

Systematic Assessment of Seven Solvent and Solid-Phase Extraction Methods for Metabolomics Analysis of Human Plasma by LC-MS

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SUPPLEMENTARY MATERIALS

EXPERIMENTAL – SUPPLEMENTARY METHODS

Plasma extractions were analyzed on C18 or Scherzo columns coupled to QTOF via positive and negative ESI. Therefore, each sample was analyzed in four LC-MS modes: (i) reversed-phase C18 UPLC in positive and negative ESI and, (ii) mixed reversed-phase/ion-exchange Scherzo HPLC in positive and negative ESI. Buffer extractions were analyzed on Orbitrap Velos™ mass spectrometer in positive ESI.

LC-MS reversed phase method for the analysis of extractions in plasma

C18 separation was executed at 0.4 mL/min flow rate at 35°C using binary solvent system consisting of 0.1% formic acid, 2% acetonitrile in water (solvent A) and 0.1 % formic acid in 100% acetonitrile (solvent B). For LC-MS analysis in negative ESI, concentration of formic acid in both solvents was reduced to 0.05 %. The following gradient was used for separation: 2 % B for 3 min, then increase of B from 2 to 100 % for 20 min followed by 2 min hold at 100 % B and 4 min of re-equilibration to 100 % A.

Positive ESI QTOF settings were: capillary voltage of 3800 V for the entire run and nozzle voltage of 200 V for the first 4 min and 1500 V from 4th to 28th min of the analysis. In negative ESI, capillary and nozzle voltages were set to 3500 and 500 V, respectively during the first 5.5 minutes of run and to 4200 and 800 V, respectively between 5.5 and 29th minutes. For both positive and negative ESI, drying and sheath gas temperatures were set to 250 and 275°C and flow rates to 15 and 12 L/minutes, respectively. Nebulizer pressure was set to 30 psig and fragmentor voltage to 175 V. Data was acquired in both centroid and profile mode at the rate of 3 spectra per second in the extended dynamic range mode (2 GHz). Resolution of 12,000 FWHM (full width at half maximum) at m/z 121 and 24,000 FWHM at m/z 922 was achieved. To assure the desired mass accuracy of recorded ions, continuous internal calibration was executed using signals at m/z 121.0509 (protonated purine) and m/z 922.0098 (protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine (HP-921)) in positive ion mode. For negative ESI analysis, ions with m/z 119.0363 (deprotonated purine) and m/z 966.0007 (formate adduct of HP-921) were used.

LC-MS mixed-mode method for the analysis of extractions in plasma

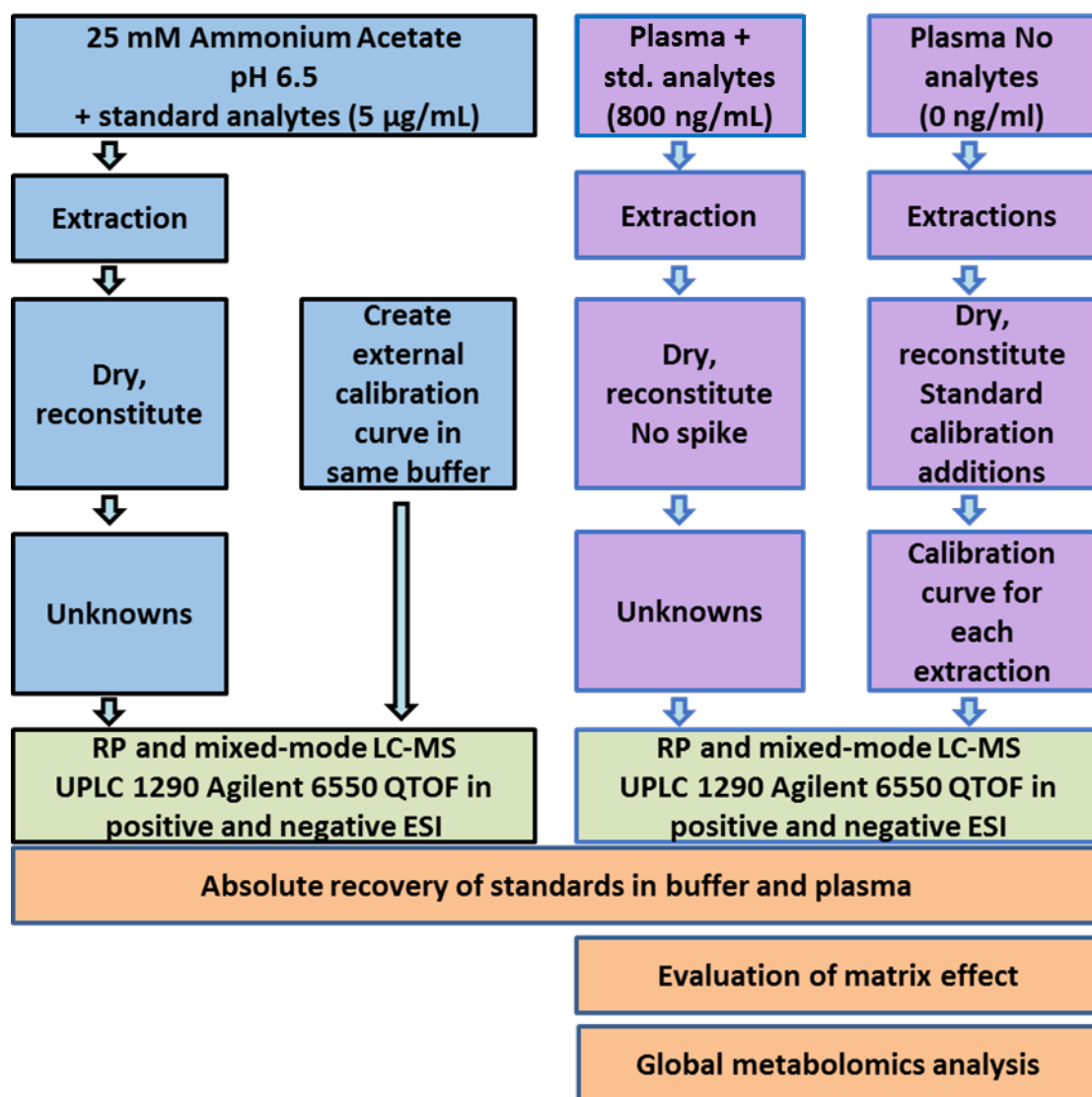
The stationary phase of Scherzo SM-C18 HPLC column is composed of C18 alkyl and weak cation and anion moieties. Therefore, solvent was supplemented with ammonium acetate buffer to execute mixed mode (reversed-phase/ion exchange) chromatography and elute ionic species from the column. The binary solvent system consisted of 2 mM ammonium acetate buffer, pH 3.5 in 2% acetonitrile and 98% of water (solvent A) and 100 mM ammonium acetate buffer, pH 3.5 in 96% acetonitrile and 4% of water (solvent B). The samples were separated at 0.22 mL/min flow rate at 35°C using the following gradient: 0 % B for the first 2 min, increase of B from 0 to 20% between 2nd and 10th min, increase of B from 20 to 100% between 10th and 25th min, isocratic hold at 100% B for 6 min and 6 min of re-equilibration to 100% A. In positive ESI, the capillary voltage was kept at 3500 V for the entire run, while nozzle voltage was set to 200 V for the first 5 minutes of run and to 800 V between 5th and 35th minutes. In negative ESI, nozzle and capillary voltages were held at 250 V and 3750 V for the entire run. All other MS settings were the same as described for LC-MS RP in previous section.

