Supplementary Data

Development of nanoelectrospray high resolution isotope dilution mass spectrometry for targeted quantitative analysis of urinary metabolites: Application to population profiling and clinical studies

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Supplementary Table S1. Pregnancy outcomes in each of three consecutive cycles. For each outcome three samples have been collected on the days 18, 22, and 26.

Patient ID	Cycle 1	Cycle 2	Cycle 3
39C	NP	P	
22C	P		
21C	NP	NP	
5C	NP	P	
19C	P		
43C	NP	P	
8C	NP	NP	P
53C	P		
54C	NP	NP	P
1C	NP	NP	P
42C	P		
13C	P		
18C	P		
40C	P		
32C			EPL
59C		EPL	EPL
16C	EPL	EPL	
61C	EPL	EPL	EPL
57C	EPL	EPL	

Supplementary Table S2. Calibration curves **ax+b=y** (average of 3 replicates), linear range and LOQ of the metabolites obtained in water.

Metabolite	a	b	R^2	Linear range (μg/mL) [*]	LOQ μg/mL [**] in a well (*μmol/mmol creatinine*)	Literature Value[***] (*μmol/mmol creatinine*)
Succinic acid	0.0612	0.0050	0.998	0 – 0.833	0.18 (*5.87*)	*0.3-33.3*
D,L-Leucine	0.1810	-0.0091	0.997	0.083-0.833	0.19 (*8.57*)	*1.2-19.07*
Glutaric acid	0.0769	0.0037	0.999	0.008-0.833	0.16 (*5.55*)	*0.7-3.6*
Adipic acid	1.1802	0.0297	0.999	0-0.167	0.08 (*3.24*)	*1.6-14.3*
D,L-Phenylalanine	0.0703	0.0073	0.999	0.083-0.833	0.24 (*8.97*)	*3.5-11.2*
Palmitic acid	0.0897	0.0657	0.993	0.008-0.833	0.38 (*8.15*)	*2.6-24.3*
Nicotinamide	0.0704	-0.0005	0.995	0-0.167	0.09 (*4.62*)	*<1*
Acetylcarnitine	0.0588	-0.0007	0.997	0-0.167	0.12 (*3.50*)	*0.4-5.9*

[*] expressed as the concentration of a calibrator in a well

[**] LOQ is presented as concentration in a well. In parenthesis: estimated concentration in *μmol/mmol creatinine* recalculated using an average creatinine concentration and taking into account the urine dilution in a well

Supplementary Table S3. Assay of accuracy and precision of the QC samples (replicates of calibrations) performed in the pooled urine sample.

Metabolite	Accuracy			Precision		
	Target Concentration, µg/mL	Measured Concentration (average of n=5), µg/mL	Accuracy % (n=5)	Concentration, µg/mL	Intra-day (n=5) RSD %	Inter-day (n=9) RSD %
Succinic acid						
	16.67	17.96	107.8	**16.67**	9.5	12.3
	1.67	2.32	133.9	**1.67**	6.0	21.3
	0.83	1.02	122.4	**0.83**	19.6	37.5
D,L-Leucine						
	16.67	15.589	93.5	**16.67**	7.6	8.3
	1.67	1.9	114.0	**1.67**	6.7	13.8
	0.83	0.747	89.6	**0.83**	12.4	11.1
Glutaric acid						
	16.67	17.411	104.5	**16.67**	5.0	5.4
	1.67	1.867	112.0	**1.67**	8.3	11.9
	0.83	0.802	96.3	**0.83**	14.6	27.8
Adipic acid	**16.67**	16.033	96.2	**16.67**	7.1	8.6
	1.67	1.613	96.8	**1.67**	6.7	8.4
	0.83	0.681	81.7	**0.83**	9.2	10.3
	0.17	0.191	114.7	**0.17**	4.9	8.2
D,L-Phenylalanine						
	1.67	1.684	101.0	**1.67**	5.2	4.8
	0.83	0.773	92.8	**0.83**	9.0	15.6
	0.17	0.228	136.7	**0.17**	22.1	22.8

