*Supporting Information for Publication*

# Toward 'Omic' Scale Metabolite Profiling: A Dual Separation – Mass Spectrometry Approach for Coverage of Lipids and Central Carbon Metabolism

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**Sample preparation.** For untargeted analysis of the bacterial metabolic response to nitric oxide, *Shewanella oneidensis* MR-1 samples were cultured in MM mineral medium, supplemented with 0.1 % casamino acids, 50 mM sodium fumarate and 50 mM sodium D/L-lactate.[1-2] Replicate batch cultures were inoculated at a 1:100 dilution from an overnight culture in LB medium, and were grown anaerobically in sealed serum vials at 30 ºC. When cultures reached mid-log phase ($OD_{600}$ between 0.2 – 0.3), each sample was split into two separate Hungate tubes. One half was treated with the slow-release nitric oxide donor PAPA NONOate (10 µM, Cayman Chemicals). Anaerobic NONOate stocks were freshly prepared in 10 mM NaOH inside an anaerobic glovebox (MBraun). The second half of each sample was treated with 0.5 mM of the nitric oxide scavenger 2-Phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (PTIO, TCI America). The cultures were incubated at 30 ºC for an additional 60 min, and subsequently chilled on ice. The supernatant was removed by centrifugation at 2,000 x g for 20 min at 4 ºC. The cell pellets were rinsed once with ice-cold 0.6 % NaCl and then flash-frozen in liquid nitrogen. Pellets were stored at -80 ºC until extraction of metabolites.

For the evaluation of comprehensive, single extraction – dual separation workflow, the following three types of samples were used: standard human plasma samples, *Escherichia coli* cell pellets and one human cancer (Raji Burkitt's) cell line.

The *E. coli* strain W3110 was cultured in three batch culture replicates in complete LB medium from a single overnight culture. The cells were grown at 37 °C, and 15 mL aliquots were harvested from each culture at mid-exponential growth ($OD_{600}$ nm = 0.2 - 0.3). The supernatant was removed by centrifugation for 5 min at 3000 rpm and 4 °C and the cells were rinsed once with 1.5 mL of 0.6 % NaCl solution to wash off the media. Cell pellets were stored at -80 °C until metabolite extraction.

Raji Burkitt's lymphoma cells (CCL-86; ATCC, Manassas, VA, USA) were propagated at a density of 150,000 to 2.0 million cells per mL in complete growth medium consisting of RPMI-1640 medium (#A10491-01; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (PAA #A15-704; GE Healthcare Bio-Sciences, Piscataway, NJ, USA), 100 I.U./ml penicillin, 100 µg/ml streptomycin, and 2mM L-Glutamine (Invitrogen #15070-063) at 37 °C in a 5 % $CO_2$ incubator. Cell count and viability was monitored daily using the Guava Viacount® assay (#4000-0040; Millipore, Billerica, MA, USA). The day before harvest, cells were placed in fresh growth medium, and were greater than 93% viable at the time of sample collection. Samples within each experiment were collected from a common batch of cells as follows: 50 million cells were collected in 50 mL polypropylene centrifuge tubes (# 352070; Becton Dickinson, Franklin Lakes, NJ), and immediately centrifuged for 3 minutes, at 450 x g and 4 °C. Supernatants were immediately decanted by inverting the tubes, and cell pellets were gently but rapidly re-suspended by pipetting in 50 mL of ice cold, cell culture-grade Dulbecco's phosphate buffered saline (PBS, #14190; Invitrogen). After centrifugation as above, cell pellets were re-suspended in 25 mL ice cold PBS and 5 mL aliquots (~ 10 million cells per replicate) were immediately dispensed into 15 mL polypropylene tubes (#352097, Becton Dickinson), centrifuged and immediately decanted as above. Residual PBS from this final wash was removed by gently tapping each tube on paper towels to reduce residual PBS without disturbing cell pellets. Samples were snap-frozen in a dry ice-ethanol bath, and stored at -80 °C until extraction.

