SYSTEMATIC EVALUATION OF SOLID-PHASE MICROEXTRACTION COATINGS FOR UNTARGETED METABOLOMIC PROFILING OF BIOLOGICAL FLUIDS BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

SUPPLEMENTARY INFORMATION

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SUMMARY

This supporting information file includes additional results and information as described in the text of the main article including (1) list of SPE sorbents and their properties used in the coating evaluation, (2) summary of physicochemical properties of metabolites included in standard metabolite mixture, (3) summary of optimized LC-MS conditions used on Varian 500 ion trap instrument during the coating evaluation experiments, (4) detailed description of procedures and calculations for the determination of correction factors to use for coating comparison, (6) results for the dependence of the amount extracted on extraction time for the set of known metabolites after extraction of human plasma, (7) results for the carryover determination for the set of known metabolites after extraction of human plasma, (8) overview of proposed workflow for metabolomics using SPME, (9) results for the determination of absolute matrix effects (ionization suppression) using both reverse phase and HILIC methods for the analysis of human plasma using mixed-mode SPME fibre and (10) metabolite coverage obtained using plasma protein precipitation with acetonitrile (PP), plasma protein precipitation with methanol/ethanol (PM) and ultrafiltration (UF) in combination with negative reverse phase LC-MS method with pentafluorophenyl column.

Supplementary Table 1. Summary of SPE sorbents and their properties.

Sorbent	Manufacturer	Support	Type	Functional group	Particle size (µm)	Surface area (m^2/g)	Pore size (Å)
Clean Screen DAU	UCT	silica	reverse phase+SCX	proprietary and benzenesulfonic acid	40-63	NA	NA
Clean Screen GHB	UCT	silica	proprietary mixed-mode		NA	NA	NA
SSBCX	UCT	silica	SCX	benzenesulfonic acid	40-63	NA	NA
C18+B	UCT	silica	reverse phase+SCX	C_{18} and benzenesulfonic acid	40-63	NA	NA
C8+B	UCT	silica	reverse phase+SCX	C_8 and benzenesulfonic acid	40-63	NA	NA
RPA	Supelco	silica	reverse phase	C_{16} with embedded amide	3	450	100
HiSEP	Supelco	silica	reverse phase	Surface modified with hydrophilic polymer	5	NA	120
PEG	Supelco	silica	reverse phase	polyethylene glycol	5	NA	120
Discovery MCAX	Supelco	silica	reverse phase+SCX	C_8 and benzenesulfonic acid	50	480	70
DPA 6S	Supelco	polymer	adsorption of compounds containing -OH and -COOH	polyamide resin	50-120	NA	NA
Oasis MCX	Waters	polymer	reverse phase + SCX	N-vinylpyrrolidone divinyl benzene copolymer + sulfonic acid	30	810	80
Oasis WAX	Waters	polymer	reverse phase + WAX	N-vinylpyrrolidone divinyl benzene copolymer + piperazine	30	810	80
Oasis WCX	Waters	polymer	reverse phase + WCX	N-vinylpyrrolidone divinyl benzene copolymer + carboxylic acid	30	810	80

Sorbent	Manufacturer	Support	Type	Functional group	Particle size (µm)	Surface area (m^2/g)	Pore size (Å)
Oasis MAX	Waters	polymer	reverse phase + SAX	N-vinylpyrrolidone divinyl benzene copolymer + quartenary amine	30	810	80
HRP	Macherey Nagel	polymer	reverse phase	highly porous styrene divinylbenzene polymer	50-100	1200	NA
HRX	Macherey Nagel	polymer	reverse phase	hydrophobic styrene divinylbenzene polymer	85	1000	55-60
Carboxen-1016	Supelco	carbon	adsorption	graphitized carbon, 60/80 mesh	177-250	NA	NA
Diamino	Macherey Nagel	silica	special	primary and secondary amine	45	500	60
Easy	Macherey Nagel	polymer	reverse phase + WAX	polar-modified styrene divinylbenzene polymer + unknown WAX group	80	650-700	50
AccuCAT	Varian	silica	SAX + SCX	sulfonic acid and quartenary amine	40	NA	60
Spe-ed Advanta	Applied Separations	polymer	reverse phase + WCX	polar-modified styrene divinylbenzene polymer (carboxylic acid modification)	NA	NA	NA
Certify	Varian	silica	reverse phase + SCX	C_8 and benzenesulfonic acid	40	NA	60
Certify II	Varian	silica	reverse phase + SAX	C_8 and quartenary amine	40	NA	60
CH	Applied Separations	silica	reverse phase	cyclohexyl	40	NA	60
Focus	Varian	polymer	normal + reverse phase	polar-modified styrene divinylbenzene polymer	NA	NA	NA
Screen A	Phenomenex	silica	reverse phase + SAX	C_8	55	500	70

