Electronic Supplementary Material (ESI) for Analytical Methods.

Effects of harvesting and extraction methods on metabolite recovery from adherently growing mammalian cells

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Table S2 List of statistical results for differential metabolites harvested using four different extraction methods

Text S1 Experimental procedures for cell harvesting and extraction

Methods for cell harvesting

For trypsin/EDTA treatment sample collection, after removing the cell culture medium, cells were rapidly washed twice with 2 mL PBS, and 200 μL trypsin/EDTA solution was added to the plates. After incubation at room temperature for 2 min and tapping of the plates, 400 μL medium and 400 μL PBS were added to dilute the trypsin, and the media containing the detached cells was transferred into an Eppendorf tube. Next, the cell suspension was centrifuged at 1,000×g and 4 °C for 10 min, the media was removed and the cell pellet was washed twice with 1 mL cold PBS to remove extracellular metabolites. Afterwards, PBS was removed by centrifugation (3,000×g and 4 °C for 10 min), and cell pellets were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

For scraping in PBS sample collection, after removing the cell culture medium, cells were rapidly washed twice with 2 mL PBS, then scraped with 1 mL PBS and the PBS containing the detached cells was transferred into an Eppendorf tube. Finally, the wells were washed one time with 0.5 mL PBS and the wash solution was added to the Eppendorf tube. After centrifugation at 3, 000×g and 4 °C for 10 min, the PBS was removed, and then cell pellets were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

For direct solvent scraping sample collection, after removing the cell culture medium, cells were rapidly rinsed twice with 2 mL cold water and immediately frozen using liquid nitrogen. Approximately 5 s passed between addition of water and quenching by addition of liquid nitrogen. The quenched cells in plates were stored at −80 °C until analysis. Afterwards, cells were scraped in the presence of 0.4 mL of ice-cold 80% methanol and transferred into an Eppendorf tube.

For water disruption sample collection，after removing the cell culture medium, cells were rapidly rinsed twice with 2 mL cold water and immediately frozen using liquid nitrogen. Approximately 5 s passed between addition of water and quenching by addition of liquid nitrogen. The quenched cells in plates were stored at −80 °C until analysis. Then, 1 mL of ultrapure water was added to each well, followed by ultrasonic disruption in an ice-water bath for 3 min. Cells were disrupted in water and detached from the plates.

Afterwards, the suspension containing cell fragments was transferred into an Eppendorf tube. The freeze-drying of the samples was then performed for the disrupted cells.

The extraction steps were exactly the same in the four methods for cell harvesting. Cell samples were extracted with 0.4 mL of ice-cold 80% methanol including six internal standards (L-phenylalanine-d_5, octanoyl (8,8,8-D_3)-L-carnitine, 1-lauroyl-2-hydroxy-sn-glycero3-phosphocholine, hendecanoic acid, nonadecanoic acid and 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine) which served as quality controls for sample preparation and instrumental analysis. First, the cell samples were sonicated for 3 min in an ice-water bath and vortexed for 2 min. Two cycles of sonicating / vortexing were performed. Then, centrifugation was performed for 10 min at 13,000 × g and 4 °C. Finally, the supernatant was filtered by an organic phase filter and transferred to a vial for metabolite analysis. The evaluation of cell harvesting methods was conducted with six duplicates for each method.

Methods for metabolite extraction

The steps for cell harvesting were exactly the same in the four methods for metabolite extraction. Cells were harvested via water disruption sample collection method and the details were described above. All the samples below were assayed within 10 min of extraction. In all the cases cold solvents, either −20 °C or ice cold, were employed to perform the extractions. The evaluation of metabolite extraction methods was conducted with four duplicates for each method.

For 80% methanol and 75% 9:1 MC extraction, 495 μL of organic solvent containing six internal standards was added and the same extraction procedure was performed (by the methods described above) as the extraction procedure in the study of different cell harvesting methods.