**Table S1.** List of metabolites in hydrophilic standard mixture.

| NAME | EXACT MASS | [M+H]+ | [M-H]- | FORMULA |
|---|---|---|---|---|
| L-Glutamic Acid | 147.0532 | 148.0602 | 146.0454 | C5H9NO4 |
| D-Aspartic acid | 133.0375 | 134.0445 | 132.0297 | C4H7NO4 |
| D-Ala-D-Ala | 160.0848 | 161.0918 | 159.0770 | C6H12N2O3 |
| L-Arginine | 174.1117 | 175.1187 | 173.1039 | C6H14N4O2 |
| DL-Homoserine | 119.0582 | 120.0652 | 118.0504 | C4H9NO3 |
| Phenylalanine | 165.0790 | 166.0860 | 164.0712 | C9H11NO2 |
| S-(5'-Adenosyl)-L-Homocysteine | 384.1216 | 385.1286 | 383.1138 | C14H20N6O5S |
| L-Tryptophan | 204.0899 | 205.0969 | 203.0821 | C11H12N2O2 |
| Taurine | 125.0147 | 126.0217 | 124.0069 | C2H7NO3S |
| Spermine | 202.2157 | 203.2227 | 201.2079 | C10H26N4 |
| Melatonine | 232.1212 | 233.1282 | 231.1134 | C13H16N2O2 |
| Adenine | 135.0545 | 136.0615 | 134.0467 | C5H5N5 |
| Adenosine | 267.0967 | 268.1037 | 266.0889 | C10H13N5O4 |
| Inosine | 268.0808 | 269.0878 | 267.0730 | C10H12N4O5 |
| Guanosine | 283.0917 | 284.0987 | 282.0839 | C10H13N5O5 |
| Thymidine | 242.0903 | 243.0973 | 241.0825 | C10H14N2O5 |
| Deoxyuridine | 228.0746 | 229.0816 | 227.0668 | C9H12N2O5 |
| 5'-CMP | 323.0519 | 324.0589 | 322.0441 | C9H14N3O8P |
| ATP | 506.9958 | 508.0028 | 505.9880 | C10H16N5O13P3 |
| AMP | 347.0631 | 348.0701 | 346.0553 | C10H14N5O7P |
| UMP | 324.0359 | 325.0429 | 323.0281 | C9H13N2O9P |
| D-Erythrose-4-phosphate | 200.0086 | 201.0156 | 199.0008 | C4H9O7P |
| D-Ribose 5-phosphate | 230.0192 | 231.0262 | 229.0114 | C5H11O8P |
| D-glucose-6-phosphate | 260.0297 | 261.0367 | 259.0219 | C6H13O9P |
| D-Fructose-6-Phosphate | 260.0297 | 261.0367 | 259.0219 | C6H13O9P |
| Fructose-1,6-bisphosphate | 339.9961 | 341.0031 | 338.9883 | C6H14O12P2 |
| Phosphoenolpyruvate | 167.9824 | 168.9894 | 166.9746 | C3H5O6P |
| UDP-D-glucose | 566.0550 | 567.0620 | 565.0472 | C15H24N2O17P2 |
| D-Ribulose-5-phosphate | 230.0192 | 231.0262 | 229.0114 | C5H11O8P |
| Nicotinuric acid | 180.0535 | 181.0605 | 179.0457 | C8H8N2O3 |
| 2-Ethyl-2-Hydroxybutyric acid | 132.0786 | 133.0856 | 131.0708 | C6H12O3 |
| Citric Acid | 192.0270 | 193.0340 | 191.0192 | C6H8O7 |
| Retinoic acid | 300.2089 | 301.2159 | 299.2011 | C20H28O2 |
| Succinic Acid | 118.0266 | 119.0336 | 117.0188 | C4H6O4 |
| Ascorbic acid | 176.0321 | 177.0391 | 175.0243 | C6H8O6 |
| Nicotinic acid | 123.0320 | 124.0390 | 122.0242 | C6H5NO2 |
| Aconitic acid | 174.0164 | 175.0234 | 173.0086 | C6H6O6 |
| Oxaloacetic acid | 132.0059 | 133.0129 | 130.9981 | C4H4O5 |
| Malic acid | 134.0215 | 135.0285 | 133.0137 | C4H6O5 |
| Lactic acid | 90.0317 | 91.0387 | 89.0239 | C3H6O3 |
| Acetyl-CoA | 809.1257 | 810.1327 | 808.1179 | C23H38N7O17P3S |
| Malonyl-CoA | 853.1156 | 854.1226 | 852.1078 | C24H38N7O19P3S |
| NAD+ | 663.1091 | 664.1161 | 662.1013 | C21H27N7O14P2 |
| NADPH | 743.0755 | 744.0825 | 742.0677 | C21H28N7O17P3 |
| Glutathione oxidized | 612.1520 | 613.1590 | 611.1442 | C20H32N6O12S2 |
| Alpha ketoglutarate | 146.0215 | 147.0285 | 145.0137 | C5H6O5 |
| FAD | 785.1571 | 786.1641 | 784.1493 | C27H33N9O15P2 |
| R-(-)-Mandelic acid | 152.0473 | 153.0543 | 151.0395 | C8H8O3 |
| PGA1 | 336.2301 | 337.2371 | 335.2223 | C20H32O4 |
| Palmitoyl dopamine | 391.3086 | 392.3156 | 390.3008 | C24H41NO3 |