Sorbent	Manufacturer	Support	Type	Functional group	Particle size (µm)	Surface area (m^2/g)	Pore size (Å)
Screen C	Phenomenex	silica	reverse phase + SCX	C_8 and benzenesulfonic acid	55	500	70
Strata X	Phenomenex	polymer	reverse phase	surface modified styrene divinylbenzene polymer with pyrrolidone group	33	800	85
Strata XAW	Phenomenex	polymer	reverse phase + WAX	surface modified styrene divinylbenzene polymer + diamine group	33	800	85
Strata XCW	Phenomenex	polymer	reverse phase + WCX	surface modified styrene divinylbenzene polymer + carboxylic acid	33	800	85
PBA	Varian	silica	covalent	phenylboronic acid	40	NA	60
PH	Applied Separations	silica	reverse phase	phenyl	40	NA	60
Plexa	Varian	polymer	reverse phase	proprietary highly polar hydroxylated polymer	NA	NA	NA
Plexa PCX	Varian	polymer	reverse phase + SCX		NA	NA	NA

Supplementary Table 2 Physicochemical properties of metabolites included in standard metabolite mixture.

Analyte	Formula	Molecular Weight (MW)	pKa[1]	Log P[1]
3-hydroxybutyric acid (HBA)	$C_4H_8O_3$	104.1	4.41	-0.47
Adenine	$C_5H_5N_5$	135.1	4.15	-0.09
Adenosine	$C_{10}H_{13}N_5O_4$	267.2	NA	-1.05
Adenosine diphosphate (ADP)	$C_{10}H_{15}N_5O_{10}P_2$	427.2	NA	-2.64
Adenosine monophosphate (AMP)	$C_{10}H_{14}N_5O_7P$	347.2	NA	-1.68
Adenosine triphosphate (ATP)	$C_{10}H_{16}N_5O_{13}P_3$	507.2	NA	-3.61
β-Estradiol	$C_{18}H_{24}O_2$	272.4	NA	4.01
β-NAD	$C_{21}H_{27}N_7O_{14}P_2$	663.4	NA	-3.68
Cholic acid	$C_{24}H_{40}O_5$	408.6	4.98	2.02
Choline	$C_5H_{14}NO^+$	104.1	NA	-5.16
Citric acid	$C_6H_8O_7$	192.1	2.79	-1.64
Fructose	$C_6H_{12}O_6$	180.2	12.1	-1.55
Fumaric acid	$C_4H_4O_4$	116.1	3.03	0.46
Glucose	$C_6H_{12}O_6$	180.2	12.9	-3.24
Glucose 6-phosphate	$C_6H_{13}O_9P$	260.1	1.11	-3.79
Glutamic acid	$C_5H_9NO_4$	147.1	2.23	-3.69
Glutathione (oxidized)	$C_{20}H_{32}N_6O_{12}S_2$	612.6	NA	-7.89
Glutathione (reduced)	$C_{10}H_{17}N_3O_6S$	307.3	NA	-5.41
Histamine	$C_5H_9N_3$	111.1	9.8	-0.7
Histidine	$C_6H_9N_3O_2$	155.2	2.76	-3.32
Hydrocortisone (cortisol)	$C_{21}H_{30}O_5$	362.5	NA	1.61
Linoleic acid	$C_{18}H_{32}O_2$	280.4	4.77	7.05
Lysine	$C_6H_{14}N_2O_2$	146.2	3.12	-3.05
Maleic acid	$C_4H_4O_4$	116.1	1.83	-0.48
Nicotinamide	$C_6H_6N_2O$	122.1	3.35	-0.37
Phenylalanine	$C_9H_{11}NO_2$	165.2	1.24	-1.38
Progesterone	$C_{21}H_{30}O_2$	314.5	NA	3.87
Protoporphyrin IX	$C_{34}H_{34}N_4O_4$	562.7	NA	7.43
Pyruvic acid	$C_3H_4O_3$	88.1	2.45	-1.24
Riboflavin	$C_{17}H_{20}N_4O_6$	376.4	10.2	-1.46
Ribose-5-phosphate	$C_5H_{11}O_8P$	230.1	NA	-2.65
Sucrose	$C_{12}H_{22}O_{11}$	342.3	12.6	-3.7
Taurocholic acid	$C_{26}H_{45}NO_7S$	515.7	NA	0.01
Thyroxine	$C_{15}H_{11}I_4NO_4$	776.8	NA	4.12
Tryptophan	$C_{11}H_{12}N_2O_2$	204.2	7.38	-1.06
Uridine diphosphate glucose (UDPG)	$C_{15}H_{24}N_2O_{17}P_2$	566.3	NA	-5.8

1 Syracuse Research Corporation, *PhysProp Database, accessed May 2009.*

Supplementary Table 3. Summary of optimized LC-MS parameters on Varian 500 iontrap LC-MS instrument