For pre-MTBE-based metabolomic extraction, freeze-dried cell samples were firstly ice-bath sonicated for 2 min with 120 μL 75% methanol including standards to break up the cells. Next, 300 μL MTBE was added and the samples were vortexed for 6 min at room temperature. The one-phase solvent system in this step allows for an optimal contact between extraction solvent and cell material. Next, phase separation was induced by adding 75 μL water and the samples were vortexed for 2 min at room temperature, letting sit for 10 min at room temperature and centrifuging for 10 min at 13,000 × g, 4 °C. Afterwards, 200 μL upper plus 100 μL lower fractions were pooled and evaporated before the samples were reconstituted in 300 μL 80% methanol.

For novel two-phase solvent system extraction procedure, freeze-dried cell samples were firstly vortexed for 3 min at room temperature and ice-bath sonicated for 3 min with 300 μL MTBE to break up the cells. In this step, lipids and lipophilic metabolites can be extracted maximally to the MTBE phase. After being let sit for 10 min at 4 °C, cell samples sank to the bottom. Then, 105 μL water was added to form two-phases solvent system and the samples were ice-bath sonicated for 2 min. Polar and semi-polar metabolites in this step are mainly extracted to the lower water phase. Next, 90 μL methanol including internal standards was added and the samples were vortexed for 2 min at room temperature. Then, centrifugation was performed for 10 min at 13,000 × g, 4°C. 200 μL upper plus 100 μL lower fractions were pooled and evaporated before the samples were reconstituted in 300 μL 80% methanol.

For comparison of pre-MTBE-based method and novel two-phase solvent system extraction with MTBE method, the methanol/water phase was injected into the LC-MS system directly and the MTBE−phase(100 μL) were evaporated before the samples were dissolved in 50 μL dichloromethane/ methanol (2:1) and finally resuspended (vortex for 2 min) in 50μL of 80% methanol before being transferred to a HPLC vial and injected into the LC-MS system.

Text S2. **UHPLC/Q-Trap MRM MS for targeted Metabolomic Analysis**

A UHPLC/Q-Trap MS-based targeted metabolomic analysis was conducted multiple reaction monitoring (MRM) mode. The MRM ion pair informations were got from a pseudo-targeted metabolomic study based on HepG2 cell. Metabolite identification was carried out in reference to authentic standards or data from MS/MS libraries including HMDB (http://www.hmdb.ca/), METLIN (https://metlin.scripps.edu/index.php) and KEGG (http://www.kegg.jp/kegg/).

In positive ion mode, 10 μL of extract containing metabolites was injected into the UHPLC/Q-Trap MS system with an ACQUITY UPLC BEH C8 column (2.1 mm × 100 mm × 1.7 μm, Waters, USA) maintained at 50 °C. Water and acetonitrile both containing 0.1% (v/v) formic acid were used as mobile phases A and B, respectively. For comparison of cell harvesting methods, the flow rate was 0.35 mL/min, and the gradient elution was as follows (time, %B): 0 min, 10%; 3 min, 40%; 15 min, 100%, and maintained for 5 min; 20.1 min, 10%, and re-equilibrated for 2.9 min. For comparison of the extraction methods, the flow rate was 0.35 mL/min, and the gradient elution was as follows (time, %B): 0 min,

5%; 1min, 5%; 23 min, 100%, and maintained for 4 min; 27.1 min, 5%, and re-equilibrated for 2.9 min. The MS instrumental parameters were set as follows: source temperature, 550 °C; gas I, 40 arbitrary units; gas II, 40 arbitrary units; curtain gas, 35 arbitrary units; ion spray voltage, 5500 V. Four internal standards containing lysphosphatidyl cholines LPC (12: 0), phosphatidyl ethanolamine PE (17: 0), octanoyl (8,8,8-D3)-L-carnitine, and L-phenylalanine-d5 were used for normalizing the peak areas of all other analytes.