**Table S2.** List of metabolites in hydrophobic standard mixture.

| NAME | EXACT MASS | [M+H]+ | [M-H]- | FORMULA |
|---|---|---|---|---|
| Creatinine | 113.0589118 | 114.0662118 | 112.0516118 | C4H7N3O |
| Caffeic Acid | 180.04226 | 181.04956 | 179.03496 | C9H8O4 |
| Caffeine | 194.080376 | 195.087676 | 193.073076 | C8H10N4O2 |
| 1-Tetradecanol | 214.2297 | 215.237 | 213.2224 | C14H30O |
| Acetyl-DL-Glucosamine | 221.089933 | 222.097233 | 220.082633 | C8H15NO6 |
| Melatonin | 232.1211716 | 233.1284716 | 231.1138716 | C13H16N2O2 |
| Alizarin | 240.0422587 | 241.0495587 | 239.0349587 | C14H8O4 |
| Palmitic Acid | 256.2402172 | 257.2475172 | 255.2329172 | C16H32O2 |
| 1-Octadecanol | 270.2923 | 271.2996 | 269.285 | C18H38O |
| Estradiole | 272.17763 | 273.18493 | 271.17033 | C18H24O2 |
| Linoleic Acid | 280.2402 | 281.2475 | 279.2329 | C18H32O2 |
| Oleic Acid | 282.25588 | 283.26318 | 281.24858 | C18H34O2 |
| Stearamide | 283.2874992 | 284.2947992 | 282.2801992 | C18H37NO |
| Stearic Acid | 284.2715 | 285.2788 | 283.2642 | C18H36O2 |
| trans-Retinol( Vitamin A) | 286.229653 | 287.236953 | 285.222353 | C20H30O |
| B-Octylglucoside | 292.1885788 | 293.1958788 | 291.1812788 | C14H28O6 |
| Sphingosine | 299.282429 | 300.289729 | 298.275129 | C18H37NO2 |
| Retinoic acid | 300.2089188 | 301.2162188 | 299.2016188 | C20H28O2 |
| Arachidonic Acid | 304.24023 | 305.24753 | 303.23293 | C20H32O2 |
| Progesterone | 314.2245802 | 315.2318802 | 313.2172802 | C21H30O2 |
| Decanoyl-DL-Carnitine | 315.241 | 316.2483 | 314.2337 | C17H33NO4 |
| Docosahexanoic Acid | 328.2402 | 329.2475 | 327.2329 | C22H32O2 |
| Eicosapentaenoic Acid | 334.2144 | 335.2217 | 333.2071 | C20H30O4 |
| PGA1 | 336.23006 | 337.23736 | 335.22276 | C20H32O4 |
| Erucamide | 337.334464 | 338.341764 | 336.327164 | C22H43NO |