LC parameter	Reverse phase LC method	HILIC LC method
Analytical column	HS F5 Pentafluorophenyl (Supelco)	Ascentis Si (Supelco)
Column dimensions	2.1 x 10 mm	2.1 x 10 mm
Particle size	3 µm	3 µm
Mobile phase A	Water/acetic acid (99.9/0.1, v/v)	ammonium formate/acetonitrile (5/95, 2 mM total)
Mobile phase B	water/acetonitrile/acetic acid (89.9/10/0.1)	ammonium formate/acetonitrile (40/60, 2 mM total)
Flow rate	200 µL/min	200 µL/min
Injection volume	20 µL	20 µL
Run time	35 min	30 min
Gradient program	0-3 min 100% A, 3-20 min linear gradient to 10%A, 20-30 min hold at 10% A, 5 min re-equilibration at 100% A	0-2 min 100% A, 2-14 min linear gradient to 65%A, 14-18 min hold at 65% A, 12 min re-equilibration at 100% A
Nebulizer pressure	60 psi	60 psi
Drying gas pressure	22 psi	22 psi
Temperature	400℃	400℃

Determination of correction factors to use for coating comparison

To prepare coatings with commercial sorbents, particles of different size were immobilized because of limited commercial availability of 5 µm particles in SPE cartridge format, while coating length and solid support core were kept constant at 15 mm and 1.55 mm, respectively for all lab-made coatings in this study. The coatings obtained from Supelco (both commercial and prototype) had different dimensions than lab-made coatings, so appropriate correction factors were needed to enable direct comparison of all coatings. Coating volume was estimated by simply treating all types of coatings as cylinders and assuming the entire volume of immobilized phase could act as sorbent. Based on SEM results, it was determined that a single layer of sorbent particles was immobilized using the described procedure for lab-made coatings for all particle sizes ≥ 5 µm. Therefore, particle size was used as an approximation of coating thickness. However, particle size of 3 µm resulted in multiple layer coverage with estimated coating thickness of 10 µm, so this was used as coating thickness for this particular type of coating. In the next step, the volume of two cylinders was calculated: (i) the volume of fibre core cylinder only (radius = core diameter/2) and (ii) the volume of entire fibre (radius = fibre core diameter/2 + coating thickness). The volume of the coating is then determined by the subtraction of fibre core volume (i) from the total volume of entire fibre (ii). Full calculations for all types of coatings used in current study are shown in Supplementary Table 2. For sorbents where a range of particle sizes was given, the mean size was used as coating thickness for volume estimation. For example, for HR-P sorbent where size was reported as 50-100 µm, the value of 75 µm was used to estimate the coating volume. Clearly, the correction factors are only approximate because they assume (i) tight packing of spherical particles within the coating (ii) uniform size of all particles (iii) same extraction efficiency for sorbent and binder and (iv) do not take into account different surface areas and porosity of particles. Despite of this, the approximation is useful to give an estimate of how the performance of new coatings compares to existing coatings and to ensure coatings are not accidentally rejected simply based on different dimensions. Particle size information for Plexa, Plexa PCX and Focus sorbents from Varian and for Speed Advanta sorbent from Applied Separations could not be obtained as shown in Table 1 so no correction factor was applied to these coatings. This is in agreement with visual examination (< 50 µm), so 40 µm particle size appears to be a reasonable assumption for these coatings. No correction factor was applied for carbon tape coatings as the proportion of carbon was unknown. Therefore, the results for this coating are reported "as is", whereas the results for all other coatings are reported with respect to performance of 40 µm particle size which was arbitrarily selected as the reference point as shown in Supplementary Table 3.

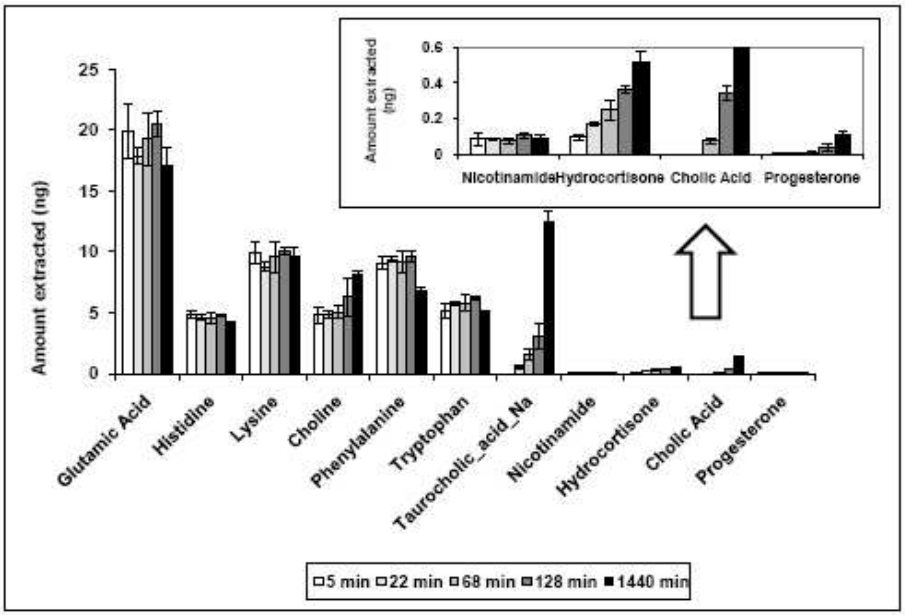
Supplementary Table 4. Determination of correction factors to use during coating comparison.

