Supporting Information:

Solid phase extraction and nanoflow liquid chromatography-nanoelectrospray ionisation mass spectrometry for improved global urine metabolomics

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Contents:

Additional methodology details

UHPLC-ESI-TOFMS analysis

Samples (5 µL) were analysed in both ionisation modes in a randomised block design using a Waters Acquity UHPLC coupled with a Waters Ultima TOFMS system (Waters Ltd, Manchester, UK). The MS system was conditioned using repeated analyses of a quality control (QC) sample prior to analysis of the test replicates. Separation was carried out on an Acquity BEH C18 column (2.1 x 100 mm, 1.7 µm) at 25^{o}C, with a C18 vanguard (2.1 x 50 mm) column. Mobile phase A consisted of water (0.2% FA) and B acetonitrile (0.2% FA). The gradient was 0-1 min 5% B, 1-5 min 15% B, 5-18 min 40% B, 18-25 min 100% B, 25-26 min 100% B at a flow rate of 0.2 mL/min. Prior to each injection, the column was equilibrated in initial conditions for 5 minutes. The mass spectrometer was tuned to a mass resolution of 9000 and mass spectra data were collected between 100 to 1000 *m/z*. A lockmass solution was infused during analysis, and comprised sulfadimethoxine in 1:1 v/v methanol: water (plus 0.1% FA for acquisition in positive ESI mode). The source temperature and desolvation temperatures were 100 ^{o}C and 250 ^{o}C respectively, with a desolvation gas flow of 400 L/h nitrogen.

NanoflowUHPLC-nanospray TOFMS

nUHPLC-nESI-TOFMS methodology for small molecule analyses has recently been established in our laboratory (see ref 30 in main manuscript). SPE extracts were analysed on a Waters nanoAcquity UPLC, and 0.5 µL aliquots of extract were injected on a Waters UHPLC HSS-T3 nanoAcquity column (100Å, 1.8 µm, 100 µm x 100 mm) maintained at 25 ^{o}C. Mobile phase A was water (0.01% FA) and B acetonitrile (0.01% FA) at a flow rate of 700 nL/min. Initial conditions were 10% B with a gradient of 0-4 min to 30% B, 4-18 min 50% B, 18-28 mins 100% B. The column was maintained at 100% B for 10 minutes to and then re-equilibrated in initial conditions for 15 minutes before the next injection. The sample needle was washed in 1.5 mL water and 1.5 mL acetonitrile to eliminate carry over in subsequent injections. SPE samples were analysed after conditioning the MS system with QC samples. The Waters Xevo G2 with nanospray

source was tuned to a mass resolution of 20,000 with a mass spectra range of 50-1000 *m/z*. A capillary voltage of 3.5 kV was used in both ESI modes, with collision energy of 10 eV and a flow of 61 L/hr for the cone and 300 L/hr for desolvation nitrogen gas. A leucine enkephalin lockspray standard in 1:1 methanol:water (2 ng/µL) was infused through a separate lockspray probe at 700 nL/min in both ionisation modes. A baffle was used which acquired from the lockspray probe for 1 second out of every 30 seconds for sample mass correction. Mass fragmentation information was obtained by further analysis of selected samples in MS^e mode with a scan at 10 eV followed by a scan with a ramp of 20 to 30 eV.

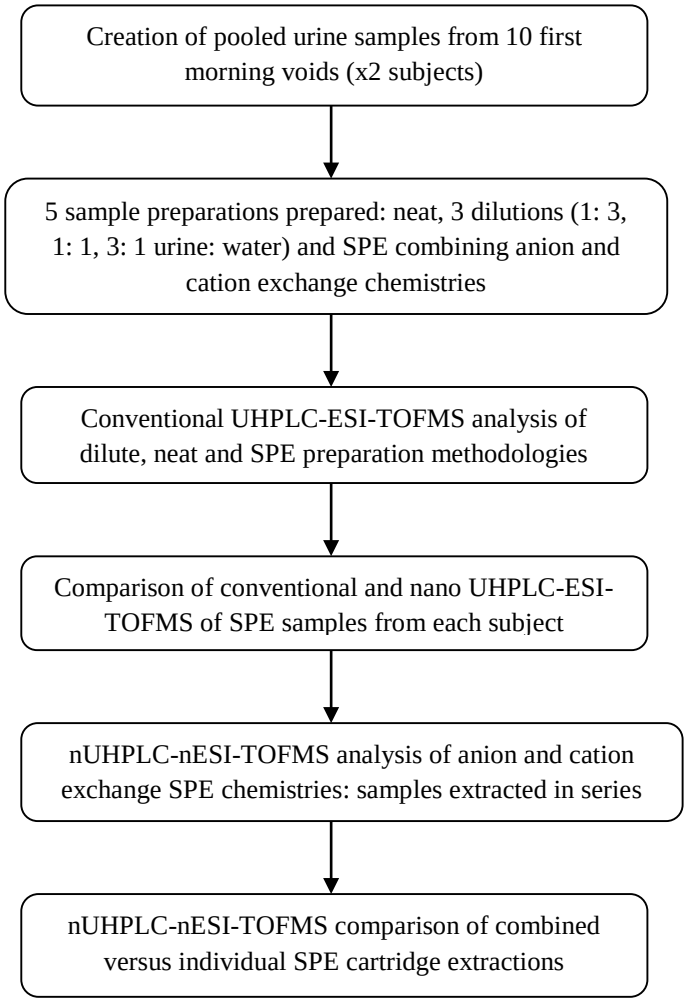

Figure S-1. Flow diagram of methodologies used to assess sample preparation and analytical methodologies.