Palmitic acid	**1.67**	2.244	134.6	**1.67**	15.6	18.6
	0.83	0.866	103.9	**0.83**	11.9	16.6
	0.17	0.175	104.9	**0.17**	8.6	10.9
Nicotinamide	**16.67**	16.178	97.1	**16.67**	7.7	15.7
	1.67	1.934	116.0	**1.67**	7.8	7.3
	0.83	0.783	94.0	**0.83**	10.1	10.8
	0.17	0.174	104.4	**0.17**	10.4	11.0
	0.08	0.086	103.6	**0.08**	11.0	21.6
Acetylcarnitine						
	1.67	2.265	135.9	**1.67**	4.5	13.6
	0.83	0.866	103.9	**0.83**	8.4	7.2
	0.17	0.167	100.4	**0.17**	10.5	13.7
	0.08	0.071	85.5	**0.08**	0.0	23.8

**Supplementary Table S4.** Results of metabolite quantification in the urine samples in µg/mL (from the calibrartion curve) and normalized by creatinine quantified (by relative quantification) in each samples in µg/(µg creatinine)

| Metabolite	Median value of absolute concentration in non-diluted urine, µg/mL			Mean value of absolute concentration in non-diluted urine, µg/mL			Median value of concentration normalized by creatinine in non-diluted urine, µg/(µg creatinine)			Mean value of concentration normalized by creatinine in non-diluted urine, µg/(µg creatinine)		
	NP	P	EPL	NP	P	EPL	NP	P	EPL	NP	P	EPL
Succinic acid	45.10	40.05	77.21	60.06	73.99	102.01	0.91	0.72	1.04	1.18	0.80	1.18
D,L-Leucine	16.61	13.75	26.22	17.63	17.01	25.85	0.29	0.24	0.29	0.29	0.27	0.32
Glutaric acid	35.83	44.44	60.17	43.82	46.41	66.05	0.67	0.48	0.74	0.76	0.71	0.84
Adipic acid	9.76	10.37	12.16	9.86	10.96	12.99	0.16	0.14	0.16	0.17	0.17	0.17
D,L-Phenylalanine	12.62	12.18	22.19	22.92	30.56	30.79	0.27	0.18	0.24	0.47	0.28	0.52
Palmitic acid	11.61	10.73	12.67	14.99	13.01	18.22	0.18	0.24	0.17	0.23	0.31	0.25
Nicotinamide	8.76	4.00	10.36	10.36	5.68	11.26	0.12	0.06	0.11	0.15	0.14	0.15
Acetylcarnitine	2.29	1.53	3.40	4.42	5.14	4.65	0.05	0.04	0.03	0.06	0.07	0.06