	Thickness (µm)	Length (cm)	Core (µm)	Fibre diameter (µm)	Fibre radius (µm)	Core radius (µm)	Total volume ($\mu m^2 cm$)	Core volume ($\mu m^2 cm$)	Coating volume ($\mu m^2 cm$)	Correction factor
lab-made	213.5	1.5	1550	1977	988.5	775	4.60E+07	2.83E+07	1.77E+07	0.17
lab-made	85	1.5	1550	1720	860	775	3.49E+07	2.83E+07	6.55E+06	0.46
lab-made	80	1.5	1550	1710	855	775	3.44E+07	2.83E+07	6.14E+06	0.49
lab-made	51	1.5	1550	1652	826	775	3.22E+07	2.83E+07	3.85E+06	0.78
lab-made	55	1.5	1550	1660	830	775	3.25E+07	2.83E+07	4.16E+06	0.72
lab-made	75	1.5	1550	1700	850	775	3.40E+07	2.83E+07	5.74E+06	0.52
lab-made	40	1.5	1550	1630	815	775	3.13E+07	2.83E+07	3.00E+06	1.00
lab-made	45	1.5	1550	1640	820	775	3.17E+07	2.83E+07	3.38E+06	0.89
RPA 3 µm	10	1.5	1550	1570	785	775	2.90E+07	2.83E+07	7.35E+05	4.08
lab-made	5	1.5	1550	1560	780	775	2.87E+07	2.83E+07	3.66E+05	8.18
lab-made	33	1.5	1550	1616	808	775	3.08E+07	2.83E+07	2.46E+06	1.22
lab-made	30	1.5	1550	1610	805	775	3.05E+07	2.83E+07	2.23E+06	1.34
lab-made	50	1.5	1550	1650	825	775	3.21E+07	2.83E+07	3.77E+06	0.80
CW TPR	50	1.0	160	260	130	80	5.31E+05	2.01E+05	3.30E+05	9.09
biocompatible prototypes	45	1.5	200	290	145	100	9.91E+05	4.71E+05	5.20E+05	5.77
PA	85	1.0	160	330	165	80	8.55E+05	2.01E+05	6.54E+05	4.58
PDMS	100	1.0	160	360	180	80	1.02E+06	2.01E+05	8.17E+05	3.67
PDMS DVB	60	1.0	160	280	140	80	6.16E+05	2.01E+05	4.15E+05	7.23

Supplementary Table 5. Dependence of the extraction efficiency of Waters Oasis MAX, MCX, WAX and WCX coatings on sample pH.

pH	Coating type	ADP	AMP	ATP	Beta NAD	HBA	Sucrose
pH 3.0	WAT MAX	ND	0.01	ND	0.13	23	0.54
	WAT MCX	ND	0.04	ND	0.38	32	0.55
	WAT WAX	ND	0.01	ND	0.15	24	0.48
	WAT WCX	ND	0.01	ND	0.15	30	0.53
pH 5.0	WAT MAX	ND	0.19	ND	ND	39	0.22
	WAT MCX	0.03	0.16	ND	ND	35	0.30
	WAT WAX	ND	0.19	ND	ND	48	0.28
	WAT WCX	ND	0.15	ND	ND	49	0.29
pH 7.4	WAT MAX	ND	0.15	ND	1.1	27	0.31
	WAT MCX	0.05	0.12	ND	0.72	29	0.26
	WAT WAX	ND	0.21	ND	0.86	39	0.29
	WAT WCX	0.02	0.13	ND	0.64	33	0.32
pH 9.5	WAT MAX	0.05	0.32	ND	8.7	2.2	0.78
	WAT MCX	0.09	0.27	0.05	2.8	2.5	0.41
	WAT WAX	0.17	0.74	ND	7.5	0.78	0.91
	WAT WCX	0.31	0.62	0.15	5.8	1.4	0.87

Supplementary Figure 1. Dependence of the amount extracted of selected metabolites on extraction time using Supelco mixed-mode fibres (n=3 at each time point) after positive ESI pentafluorophenyl LC-MS analysis. The inset graph shows expanded region to facilitate the comparison of analytes with sub-ng amounts extracted. Polar metabolites (glutamic acid, histidine, lysine, choline, phenylalanine, tryptophan, nicotinamide) reached equilibrium within the shortest extraction time tested (≤ 5 min). More hydrophobic metabolites (taurocholic acid, cholic acid, hydrocortisone and progesterone) required longer extraction times (≥ 1440 min) to reach equilibrium. Table insert shows the results for ANOVA test indicating extraction time is not significant variable at 95% confidence for glutamic acid, histidine, lysine and nicotinamide when all time points are used. However, further examination of this data excluding the last time point (1440 min) indicates that equilibrium is in fact reached for choline, phenylalanine and tryptophan as well, because no statistically significant increases are observed as the extraction time is increased from 5-128 min. We attribute the discrepancy in P-values for the two ANOVA tests (including and excluding 1440 min extraction) to possible changes in the composition of plasma sample during 1440 min room temperature extraction due to degradation.

