

A novel UHPLC-MS method targeting urinary metabolomic markers for Autism Spectrum disorder

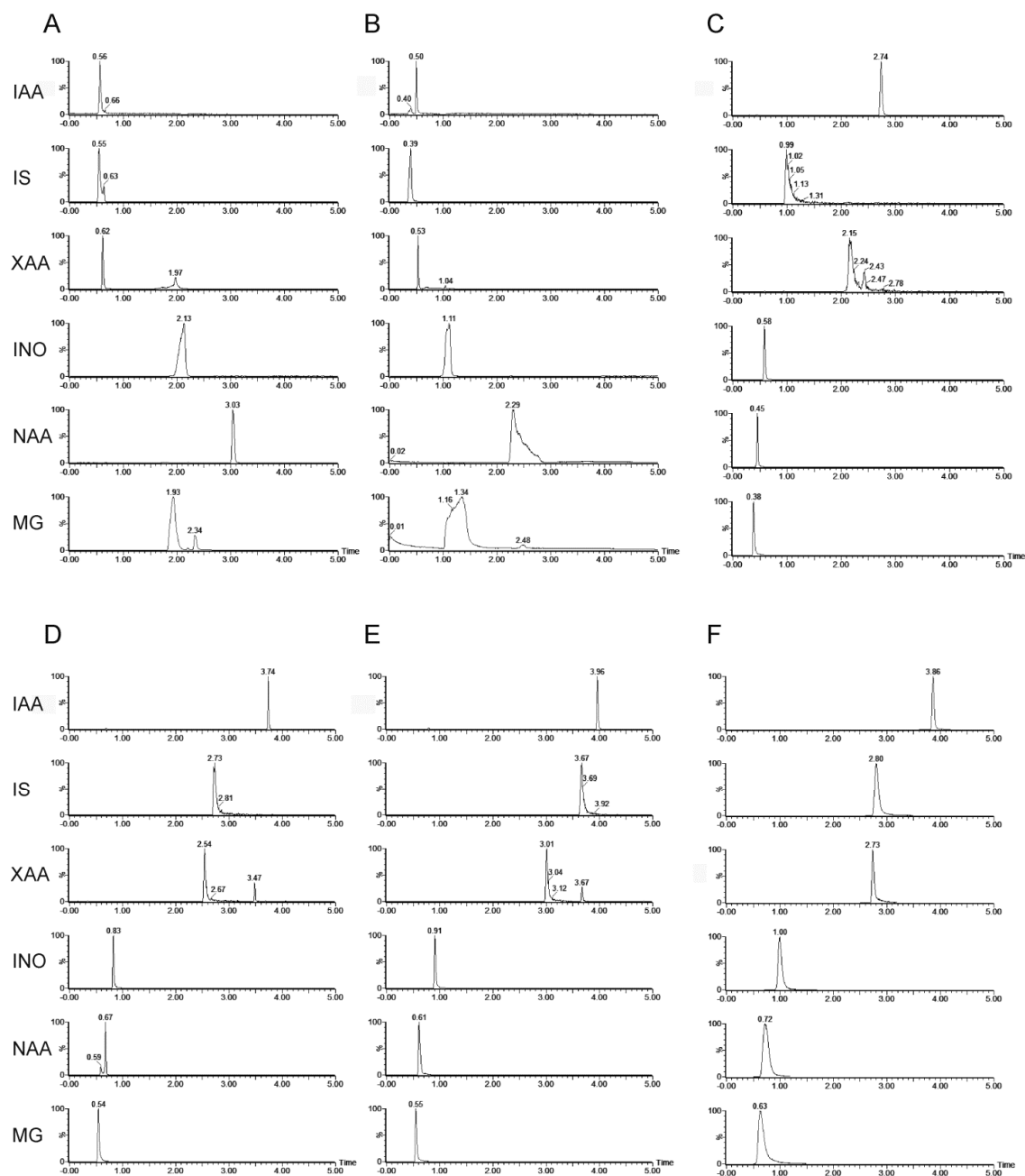


Figure S1 - Representative MRM ion-chromatograms of different columns – **HILIC** (amide – A, diol - B), **RP** (CSH18 – C, Cortecs T3 – D, Shield RP18 – E, Luna Polar C18 - F)