| Erucic acid (13(Z)-Docosenoic Acid) | 338.3184632 | 339.3257632 | 337.3111632 | C22H42O2 |
| Lauroylcarnitine | 343.2722 | 344.2795 | 342.2649 | C19H37NO4 |
| Rosmarinic Acid | 360.0845136 | 361.0918136 | 359.0772136 | C18H16O8 |
| Hydrocortisone | 362.209313 | 363.216613 | 361.202013 | C21H30O5 |
| Misoprostol | 368.256275 | 369.263575 | 367.248975 | C21H36O5 |
| 7-dehydrocholesterol | 384.3391974 | 385.3464974 | 383.3318974 | C27H44O |
| Cholesterol | 386.3548661 | 387.3621661 | 385.3475661 | C27H46O |
| Palmitoyl Dopamine | 391.3086276 | 392.3159276 | 390.3013276 | C24H41NO3 |
| C-6 Ceramide | 397.3555944 | 398.3628944 | 396.3482944 | C24H47NO3 |
| Palmitoyl-DL-Carinitine | 399.3349 | 400.3422 | 398.3276 | C23H45NO4 |
| Delta-Tocopherol | 402.34978 | 403.35708 | 401.34248 | C27H46O2 |
| Cholic Acid | 408.2876 | 409.2949 | 407.2803 | C24H40O5 |
| Squalene | 410.39123 | 411.39853 | 409.38393 | C30H50 |
| alpha-Tocopherol | 430.3810808 | 431.3883808 | 429.3737808 | C29H50O2 |
| Folic Acid | 441.1397 | 442.147 | 440.1324 | C19H19N7O6 |
| Geranylgeranyl pyrophosphate | 450.1936166 | 451.2009166 | 449.1863166 | C20H36O7P2 |
| PC (16:0/0:0) | 495.332492 | 496.339792 | 494.325192 | C24H50NO7P |
| N-Oleoyl-D-Erythro-Sphingosine (C18:1 Ceramide) | 563.5277164 | 564.5350164 | 562.5204164 | C36H69NO3 |
| Cholesteryl oleate | 650.6002 | 651.6075 | 649.5929 | C45H78O2 |
| PS (16:0/16:0) | 735.505 | 736.5123 | 734.4977 | C38H74NO10P |
| Palmitoyl Thio-PC | 749.539282 | 750.546582 | 748.531982 | C40H80NO7PS |
| L-Thyroxine | 776.6867126 | 777.6940126 | 775.6794126 | C15H11I4NO4 |
| Coenzyme Q10 | 862.6839114 | 863.6912114 | 861.6766114 | C59H90O4 |
| Phosphatidylinositol 3-phosphate, dipalmitoyl | 890.492134 | 891.499434 | 889.484834 | C41H80O16P2 |
| Decanoyl-CoA | 921.2509 | 922.2582 | 920.2436 | C31H54N7O17P3S |