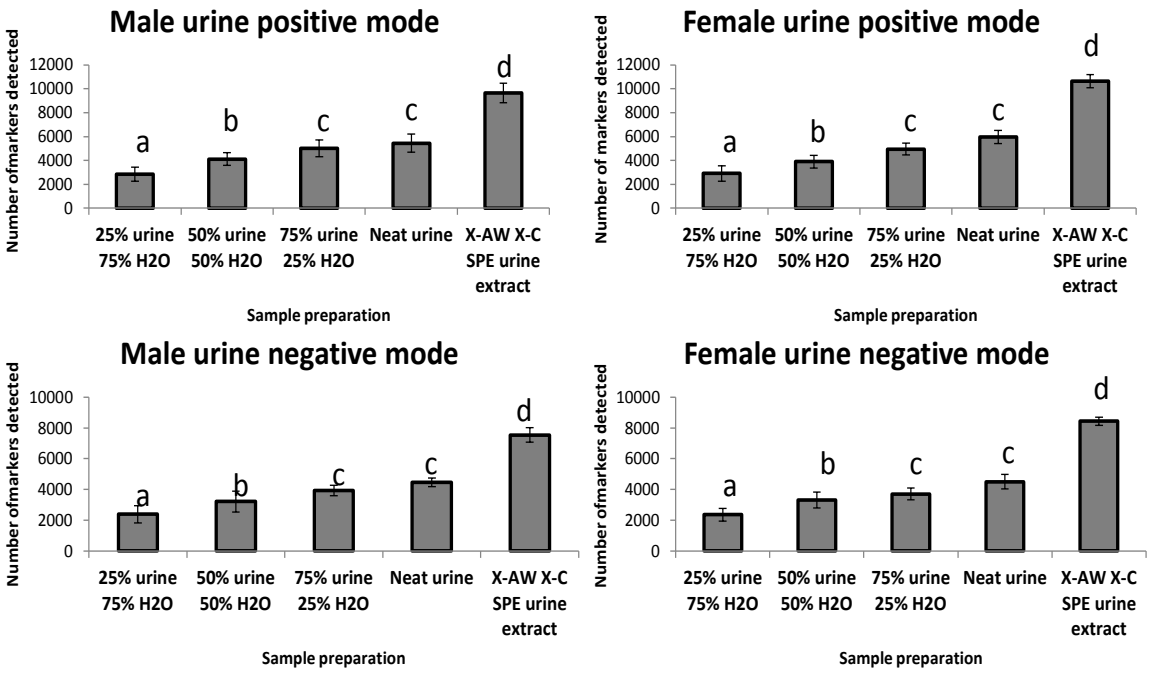

Figure S-2. Numbers of markers detected using different urine sample preparation methods. Number of markers are given as mean ±SD (n=5) after removal of workup blank signals which were 305 ±47 (+ESI) and 140±9 (-ESI). Letters above histogram indicate significant differences after ANOVA analyses and post hoc Tukey's test. Same letters indicate no significant difference ($p \leq 0.05$), different letters mark a significant difference compared to neat urine ($p \leq 0.01$).

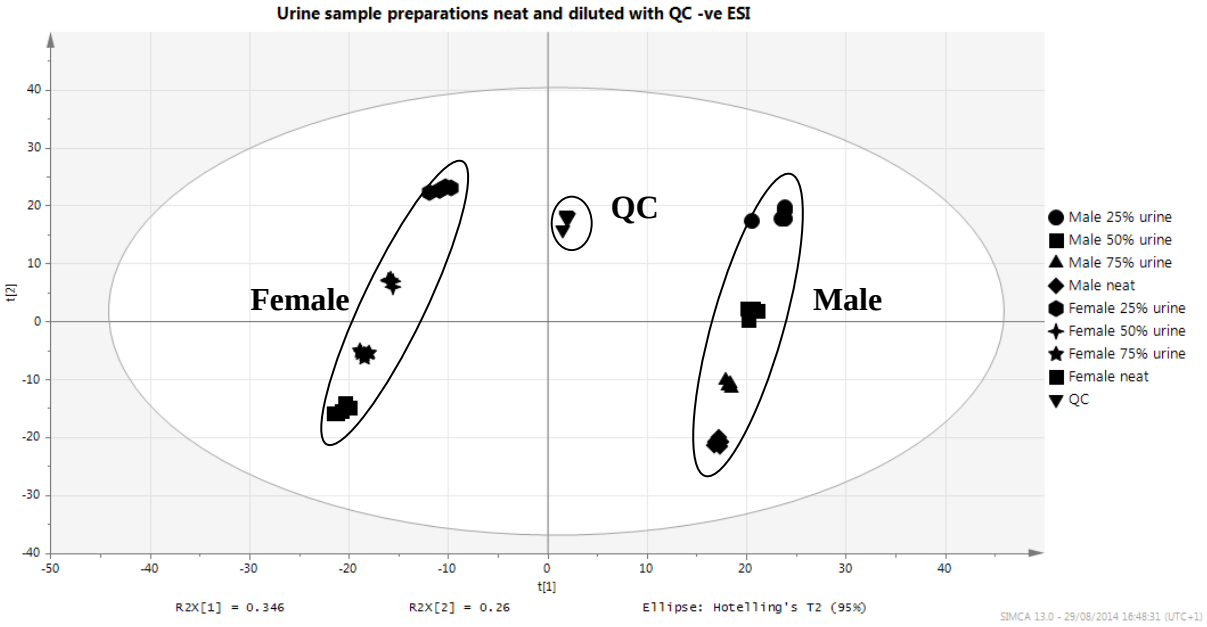

Figure S-3: . PCA scores plot of the metabolomic profiles from neat, diluted and QC urine sample preparations.

Neat urine from either a male or female subject was diluted with water to give 25%, 50% and 75% urine concentrations. An aliquot (5 µL) of each of the 5 replicates was analysed by UHPLC-ESI-TOFMS (-ESI).

Table S-1. Metabolites unique to neat urine compared with samples diluted to 50% concentration.

Results shown were obtained from analysis of male and female subjects. Metabolite identities confirmed with standards or comparison with fragmentation pattern in databases. [1]Putative identity based upon accurate mass measurement of molecular ion. nd = metabolite signal too weak for detection of fragments.