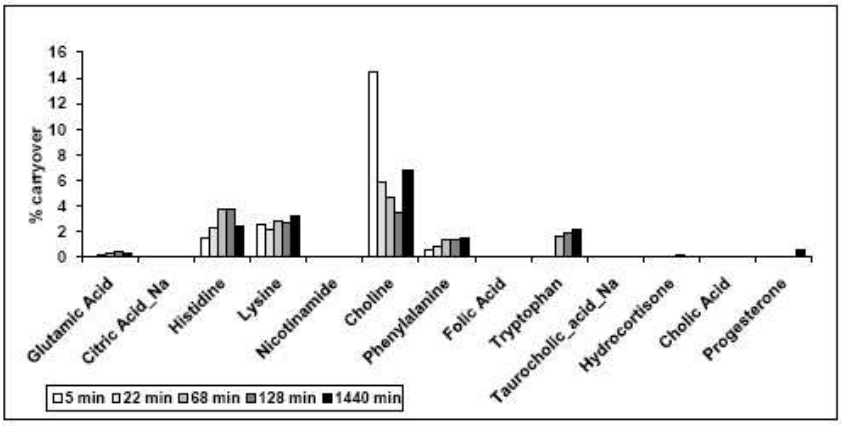


	All time points P-value	5-128 min timepoints P-value	Conclusion
Glutamic acid	0.13	0.32	Equilibrium reached within 5 min
Histidine	0.07	0.65	Equilibrium reached within 5 min
Lysine	0.36	0.27	Equilibrium reached within 5 min
Choline	2.0E-03	0.23	Equilibrium reached within 5 min
Phenylalanine	4.9E-04	0.63	Equilibrium reached within 5 min

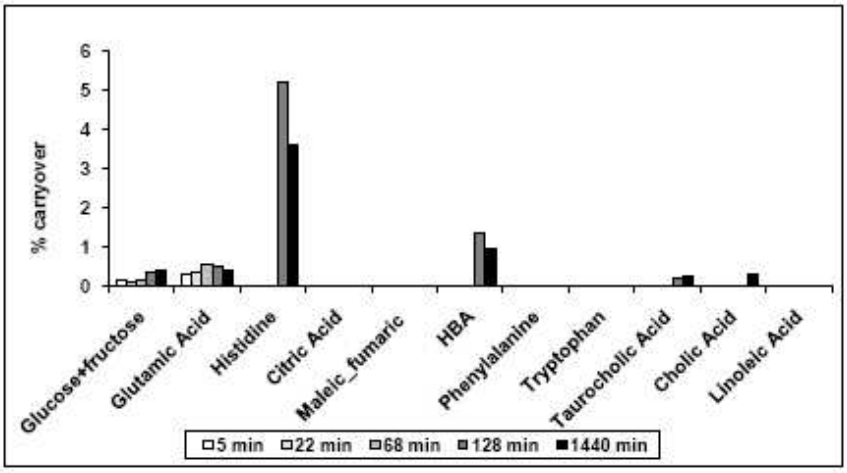
Tryptophan	0.02	0.10	Equilibrium reached within 5 min
Taurocholic acid	1.6E-09	7.7E-04	Equilibrium not reached
Nicotinamide	0.38	0.28	Equilibrium reached within 5 min
Hydrocortisone	8.5E-07	1.8E-05	Equilibrium not reached
Cholic acid	3.4E-08	1.1E-07	Equilibrium not reached
Progesterone	3.4E-08	0.03	Equilibrium not reached

Supplementary Figure 2. Amount of carryover observed for a set of identified metabolites after extraction of a human plasma sample for 5, 22, 68, 128 and 1440 min. In all cases, desorption was performed using 300 µL of acetonitrile/water (1/1, desorption solvent, 1 hr, 1000 rpm vortex agitation), and carryover was evaluated by performing a second desorption using a fresh portion of desorption solvent. Samples were analyzed using reverse-phase LC-MS method with a pentafluorophenyl column (a) in positive ESI mode and (b) in negative ESI mode. HBA stands for hydroxybutyric acid.

