Materials & Methods

Bacterial Growth

The chosen biological matrix was E.*coli* DH5 cultured in LB broth. The cells were sub-cultured from a seed stock into 50 mL flasks and cultured overnight (180 rpm, 37**°**C) as five biological replicates. After 18 h the five replicates were pooled and the final OD600 was recorded. The two control media were combined.

Metabolite Extraction

A large batch of samples were extracted and prepared at once. The cell culture was aliquoted (10 mL) into respective centrifuge tubes. To rapidly quench the metabolism of the cells methanol (60%, 20 mL, -48C) was added to each sample. The sample was manually mixed, centrifuged (4800 *g*, 4C, 10 min) and the supernatant discarded. To extract the cells, methanol (80%, 1 mL, -48C) was added to the cell pellet. The sample was vortex mixed (15 s) to fully disrupt the pellet before transferring the suspension to Eppendorf tubes (2 mL). The cells were then extracted by three freeze thaw cycles (liquid nitrogen for one minute followed by thawing on ice). After the final thaw, the sample was vortex mixed (15 s) and centrifuged (2000 *g*, -9C, 5 min). The supernatant was transferred to new Eppendorf tubes and dried under vacuum concentration (Eppendorf Concentrator Plus , 5 h, RT). The samples were stored at -80**°**C until LC-MS analysis.

Standard Mixes

Spiking solutions were prepared separately for each of 30 standards at concentration 0.03 – 17.08 mg/mL (depending on solubility and required concentration) in 20% methanol/water (for details see Supplement S1; Table 1). In order to achieve final fold concentrations (Supplement S1; Table 2), 3 standard mixes were prepared and used to reconstitute the samples (to a total volume of 200 µl per sample). Each of these standard mixes contained 10 standards (for composition see Supplementary, Table 1).

After reconstitution 10 L of each sample was pooled together to provide a pooled QC sample.

Mass Spectrometry

All analysis was conducted on a QExactive Plus equipped with an Ultimate 3000 UHPLC (Thermo, UK). The UHPLC was equipped with a Hypersil Gold reverse phase column (C18 -2.1 mm x 150 mm; 1.9 m particle size). The solvents employed were (A) water + 0.1% formic acid and (B) methanol + 0.1 % formic acid. The flow gradient was programmed to equilibrate at 95% A for 2 min followed by a linear gradient to 95% B over 8 min and held at 95% B for 2 min before returning to 95% A for 2 min. The column was maintained at 40 C and the samples chilled in the autosampler at 4 C. A sample volume of 5 L was injected onto the column. Blank injections were analysed at the start and end of the analytical batch to assess the background and carryover. In addition pooled QC samples were analysed at every 6th injection to assess for analytical drift over time. Data acquisition was conducted in full MS mode in the scan range of 70-1050 m/z with a resolution of 70,000, an AGC target of 3e6 and a maximum integration time of 200 ms. The samples were analysed in positive and negative mode in separate acquisitions.