In negative ion mode, 10 μL of extract containing metabolites was injected into the UHPLC/Q- Q-Trap MS system with an ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm × 1.8 μm, Waters, USA) maintained at 50 °C. Water and methanol both containing 5 mmol/L ammonium bicarbonate were used as mobile phases A and B, respectively. The flow rate was also 0.35 mL/min, and the gradient elution was as follows (time, %B): 0 min, 2%; 3 min, 42%; 12 min, 100%, and maintained for 4 min; 16.1 min, 2%, and re-equilibrated for 3.9 min. The MS instrumental parameters were set as follows: source temperature, 550 °C; gas I, 40 arbitrary units; gas II, 40 arbitrary units; curtain gas, 35 arbitrary units; ion spray voltage, −4500 V. Octanoyl (8,8,8-D3)-L-carnitine, L-phenylalanine-d5, hendecanoic acid and nonadecanoic acid served as internal standards to normalize the peak areas of all other analytes.

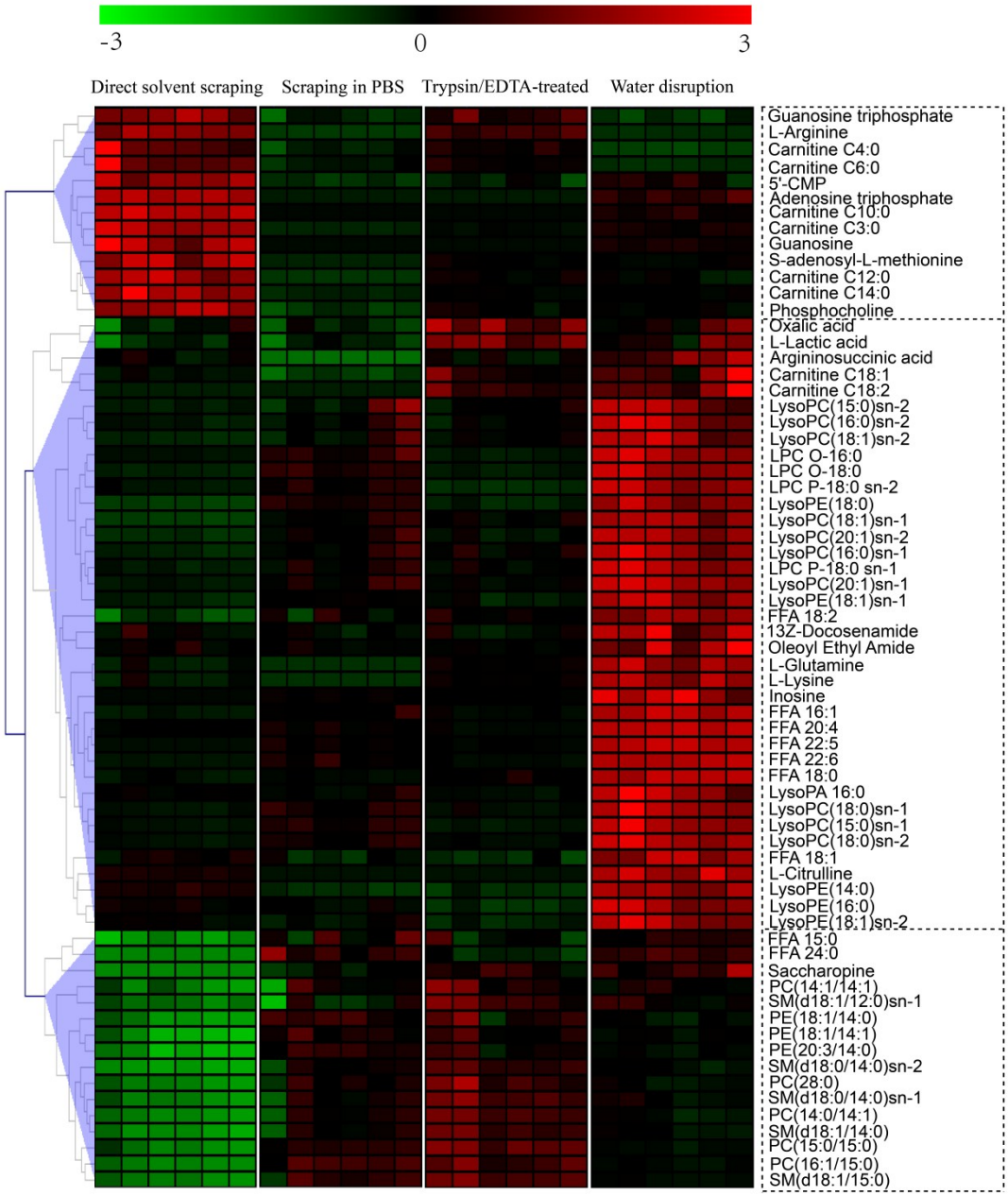

Figure. S1 Heat map of 67 metabolites with most significant change (PLS-DA, VIP > 1 and FDR < 0.05) among the different harvesting method groups.