Observed m/z	Empirical formula	Theoretical mass	Q-TOF Fragments	Identity
Amino acids and metabolites				
M+H 156.0762	$C_6H_9N_3O_2$	156.0773	110.0685	Histidine
M-H 245.0930	$C_{13}H_{14}N_2O_3$	245.0926	203.0801, 74.0254	N-acetyltryptophan
M+H 195.0769	$C_9H_{10}N_2O_3$	195.0770	149.071, 120.045	Aminohippuric acid
Neurotransmitter/ metabolites				
M-H 182.0822	$C_9H_{13}NO_3$	182.0817	166.084	Normetanephrine
Vitamins				
M+H 377.1456	$C_{17}H_{20}N_4O_6$	377.1461	243.0867	Riboflavin (Vitamin B2)
M-H 297.1128	$C_{18}H_{18}O_4$	297.1127	nd	7C-aglycone[1] (a vitamin K metabolite)
Conjugated androgen				
M-H 479.2279	$C_{25}H_{36}O_9$	479.2281	175.0224, 113.0223	11-Oxo-androsterone glucuronide[1]
M-H 467.2641	$C_{25}H_{40}O_8$	467.2645	175.0239, 157.0145, 113.0241	Androstanediol glucuronide[1]
Glucocorticoids				
M-H 535.2170	$C_{27}H_{36}O_{11}$	535.2179	175.0241, 157.0138, 113.0242	Aldosterone glucuronide[1]
Bile acids				
M-H 407.2798	$C_{24}H_{40}O_5$	407.2797	343.2623, 289.2140, 251.2000	Cholic acid
M-H 567.3167	$C_{30}H_{48}O_{10}$	567.3169	175.0265, 113.0318	Deoxycholic acid glucuronide[1]
M-H 624.3390	$C_{32}H_{51}NO_{11}$	624.3384	175.02298, 113.229	Glycochenodeoxycholic acid glucuronide[1]
Diet compounds				
M-H 395.0063	$C_{12}H_{19}Cl_3O_8$	395.0067	359.0368	Sucralose
M-H 297.0979	$C_{14}H_{18}O_7$	297.0974	175.0241,157.0142, 113.0242	Phenylethanol glucuronide[1]
M-H 507.2234	$C_{26}H_{36}O_{10}$	507.2230	nd	Unidentified dietary compound[1]
Smoking related compounds				
M+H 177.1031	$C_{10}H_{12}N_2O$	177.1028	137.0027	Cotinine
M+H 179.1177	$C_{10}H_{14}N_2O$	179.1184	nd	Nicotine-1'-N-oxide[1]
Organic acids ≤10 carbons				
M-H 201.1127	$C_{10}H_{18}O_4$	201.1127	183.1018, 139.1119	Sebacic acid
M+H 291.1312	$C_{10}H_{18}N_4O_6$	291.1305	nd	Argininosuccinic acid[1]
M-H 187.0973	$C_9H_{16}O_4$	187.0970	125.097,97.066, 57.036	Azelaic acid
M-H 266.0869	$C_9H_{17}NO_8$	266.0876	nd	Neuraminic acid[1]
M+H 146.0929	$C_5H_{11}N_3O_2$	146.0930	104.0701, 87.0450, 86.1601	Guanidinobutanoic acid
Organic acids >10 carbons				
M+H 449.2554	$C_{18}H_{36}N_6O_5S$	449.2546	nd	Glutathionylaminopropylcadaverine[1]

Table S-2. Metabolites unique to either SPE or neat urine preparations

Results shown were obtained from analysis of male and female subjects. Metabolite identities were confirmed with standards or Q-TOF fragmentation pattern. [1]Putative identity based upon accurate mass measurement of molecular ion.

Experimental mass and ion species.	Putative Structure	Theoretical mass	Fragments	Identity
Metabolites unique to SPE				
Amino acids and metabolites				
M-H 192.0664	$C_{10}H_{11}NO_3$	192.0661	148.0752, 91.0573	Methylhippuric acid
M+H 170.0925	$C_7H_{11}N_3O_2$	170.0930	126.1021, 109.0751, 96.0683	Methyl histidine
M+H 295.1292	$C_{14}H_{18}N_2O_5$	295.1294	nd	Glutamylphenylalanine[1]
Carnitines				
M+H 372.3108	$C_{21}H_{41}NO_4$	372.3114	85.0272	Tetradecanoylcarnitine
Neurotransmitter/ metabolites				
M+H 249.1231	$C_{13}H_{16}N_2O_3$	249.1239	190.0858	Hydroxymelatonin
Vitamins				
M-H 605.3331	$C_{33}H_{50}O_{10}$	605.3326	175.0238,157.0146, 113.0243	Dihydroxy-oxovitamin D3-glucuronide[1]
Bile acids and metabolites				
M-H 611.3795	$C_{33}H_{56}O_{10}$	611.3799	175.0242,157.0149, 113.0244	Cholestane-tetrol-glucuronide
M-H 391.2843	$C_{24}H_{40}O_4$	391.2848	nd	Chenodeoxycholic acid
M-H 514.2836	$C_{26}H_{45}NO_7S$	514.2838	124.0071	Taurocholic acid
M-H 391.2848	$C_{24}H_{40}O_4$	391.2848	327.271, 329.2836, 345.2793, 347.2956	Deoxycholic acid
M-H 455. 2478	$C_{24}H_{40}O_6S$	455.2478	nd	Sulfolithocholic acid[1]
M-H 512.2692	$C_{26}H_{43}NO_7S$	512.2682	nd	Unidentified bile acid[1]
Lipid metabolites				
M+H 288.2911	$C_{13}H_{37}NO_2$	288.2903	nd	C17 Sphinganine[1]
M+H 542.2999	$C_{24}H_{47}NO_{10}S$	542.2978	nd	Psychosine sulfate[1]
M+H 358.2957	$C_{20}H_{39}NO_4$	358.2963	nd	Palmitoyl threonine[1]
Dietary compounds				
M-H 415.1243	$C_{18}H_{24}O_{11}$	415.1240	nd	Hydroxybenzaldehyde-xylosyl-glucoside[1]
M-H 212.0024	$C_8H_7NO_4S$	212.0018	132.0455	Indoxylsulfuric acid
M-H 258.9910	$C_9H_8O_7S$	258.9912	nd	Caffeic acid sulfate[1]
Smoking related compounds				
M+H 163.1235	$C_{10}H_{14}N_2$	163.1238	132.0785, 130.0785	Nicotine
M+H 163.1229	$C_{10}H_{14}N_2$	163.1235	146.0951	Anabasine
Free pharmaceuticals				
M+H 223.1328	$C_{13}H_{18}O_3$	223.1334	nd	Hydroxyibuprofen[1]
M+H 152.0704	$C_8H_9NO_2$	152.0712	nd	Acetaminophen

Conjugated pharmaceuticals				
M-H 285.0789	$C_{13}H_{18}O_5S$	285.0797	205.1225, 79.9575	Ibuprofen Sulfate
Organic acids ≤10 carbons				
M+H 138.0915	$C_8H_{11}NO$	138.0919	121.065, 93.0690, 91.0534	m-Tyramine
Metabolites unique to neat urine				
Amino acids/ metabolites				
M-H147.0301	$C_5H_8O_5$	147.0293	129.0204,101.0261, 85.0322	Hydroxyglutamate
Nucleotides/ metabolites				
M-H 243.0631	$C_9H_{12}N_2O_6$	243.0630	200.0556	Uridine
M-H 195.0522	$C_7H_8N_4O_3$	195.0518	nd	Dimethyl uric acid
M+H 183.0521	$C_6H_6N_4O_3$	183.0518	191.9606	Methyl uric acid
Vitamins				
M-H 135.0302	$C_4H_8O_5$	135.0293	nd	Threnoic acid
Dietary compounds				
M-H 221.0670	$C_8H_{14}O_7$	221.0661	175.0237, 157.0116	Ethyl glucuronide
M-H 189.0411	$C_8H_{14}OS_2$	189.0408	nd	Methialdol[1]
M+H 167.0573	$C_6H_6N_4O_2$	167.0569	110.0338	Methylxanthine
M-H 383.1201	$C_{14}H_{24}O_{12}$	383.1190	324.0712	Acetyl maltose
M-H 179.0571	$C_6H_{12}O_6$	179.0556	161.0463	Unidentified sugar
Organic acids ≤10 carbons				
M-H 161.0460	$C_6H_{10}O_5$	161.0450	nd	Unidentified organic acid
M-H 191.0195	$C_6H_8O_7$	191.0192	147.307,129.0195, 111.0088	Citric acid