For targeted analysis, all samples were analyzed in random order with QC samples (mixture of all analyzed samples (iii) loaded at each 11th injection. XICs were extracted with 15 ppm accuracy and aligned within ± 0.15 minutes retention time interval. Metabolite identity was confirmed by the match of retention time and m/z between signals in analyzed samples and signals in calibration points. The concentrations of standard analytes were quantitated using external calibration curves obtained as described in materials and methods. For both targeted and global analyses, at least 10 runs of QC samples preceded batch runs in order to stabilize chromatography and ESI performance. For the global analysis, to ensure confident comparisons across extraction methods in global metabolomics analysis, samples from solvent, LLE and SPE extractions were analyzed side by side in 6 randomly organized sub-batches containing a single replicate from each of 7 extractions and one QC

sample. In order to analyze seven blank samples (type i) from seven methods in six sub-batches, five of sub-batches comprised one and the sixth sub-batch – two blank samples. PCA was performed using SIMCA 14 (Umetrics, Sweden) on all high-quality data described in Methods section, after Pareto scaling. PCA was used to verify the stability of LC-MS signals throughout the analysis by checking the clustering of QC signals for each analytical batch (Supplementary Figure 3). It can also be used to visualize repeatability and similarity between different extraction methods.

LC-MS mixed-mode method for the analysis of extractions in buffer

The column and buffer composition were identical to the analysis in plasma. Samples were separated at 0.22 mL/min flow rate at 35 °C using the following gradient: 0 % B for the first 2 min, increase of B from 0 to 100% between 2nd and 18th min, isocratic hold at 100% B for 6 min and 7 min of re-equilibration to 100 % A. Column was coupled to Orbitrap VelosTM mass spectrometer in positive ESI, at the following settings: ESI voltage 3.25 kV, heater and capillary temperature at 350 °C, sheet gas, auxiliary gas and sweep gas flow rates at 40, 10 and 0 arbitrary units, respectively; detection of signals was executed in the range 50-1000 m/z at 250 ms activation time, normalized collision energy 35 V, resolution 60,000 and mass accuracy 10 ppm. Deconvolution of raw data and quantitative analysis was executed using XcaliburTM, v. 2.0 (Thermo Fisher Scientific, Waltham, MA, USA) and statistical analysis in Microsoft Excel unless otherwise specified.



Supplementary Figure 1. Study design for the targeted and global metabolomics analysis of extraction methods. Blue and purple colors designate experiments in buffer and blood plasma, respectively. Green color designates LC-MS analyses step executed on RP (reversed phase) and mixed mode (weak anion/weak cation/RP) mode columns. Peach color designates major data analysis blocks.