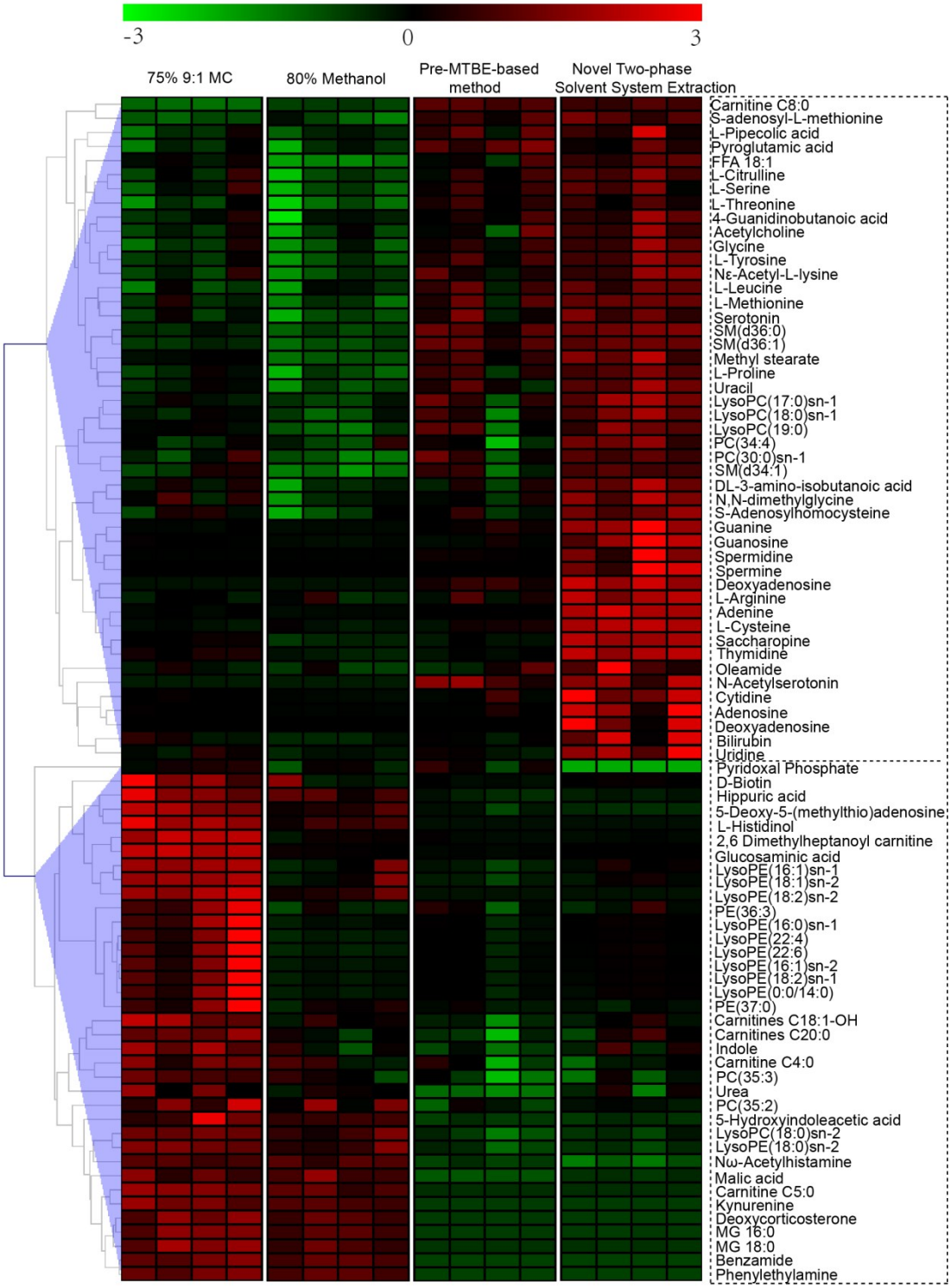

Figure. S2 Heat map of 84 metabolites with most significant change (PLS-DA, VIP > 1 and FDR < 0.05) among the different extraction method groups.

Table S1 List of statistical results for differential metabolites harvested using four different harvesting methods

Metabolites	Parent Ion	Product Ion	VIP	P	FDR
Adenosine triphosphate	505.9875	408.0105	1.0137	1.01E-16	2.46E-15
PC(16:1/15:0)	718.538	184.073	1.0345	9.49E-11	2.18E-10
PE(18:1/14:0)	690.507	549.488	1.0409	3.03E-07	4.12E-07
LysoPE(14:0)	426.262	285.242	1.0573	1.62E-14	1.25E-13
PC(15:0/15:0)	706.538	184.073	1.0752	3.14E-11	7.84E-11
5'-CMP	322.0426	78.9586	1.076	2.07E-09	3.71E-09
Oxalic acid	88.9875	44.9977	1.096	1.24E-04	1.39E-04
SM(d18:1/15:0)	689.56	184.073	1.1189	7.97E-10	1.63E-09
LysoPE(16:0)	454.293	313.274	1.1547	6.74E-11	1.57E-10
FFA 18:1	281.2492	281.2492	1.191	7.56E-10	1.57E-09
PC(28:0)	678.507	184.073	1.1913	1.16E-07	1.63E-07
Carnitine C3:0	218.139	85.0284	1.2045	9.96E-15	8.06E-14
Phosphocholine	184.073	86.0966	1.2079	3.38E-13	1.69E-12
L-Arginine	175.1187	70.0658	1.209	3.30E-16	5.86E-15
Argininosuccinic acid	289.1124	271.103	1.2112	2.85E-08	4.29E-08
PC(14:0/14:1)	676.491	184.073	1.2356	3.72E-09	6.39E-09