**Table S3**. List of tested mobile phase conditions**.**

| HILIC ESI±MS | | |
|---|---|---|
| **Additives in A = 95% $H_2O$** | **Additives in B = 95% ACN** | **pH** |
| 20 mM $NH_4Ac$ + 0.1% FA | / | 4-4.5 |
| 20 mM $NH_4Ac$ | / | 6.5-7 |
| 20 mM $NH_4OH$ / 20 mM $NH_4Ac$ | / | 9.8 |
| 50 mM NH4OH / 50 mM $NH_4Ac$ | / | 9.8 |
| 10 mM $NH_4OH$ / 10 mM $NH_4Ac$ | / | 9.8 |
| 5 mM $NH_4OH$ / 5 mM $NH_4Ac$ | / | 9.8 |
| **HILIC ESI-MS** | | |
| **Additives in A = 95% $H_2O$** | **Additives in B = 95% ACN** | **pH** |
| 10 mM $NH_4OH$ / 10 mM $NH_4HCOO$ | / | 9.6 |
| 10 mM $NH_4OH$ / 10 mM $(NH4)_2CO_3$ | / | 9.6 |
| 10 mM $NH_4OH$ / 10 mM $NH_4Ac$ + 0.1m $MNH_4F$ | 0.1m $MNH_4F$ | 9.7 |
| 10 mM $NH_4OH$ / 9 mM $NH_4Ac$ + 1 mM $NH_4F$ | / | 9.5 |
| 5 mM $NH_4OH$ / 5 mM $NH_4Ac$ | 5 mM $NH_4OH$ / 5 mM $NH_4Ac$ | 9.7 |
| **RPLC ESI-MS** | | |
| **Additives in A = 100% $H_2O$** | **Additives in B = 100% ACN** | |
| 5 mM $NH_4Ac$ | / | 7.8 |
| 1 mM $NH_4F$ | / | 7.5 |
| 0.5 mM $NH_4F$ | / | 7.5 |
| 0.1 mM $NH_4F$ | / | 7.9 |
| 0.5 mM $NH_4F$ | 0.5 mM $NH_4F$ | 8.5 |
| 0.1 mM $NH_4F$ | 0.1 mM $NH_4F$ | 8.6 |

**Further optimization of HILIC/MS mobile phase.** To further examine the effect of salt additives, ammonium acetate was replaced by ammonium formate and ammonium carbonate in the same concentration mixture with ammonium hydroxide at high pH (Table S3). Both ammonium formate and ammonium carbonate buffers resulted in longer retention times, deterioration of peak shape, and signal suppression on the MS detection in comparison to ammonium acetate, thus giving significantly lower performance scores (Figure S1).