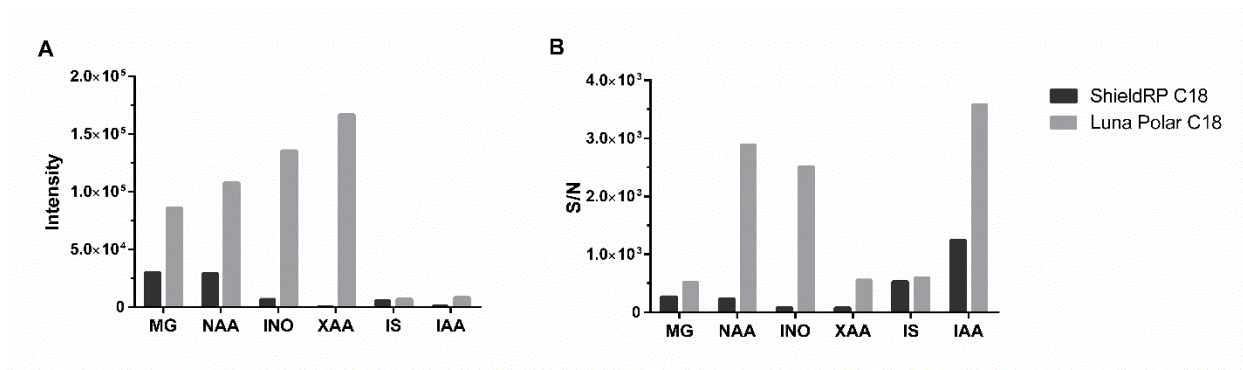


Figure S2 - Comparison of intensities (A) and signal to noise ratios (B) of two C18 columns tested under reverse phase mode

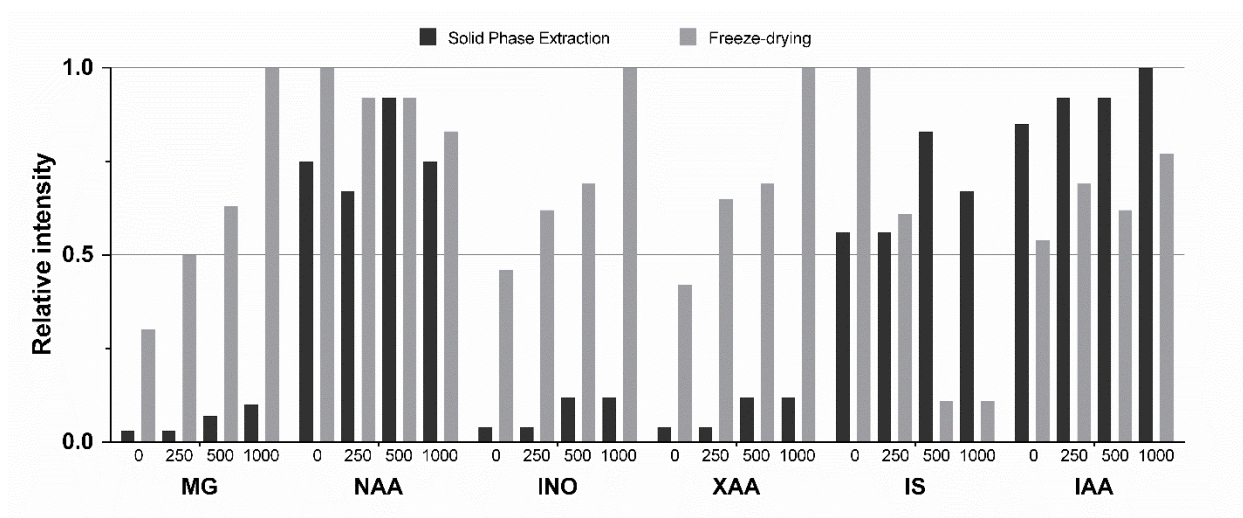


Figure S3 - Comparison of relative intensities of compounds in spiked urine samples after Solid phase extraction (black) and freeze-drying (grey)

Table S1 – Recovery after different sample preparation procedures

Compound	Recovery (%)		
	OASIS MCX	OASIS HLB	Dilution 1:2
Methylguanidine	45.7	69.6	91.1
Indole-3-acetic acid	80.9	102.3	109.2
N-acetylarginine	43.5	96.3	104.0
Indoxyl sulphate	7.9	81.8	108.7
Xanthurenic Acid	99.5	110.2	96.5
Inosine	109.3	99.3	101.8

Table S2 – Changes of the signal intensity after 1:1 and 1:2 sample dilution

Compound	Intensity (AUC)		$\Delta\%$
	1:2	1:1	
Methylguanidine	2 302.5	2 303.1	0.03
N-acetylarginine	136 173.0	158 869.2	16.67
Inosine	448.3	428.6	-4.38
Xanthurenic Acid	901.9	1 088.1	20.65
Indoxyl sulphate	32 885.4	39 852.8	21.19
Indole-3-acetic acid	3 986.7	4 526.9	13.55

Table S1 - Intra-day and inter-day accuracy and precision statistics from analysis of QC samples.