Target analyte	Chemical group	Monoisotopic mass	RT on RP column (min)	RT on Scherzo column (min)	ACD/LogP	Buffer composition in the Individual stock
4-aminobutanoic acid	Zwitterion	103.0633	0.7	1.4	-0.9	50% methanol, 2 mM AmAc
4-aminobutanoic acid (d6)	Zwitterion	109.1043	0.6	1.4	-0.9	50% methanol, 2 mM AmAc
5-methoxytryptamine	Zwitterion	190.1106	6.6	8.6	1.3	Water
ACTH	Zwitterion	4508.0410	ND	ND	N/A	Water
Adenine	Positive	135.0545	0.9	2.8	-2.1	Water
Cholic acid	Negative	408.2875	13.2(-ve)	19.1 (-ve)	2.3	0.1% NH ₄ OH
Cholic acid (d4)	Negative	412.3167	13.2(-ve)	19.1 (-ve)	2.3	0.1% NH ₄ OH
Cortisol	Neutral	362.2093	10.7	16.8	1.4	50% methanol
Cortisol (d4)	Neutral	366.2366	10.7	16.8	1.4	50% methanol
Cortisone	Neutral	360.1937	10.8	16.9	1.4	50% methanol
Creatinine	Zwitterion	113.0589	0.7	1.6	-1.6	50% methanol
Sphingosine	Zwitterion	299.2824	14.6	20.4	6.4	50% methanol
Dopamine	Positive	153.0790	0.7	1.7	0.1	20% methanol, 0.1 % FA
Dopamine (d4)	Positive	157.1063	0.7	1.7	0.1	See Dopamine
Epinephrine	Negative	183.0895	ND	ND	-0.6	See Dopamine
Epinephrine (d3)	Negative	186.1100	ND	ND	-0.6	See Dopamine
Folic acid	Zwitterion	441.1397	6.6	14.7	-3	0.1% NH ₄ OH, 50% methanol
Glutamic acid	Zwitterion	147.0531	1.1	6.4	-3.9	water
Histamine	Zwitterion	111.0796	1.5	2	-0.7	water
Homovanillic acid	Negative	182.0579	7.3	14.2	1.1	as Folic acid
Homovanillic acid (d3)	Negative	185.0784	7.3	14.2	1.1	as Folic acid
Kynurenine	Zwitterion	208.0848	2.9	5.8	1.1	50% methanol, 0.1 % FA
Melatonin	Neutral	232.1210	9.2	15.5	1.2	50% methanol
Melatonin (d4)	Neutral	236.1485	9.2	15.5	1.2	50% methanol
Neurotensin	Zwitterion	1671.9097	8.2	12.7	N/A	0.1 % FA
Norepinephrine	Positive	169.0740	ND	ND	-0.9	see epinephrine
Norepinephrine (d6)	Positive	175.1149	ND	ND	-0.9	see epinephrine
Pantothenic acid	Negative	219.1107	5.0	8.6	-0.4	See Folic
PC (19:0/19:0)	Positive	818.5540	ND	ND	11.5	90% methanol, 10% IPA
PE (17:0/17:0)	Positive	719.5465	21.1	ND	11.5	
Phenylalanine (d5)	Zwitterion	170.1155	3.0	5.3	1.1	See Dopamine
Angiotensin II	Zwitterion	1045.5345	8.9	ND	N/A	See Dopamine
PI (18:3/22:4)	Neutral	908.5391	21.4	ND	11.5	See PC
Serotonin	Zwitterion	176.0950	2.5	6.8	0.2	See Dopamine
Thyroxine	Negative	776.6867	11.8	18.2	5.9	50-50 DMSO-methanol
Thyroxine (13C6)	Negative	782.6860	11.8	18.2	5.9	
Triiodothyronine	Negative	650.7900	11	17.2	5.1	
Tyrosine	Zwitterion	181.0739	1.4	3	0.4	See Dopamine
Thyrotropin releasing hormone	Positive	362.1703	1.5	2.6	N/A	See Dopamine

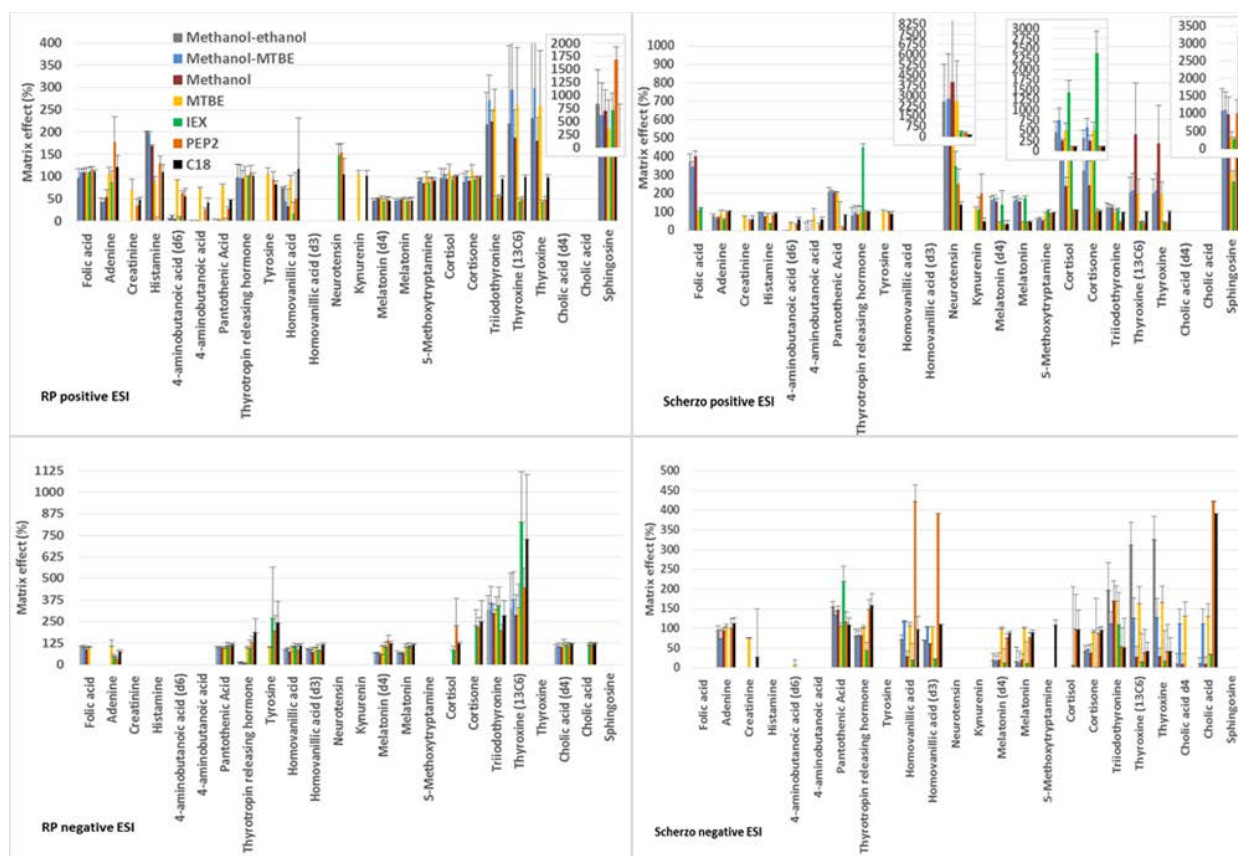
Supplementary Table 1 Standard analytes used in the study. Monoisotopic masses and predicted ACD/LogP values were obtained from ChemSpider (<http://www.chemspider.com>). LogP values predicted by ACDLabs algorithm (See Table 1 in the text for details). LogP predicted values could not be found for some metabolites in ChemSpider database and these entries are shown as not available (N/A) in the table. Retention times provided are for 500 ng/mL buffer calibration (= highest concentration analyzed) point analyzed in either ESI mode. ND stands for not detected and (-ve) designates analytes detected at negative ESI mode. ACTH, epinephrine, norepinephrine and PC (19:0/19:0) were not detected in any conditions and are removed from subsequent tables. For details on the usage and fate of analytes see Supplementary Tables 2, 3 and 7. Other abbreviations: AmAc - ammonium acetate, FA- formic acid, NH₄OH-ammonium hydroxide, IPA-isopropanol, DMSO-dimethylsulfoxide.