Supplementary Figure S1. General nESI-HRMS method workflow of data acquisition and processing
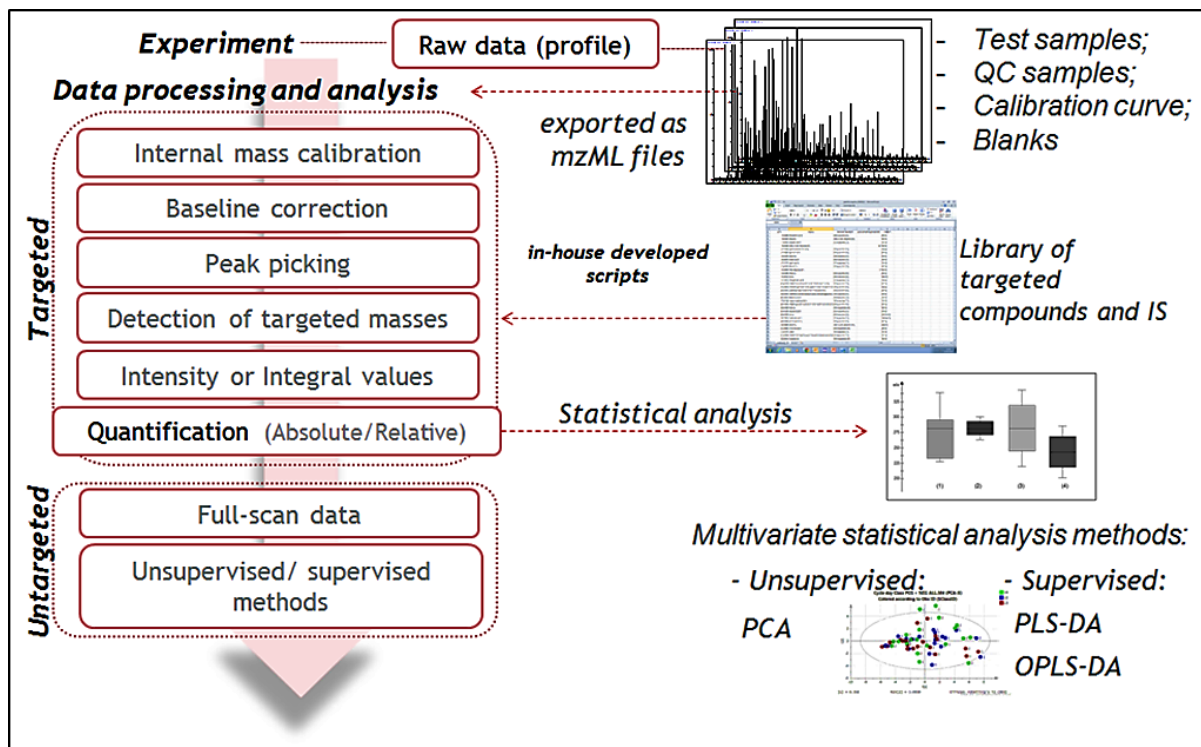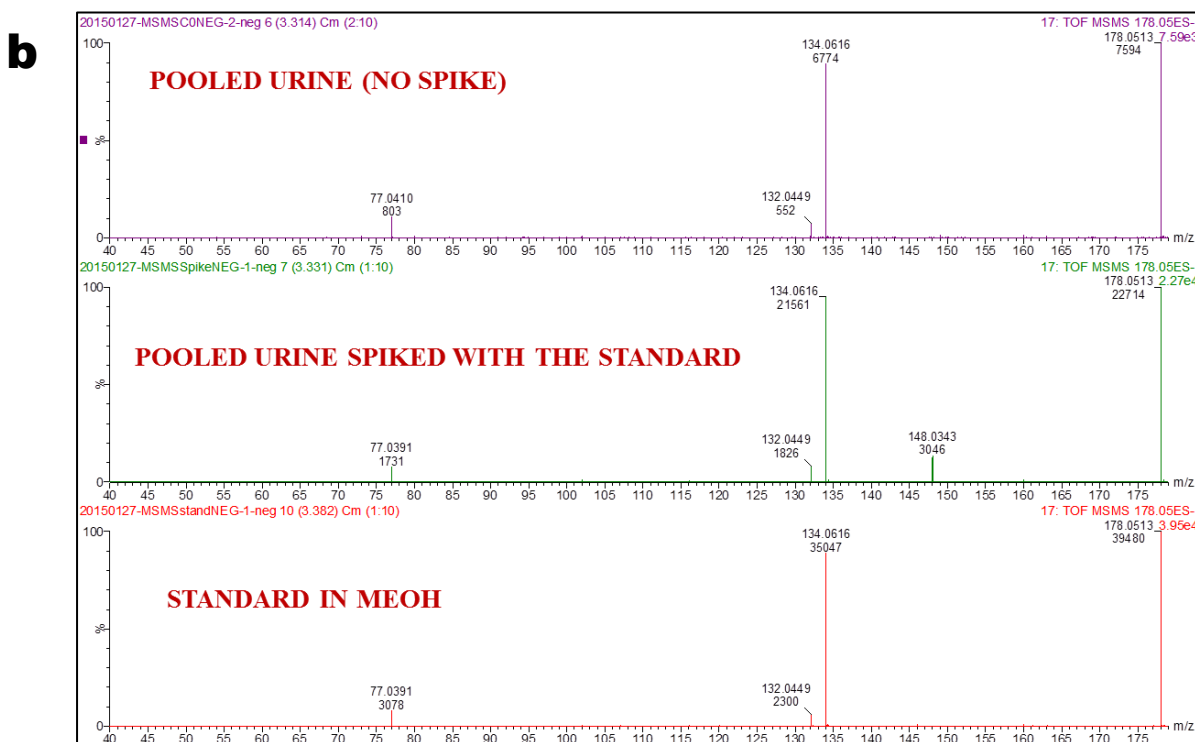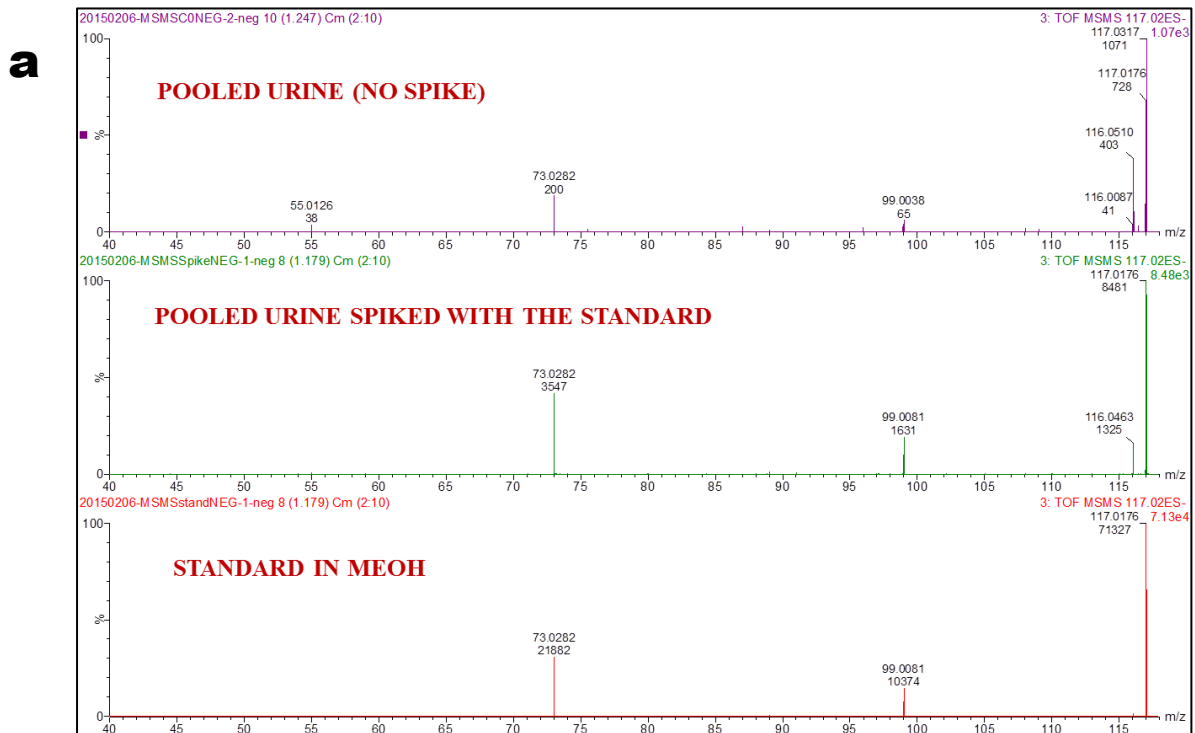
Supplementary Figure S2. Full-scan data obtained for three individual samples from each outcome on the same cycle