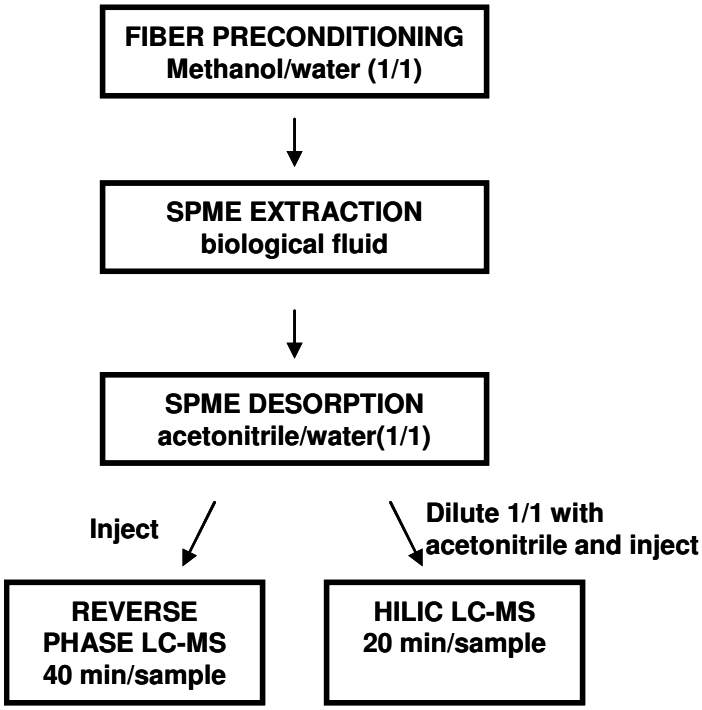
(a)



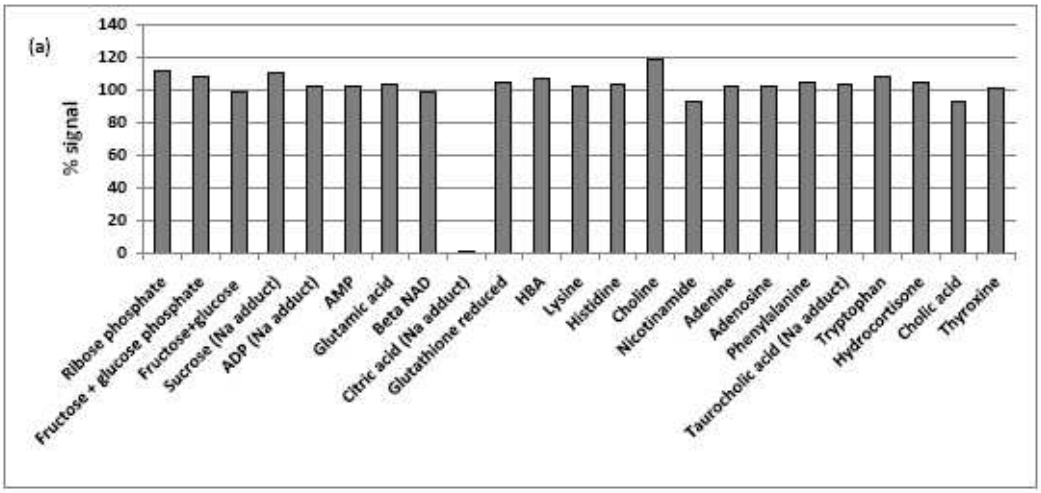
(b)