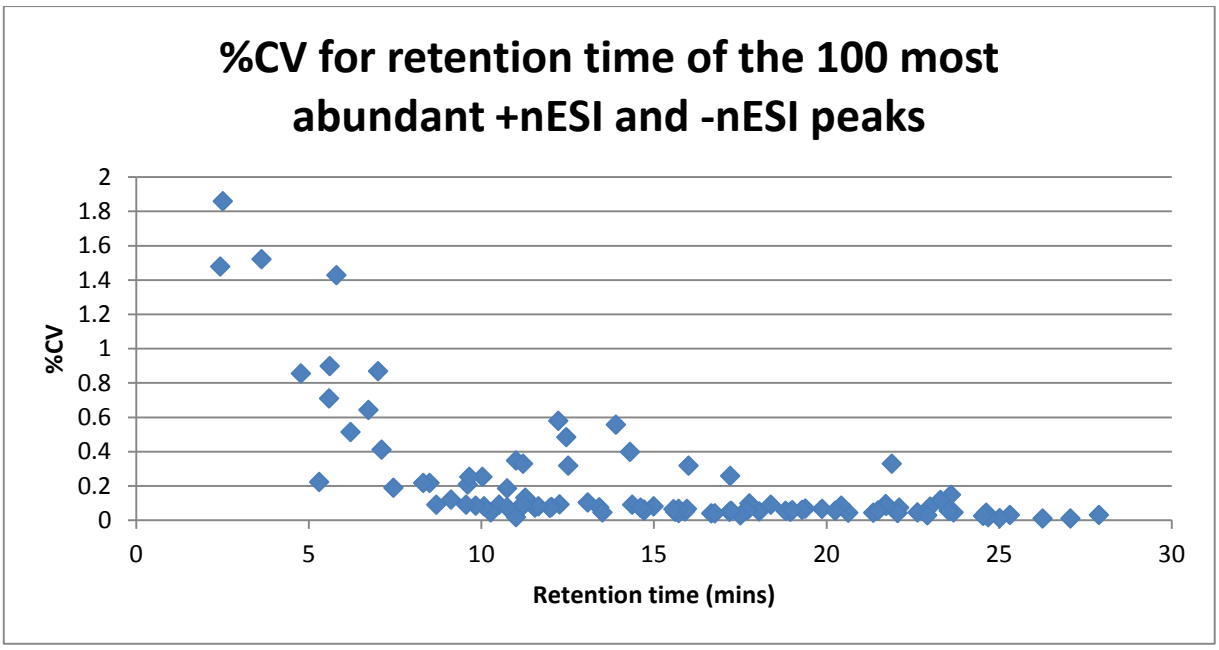

Figure S-4. Retention time repeatability of nUHPLC-nESI-TOFMS analyses

Retention time variability calculated as %CV from the mean retention time of the 100 most abundant peaks (50 +nESI and 50-nESI) present in the 10 QC injections. All values are < 1% for resolved peaks.

Table S-3: Comparison of UHPLC-ESI-TOF-MS and nUHPLC-nESI-TOFMS platforms for detection of discriminatory metabolites

Metabolites unique to samples from either a male or female subject were detected using OPLS-DA analysis of datasets from the ESI or nESI platforms. Additional signalling molecules and other metabolites unique to the nano platform were also detected after further targeted examination of the datasets. Compound identities were confirmed by comparison of fragments with databases or standards. [1]Putative identity based upon accurate mass measurement of molecular ion.

Observed m/z	Structure	Theoretical mass	Fragments	Unique to male /female samples	Identity
Discriminatory metabolites of subject detected by UPLC-ESI-TOF-MS					
Neurotransmitter/ metabolites					
M-H 359.0979	$C_{15}H_{20}O_{10}$	359.0978	175.0230, 113.0224	Female	Methoxy-hydroxyphenylglycol glucuronide[1]
M-H 263.0231	C9H12O7S	263.0225	183.0682	Male	Methoxy hydrophenylglycol sulfate
Bile acids and metabolites					
M-H 391.2843	$C_{24}H_{40}O_4$	391.2848	nd	Female	Chenodeoxycholic acid
M-H 498.2886	$C_{26}H_{45}NO_6S$	498.2889	nd	Male	Taurodeoxycholic acid
Lipid metabolites					
M+H 542.2975	$C_{24}H_{47}NO_{10}S$	542.2990	463.092	Female	Psychosine sulfate
Dietary compounds					
M-H 445.1129	$C_{22}H_{22}O_{10}$	445.1135	nd	Female	Unidentified glucoside[1]
M+H 304.0930	$C_{16}H_{15}O_6$	304.0947	nd	Female	Unidentified flavanoid[1]
M+H 401.1791	$C_{19}H_{28}O_9$	401.1812	nd	Female	Corchoionoside B[1]
M-H 557.0580	$C_{22}H_{22}O_{15}S$	557.0601	nd	Female	Unidentified sulfated glucoside[1]
M-H 387.1831	$C_{22}H_{28}O_6$	387.1808	nd	Female	Unidentified dietary sugar[1]
M-H 381.0291	$C_{16}H_{14}O_9S$	381.0280	nd	Female	Hesperetin sulfate[1]
M-H 477.1040	$C_{22}H_{22}O_{12}$	477.1033	nd	Female	Unidentified glucoside[1]
M-H 321.1554	$C_{14}H_{26}O_8$	321.1549	nd	Female	Butyl hydroxyl butyrate glucoside[1]
M-H 395.0069	$C_{12}H_{19}Cl_3O_8$	395.0067	359.0368	Male	Sucralose
M-H 217.0185	$C_8H_{10}O_5S$	217.0171	nd	Female	Tyrosol sulfate[1]
M-H 571.1451	$C_{28}H_{28}O_{13}$	571.1452	nd	Male	Triacetylglycitin[1]
M-H 313.0552	$C_{13}H_{14}O_9$	313.0560	175.0216, 113.0267	Male	Salicylate glucuronide[1]
M-H 427.1959	$C_{21}H_{32}O_9$	427.1968	nd	Male	Taraxacolide glucopyranoside[1]
Smoking related compounds					
M+H 177.1029	$C_{10}H_{12}N_2O$	177.1028	80.049	Female	Cotinine[1]
M+H 193.0974	$C_{10}H_{12}N_2O_2$	193.0977	143.117	Female	Cotinine N-Oxide

