate IS.

Abbreviations: Std. Dev., standard deviation; LOD, limit-of-detection; LOQ, limit-of-quantitation.

Table S10: Accuracy (%Bias) and Precision (%CV) and assessments for the Tyrosine Method.

	Tyramine				**Dopamine**				**L-DOPA**			
QC	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**
Conc. (ng/mL)	**9.77**	**29.3**	**484**	**8,000**	**9.77**	**29.3**	**484**	**8,000**	**9.77**	**29.3**	**484**	**8,000**
QC 1	8.94	31.80	538	8,467	7.87	27.52	494	7,511	7.97	25.81	523	8,298
QC 2	9.45	31.44	488	8,154	9.15	29.49	478	7,671	8.43	26.73	493	8,704
QC 3	9.04	30.78	481	8,208	9.33	28.12	485	7,812	7.90	28.44	494	8,253
QC 4	9.60	31.91	531	8,223	8.82	26.92	479	7,742	**6.84**	26.69	499	8,010
QC 5	8.62	31.60	509	8,097	8.42	30.13	473	7,917	7.88	25.37	491	8,096
QC 6	8.67	30.93	523	8,714	8.49	29.03	508	7,677	**6.14**	25.56	514	8,553
Pass Incidence	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	4/6	6/6	6/6	6/6
Mean Conc. (ng/mL)	9.05	31.41	512	8,311	8.68	28.53	486	7,722	7.53	26.43	502	8,319
Std. Dev. (ng/mL)	0.40	0.46	23.19	234.82	0.53	1.23	12.70	138.44	0.86	1.14	13.15	265.50

Accuracy (% Bias)	92.7%	107.2%	105.7%	103.9%	88.8%	97.4%	100.4%	96.5%	77.0%	90.2%	103.8%	104.0%
Precision (% CV)	4.4%	1.5%	4.5%	2.8%	6.2%	4.3%	2.6%	1.8%	11.4%	4.3%	2.6%	3.2%
	Tyrosine[A]				**Norepinephrine[B]**				**Epinephrine**			
QC	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**	**LCS**	**Low**	**Medium**	**High**
Conc. (ng/mL)	**9.77**	**29.3**	**484**	**8,000**	**9.77**	**29.3**	**484**	**8,000**	**9.77**	**29.3**	**484**	**8,000**
QC 1	8.50	26.89	439	8,271	8.71	29.03	500	8,208	8.91	28.85	502	8,327
QC 2	8.25	25.37	430	8,520	9.75	31.98	508	8,320	8.83	28.92	507	8,436
QC 3	8.07	28.35	427	8,034	8.96	30.36	510	8,443	8.13	31.90	523	8,190
QC 4	11.29	30.69	427	8,587	9.70	30.87	532	8,251	8.86	30.28	529	8,134
QC 5	8.06	26.83	444	8,360	8.62	29.17	527	8,395	7.83	31.84	529	8,256
QC 6	8.24	27.71	423	8,691	9.81	29.81	536	8,490	7.90	31.49	530	8,423
Pass Incidence	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
Mean Conc. (ng/mL)	8.73	27.64	432	8,411	9.26	30.20	519	8,351	8.41	30.55	520	8,294
Std. Dev. (ng/mL)	1.26	1.80	8.04	238.96	0.55	1.12	14.54	110.76	0.51	1.41	12.34	123.08
Accuracy (% Bias)	89.4%	94.3%	89.2%	105.1%	94.8%	103.1%	107.2%	104.4%	86.1%	104.3%	107.4%	103.7%
Precision (% CV)	14.4%	6.5%	1.9%	2.8%	6.0%	3.7%	2.8%	1.3%	6.1%	4.6%	2.4%	1.5%
	Anthranilic Acid[C]											
QC	**LCS**	**Low**	**Medium**	**High**								
Conc. (ng/mL)	**9.77**	**29.3**	**484**	**8,000**								
QC 1	8.31	27.98	**563**	**9,792**								
QC 2	9.08	29.67	541	**10,042**								
QC 3	9.93	31.70	527	**10,057**								
QC 4	9.92	31.70	550	**10,495**								
QC 5	9.98	30.05	**566**	**10,006**								
QC 6	10.10	33.00	534	**10,164**								
Pass Incidence	6/6	6/6	6/6	**0/6[D]**								
Mean Conc. (ng/mL)	9.55	30.69	547	**10,093**								
Std. Dev. (ng/mL)	0.71	1.80	15.69	231.80								
Accuracy (% Bias)	97.8%	104.7%	113.0%	**126.2%[D]**								
Precision (% CV)	7.5%	5.9%	2.9%	2.3%								

Notes: [A]D4-Tyramine was used as a surrogate IS for Tyrosine; [B]D3-L-DOPA was used as a surrogate IS for Norepinephrine; [C]D4-Tyramine was used as a surrogate IS; [D]Ion suppression was evident for the D4-Tyramine IS response in the QC-High Standards – this suppression causes a slight elevation in the measured concentrations of anthranilic acid measured in the QC-High standards; Bolded and underlined metabolite concentration values fail acceptance criteria.

Abbreviations: QC, quality control standard; LCS, lowest calibration standard; Conc., concentration; Mean Conc., mean concentration; Std Dev., standard deviation.