A. Recovery (% of input) of standard analytes from buffer							
Analytes	Methanol-Ethanol	Methanol	Methanol-MTBE	MTBE	C18	PEP2	IEX
Glutamic acid	79.0	106.3	70.8	ND	ND	25.7	73.4
Tyrosine	73.9	79.1	71.4	ND	ND	2.3	70.0
Creatinine	83.7	91.6	87.6	ND	ND	ND	ND
Thyrotropin releasing hormone	68.8	74.1	66.9	10.4	10.2	39.3	57.7
Pantothenic Acid	73.2	79.5	73.9	13.1	9.1	27.7	120.9
Histamine	112.2	122.4	98.7	ND	ND	133.6	67.0
4-aminobutanoic acid	58.6	123.7	61.7	ND	55.5	141.9	ND
Adenine	75.9	80.4	73.5	44.6	ND	46.1	108.4
Dopamine	35.2	34.4	38.0	82.0	8.1	55.5	9.8
Serotonin	106.2	117.5	110.5	54.7	35.3	107.0	31.2
Homovanillic acid	56.0	50.3	51.6	102.7	60.0	122.2	ND
Phenylalanine (d5)	64.7	69.2	66.5	14.3	11.1	22.8	128.8
5-methoxytryptamine	65.9	71.7	70.3	71.4	54.6	134.2	ND
Cortisol	26.7	33.6	29.1	112.6	108.9	70.3	ND
Cortisone	25.9	31.6	28.0	101.1	113.7	71.1	ND
Melatonin	93.6	58.8	99.0	104.1	109.8	122.4	ND
Triiodothyronine	76.3	83.3	75.4	90.5	105.4	142.8	ND
Thyroxine	18.8	25.9	10.3	57.2	18.6	ND	ND
Sphingosine	33.7	34.4	34.7	62.6	74.4	46.8	46.5
PI (19:0)	64.5	66.9	85.0	83.8	ND	ND	ND
PE (17:0)	89.6	103.6	81.7	45.5	ND	ND	ND
Angiotensin II	67.6	70.4	71.9	28.4	19.5	74.7	ND
B. Repeatability (RSD %) of extractions of standard analytes from buffer							
Glutamic acid	25.5	26.4	6.2	N/A	N/A	14.6	41.5
Tyrosine	4.6	12.2	5.7	N/A	N/A	9.0	30.8
Creatinine	9.0	8.7	5.8	N/A	N/A	N/A	N/A
Thyrotropin releasing hormone	4.0	12.6	4.0	42.0	3.0	14.0	11.2
Pantothenic Acid	3.9	8.6	1.5	65.0	26.8	13.2	18.9
Histamine	7.1	3.3	3.6	N/A	N/A	10.4	27.5
4-aminobutanoic acid	5.5	8.6	5.4	N/A	31.0	14.7	N/A
Adenine	14.0	20.4	14.1	46.9	N/A	17.1	13.1
Dopamine	8.4	11.0	23.4	24.6	25.9	20.8	40.5
Serotonin	6.4	2.9	4.1	17.3	22.6	11.7	10.9
Homovanillic acid	19.1	6.0	16.1	25.2	52.3	10.2	N/A
Phenylalanine (d5)	4.9	2.8	3.0	61.0	21.3	16.1	17.6
5-methoxytryptamine	5.5	3.2	4.6	12.3	25.2	3.6	N/A
Cortisol	24.6	23.8	27.3	6.8	18.6	29.4	N/A
Cortisone	16.9	24.9	18.9	8.7	13.9	27.1	N/A
Melatonin	14.4	9.6	12.1	9.0	20.5	16.8	N/A
Triiodothyronine	5.2	3.5	2.8	6.1	11.7	6.6	N/A
Thyroxine	15.0	56.3	15.6	55.6	18.3	N/A	N/A
Sphingosine	1.2	3.0	3.5	3.0	31.9	1.0	0.3
PI (19:0)	16.0	10.4	20.4	25.0	N/A	N/A	N/A
PE (17:0)	46.8	21	63.7	16.8	N/A	N/A	N/A
Angiotensin II	3.9	5.3	3.0	10.5	6.8	2.7	N/A

Supplementary Table 2. Recovery and precision of extractions of standard analytes from buffer. The table displays average recovery calculated from individual replicates (n=6, amount of standard analyte spiked before extraction = 100%) and a relative standard deviation (standard deviation/mean*100%, n=6) of recovered amounts. “ND” stands for “not detected”, “N/A” stands for “not applicable. LogP was obtained from ChemSpider (<http://www.chemspider.com/Default.aspx>) and represents predicted octanol-water partition coefficients (predicted ACD/LogP, ACD/Labs, Toronto, Canada. For details on the usage and fate of analytes see Supplementary Table 7.