M+H 163.1238	$C_{10}H_{14}N_2$	163.1235	132.0785, 130.0785	Female	Nicotine
Organic acids ≤10 carbons					
M-H 211.0607	$C_{10}H_{12}O_5$	211.0606	123.046	Female	Unidentified acid[1]
Organic acids >10 carbons					
M-H 224.0599	$C_{11}H_{13}O_5$	224.0685	nd	Male	Unidentified acid[1]
Additional discriminatory metabolites of subject detected by nUPLC-nESI-TOF-MS:					
Amino acids/ metabolites					
M-H165.0547	$C_9H_{10}O_3$	165.0552	147.042, 119.0504	Female	Phenyllactic acid
Neurotransmitter/ metabolites					
M+H 191.1185	$C_{11}H_{14}N_2O$	191.1184	174.092, 159.068	Male	5-Methoxytryptamine
Conjugated androgens					
M-H 383.1525	$C_{19}H_{28}O_6S$	383.1528	96.9595, 79.9571	Female	Unidentified sulfated androgen
M-H 467.2641	$C_{25}H_{40}O_8$	467.2645	175.0240, 157.0141, 113.0241	Male	Unidentified androgen glucuronide[1] (retention time 12.89 mins)
Free estrogens					
M-H 269.1545	$C_{18}H_{22}O_2$	269.1542	221.1563, 145.0656	Female	Estrone
Conjugated estrogens					
M-H 445.1871	$C_{24}H_{30}O_8$	445.1862	175.0231, 157.1220, 113.0231	Female	Estrone glucuronide
M-H 477.2133	$C_{25}H_{34}O_9$	477.2125	175.0223, 113.0225	Female	Methoxy-estradiol-glucuronide[1]
Progestogens					
M+H 315.2332	$C_{21}H_{30}O_2$	315.2324	nd	Female	Progesterone
M-H 395.1888	$C_{21}H_{32}O_5S$	395.1882	79.9579	Female	Pregnenolone sulfate[1]
Eicosanoids					
M-H 319.2275	$C_{20}H_{32}O_3$	319.2273	285.2281, 303.2351	Female	Unidentified HETE
M+H 440.2477	$C_{23}H_{37}NO_5S$	440.2471	319.2250, 189.165	Male	Leukotriene E4
Lipid metabolites					
M+H 282.2790	$C_{18}H_{35}NO$	282.2797	nd	Male	Oleamide[1]
Dietary compound					
M-H 533.1382	$C_{25}H_{26}O_{13}$	533.1373	nd	Female	Unidentified glucoside
M-H 367.1421	$C_{18}H_{24}O_8$	367.1393	nd	Female	Unidentified glucoside
M-H 204.0665	$C_{11}H_{11}NO_3$	204.0661	160.0763,132.0811,130.0652, 117.07, 103.0546	Female	Cinnamoylglycine
M+H 261.1231	$C_{14}H_{16}N_2O_3$	261.1239	nd	Male	Maculosin L,L-Cyclo(leucylprolyl)[1]
M-H 359.1141	$C_{19}H_{20}O_7$	359.1131	nd	Male	Unidentified flavonoid[1]
Organic acids ≤10 carbons					
M-H 147.0440	$C_9H_8O_2$	147.0446	103.0554	Male	Cinnamic acid
Organic acids >10 carbons					

M-H 427.1840	$C_{18}H_{28}N_4O_8$	427.1829	nd	Male	Pyridinoline[1]
Additional metabolites detected in both subjects and unique to nUPLC-nESI-TOF-MS:					
Amino acids/ metabolites					
M+H 205.0973	$C_{11}H_{12}N_2O_2$	205.0977	188.0625	-	Tryptophan
M+H 225.0884	$C_{10}H_{12}N_2O_4$	225.0875	208.0620	-	Hydroxykynurenine
M-H 159.0293	$C_6H_8O_5$	159.0293	59.014	-	Oxoadipic acid
M+H 175.0721	$C_6H_{10}N_2O_4$	175.0719	nd	-	Formiminoglutamic acid[1]
M+H 145.1332	$C_7H_{16}N_2O$	145.1341	nd	-	Acetylcadaverine[1]
M-H 143.0347	$C_6H_8O_4$	143.0344	nd	-	Methylglutaconic acid[1]
M-H 138.0194	$C_6H_5NO_3$	138.0191	94.0320	-	Hydroxypicolinic acid
Neurotransmitter/ metabolite					
M+H 154.0841	$C_8H_{11}NO_2$	154.0868	137.0495, 113.0630	-	Dopamine
M+H 233.1295	$C_{13}H_{16}N_2O_2$	233.1290	nd	-	Melatonin
Free androgens					
M+H 289.2162	$C_{19}H_{28}O_2$	289.2168	nd	-	Testosterone
M+H 287.2016	$C_{19}H_{26}O_2$	287.2011	nd	-	Androstenedione
Conjugated androgens					
M-H 467.2641	$C_{25}H_{40}O_8$	467.2645	175.0240, 157.0141, 113.0241	-	Unidentified androgen glucuronide[1] (retention time 15.53 mins)
Conjugated estrogens					
M-H 447.2021	$C_{24}H_{32}O_8$	447.2019	175.0237, 157.1229, 113.0232	-	Estradiol glucuronide
Glucocorticoids					
M+H 361.2021	$C_{21}H_{28}O_5$	361.2015	326.16, 301.165	-	Cortisone
M+H 331.2278	$C_{21}H_{30}O_3$	331.2273	nd	-	11-deoxycorticosterone
M+H 335.2581	$C_{21}H_{34}O_3$	335.2586	nd	-	Tetrahydrodeoxycorticosterone
Bile acid/ metabolites					
M-H 377.2699	$C_{23}H_{38}O_4$	377.2848	nd	-	Apocholic acid[1]
M-H 583.3128	$C_{30}H_{48}O_{11}$	583.3118	407.2796, 175.0234, 157.1230, 113.0233	-	Cholic acid glucuronide
Lipid metabolites					
M+H 300.2902	$C_{16}H_{37}NO_2$	300.2903	282.2767	-	Sphingosine
M-H 295.2275	$C_{18}H_{22}O_3$	295.2273	277.2170, 195.1440, 113.0960, 71.0150	-	Hydroxyoctadecadienoic acid
M-H 199.0972	$C_{10}H_{16}O_4$	199.0970	nd	-	Decanoic acid[1]
M-H 171.1386	$C_{10}H_{20}O_2$	171.1385	68.9971	-	Capric acid
M-H 155.1075	$C_9H_{16}O_2$	155.1072	nd	-	Hydroxynonenal[1]
M+H 170.0450	$C_7H_7NO_4$	170.0453	nd	-	Furoylglycine[1]
M+H 232.1181	$C_{10}H_{17}NO_5$	232.1185	nd	-	Suberylglycine
M+H 158.0813	$C_7H_{11}NO_3$	158.0817	nd	-	Methylcrotonylglycine[1]