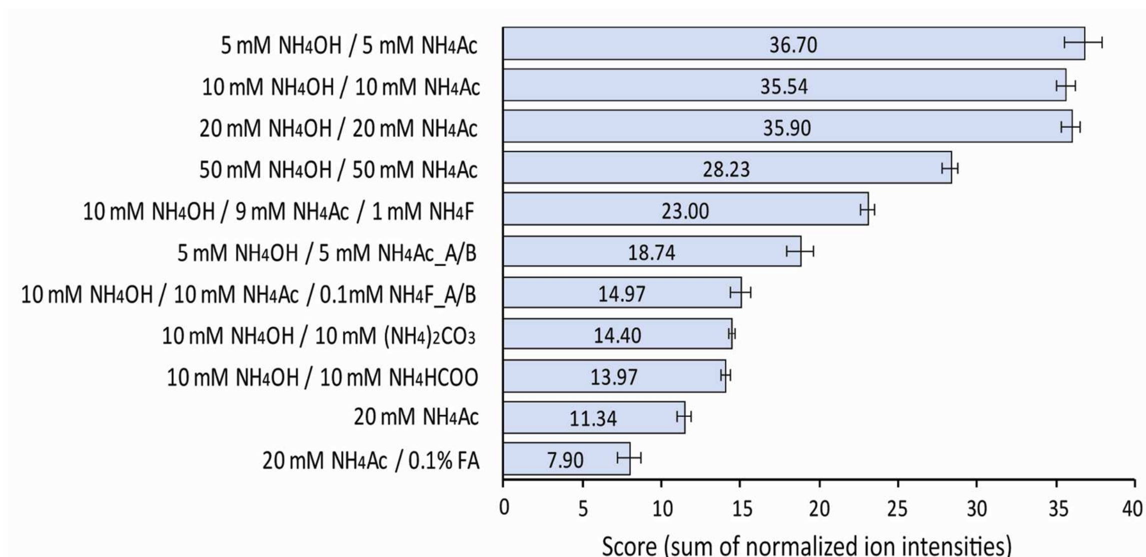


**Figure S1.** Further optimization of analysis conditions in HILIC ESI negative mode. Each tested analysis condition (Table S3) is presented by its overall score, calculated as a sum of normalized intensities of 50 metabolites from the hydrophilic standard mixture (Table S1).

Ammonium fluoride ($NH_4F$), which has previously been shown to promote ionization efficiency in negative mode in RPLC,[3] was also tested in HILIC ESI negative mode. Fluoride ions, which have stronger basicity than acetate ions in gas phase, effectively promote analyte deprotonation and enhance ionization efficiency[3]. In RPLC/MS, ammonium fluoride significantly increased the intensity of most metabolites by as much as five times compared to

ammonium acetate. As seen in Figure S2, the optimal concentration of ammonium fluoride additive in RPLC/MS was 0.5 mM. However, the addition of fluoride to the mobile phase in HILIC/MS had deleterious effects for polar metabolite ionization (Figure S1). The overall performance score decreased upon addition of $NH_4F$ from 35.5 (10 mM $NH_4Ac$ and 10 mM $NH_4OH$) to 23.0 (9 mM $NH_4Ac$ + 1 mM $NH_4F$ and 10 mM $NH_4OH$). The observed sensitivity decrease may be due to competition of ammonium fluoride with ammonium acetate which may interfere with the ionization process thus decreasing the MS signal intensity.

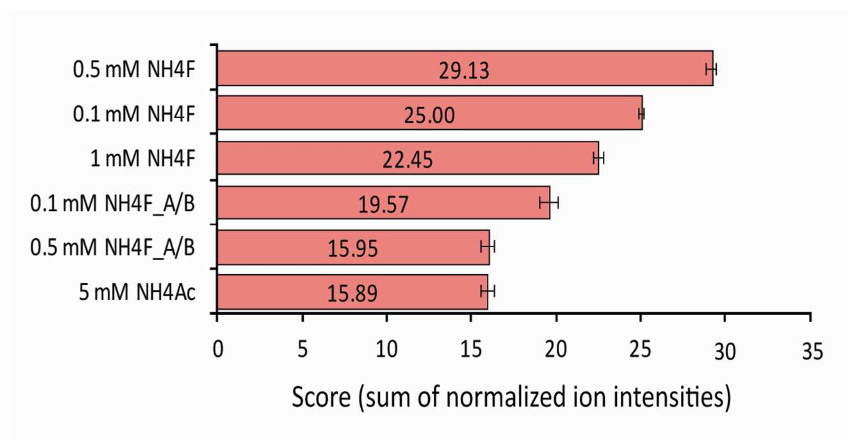


**Figure S2. O**ptimization of analysis conditions in RPLC ESI negative mode. Each tested analysis condition is presented by its overall score, calculated as a sum of normalized intensities of 50 metabolites from the hydrophobic standard mixture (Table S2).