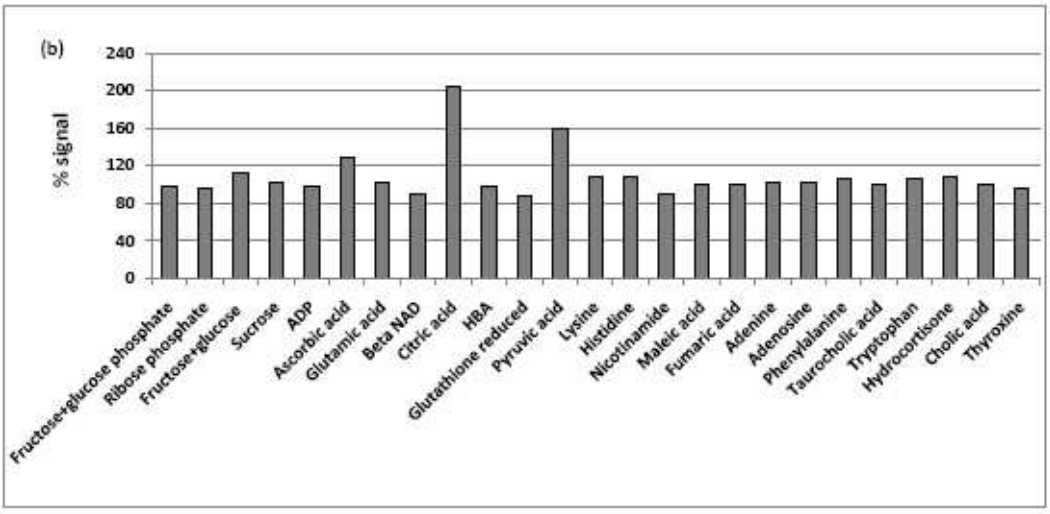


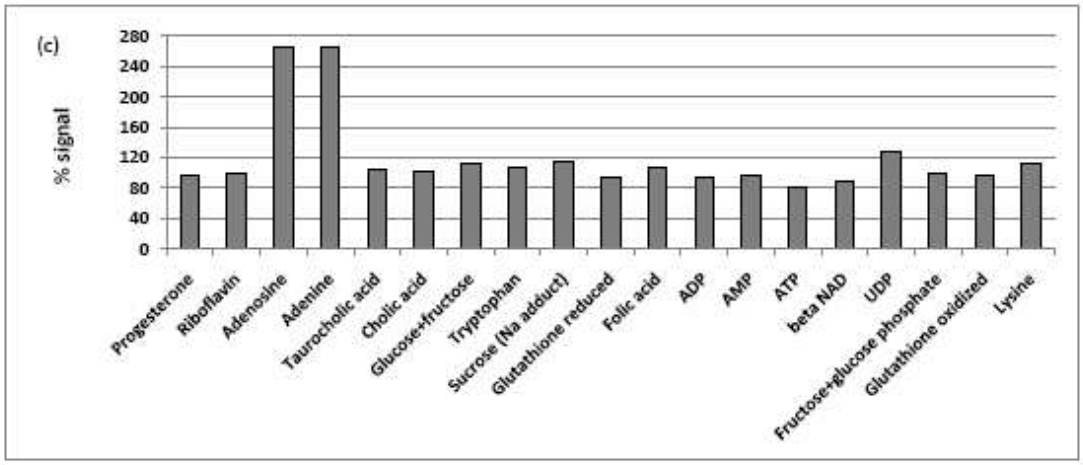
Supplementary Figure 3. Recommended SPME workflow for extraction of human plasma for metabolite profiling studies using mixed-mode (C18+ benzenesulfonic acid) coating.



Supplementary Figure 4. Absolute matrix effects for identified metabolites in human plasma using SPME as sample preparation method and (a) a positive ESI LC-MS method with pentafluorophenyl column and (b) a negative ESI LC-MS method with pentafluorophenyl column (c) a positive ESI HILIC LC-MS method and (d) a negative ESI HILIC LC-MS method.



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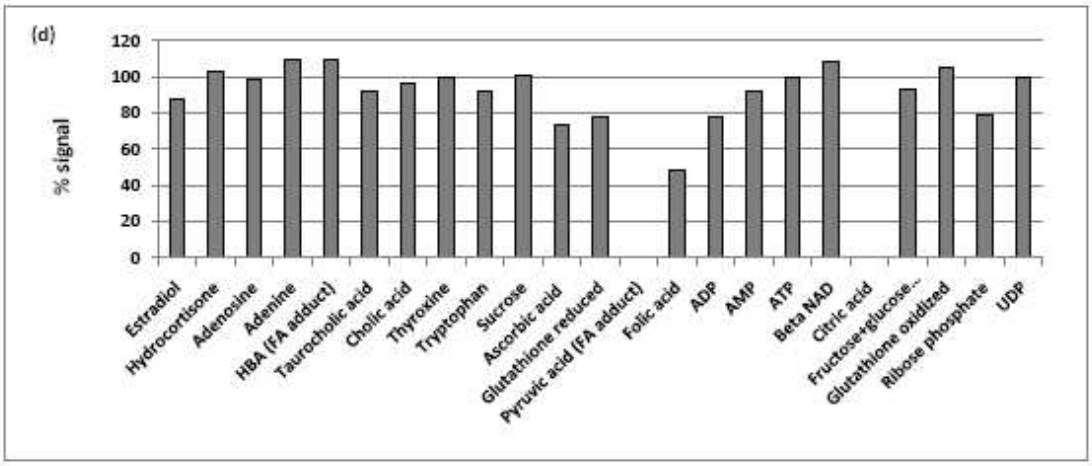
Progesterone
Riboflavin
Adenosine
Adenine
Taurocholic acid
Cholic acid
Glucose+fructose
Tryptophan
Sucrose (Na adduct)
Glutathione reduced
Folic acid
ADP
AMP
ATP
beta NAD
UDP
Fructose+glucose phosphate
Glutathione oxidized
Lysine