M-H 173.0813	$C_8H_{14}O_4$	173.0814	111.0810	-	Suberic acid
Dietary compounds					
M-H 165.0551	$C_9H_{10}O_3$	165.0552	121.0655	-	Methoxyphenylacetic acid
M-H 307.0283	$C_{14}H_{12}O_6S$	307.0276	nd	-	Resveratrol sulfate [1]
M-H 391.1230	$C_{20}H_{22}O_8$	391.1393	nd	-	Resveratrol glucoside[1]
M-H 403.1028	$C_{20}H_{20}O_9$	403.1029	175.0229, 113.0225	-	Resveratrol glucuronide[1]
M-H 155.1437	$C_{10}H_{20}O$	155.1436	nd	-	Menthol[1]
M-H 273.0765	$C_{15}H_{14}O_5$	273.0763	167.0340, 119.0490	-	Phloretin
M-H 253.0505	$C_{15}H_{10}O_4$	253.0501	91.0180	-	Daidzein
M-H 379.1392	$C_{19}H_{24}O_8$	379.1393	nd	-	Unidentified glucoside[1]
M+H 295.1300	$C_{14}H_{18}N_2O_5$	295.1294	nd	-	Aspartame[1]
M-H 297.1130	$C_{18}H_{18}O_4$	297.1127	nd	-	Enterolactone[1]
M-H 255.0660	$C_{15}H_{12}O_4$	255.0657	nd	-	Dihydrodaidzein[1]
M-H 283.0598	$C_{16}H_{12}O_5$	283.0606	268.0380	-	Glycitein
M-H 271.0612	$C_{15}H_{12}O_5$	271.0606	nd	-	Dihydrogenistein[1]
M+H 179.1065	$C_{11}H_{16}O_2$	179.1072	nd	-	Butylated hydroxyanisole[1]
M-H 137.0609	$C_8H_{10}O_2$	137.0603	119.0501	-	Tyrosol
M-H 301.1550	$C_{18}H_{22}O_4$	301.1440	nd	-	Enterodiol[1]
M-H 115.0760	$C_6H_{12}O_2$	115.0759	nd	-	Caproic acid
M-H 227.1291	$C_{12}H_{20}O_4$	227.1283	183.1380, 165.1280	-	Traumatic acid
Free pharmaceuticals					
M-H 205.1233	$C_{13}H_{18}O_2$	205.1229	161.1330, 154.9740	-	Ibuprofen
M+H 152.1068	$C_9H_{13}NO$	152.1075	134.0961, 117.0695	-	Phenylpropanolamine
Organic acids ≤10 carbons					
M+H 188.1760	$C_9H_{21}N_3O$	188.1763	171.1490, 117.1020, 100.0750	-	Acetylspermidine

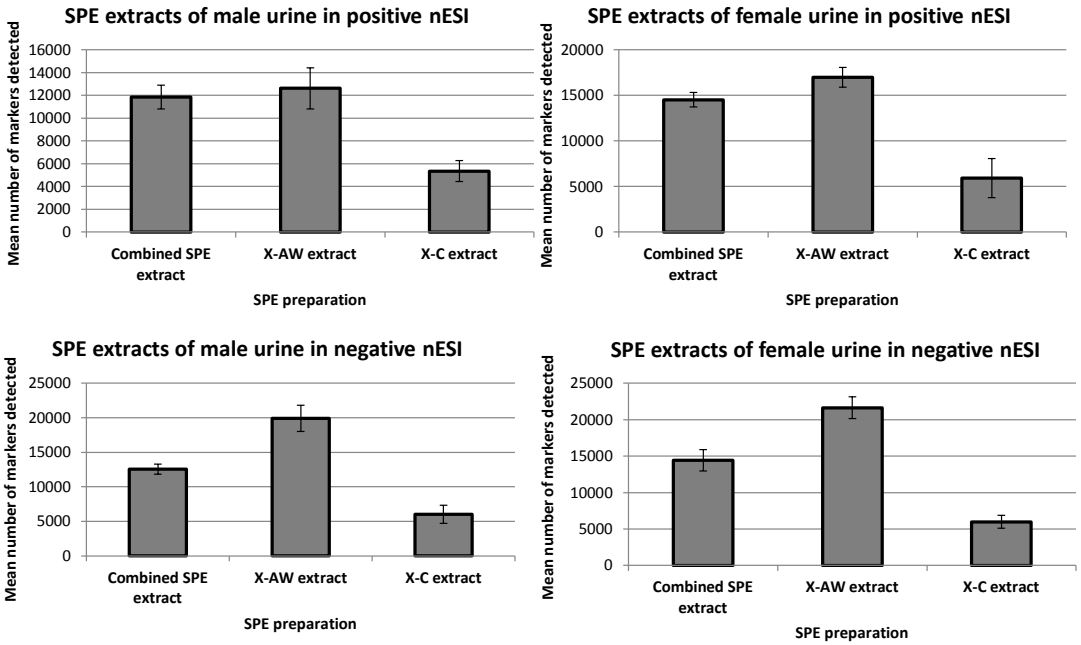

Figure S-5. Number of markers detected in profiles of individual SPE preparations compared with profiles from combined SPE samples.

Number of markers in extracts from X-AW and/or X-C SPE phases are given as mean ±SD (n=5) after removal of workup blank signals. Graphs A and B markers detected in positive nESI modes and Graphs C and D markers detected in negative nESI mode for the male and female samples.

Table S-4: Metabolites markers unique to the strong cation exchange SPE cartridge

Urine samples were sequentially extracted by anion (X-AW) followed by cation (X-C) SPE phases and the extracts analysed by UHPLC-nESI-TOFMS. Datasets were analysed by OPLS-DA and 'S' plot to determine markers retained only by X-C. Metabolite identities confirmed with standards or comparison with fragmentation pattern in databases, [1]Putative identity based upon accurate mass measurement of the molecular ion. nd = metabolite signal too weak for detection of fragments.