Table 1. Composition of the standard mixes, required concentration of the standards in the standard mix and concentration of standards in spiking.solutions.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **STANDARD MIX 1** | | | | | | | | | | | | | |
| **ECMDB id** | | **Standard** | | **Concentration in the STD mix [mM]** | | **Mass concentration [mg/mL]** | | **Supplier** | | **Product number** | | **CAS number** | **Spiking solution concentration [mg/mL]** |
| ECMDB01406 | | Nicotinamide | | 0.252825615 | | 0.030856864 | | Sigma | | N3376 | | 98-92-0 | 1.760 |
| ECMDB00034 | | Adenine | | 0.009480961 | | 0.001280446 | | Sigma | | A8626 | | 73-24-5 | 0.360 |
| ECMDB00133 | | Guanosine | | 0.010113025 | | 0.002862913 | | Acros Organics | | 411130050 | | 118-00-3 | 0.128 |
| ECMDB00131 | | Glycerol | | 0.063206404 | | 0.005817982 | | Fisher Scientific | | G/0650 | | 56-81-5 | 1.504 |
| ECMDB00283 | | D-(-)-Ribose | | 0.063206404 | | 0.009484299 | | Sigma | | R1757 | | 50-69-1 | 1.940 |
| ECMDB00161 | | L-Alanine | | 15.80160096 | | 1.407095882 | | Sigma | | A-7627 | | 56-41-7 | 8.400 |
| ECMDB00244 | | (-)-Riboflavin | | 0.120092167 | | 0.045171262 | | Sigma | | R-4500 | | 83-88-5 | 0.156 |
| ECMDB04087 | | Myo-inositol | | 0.063206404 | | 0.011381159 | | Sigma | | I5125 | | 87-89-8 | 1.940 |
| ECMDB00883 | | L-Valine | | 25.28256154 | | 2.960056481 | | Sigma | | V-0500 | | 72-18-4 | 10.960 |
| ECMDB00930 | | Trans Cinnamic Acid | | 0.063206404 | | 0.009357862 | | Sigma | | 133760-0 | | 140-10-3 | 0.076 |
| **STANDARD MIX 2** | | | | | | | | | | | | | |
| **ECMDB id** | **Standard** | | **Concentration in the STD mix [mM]** | | **Mass concentration [mg/mL]** | | **Supplier** | | **Product number** | | **CAS number** | | **Spiking solution concentration [mg/mL]** |
| ECMDB00044 | L-Ascorbic acid | | 0.063206404 | | 0.011126355 | | BDH | | 103033E | | 50-81-7 | | 2.056 |
| ECMDB04073 | D-(+)-Glucuronic acid | | 0.063206404 | | 0.012264738 | | Acros Organics | | 204570250 | | 14984-34-0 | | 2.000 |
| ECMDB00122 | D(+)-Glucose | | 0.063206404 | | 0.011381159 | | Sigma | | G8270 | | 50-99-7 | | 2.328 |
| ECMDB00176 | Fumaric Acid | | 0.695270442 | | 0.080658991 | | Sigma | | 24074-5 | | 110-17-8 | | 2.100 |
| ECMDB00696 | L-Methionine | | 0.884889654 | | 0.131893731 | | Sigma | | M9625 | | 63-68-3 | | 2.188 |
| ECMDB00167 | L-Threonine | | 1.137715269 | | 0.135454381 | | Sigma | | T-8625 | | 72-19-5 | | 1.976 |
| ECMDB00574 | L-Cysteine | | 1.289410638 | | 0.156044152 | | Sigma | | C-7352 | | 52-90-4 | | 2.024 |
| ECMDB00296 | Uridine | | 13.96861525 | | 3.409313444 | | Sigma | | U-3750 | | 58-96-8 | | 9.952 |
| ECMDB00517 | L-Arginine | | 0.366597142 | | 0.063828843 | | Sigma | | 11009 | | 74-79-3 | | 1.004 |
| ECMDB00187 | L-Serine | | 0.429803546 | | 0.045147679 | | Sigma | | S-4500 | | 56-45-1 | | 2.008 |

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **STANDARD MIX 3** | | | | | | | |
| **ECMDB id** | **Standard** | **Concentration in the STD mix [mM]** | **Mass concentration [mg/mL]** | **Supplier** | **Product number** | **CAS number** | **Spiking solution concentration [mg/mL]** |
| ECMDB00094 | Citric acid | 12.64128077 | 2.427467255 | BDH | 10081314 | 77-92-9 | 17.084 |
| ECMDB00121 | Folic Acid | 0.063206404 | 0.013 | Sigma | F7876 | 59-30-3 | 0.033 |
| ECMDB00929 | L-Tryptophan | 0.075847685 | 0.015479745 | Sigma | T-0254 | 73-22-3 | 0.396 |
| ECMDB00630 | Cytosine | 0.088488965 | 0.009826103 | Sigma | C3506 | 71-30-7 | 0.908 |
| ECMDB00687 | L-Leucine | 1.390540884 | 0.182292441 | Acros Organics | 125121000 | 61-90-5 | 4.548 |
| ECMDB00162 | L-Proline | 2.401843346 | 0.27636409 | Sigma | P0380 | 147-85-3 | 4.428 |
| ECMDB00168 | L-Asparagine | 3.229847236 | 0.426512607 | Sigma | A-0884 | 70-47-3 | 1.992 |
| ECMDB00904 | L-Citrulline | 3.261450438 | 0.571065919 | Sigma | C-7629 | 372-75-8 | 7.876 |
| ECMDB00159 | L-Phenylalanine | 0.113771527 | 0.018781287 | Sigma | P2126 | 63-91-2 | 0.860 |
| ECMDB00300 | Uracil | 0.01 | 0.001120868 | Sigma | U-0750 | 66-22-8 | 3.774 |

Table 2. Setting of reconstitution of bacterial extracts.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Standard mix 1** | **Standard mix 2** | **Standard mix 3** |
| **group A** | -- | 3 Fold | 5 Fold |
| **group B** | 3 Fold | 5 Fold | -- |
| **group C** | 5 Fold | -- | 3 Fold |
| **group D / Controls** | -- | -- | -- |