PC(14:1/14:1)	674.476	184.073	1.2467	4.91E-04	5.35E-04
PE(20:3/14:0)	714.507	573.488	1.2548	3.29E-10	7.17E-10
L-Lactic acid	89.024	43.0196	1.2625	1.95E-05	2.34E-05
Carnitine C12:0	344.2803	85.0284	1.2699	2.93E-12	9.98E-12
Guanosine triphosphate	521.9797	158.9248	1.2779	6.17E-09	1.02E-08
L-Citrulline	176.1019	70.0657	1.2865	5.55E-14	3.78E-13
SM(d18:0/14:0)sn-1	677.559	184.073	1.2968	6.46E-08	9.24E-08
PE(18:1/14:1)	688.491	547.472	1.3151	8.92E-09	1.43E-08
SM(d18:1/14:0)	675.544	184.073	1.3179	5.84E-09	9.73E-09
Guanosine	152.0564	135.03	1.3184	1.04E-09	2.06E-09
Carnitine C18:1	426.358	85.0284	1.327	2.98E-04	3.29E-04
LysoPE(18:1)sn-2	480.308	339.289	1.3278	3.66E-10	7.78E-10
FFA 24:0	309.2779	309.2779	1.3418	3.99E-10	8.37E-10
Oleoyl Ethyl Amide	310.3097	69.0705	1.3484	4.14E-06	5.06E-06
Carnitine C14:0	372.311	85.029	1.3501	3.02E-12	1.01E-11
S-adenosyl-L-methionine	399.1434	250.0929	1.3506	3.70E-11	8.87E-11
LPC P-18:0 sn-2	508.376	104.107	1.3597	1.39E-12	4.94E-12
Carnitine C4:0	232.1555	85.0296	1.3695	1.58E-05	1.91E-05
Carnitine C6:0	260.186	85.03	1.3763	4.61E-05	5.48E-05
13Z-Docosenamide	338.342	69.0711	1.3986	6.94E-07	9.00E-07
SM(d18:0/14:0)sn-2	677.559	184.073	1.4006	4.57E-11	1.08E-10
SM(d18:1/12:0)sn-1	647.5	184.073	1.4022	4.93E-05	5.83E-05