A. Recovery (% of input) of standard analytes from plasma							
Analytes	Methanol-Ethanol	Methanol	Methanol-MTBE	MTBE	C18	PEP2	IEX
Folic acid	81.5	99.2	44.2	10.8	73.1	139.0	185.7
Adenine	91.1	75.3	96.6	13.0	244.0	245.6	ND
Histamine	28.8	68.0	46.3	ND	23.2	53.2	129.7
4-aminobutanoic acid (d6)	65.6	86.3	74.5	ND	2.4	ND	33.2
4-aminobutanoic acid	ND	ND	ND	ND	15.5	ND	ND
Pantothenic Acid	110.3	109.3	102.9	7.9	12.6	5.4	95.1
Tyrosine	ND	ND	ND	101.6	53.7	18.6	ND
Homovanillic acid	98.0	111.6	92.7	8.5	ND	115.6	99.1
Homovanillic acid (d3)	105.1	115.0	101.7	8.7	5.0	108.5	96
Kynurenine	ND	ND	ND	1.1	152.7	ND	ND
Melatonin (d4)	118.0	121.0	119.0	103.9	163.9	158.8	2.2
Melatonin	106.1	106.8	104.1	92.6	150.7	157.3	1.5
5-Methoxytryptamine	73.1	80.4	66.8	18.8	107.1	109.4	ND
Cortisol (d4)	93.4	90.7	80.7	69.4	5.2	2.7	ND
Cortisol	98.1	96.4	86.2	73.7	7.3	6.4	ND
Cortisone	69.3	74.0	38.3	74.5	2.9	0.4	ND
Cholic acid	114.4	123.5	121.4	ND	19.6	138.8	142.9
Cholic acid (d4)	114.3	114.6	110.6	ND	12.7	139.7	147.6
Triiodothyronine	46.7	56.8	58.8	11.7	90.0	125.3	6.9
Thyroxin (13C6)	56.7	77.5	68.5	3.8	84.9	117.0	6.2
Thyroxine	60.3	80.8	72.4	4.6	77.1	105.7	5.8
Sphingosine	3.9	27.4	10.5	56.6	56.0	75.0	21.4
Neurotensin	62.0	68.9	39.3	6.9	143.3	154.7	5.2
Thyrotropin releasing hormone	90.7	98.0	82.6	ND	102.9	125.8	134.9
B. Repeatability (RSD %) of extractions of standard analytes from plasma							
Folic acid	12.8	8.5	15.3	103.0	110.0	46.0	40.0
Adenine	8.7	16.3	15.6	69.2	81.2	46.0	N/A
Histamine	26.9	13.4	13.7	N/A	60.1	46.0	20.6
4-aminobutanoic acid (d6)	8.4	20.8	7.7	N/A	12.1	N/A	14.2
4-aminobutanoic acid	N/A	N/A	N/A	N/A	59.0	N/A	N/A
Pantothenic Acid	5.4	3.0	3.6	123.8	38.9	42.7	18.0
Tyrosine	N/A	N/A	N/A	115.6	61.4	63.8	N/A
Homovanillic acid	8.7	5.8	4.0	245.0	N/A	52.1	11.4
Homovanillic acid (d3)	5.9	7.0	8.7	235.0	0.5	48.7	12.8
Kynurenine	N/A	N/A	N/A	161.9	143.5	N/A	N/A
Melatonin (d4)	9.7	6.4	3.1	18.8	6.3	9.5	244.9
Melatonin	8.6	4.5	3.4	19.1	6.6	9.9	244.9
5-methoxytryptamine	7.0	4.1	5.3	58.2	14.6	22.6	N/A
Cortisol (d4)	5.2	5.4	8.9	35.0	91.6	131.4	N/A
Cortisol	6.2	7.8	8.8	36.7	58.5	53.8	N/A
Cortisone	9.2	7.4	42.5	29.4	93.0	N/A	N/A
Cholic acid	11.8	10.7	9.9	N/A	113.3	9.1	40.6
Cholic acid (d4)	12.6	9.0	9.8	N/A	110.6	9.6	38.0
Triiodothyronine	36.0	23.8	10.4	23.3	10.1	32.4	28.6
Thyroxin (13C6)	32.0	27.6	27.1	51.2	13.5	40.9	2.7
Thyroxine	33.0	28.8	27.1	45.3	13.7	41.7	1.9
Sphingosine	18.2	15.4	20.1	55.6	19.2	81.0	54.3
Neurotensin	10.7	7.4	11.0	111.2	43.1	24.7	52.3
Thyrotropin releasing hormone	23.8	27.1	22.8	N/Ap	68.4	37.1	10.5

Supplementary Table 3. Recovery and precision of extraction of standard analytes from plasma. The table displays average recovery calculated from individual replicates after subtraction of any endogenous level of metabolite present (n=6, amount of standard analyte spiked before extraction = 100%) and a relative standard deviation (standard deviation/mean*100%, n=6) of recovered amounts. LogP was obtained online from ChemSpider website (<http://www.chemspider.com/Default.aspx>) and represents predicted octanol-water partition coefficients (predicted ACD/LogP, ACD/Labs, Toronto, Canada). “ND” stands for “not detected”, “N/A” stands for “not applicable”. For details on the usage and fate of analytes see Supplementary Table 8.