Experimental mass	Structure	Theoretical mass	Q-TOF Fragments	Identity
Amino acids/ metabolites				
M+H 156.0769	$C_6H_9N_3O_2$	156.0773	110.0719	Histidine
M+H 195.0769	$C_9H_{10}N_2O_3$	195.0770	149.071, 120.0450	Aminohippuric acid
M-H 192.0664	$C_{10}H_{11}NO_3$	192.0661	148.075, 91.057	Methylhippuric acid
M+H 170.9250	$C_7H_{11}N_3O_2$	170.0930	126.1021, 109.0751, 96.0683	Methyl histidine
M+H 166.0861	$C_9H_{11}NO_2$	166.0868	120.0801	Phenyl alanine
M-H 173.0918	$C_7H_{12}N_2O_3$	173.0926	116.0710, 70.0660	Glycylproline
M+H 182.0824	$C_9H_{11}NO_3$	182.0817	165.0560 136.0764	Tyrosine
M+H 291.1314	$C_{10}H_{18}N_4O_6$	291.1305	nd	Argininosuccinic acid[1]
M+H 203.1500	$C_8H_{18}N_4O_2$	203.1508	nd	Symmetric dimethylarginine[1]
M+H 295.1292	$C_{14}H_{18}N_2O_5$	295.1294	nd	Glutamylphenylalanine[1]
M-H 269.0603	$C_{11}H_{14}N_2O_4S$	269.0596	nd	Hydroxyphenylacetothiohydroximoyl cysteine[1]
M+H 139.0502	$C_6H_6N_2O_2$	139.0508	121.0400	Urocanic acid
M+H 118.0860	$C_5H_{11}NO_2$	118.0868	101.0600	Aminopentanoic acid
M+H 145.1332	$C_7H_{16}N_2O$	145.1341	nd	N-Acetylcadaverine[1]
M+H 138.0919	$C_8H_{11}NO$	138.0919	121.065, 93.0690, 91.0534	m-Tyramine
Carnitines				
M+H 162.1121	$C_7H_{15}NO_3$	162.1130	nd	Carnitine
M+H 232.1549	$C_{11}H_{21}NO_4$	232.1549	173.0808	Butyrlcarnitine
M+H 232.1549	$C_{11}H_{21}NO_4$	232.1549	173.0808	Isobutyrlcarnitine
M+H 244.1547	$C_{12}H_{21}NO_4$	244.1549	nd	Ethylacrylcarnitine[1]
M+H 246.1706	$C_{12}H_{23}NO_4$	246.1705	187.0985	Methylbutyroylcarnitine
Neurotransmitter/ metabolites				
M+H 191.1185	$C_{11}H_{14}N_2O$	191.1184	174.092, 159.068	Methoxytryptamine
M+H 178.0858	$C_{10}H_{11}NO_2$	178.0868	nd	Hydroxytryptophol[1]
M+H 249.1231	$C_{13}H_{16}N_2O_3$	249.1239	190.0858	Hydroxymelatonin
M+H 285.1924	$C_{13}H_{24}N_4O_3$	285.1927	nd	Melanostatin[1]
M+H 152.1068	$C_9H_{13}NO$	152.1075	134.0961, 117.0695	Phenylpropanolamine
M+H 176.0715	$C_{10}H_9NO_2$	176.0712	nd	Hydroxyindoleacetaldehyde[1]
Nucleotides/metabolties				

M+H 166.0720	$C_6H_7N_5O$	166.0729	149.0459	Methylguanine
M-H 310.1163	$C_{12}H_{17}N_5O_5$	310.1151	nd	Dimethylguanosine[1]
M+H 268.1066	$C_{10}H_{13}N_5O_4$	268.1046	136.0615	Deoxyguanosine
M+H 282.1199	$C_{11}H_{15}N_5O_4$	282.1202	136.0620, 119.0350	Methyladenosine
M-H 167.0223	$C_5H_4N_4O_3$	167.0205	124.0163	Uric acid
Vitamins				
M+H 245.0953	$C_{10}H_{16}N_2O_3S$	245.0960	227.0860	Biotin
M-H 213.0699	$C_9H_{14}N_2O_2S$	213.0698	nd	Methyl bisnorbiotinyl ketone[1]
M-H 215.0483	$C_8H_{12}N_2O_3S$	215.0490	nd	Bisnorbiotin[1]
M+H 220.1185	$C_9H_{17}NO_5$	220.1186	202.1088	Pantothenic acid
Lipid metabolites				
M+H 185.0815	$C_5H_{15}NO_4P$	185.0817	nd	Phosphorylcholine[1]
Dietary compounds				
M-H 195.0518	$C_6H_{12}O_7$	195.0505	129.0197, 75.0090	Gluconic acid
M-H 165.0428	$C_6H_6N_4O_2$	165.0413	nd	Methylxanthine[1]
M+H 153.0665	$C_7H_8N_2O_2$	153.0664	nd	Methyl pyridonecarboxamide[1]
Smoking related compounds				
M+H 177.1029	$C_{10}H_{12}N_2O$	177.1028	80.049	Cotinine
M+H 193.0974	$C_{10}H_{12}N_2O_2$	193.0977	143.117	Cotinine N-Oxide
M+H 149.1074	$C_9H_{12}N_2$	149.1079	nd	Nornicotine[1]
M+H 193.0976	$C_{10}H_{12}N_2O_2$	193.0977	nd	Hydroxycotinine[1]
M+H 179.1176	$C_{10}H_{14}N_2O$	179.1184	nd	Nicotine-N-oxide[1]
M+H 163.1227	$C_{10}H_{14}N_2$	163.1235	146.095	Anabasine
Organic acids ≤10 carbons				
M+H 130.0862	$C_6H_{11}NO_2$	130.0868	84.0814, 56.0490	Pipecolic acid
M-H 138.0194	$C_6H_5NO_3$	138.0191	94.032	Hydroxypicolinic acid
M-H 115.0028	$C_4H_4O_4$	115.0031	71.0140	Maleic acid
M-H 182.0480	$C_5H_{13}NO_4S$	182.0487	nd	Choline sulfate[1]
M-H 111.0085	$C_5H_4O_3$	111.0082	67.0190	Furoic acid
M+H 175.0721	$C_6H_{10}N_2O_4$	175.0719	nd	Formiminoglutamic acid[1]
M+H 149.0929	$C_5H_{11}N_3O_2$	146.0930	104.0700, 87.0450, 86.0600	Guanidinobutanoic acid
M+H 106.0868	$C_4H_{11}NO_2$	106.0868	88.0760	Diethanolamine
M+H 144.0668	$C_4H_7N_3O$	144.0667	nd	Creatinine
Other				
M-H 308.0782	$C_{14}H_{15}NO_7$	308.0770	175.0243, 157.0147, 113.0241	Inodxyl glucuronide[1]

Table S-5. Metabolites not detected from analysis of combined SPE extracts compared with extracts from X-AW and X-C phases analysed separately

Urine samples were sequentially extracted by anion (X-AW) followed by cation (X-C) SPE phases and the SPE extracts analysed individually or as a combined extract by nUHPLC-nESI-TOFMS. Datasets were analysed by OPLS-DA and 'S' plot to determine which markers were not detected after combination of the SPE extracts. Metabolite identities confirmed with standards or comparison with fragmentation pattern in databases, [1]Putative identity based upon accurate mass measurement of the molecular ion. nd= metabolite signal too weak for detection of fragments.