Carnitine C10:0	316.2478	85.029	1.4056	6.24E-17	1.77E-15
LysoPA 16:0	409.4825	153.1	1.4167	1.75E-08	2.73E-08
LysoPC(15:0)sn-2	482.324	184.073	1.424	1.23E-04	1.39E-04
FFA 15:0	241.2171	241.2171	1.4283	2.81E-06	3.46E-06
LPC O-16:0	482.361	104.107	1.4297	2.04E-11	5.33E-11
LysoPC(15:0)sn-1	482.324	184.073	1.4891	4.47E-12	1.36E-11
LPC O-18:0	510.392	104.107	1.5257	1.64E-13	9.03E-13
LysoPC(16:0)sn-2	496.34	184.073	1.5372	3.36E-07	4.54E-07
Inosine	267.0719	135.0298	1.5553	1.95E-09	3.61E-09
LysoPC(18:0)sn-1	524.371	184.073	1.5614	1.20E-10	2.69E-10
FFA 20:4	303.2311	303.2311	1.5867	2.97E-19	2.52E-17
LysoPE(18:0)	482.324	341.305	1.5919	1.65E-13	9.03E-13
LysoPC(18:0)sn-2	524.371	184.073	1.6067	8.64E-13	3.58E-12
Carnitine C18:2	424.342	85.0284	1.6273	5.27E-05	6.18E-05
LysoPC(18:1)sn-2	522.355	184.073	1.6293	6.13E-07	8.07E-07
LysoPE(18:1)sn-1	480.308	339.289	1.6302	5.20E-12	1.55E-11
LysoPC(20:1)sn-1	550.387	184.073	1.6441	3.18E-11	7.84E-11
FFA 22:6	327.2349	327.2349	1.6523	1.54E-17	5.22E-16
FFA 22:5	329.2461	329.2461	1.6611	4.22E-20	7.17E-18
LPC P-18:0 sn-1	508.376	104.107	1.6622	1.68E-10	3.70E-10
LysoPC(20:1)sn-2	550.387	184.073	1.6877	2.27E-11	5.77E-11
L-Lysine	147.113	84.0808	1.692	5.12E-13	2.29E-12
L-Glutamine	147.0762	84.0448	1.6985	1.25E-12	4.93E-12
FFA 16:1	253.2172	253.2172	1.7002	7.20E-15	6.45E-14
LysoPC(16:0)sn-1	496.34	184.073	1.7314	6.34E-09	1.04E-08
FFA 18:0	283.2645	283.2645	1.7581	1.06E-17	4.49E-16
Saccharopine	275.1255	257.1147	1.8353	9.52E-08	1.35E-07
LysoPC(18:1)sn-1	522.355	184.073	1.8428	1.26E-11	3.51E-11
FFA 18:2	279.2336	279.2336	1.8752	1.65E-09	3.11E-09

Table S2 List of statistical results for differential metabolites harvested using four different extraction methods