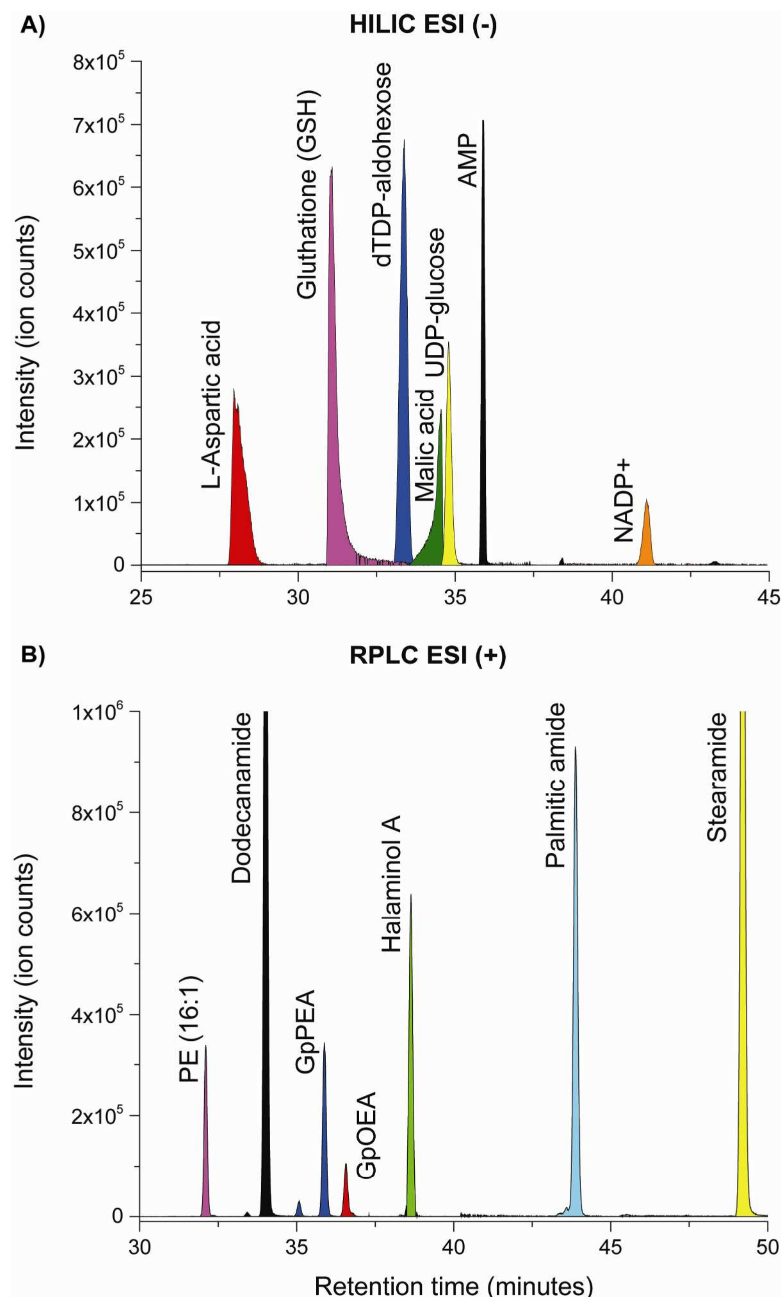


**Figure S3.** Extracted ion chromatograms from HILIC ESI negative and RPLC ESI positive mode profiling of *E. coli* extract. Indicated compounds have been putatively identified using METLIN database (Δ m/z < 5 ppm and/or MS-MS spectra matching) and retention time. GSH = Glutathione reduced, dTDP-aldohexose = Deoxythymidine diphosphate aldohexose, UDP-glucose = Uridine diphosphate glucose, AMP = Adenosine monophosphate, $NADP^+$ = Nicotinamide Adenine Dinucleotide Phosphate, PE = Phosphoethanolamine, GpPEA = Glycerophospho-N-Palmitoyl Ethanolamine, GpOEA = Glycerophospho-N-Oleoyl Ethanolamine

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(2) Thormann, K. M.; Duttler, S.; Saville, R. M.; Hyodo, M.; Shukla, S.; Hayakawa, Y.; Spormann, A. M. *J. Bacteriol.* **2006**, *188*, 2681-2691.
(3) Yanes, O.; Tautenhahn, R.; Patti, G. J.; Siuzdak, G. *Anal. Chem.* **2011**, *83*, 2152-2161.