Experimental mass	Structure	Theoretical mass	Fragments	Presence in SPE phase	Identity
Amino acid/ metabolites					
M-H 193.0608	$C_9H_{10}N_2O_3$	193.0613	149.071, 120.045	X-C	Aminohippuric acid
M-H 192.0664	$C_{10}H_{11}NO_3$	192.0661	148.075, 91.057	X-C	Methylhippuric acid
M-H 129.0546	$C_6H_{10}O_3$	129.0552	nd	X-AW	Ketoleucine[1]
M+H 295.1292	$C_{14}H_{18}N_2O_5$	295.1294	nd	X-C	Glutamylphenylalanine[1]
M+H 291.1314	$C_{10}H_{18}N_4O_6$	291.1305	nd	X-C	Argininosuccinic acid[1]
M+H 138.0915	$C_8H_{11}NO$	138.0919	121.065, 93.0690, 91.0534	X-C	m-Tyramine
M+H 304.1296	$C_{15}H_{17}N_3O_4$	304.1297	nd	Both	Indoleacetyl glutamine[1]
M+H 203.1502	$C_8H_{18}N_4O_2$	203.1508	nd	X-C	Symmetric dimethylarginine[1]
M+H 118.0861	$C_5H_{11}NO_2$	118.0868	101.06	X-C	Aminopentanoic acid
M+H 241.1296	$C_{10}H_{16}N_4O_3$	241.1301	nd	Both	Homocarnosine[1]
M+H 130.0862	$C_6H_{11}NO_2$	130.0868	84.0814, 56.049	X-C	Pipecolic acid
M+H 146.0823	$C_6H_{11}NO_3$	146.0817	nd	X-AW	Acetamidobutanoic acid[1]
M+H 118.0652	C_8H_7N	118.0657	91.0554	Both	Indole
M-H 308.0782	$C_{14}H_{15}NO_7$	308.0770	175.0243, 157.0147, 113.0241	X-C	Indoxyl glucuronide[1]
Carnitines					
M+H 218.1399	$C_{10}H_{19}NO_4$	218.1392	159.0628	X-C	Propionylcarnitine
Neurotransmitter/ metabolites					
M+H 198.1131	$C_{10}H_{15}NO_3$	198.1130	nd	X-AW	Metanephrine[1]
M+H 285.1924	$C_{13}H_{24}N_4O_3$	285.1927	nd	X-C	Melanostatin[1]
M+H 176.0712	$C_{10}H_9NO_2$	176.0712	nd	X-C	Hydroxyindoleacetaldehyde[1]
M+H 141.0654	$C_6H_8N_2O_2$	141.0664	nd	Both	Methylimidazoleacetic acid[1]
Vitamins					
M+H168.0665	$C_8H_9NO_3$	168.0661	150.0548, 127.0119	X-AW	Pyridoxal (Vit B6)
M+H 184.0610	$C_8H_9NO_4$	184.0606	166.0507, 148.0397	Both	Pyridoxic acid (Vit B6)
M+H 245.0953	$C_{10}H_{16}N_2O_3S$	245.0960	227.086	X-AW	Biotin
M-H 215.0483	$C_8H_{12}N_2O_3S$	215.0490	nd	X-C	Bisnorbiotin[1]
Lipid metabolites					
M+H 300.2902	$C_{18}H_{37}NO_2$	300.2903	282.2767	X-AW	Sphingosine
M+H 256.2644	$C_{16}H_{33}NO$	256.2640	nd	Both	Palmitic amide[1]
M+H 170.0450	$C_7H_7NO_4$	170.0453	nd	X-C	Furoylglycine[1]
M+H 158.0813	$C_7H_{11}NO_3$	158.0817	nd	X-AW	Methylcrotonylglycine[1]
M+H 232.1181	$C_{10}H_{17}NO_5$	232.1185	nd	X-AW	Suberylglycine[1]
M-H 227.2013	$C_{14}H_{28}O_2$	227.2011	nd	Both	Myristic acid[1]
M+H 181.0606	$C_8H_8N_2O_3$	181.0613	135.0556	X-AW	Nicotinuric acid
Dietary compounds					
M+H 138.0555	$C_7H_7NO_2$	138.0549	94.0661	Both	Trigonelline
M-H 221.0665	$C_8H_{14}O_7$	223.0818	175.0239, 157.0137, 113.0239	X-AW	Ethyl glucuronide[1]
M-H 149.0095	$C_4H_6O_6$	149.0086	103.0034, 87.009, 72.993, 59.015	Both	Tartaric acid

M-H 133.0138	$C_4H_6O_5$	133.0137	115.004, 71.0145	Both	Malic acid
Smoking related compounds					
M+H 163.1227	$C_{10}H_{14}N_2$	163.1235	146.095	X-C	Anabasine
Free pharmaceuticals					
M+H 152.0701	$C_8H_9NO_2$	152.0712	80.049, 110.061	Both	Acetaminophen
M-H 152.0350	$C_7H_7NO_3$	152.0348	nd	X-AW	Aminosalicylic acid[1]
Conjugated pharmaceuticals					
M+H 328.1043	$C_{14}H_{17}NO_8$	328.1032	175.0243,113.0239, 152.0701	X-AW	Acetaminophen Glucuronide[1]
Organic acid ≤10 carbons					
M-H 115.0028	$C_4H_4O_4$	115.0031	71.014	X-C	Maleic acid
M+H 168.0302	$C_7H_5NO_4$	168.0297	nd	X-AW	Quinolinic acid[1]
M+H 449.2554	$C_{18}H_{36}N_6O_5S$	449.2546	nd	Both	Glutathionylaminopropylcadaverine[1]
M-H 145.0136	$C_5H_6O_5$	145.0137	101.0241, 57.0354	Both	Oxoglutaric acid
M-H 147.0303	$C_5H_8O_5$	147.0293	129.0191, 103.0406, 85.0296, 57.0361	Both	Hydroxyglutaric acid
M-H 111.0085	$C_5H_4O_3$	111.0082	67.019	X-C	Furoic acid
M-H 129.0191	$C_5H_6O_4$	129.0188	85.03	Both	Glutaconic acid
M+H 146.1658	$C_7H_{19}N_3$	146.1657	nd	X-AW	Spermidine[1]
M-H 151.0397	$C_8H_8O_3$	151.0395	107.5	Both	Cresotinic acid
M+H 114.0659	$C_4H_7N_3O$	114.0667	nd	Both	Creatinine
M+H 106.0868	$C_4H_{11}NO_2$	106.0868	88.076	X-C	Diethanolamine