Metabolites	Parent Ion	Product Ion	VIP	P	FDR
Phenylethylamine	122.0969	105.0708	1.8399	4.15E-10	1.45E-08
2,6 Dimethylheptanoyl carnitine	302.2330	100.1120	1.6103	3.14E-10	1.45E-08
4-Guanidinobutanoic acid	146.0916	87.0446	1.1248	0.030627	0.045067
5-Deoxy-5-(methylthio)adenosine	298.0980	136.0616	1.8306	1.13E-06	1.14E-05
5-Hydroxyindoleacetic acid	192.0637	91.0543	1.6504	0.0004359	0.0020225
Acetylcholine	146.1177	87.0448	1.169	0.034353	0.048895

Adenine	136.0619	119.0359	1.5825	6.91E-11	4.01E-09
Adenosine	268.1040	136.0611	1.3422	0.0013987	0.0051508
Benzamide	122.0595	79.0545	1.8336	6.52E-10	1.68E-08
Bilirubin	585.2703	299.1389	1.1441	0.00546	0.012667
Carnitine C4:0	232.1555	85.0296	1.3443	0.024693	0.037689
Carnitine C5:0	246.1700	85.0648	1.8744	1.57E-09	3.64E-08
Carnitine C8:0	288.2170	85.0284	1.8312	9.68E-12	1.12E-09
Carnitine C18:1-OH	442.4000	85.1000	1.3422	0.0003166	0.0016324
Carnitine C20:0	456.4000	85.1000	1.0419	0.0020644	0.006386
Cytidine	244.0924	112.0512	1.4461	0.0001881	0.0010389
D-Biotin	245.0956	227.0849	1.2622	0.0085028	0.016717
Deoxyadenosine	252.1081	136.0622	1.2371	0.006251	0.013812
Deoxycorticosterone	331.2252	109.0645	1.8431	3.73E-08	5.77E-07
Deoxyguanosine	268.1031	152.0558	1.7745	2.05E-08	3.39E-07
DL-3-amino-isobutanoic acid	104.0693	45.0331	1.1918	0.0008975	0.0037182
FFA 18:1	283.2645	265.2505	1.0245	2.49E-05	0.0001861
Glucosaminic acid	196.0812	72.0444	1.5914	3.46E-13	8.02E-11
Glycine	76.0399	30.0344	1.4754	0.0014929	0.0053285
Guanine	152.0564	135.0300	1.5834	2.38E-06	2.30E-05
Guanosine	284.0983	152.0557	1.4143	4.16E-05	0.0002757
Hippuric acid	180.0650	77.0392	1.7338	3.01E-05	0.0002113
Indole	118.0646	65.0386	1.1219	0.0014913	0.0053285
Kynurenine	209.0916	192.0653	1.8568	3.36E-09	7.08E-08
L-Arginine	175.1187	70.0658	1.5943	4.61E-05	0.000297
L-Citrulline	176.1019	70.0657	1.2194	0.0018819	0.0060488
L-Cysteine	122.0289	76.0223	1.6693	4.37E-10	1.45E-08
L-Histidinol	142.0992	81.0452	1.7864	2.77E-07	3.38E-06
L-Leucine	132.1019	86.0967	1.5368	0.0049477	0.011871
L-Methionine	150.0574	133.0314	1.4338	0.0004222	0.0019991
L-Pipecolic acid	130.0859	84.0811	1.3813	0.021696	0.034242
L-Proline	116.0708	70.0659	1.3329	0.0004909	0.0021089
L-Serine	106.0496	60.0454	1.1234	0.0047226	0.011533
L-Threonine	120.0653	74.0605	1.326	0.0009264	0.0037708
L-Tyrosine	182.0864	136.0753	1.5034	0.0001454	0.0008226
LysoPC 17:0 sn-1	510.3550	184.0730	1.4498	0.0012495	0.0046757
LysoPC 18:0 sn-1	524.3710	184.0730	1.2704	0.00375	0.0098864
LysoPC 18:0 sn-2	524.3710	184.0730	1.6852	5.58E-06	4.92E-05
LysoPC 19:0	538.3870	184.0730	1.102	0.0049632	0.011871
LysoPE 16:0 sn-1	454.2930	313.2740	1.1363	0.0035029	0.0094496
LysoPE 16:1 sn-1	452.2770	311.2608	1.4056	0.0001156	0.0006703
LysoPE 16:1 sn-2	452.2770	311.2608	1.0851	0.0076285	0.015662
LysoPE 18:0 sn-2	482.3240	341.3050	1.763	4.46E-06	4.14E-05
LysoPE 18:1 sn-2	480.3080	339.2890	1.4783	0.0001142	0.0006703