day (22) in positive ion mode in the m/z range of 100-370; the most prominent identified signals correspond to creatinine (114.068 [M+H]), acetyl-d3-carnitine (207.145 [M+H]), proline betaine (144.105 [M+H]).
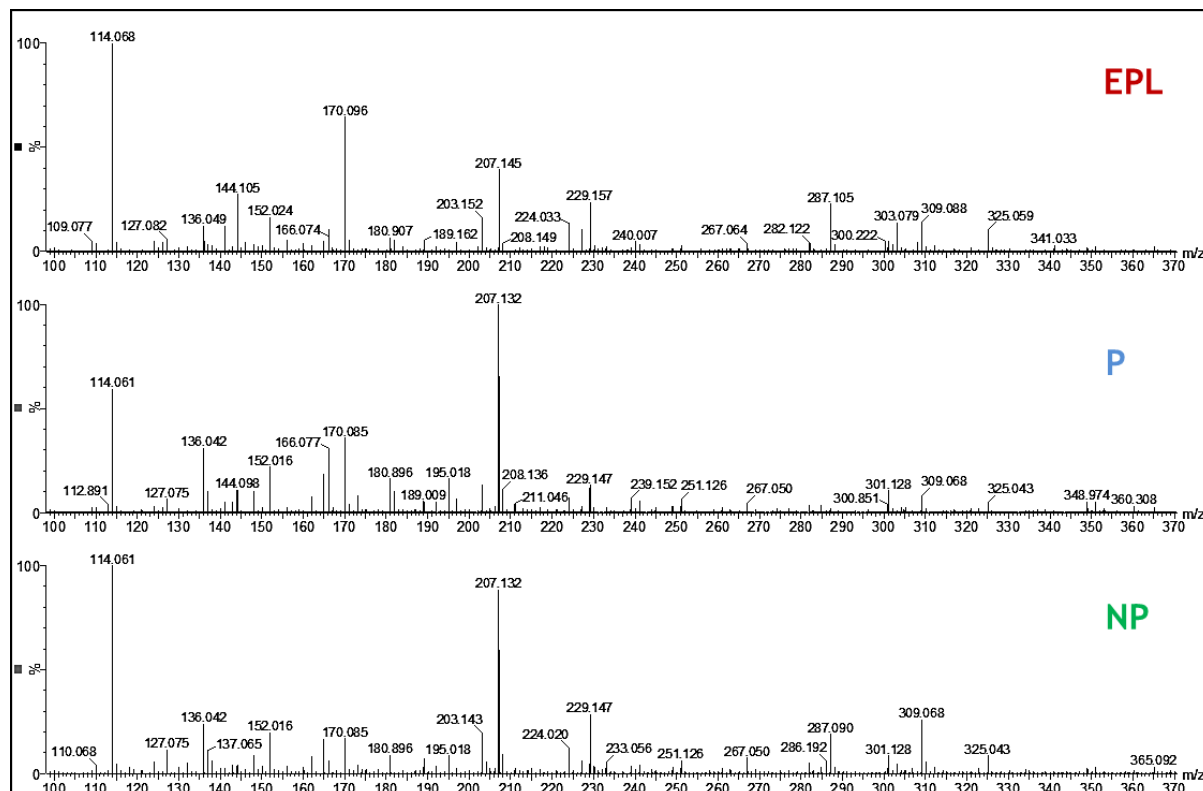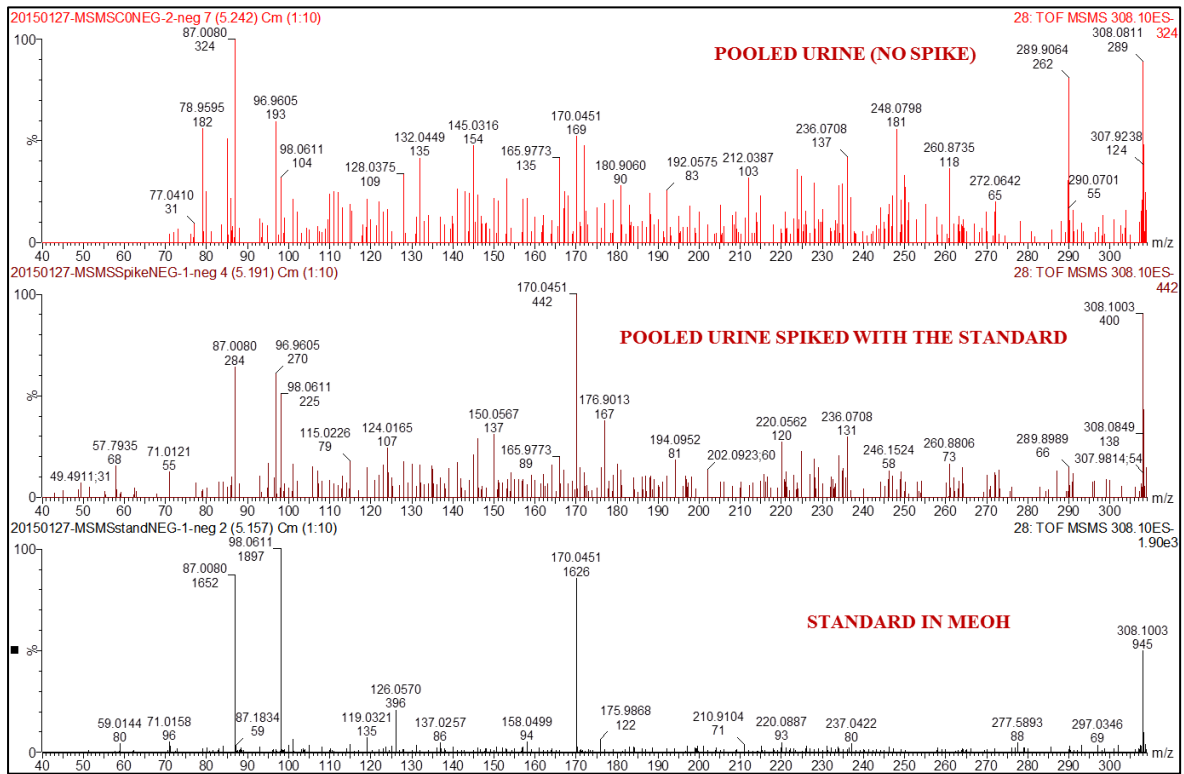