Analyte	Spiked conc. ^a	Intra-day (n= 5)			Inter-day (n= 12)		
		Mean conc. ^a	RSD (%)	Accuracy (%)	Mean conc. ^a	RSD (%)	Accuracy (%)
Methylguanidine	5000	3614.0	0.3	72.3	3807.0	13.3	76.1
	1000	697.4	0.9	69.7	702.5	10.7	70.2
	100	60.8	1.1	60.8	61.1	12.4	61.1
N-acetylarginine	5000	5080.7	1.7	101.6	5251.3	2.2	105.0
	1000	1000.8	0.6	100.1	1004.6	4.3	100.5
	100	99.0	5.9	99.0	95.9	8.0	95.9
Inosine	1500	1491.2	2.8	99.4	1506.9	3.0	100.5
	300	307.2	2.4	102.4	304.0	5.4	101.3
	30	31.9	10.1	106.3	28.9	14.8	96.4
Xanthurenic acid	1500	1534.9	1.2	102.3	1489.0	11.5	99.3
	300	302.6	3.6	100.9	282.4	10.0	94.1
	30	29.6	3.6	98.6	27.4	11.6	91.4
Indoxyl sulphate	5000	4952.8	0.8	99.1	4700.61	6.1	94.0
	1000	978.3	2.8	97.8	979.91	5.1	98.0
	100	91.8	9.3	91.8	90.29	13.2	90.3
Indole-3-acetic acid	2500	2471.4	0.7	98.9	2557.7	4.5	102.3
	500	466.2	1.4	93.2	488.1	5.9	97.6
	50	46.3	6.3	92.6	44.2	11.9	88.5

^a concentration in ng/ml

Table S4 - Matrix effects

Analyte	Matrix effect (%)
Methylguanidine	74
N-acetylarginine	68
Inosine	89
Xanthurenic acid	38
Indoxyl sulphate	17
Indole-3-acetic acid	57

Table S5 - The autosampler stability

Compound		Mean concentration ^a	Mean concentration ^a after 24h	Change %	Mean concentration ^a after three freeze/thaw cycles	Change %
Methylguanidine	QC 0	92.4	95.1	2.92	89.85	-2.71
	QC L	158.7	158.5	-0.13	161.55	1.83
	QC M	894.4	889.7	-0.53	887.6	-0.75
	QC H	4151.3	4128.2	-0.56	4215.9	1.56
N-acetylarginine	QC 0	1426.6	1436.2	0.68	1427.7	0.08
	QC L	1533.1	1530.2	-0.19	1530.7	-0.16
	QC M	2397.4	2506.4	4.55	2476.0	3.28
	QC H	6734.5	6762.8	0.42	6674.1	-0.90
Inosine	QC 0	272.4	239.6	-12.02	277.9	2.02
	QC L	293.4	271.1	-7.62	303.5	3.44
	QC M	593.7	578.3	-2.60	605.6	2.00
	QC H	1779.0	1776.7	-0.13	1784.7	0.32
Xanthurenic acid	QC 0	70.5	72.0	2.20	84.4	19.80
	QC L	94.0	95.2	1.28	111.2	18.36
	QC M	361.4	367.5	1.69	383.0	5.98
	QC H	1710.3	1743.8	1.96	1872.7	9.50
Indoxyl sulphate	QC 0	4082.5	4076.3	-0.15	4903.1	20.10
	QC L	4176.3	4145.2	-0.74	4989.2	19.47
	QC M	5054.8	5126.0	1.41	5918.2	17.08
	QC H	8301.6	8271.3	-0.36	9852.7	18.68
Indole-3-acetic acid	QC 0	185.2	171.7	-7.29	172.7	-6.78
	QC L	221.1	216.7	-1.99	211.0	-4.57
	QC M	700.5	691.8	-1.23	700.3	-0.02
	QC H	2833.6	2892.7	2.09	2905.8	2.55

^a concentration in ng/ml

Table S6 - Statistical evaluation of the influence of dietary restrictions (0-no restrictions, 1-restrictions) and gastrointestinal problems (0-no GIT problems, 1-GIT problems) on metabolite levels.

Dietary restrictions	0			1			p-value
	Mean ^a	SD	Median ^a	Mean ^a	SD	Median ^a	
Methylguanidine	0.55	0.27	0.45	0.51	0.21	0.54	0.615
N-acetyl arginine	5.96	2.43	5.73	7.57	3.66	6.85	0.181
Indole-3-acetic acid	0.99	0.50	0.86	1.35	1.03	1.18	0.260
Indoxyl sulphate	35.88	16.33	30.73	41.13	19.30	32.19	0.417
Xanthurenic acid	0.26	0.14	0.24	0.29	0.12	0.23	0.528
Inosine	0.32	0.15	0.27	0.29	0.11	0.29	0.490
^a in $\mu\text{mol}/\text{mmol Cr}$							
GI problems	0			1			p-val
	Mean ^a	SD	Median ^a	Mean ^a	SD	Median ^a	
Methylguanidine	0.47	0.21	0.42	0.57	0.27	0.58	0.183
N-acetyl arginine	6.46	3.40	6.06	6.44	2.56	6.08	0.984
Indole-3-acetic acid	1.16	0.88	0.76	1.01	0.47	0.97	0.532
Indoxyl sulphate	32.47	14.60	27.38	40.99	18.51	35.57	0.105
Xanthurenic acid	0.29	0.13	0.26	0.25	0.13	0.23	0.381
Inosine	0.28	0.13	0.23	0.34	0.14	0.31	0.156
^a in $\mu\text{mol}/\text{mmol Cr}$							