Supplementary Figure 2 Ionization effects observed in human plasma in all LC-MS analysis across all analytes and extraction methods. On Y axis, graph displays area ratios between analytes of the same concentrations in buffer and a matrix calibration points ((area in spiked matrix-area in blank matrix) / (area in buffer-area in blank)*100%) assuming the area of analyte in the buffer as 100 %. Analytes are displayed along X-axis from left to right according to the increase of their predicted ACD/LogP, ACD Laboratories Toronto, Canada). Legend designating extraction methods on RP positive panel is same for all panels. Error bars represent relative standard deviation of matrix effects between different concentration levels tested. Due to very high matrix effect of some analytes in RP and Scherzo in positive ESI, full scale inserts were placed. Missing bars indicate that matrix effects could not be calculated due to one of three possible events: (i) analyte is not detected in buffer, (ii) analyte is not detected in a particular matrix or (iii) analyte signal was saturated (signal did not increase together with increased concentration at the concentration levels tested). The differences in matrix effect between 4-aminobutanoic acid and its deuterated analog in RP positive ESI (NDM) and Scherzo in positive ESI (methanol) is most likely due to the strong suppressing matrix effect. The differences in matrix effects between cholic acid, thyroxine and their deuterated analogs in RP in negative ESI are most likely due to the error in preparation of calibration points in the correspondent samples where it was not detected. Cortisol (d4) was removed from the graph because it was not detected in neither analysis except RP in positive ESI. See supplementary Table 6 for details.

Extraction method	Buffer				Spiked plasma			
	# of analytes RSD≤20 R≥80%	# of analytes RSD≤30 R≥50 %	Mean R (%)	Mean RSD (%)	# of analytes RSD≤20 R≥80 %	# of analytes RSD≤30 R≥50 %	Mean R (%)	Mean RSD (%)
Methanol-ethanol	4	15	65.9	11.9	11	16	80.4	14.3
Methanol	5	16	73.1	12.9	11	19	89.6	12.4
Methanol-MTBE	5	15	66.2	12.0	10	19	77.1	13.3
MTBE	4	10	63.5	25.6	2	3	37.1	85.4
C18	4	5	52.9	22.0	5	7	69.8	53.4
PEP2	6	11	77.0	13.3	4	6	100.1*	42.5
IEX	3	5	71.4	21.2	4	5	69.6	52.2

Supplementary Table 4 Efficiency of extraction methods in buffer and plasma for metabolite standards using the data obtained in RP analysis in positive or negative ESI mode. The first two columns compare the extraction methods using the number of analytes recovered in extractions from buffer and plasma at different thresholds of precision and recovery (R). The number of analytes recovered with RSD less than 20% and R≥80% and can be considered as metabolites where the extraction method provides excellent performance. The metabolites which have RSD less than 30%, and recovery higher than 50% can be considered as metabolites with acceptable performance in semi-quantitative methods such as global metabolomics. The table also shows mean recovery and repeatability (expressed as mean RSD) across all metabolites observed in a given extraction method. The total number of standard analytes detected from standard mixture by at least one extraction method is 22 metabolites for buffer and 24 metabolites for plasma extractions. Supplementary Table 8 summarizes which metabolites were detected/analyzed in only one matrix. *Higher mean recovery of PEP2 is caused by the larger number of analytes with enhanced matrix effect (Table 2) in RP analysis in positive ESI comparative to other extractions.

Metabolite	1-step MTBE extraction	2-step MTBE extraction
Folic acid	8.2 ± 5.6	29.4 ± 14
4-aminobutanoic acid	ND	ND
d6 4-aminobutanoic acid	ND	ND
Histamine	1.4 ± 0.4	ND
Creatinine	ND	ND
Adenine	2.6 ± 2.8	17.9 ± 4.6
d6-Norepinephrine	ND	ND
Tyrosine	ND	ND
Thyroid releasing hormone	ND	ND
5-Methoxytryptamine	ND	ND
Pantothenic Acid	ND	ND
Cortisone	96.4 ± 7.2	122 ± 13
Cortisol	98.1 ± 7.9	125 ± 10
Melatonin	142 ± 46	144 ± 14
d4 Melatonin	121 ± 41	118 ± 13
Triiodothyronine	13.6 ± 0.2	13.2 ± 3.2
13C6 Thyroxin	8.4 ± 0.1	8.3 ± 0.1
Sphingosine	73.9 ± 14	80.2 ± 5.4
Neurotensin	ND	ND

Supplementary Table 5. Recovery and precision of extractions of standard analytes from plasma in a follow-up experiment where one-step and two-step MTBE extractions were compared. The table displays average recovery calculated from individual replicates (n=6, amount of standard analyte spiked before extraction = 100%) and standard deviation of recovered amounts. “ND” stands for “not detected”,

Analytes	Charge class	Number of methods for which metabolite had $\geq 80\%$ recovery and $\leq 20\%$ RSD:		Number of methods without matrix effect			
		in buffer	in plasma	RP +ESI	Scherzo +ESI	RP -ESI	Scherzo -ESI
Folic acid	zwit.	NA	2	7	2	4	N/Ap
Adenine	pos.	2	2	2	3	1	5
Histamine	zwit.	4	0	2	5	N/Ap	4
4-aminobutanoic acid (d6)	zwit.	NA	0	1*	0	N/Ap	N/Ap
4-aminobutanoic acid	zwit.	3	0	0	0	N/Ap	0
Pantothenic Acid	neg.	1	4	0	1	7	3
Thyrotropin releasing hormone	zwit.	0	0	7	6	2	4
Tyrosine	zwit.	0	0	3	3	1	N/Ap
Homovanillic acid	neg.	1	4	2	0	6	3
Homovanillic acid (d3)	neg.	NA	4	0	0	6	3
Neurotensin	zwit.	NA	0	1	0	N/Ap	N/Ap
Kynurenine	zwit.	NA	0	2	2	N/Ap	N/Ap
Melatonin (d4)	N	NA	6	0	0	4	2
Melatonin	N	4	6	0	0	4	2
5-Methoxytryptamine	zwit.	1	2	7	4	N/Ap	1
Cortisol	N	2	3	7	2	1	2
Cortisol (d4)	N	2	3	7	N/Ap	N/Ap	N/Ap
Cortisone	N	2	0	7	2	0	3
Triiodothyronine	neg.	4	1	1	3	0	2
Thyroxine (13C6)	neg.	NA	1**	1	1	0	0
Thyroxine	neg.	0	0**	1	1	N/Ap	0
Cholic acid	neg.	NA	4	ND	ND	3***	2
Cholic acid (d4)	neg.	NA	4	ND	ND	7	2
Sphingosine	zwit.	0	0	0	0	0	0