LysoPE 18:2 sn-1	478.2940	337.2740	1.0862	0.0061968	0.013812
LysoPE 18:2 sn-2	478.2940	337.2740	1.7805	7.09E-07	7.91E-06
LysoPE 22:4	530.3240	44.0495	1.0227	0.0079767	0.015817
LysoPE 22:6	526.2930	385.2740	1.0195	0.0060302	0.013583
LysoPE(0:0/14:0)	426.2620	285.2420	1.0766	0.0067081	0.014278
Malic acid	135.0958	89.0229	1.5692	5.73E-06	4.92E-05
Methyl stearate	299.2935	57.0698	1.4062	1.31E-05	0.0001089
MG 16:0	331.2840	57.0699	1.8597	3.92E-09	7.58E-08
MG 18:0	359.3160	95.0860	1.8434	1.73E-07	2.22E-06
N,N-dimethylglycine	104.0704	58.0061	1.0515	0.0016135	0.0053477
N-Acetylserotonin	219.1102	160.0714	1.6696	0.0002396	0.0012782
Nε-Acetyl-L-lysine	189.1228	84.0810	1.3668	0.0027702	0.0083421
Nω-Acetylhistamine	154.0972	112.0871	1.8591	1.02E-08	1.82E-07
Oleamide	282.2787	69.0711	1.172	0.030886	0.045067
PC 30:0 sn-1	706.5380	184.0730	1.0898	0.0057806	0.013148
PC 34:4	754.5380	184.0730	1.0005	0.014402	0.025505
PC 35:2	772.5850	184.0730	1.3203	0.017597	0.029025
PC 35:3	770.5690	184.0730	1.329	0.0089699	0.017198
PE 36:3	742.5380	601.5190	1.0075	0.01093	0.020285
PE 37:0	762.6010	621.5820	1.2781	0.018901	0.030664
Pyridoxal Phosphate	248.0311	150.0529	1.4936	2.91E-05	0.0002109
Pyroglutamic acid	129.8750	84.0980	1.2099	0.0036073	0.0096196
Saccharopine	277.1386	84.0811	1.3946	3.02E-11	2.33E-09
S-Adenosylhomocysteine	385.1287	136.0619	1.1751	0.0016088	0.0053477
S-adenosyl-L-methionine	399.1434	250.0929	1.7528	2.00E-05	0.000155
Serotonin	177.1020	160.0756	1.326	0.0015427	0.005342
SM 34:1	703.5750	184.0730	1.0673	0.001173	0.0045356
SM 36:0	733.6220	184.0730	1.6866	7.16E-07	7.91E-06
SM 36:1	731.6060	184.0730	1.6802	8.14E-08	1.18E-06
Spermidine	146.1645	72.0812	1.295	0.0066881	0.014278
Spermine	203.2230	112.1122	1.3265	0.0011324	0.0045298
Thymidine	243.0991	127.0499	1.3447	5.97E-10	1.68E-08
Uracil	113.0348	70.0299	1.4027	0.0002424	0.0012782
Urea	61.0396	44.0131	1.1176	0.0042185	0.010638
Uridine	245.0770	113.0349	1.3282	9.09E-05	0.000555