(d)
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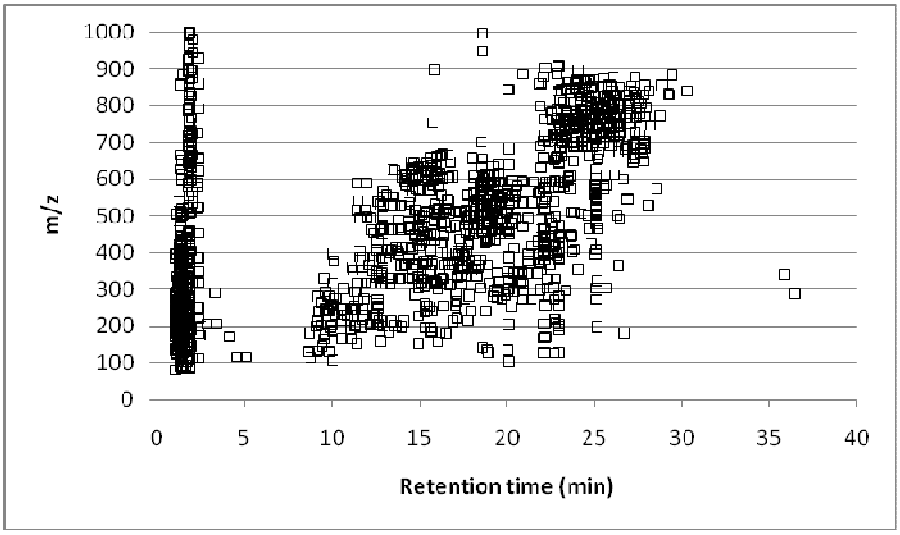
Adenine
HBA (FA adduct)
Taurocholic acid
Cholic acid
Thyroxine
Tryptophan
Sucrose
Ascorbic acid
Glutathione reduced
Pyruvic acid (FA adduct)
Folic acid
ADP
AMP
ATP
Beta NAD
Citric acid
Fructose+glucose...
Glutathione oxidized
Ribose phosphate
UDP

Supplementary Figure 5. Comparison of metabolite coverage in human plasma in the format of ion map and obtained using (a) PP (b) PM and (c) UF using negative ESI LC-MS method with a pentafluorophenyl column.

(a)



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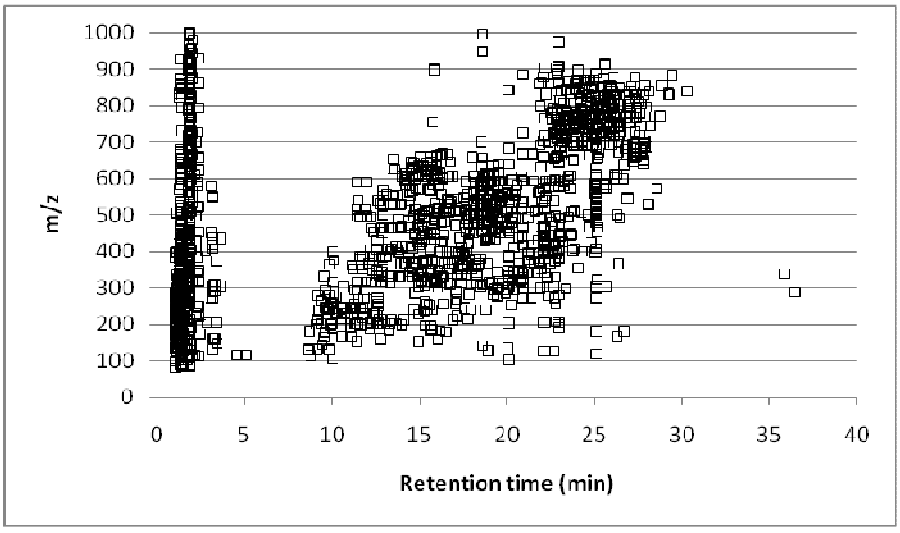

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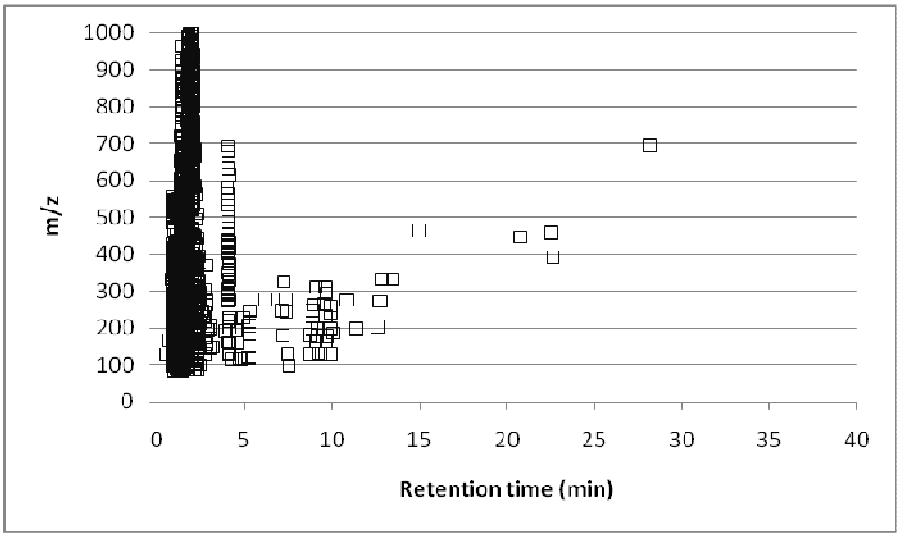
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