Supplementary Figure S3. (a) Effect of dilution of a pooled urine sample in Positive ion mode over the m/z range of 200-208; signals corresponding to acetyl-L-carnitine and its stable isotope labelled internal standard are highlighted; (b) intensities of endogenous acetyl-L-carnitine and nicotinamide and their stable isotope labelled internal standards as a function of dilution factor.
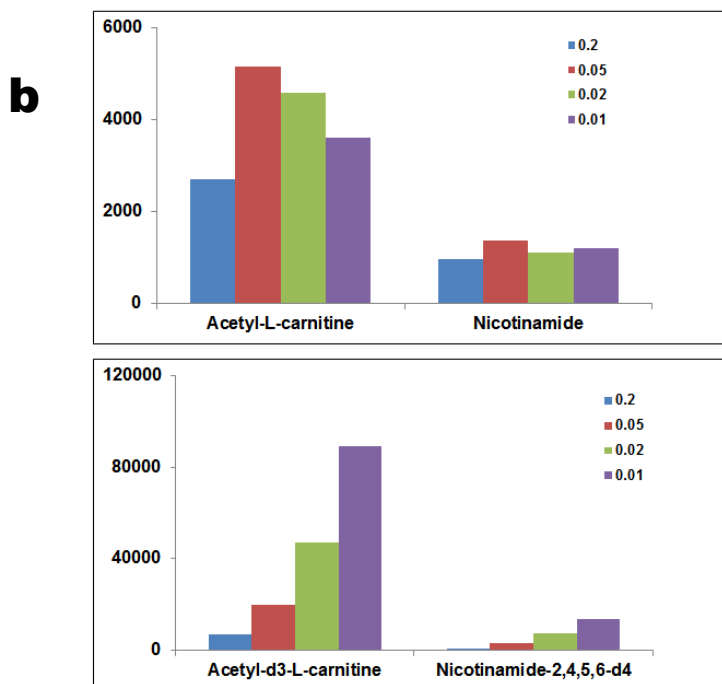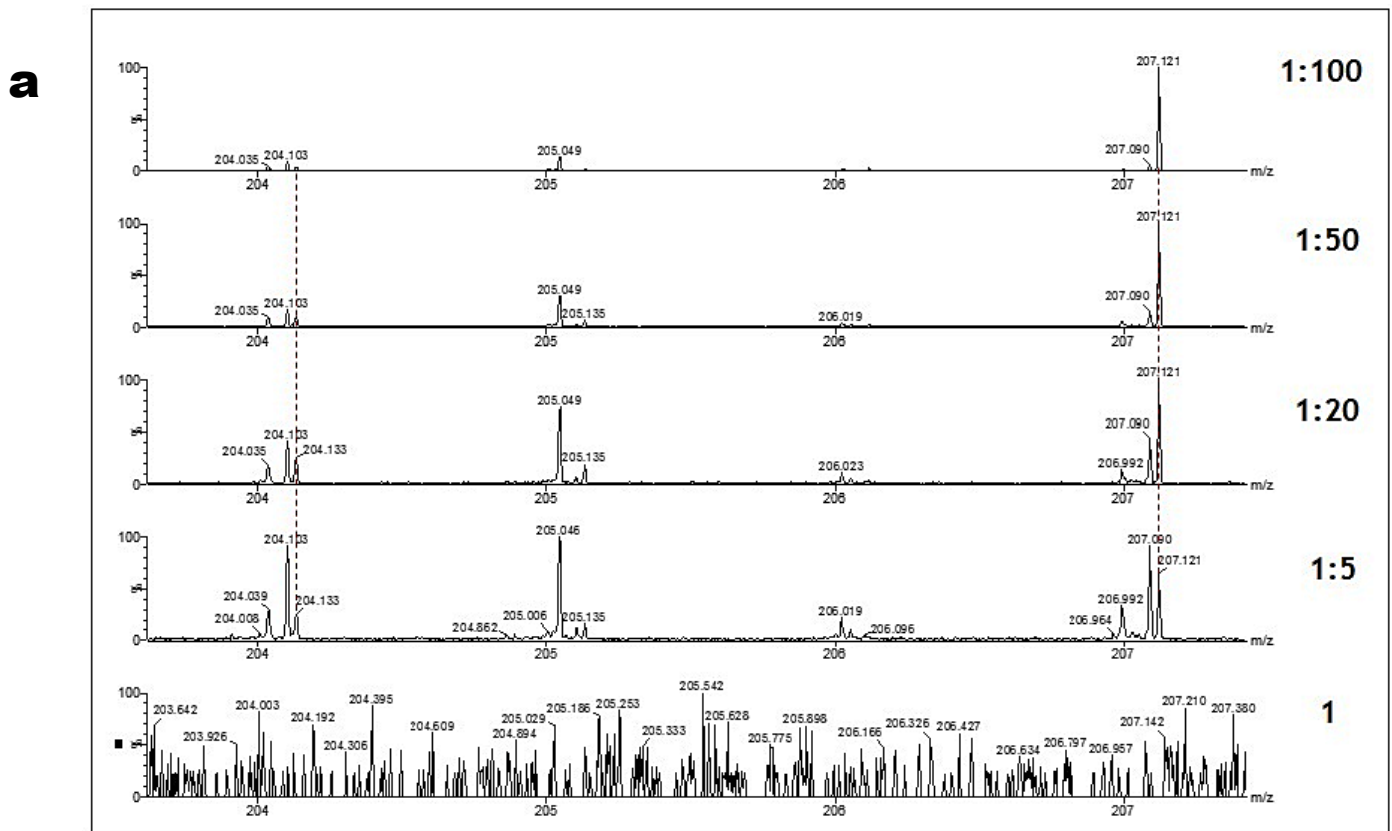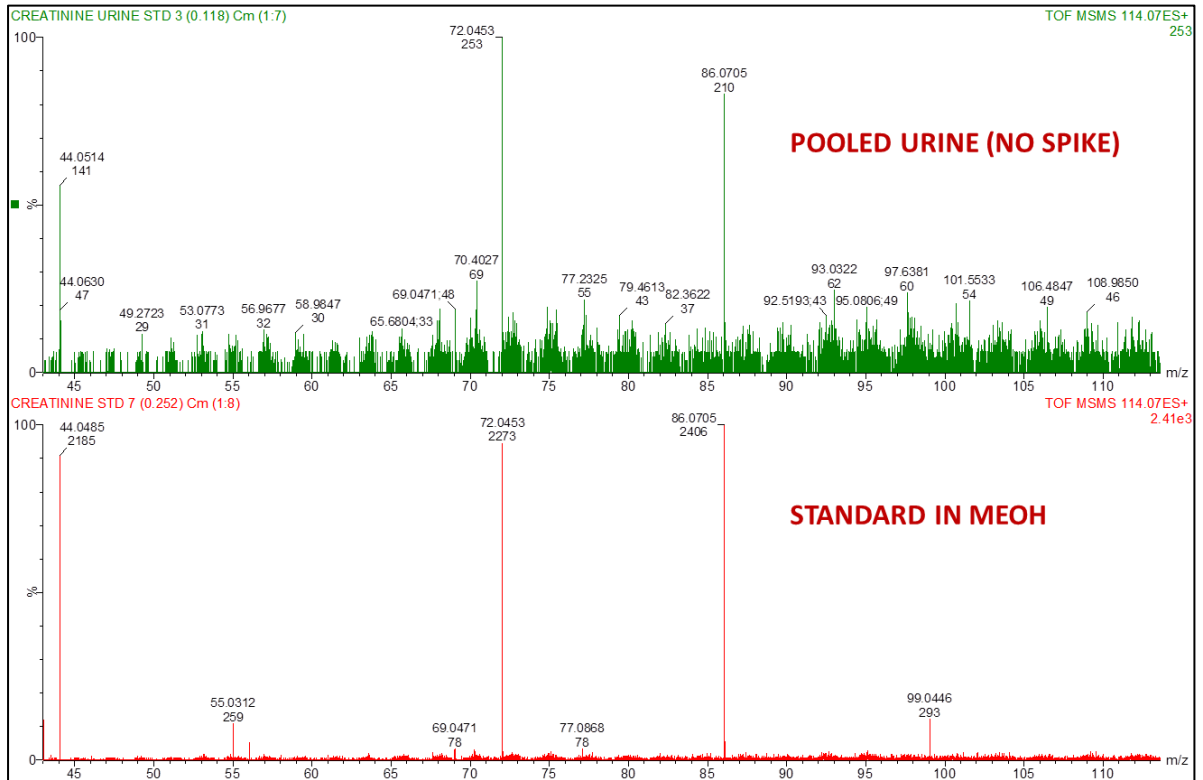
Supplementary Figure S4. Examples of CID mass spectra of succinic acid (a), hippuric acid (b), and N-acetylneurmainic acid (c) acquired in negative ion mode, and of acetylcarnitine (d) and creatinine (e) acquired in positive ion mode for the authentic chemical standard, urine spiked with the standard and non-spiked urine pooled sample.

e

20150203-MSMSC0POS-1-pos 7 (3.636) Cm (1:10)
20: TOF MSMS 204.12ES+
2.74e3

POOLED URINE (NO SPIKE)

85.0273
2740

70.0641
199

84.0811
158

117.0718
153

145.0482
187

159.1352
116

204.1218
232

20150203-MSMSspikePOS-1-pos 6 (3.619) Cm (1:10)
20: TOF MSMS 204.12ES+
1.11e4

POOLED URINE SPIKED WITH THE STANDARD

85.0273
11105

144.0999
469

204.1218
783

20150203-MSMSstandPOS-1-pos 4 (3.585) Cm (1:10)
20: TOF MSMS 204.12ES+
5.57e4

STANDARD IN MEOH

85.0293
55672

84.0811
2073

145.0508
2043

204.1218
4433