Supplementary Table 6. Extraction quality and resistance of standard analytes to matrix effects across LC-MS methods.

Analytes ordered top to down accordingly to the LogP oct/water coefficient predicted by ACD laboratories algorithm. "ND" stands for not detected. "N" stands for neutral, "NA" stands not available as standard analyte at the time of extraction, "N/Ap" designates analytes for which matrix effect could not be calculated in any extraction method, "zwit." – for zwitterions, "neg." - for negative, "pos." - for positive considering the presence of either both, acidic or basic groups, respectively. Two columns "Number of methods for which metabolite had $\geq 80\%$ recovery and $\leq 20\%$ RSD:" provide the number of extraction methods in which an analyte was detected with RSD less than 20 and recovery higher than 80% in plasma or buffer. The matrix effect section represents the number of extraction methods (total =7) in which matrix effect was not observed for the standard analyte. For the standard analytes marked with asterisk, the matrix effect for isotope analog was just slightly outside 80% limit. Thus, 4-aminobutanoic acid demonstrated 71.6 % matrix effect in MTBE, while its deuterated analog demonstrated 89.4 %, ** recovery of thyroxine was slightly lower (77.1%) then for its deuterated analog (84.9 %). Both of these results lie within normal LC-MS experimental error and are not statistically significantly different. For example, using the above table it can be seen that histamine was recovered quantitatively and reproducibly by four extraction methods from buffer but in no methods from plasma. Histamine also showed no matrix effects in 2 and 5 extraction methods in RP and Scherzo analysis in positive ESI and in 4 extraction methods in Scherzo negative ESI analysis, respectively.

*** Cholic acid was not found in any of liquid extractions (4 in total) in RP analysis at negative ESI in contrary to its deuterated analog which was detected in all extractions.

Analytes	RP +ESI							Scherzo +ESI						
	Methanol-ethanol	Methanol-MTBE	Methanol	MTBE	IEX	PEP2	C18	Methanol-ethanol	Methanol-MTBE	Methanol	MTBE	IEX	PEP2	C18
Folic acid	95.4	108.2	109.1	106.4	109.9	114.4	111.9	372.8	344.0	400.6	100.5	119.5	NDM	NDM
Adenine	42.4	43.3	52.8	106.2	87.2	176.9	121.9	77.6	62.8	68.9	101.3	57.9	97.6	103.5
Histamine	198.9	196.1	168.7	88.4	SS	129.9	110.0	99.9	98.9	75.5	82.3	36.4	84.3	93.9
4-aminobutanoic acid (d6)	6.2	12.6	4.3	89.4	0.3	62.1	55.2	0.8	1.7	0.7	41.3	NDM	30.3	60.1
4-aminobutanoic acid	0.7	0.7	0.4	71.2	NDM	24.8	40.9	0.2	0.2	NDM	75.8	NDM	29.1	59.6
Pantothenic Acid	SS	SS	SS	77.5	SS	26.7	46.6	209.0	213.7	205.9	198.9	SS	SS	SS
Thyrotropin releasing hormone	97.5	96.1	95.5	101.0	101.2	106.6	101.5	80.6	97.2	86.3	99.3	450.2	100.3	100.0
Tyrosine	SS	SS	SS	105.2	SS	92.5	82.9	NDM	NDM	NDM	101.2	NDM	99.6	91.0
Homovanillic acid	72.7	43.2	32.5	91.8	17.0	50.0	116.6	SS	SS	SS	SS	SS	SS	SS
Homovanillic acid (d3)	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB
Neurotensin	SS	SS	SS	SS	148.6	152.7	104.8	2578.3	2750.1	3976.0	2542.4	346.6	250.5	138.7
Kynurenine	SS	SS	SS	104.2	SS	SS	101.7	NDM	NDM	NDM	112.7	115.9	199.8	47.9
Melatonin (d4)	45.3	46.1	50.0	50.7	43.8	47.5	45.9	161.7	171.0	157.3	46.6	140.2	35.0	35.3
Melatonin	45.2	45.9	47.4	48.3	43.9	45.4	45.7	158.8	168.0	155.7	45.3	172.3	43.8	44.8
5-Methoxytryptamine	88.4	92.7	83.8	100.7	86.9	93.9	91.3	57.4	65.7	52.6	92.7	107.3	92.3	97.6
Cortisol	95.1	105.1	93.8	111.2	92.1	99.9	99.9	442.4	742.5	240.8	481.7	1433.7	107.3	106.0
Cortisol (d4)	93.8	100.6	95.7	108.9	92.5	87.8	94.2	NDM	NDM	NDM	NDM	NDM	NDM	NDM
Cortisone	87.4	99.7	89.4	111.6	93.7	99.3	98.6	322.6	575.1	244.8	495.4	2391.1	104.7	104.2
Triiodothyronine	215.8	270.8	223.4	253.5	51.4	53.4	94.4	123.5	124.1	123.9	102.0	114.3	44.8	96.2
Thyroxine (¹³ C6)	219.3	295.1	187.1	261.0	44.3	47.4	98.6	206.3	216.3	519.5	201.7	43.6	40.4	100.9
Thyroxine	229.7	296.7	179.2	257.5	42.5	47.2	98.1	202.5	217.2	471.9	193.6	43.2	41.6	101.8
Cholic acid (d4)	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB
Cholic acid	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB
Sphingosine	745.4	825.8	618.1	695.2	352.1	713.4	1677.1	1075.2	1102.0	962.4	313.0	265.1	989.7	3217.0
Analytes	RP -ESI							Scherzo -ESI						
	Methanol-ethanol	Methanol-MTBE	Methanol	MTBE	IEX	PEP2	C18	Methanol-ethanol	Methanol-MTBE	Methanol	MTBE	IEX	PEP2	C18
Folic acid	96.6	102.5	90.1	102.8	NDM	NDM	NDM	NDB	NDB	NDB	NDB	NDB	NDB	NDB
Adenine	NDM	NDM	NDM	108.1	47.2	32.9	75.4	95.0	74.4	94.7	104.2	NDM	102.5	112.7
Histamine	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB
4-aminobutanoic acid (d6)	SS	SS	SS	SS	SS	SS	SS	NDM	NDM	NDM	8.7	NDM	NDM	NDM
4-aminobutanoic acid	SS	SS	SS	SS	SS	SS	SS	NDB	NDB	NDB	NDB	NDB	NDB	NDB
Pantothenic Acid	94.2	95.9	92.0	97.6	108.7	106.4	115.0	155.3	134.5	147.0	105.6	219.7	116.8	108.8
Thyrotropin releasing hormone	8.1	10.2	7.4	97.7	96.5	129.6	187.3	80.7	82.3	82.0	103.3	44.3	149.3	158.2
Tyrosine	SS	SS	SS	100.9	269.4	197.6	242.5	NDM	NDM	NDM	NDM	NDM	NDM	NDM
Homovanillic acid	81.7	91.5	75.6	101.2	107.0	85.7	110.3	71.5	118.1	29.1	106.7	20.5	422.1	97.5
Homovanillic acid (d3)	87.6	87.6	72.5	101.1	81.2	85.7	116.4	70.6	104.4	61.0	105.3	22.7	392.2	111.2
Neurotensin	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB
Kynurenine	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB
Melatonin (d4)	63.3	67.7	60.2	105.6	101.5	136.4	121.8	19.3	15.5	19.9	99.5	12.9	75.8	88.3
Melatonin	63.1	64.8	62.1	100.2	106.5	103.4	116.9	15.9	13.2	19.9	100.6	11.8	76.9	89.2
5-Methoxytryptamine	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDM	NDM	NDM	NDM	NDM	NDM	109.3
Cortisol	NDM	NDM	NDM	NDM	86.5	227.1	123.6	NDM	NDM	NDM	NDM	6.1	98.3	97.5
Cortisol (d4)	NDM	NDM	NDM	NDM	NDM	NDM	NDM	NDM	NDM	NDM	NDM	NDM	NDM	NDM
Cortisone	NDM	NDM	NDM	NDM	225.9	214.8	250.8	42.8	46.8	38.1	90.9	2.8	88.3	95.9
Triiodothyronine	315.4	359.2	297.3	320.6	341.6	198.8	286.2	197.0	112.4	170.4	169.9	109.6	54.8	51.5
Thyroxine (13C6)	322.5	377.5	286.4	330.7	828.3	447.4	728.4	312.0	127.1	27.4	163.1	16.3	39.4	42.3
Thyroxine	NDM	NDB	NDB	NDB	NDB	NDB	NDB	326.2	128.0	29.6	167.2	17.8	42.3	42.5
Cholic acid (d4)	94.9	105.0	99.0	116.4	114.5	112.1	119.0	9.9	112.3	9.6	131.9	NDB	NDB	NDB
Cholic acid	NDB	NDB	NDB	NDB	115.0	116.5	121.3	10.8	112.4	10.2	130.8	34.7	422.1	392.2
Sphingosine	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB	NDB

Supplementary Table 7 Summary of matrix effects observed for all metabolites across all extraction methods and LC-MS analyses. Not all analytes were successfully detected in buffer (NDB) or in an extract (NDM) which made the calculation of matrix effect impossible as specified. Analytes which did not respond to increased concentrations of spiked standards or which exhibited negative signal response were considered to be suppressed or saturated (SS) and were not used for calculation of matrix effects.

Analytes	Detection in extractions from buffer	Detection in extractions from plasma
4-aminobutanoic acid (d6)	NA	+
Angiotensin II	+	Replaced by Neurotensin
Cholic acid	NA	Added to supplement analysis in negative ESI
Cholic acid (d4)	NA	Added to supplement analysis in negative ESI
Cortisol (d4)	NA	+
Creatinine	+	Excluded (too high endogenous concentration)
Dopamine	+	ND
Dopamine (d4)	NA	ND
Folic acid	ND	+
Glutamic acid	+	Excluded (too high endogenous concentration)
Homovanillic acid (d3)	NA	Added to supplement analysis in negative ESI
Kynurenine	NA	+
Melatonin (d4)	NA	+
Neurotensin	NA	Added to represent large peptides
Phenylalanine (d5)	+	Used as internal reference
PI (18:3/22:4)	+	ND
PE (17:0/17:0)	+	ND
Serotonin	+	ND
Thyroxine (13C6)	NA	+

Supplementary Table 8 Usage and fate of standard analytes in targeted quantitation experiments in buffer and plasma. NA- not available at the time of analysis; ND – not detected. Inability to detect PE and PI phospholipids in plasma and PC in buffer and plasma is most likely is due to a high suppression effect at the end of chromatogram caused by LC-MS methodology which was not optimized for lipid metabolites.