*Sample preparation for LC-MS analysis of polar metabolites from tissues or cultured cells (with considerations for NADH and NADPH detection)*

Metabolites should be quenched as quickly as possible by working with the samples at low temperatures. Cells can be handled at 4ºC or on dry ice, extraction buffer can be pre-chilled to -20ºC. Samples should be analyzed by LC/MS on the day of extraction. If impractical, best alternative is to store dried samples at -80ºC. Unless indicated otherwise, 1 million cells or about 2mg of tissue was extracted per condition and minimum of three replicates per condition was used in each experiment. Non-adherent cells were collected by brief centrifugation at 4ºC on a table-top centrifuge (4,500 rpm, 1.5 min) and washed briefly in 0.9% NaCl (high grade salt and LC/MS-grade water Fisher Scientific W6500 or Sigma Aldrich 1.15333). 300µl of prechilled extraction buffer were added per sample. For tissues –chunks were crushed using a hand-held homogenizer (VWR 47747-370) with several pulses while keeping the samples on ice. 300µl of prechilled extraction buffer was used per 2mg of tissue.

Extraction buffer “A”:

40:40:20 of acetonitrile:methanol:water, supplemented with 0.1M formic acid and isotopically-labeled internal standards (17 amino acids and reduced glutathione, Cambridge Isotope Laboratories, MSK-A2-1.2 and CNLM-6245-10).

Extraction buffer “B”:

80% LC/MS-grade methanol, 20% 25 mM Ammonium Acetate and 2.5 mM Na-Ascorbate prepared in LC/MS water and supplemented with isotopically labeled internal standards (17 amino acids and isotopically labelled reduced glutathione, Cambridge Isotope Laboratories, MSK-A2-1.2 and CNLM-6245-10).

Extraction buffer “C” and “C + Ellman’s”:

Solution 1: 100% LC-MS Methanol

Solution 2: 25mM Ammonium Acetate and 2.5mM Na-Ascorbate in LC-MS water supplemented with isotopically labelled reduced glutathione and isotopically labeled internal standards (17 amino acids and reduced glutathione, Cambridge Isotope Laboratories, MSK-A2-1.2 and CNLM-6245-10).

Ellman’s reagent (5,5′-Dithiobis(2-nitrobenzoic acid),D8130, Sigma Aldrich): 20 mM in “Solution 2”. Final composition is 4:1 solution 1:solution 2.

 Samples were vortexed briefly (10 sec) and sonicated for 3 min in a 4ºC water bath. Samples were then centrifuged for 10 min, 4ºC, at maximum speed on a benchtop centrifuge (Eppendorf) and the cleared supernatant was transferred to a new tube.  Samples were dried using a nitrogen dryer while on ice, and special attention was given to minimize the time of drying and to not let samples idle in the dryer (Reacti-Vap™ Evaporator, Thermo Fisher Scientific, TS-18826) once the drying process was completed. Needles were continuously adjusted to as the samples lost liquid to expedite the drying process. Samples were reconstituted in 30 µl LC/MS water by brief sonication in a 4ºC water bath. Extracted metabolites were spun for 2 min at maximum speed on a bench-top centrifuge and cleared supernatant was transferred to LC/MS micro vials (National Scientific, C5000-45B). A small amount of each sample was pooled and serially diluted 3- and 10-fold to be used as quality controls throughout the run of each batch.

*Sample preparation for LC-MS analysis of polar metabolites from CSF (with considerations for NADH and NADPH detection)*

Flash frozen, precleared CSF was used. Per condition, 3 µl were extracted by brief sonication in 200µl of indicated extraction buffers. After centrifugation for 10 min at maximum speed on a benchtop centrifuge (Eppendorf) the cleared supernatant was dried using a nitrogen dryer and reconstituted in 30 µl water by brief sonication. Extracted metabolites were spun again and cleared supernatant was transferred to LC-MS micro vials. A small amount of each sample was pooled and serially diluted 3- and 10-fold to be used as quality controls throughout the run of each batch.

*Chromatographic conditions for LC/MS*

**ZIC-pHILIC chromatography**: One ml of reconstituted sample was injected into a ZIC-pHILIC 150 × 2.1 mm (5 µm particle size) column (EMD Millipore) operated on a Vanquish™ Flex UHPLC Systems (Thermo Fisher Scientific, San Jose, CA). Chromatographic separation was achieved using the following conditions: buffer A was acetonitrile; buffer B was 20 mM ammonium carbonate, 0.1% ammonium hydroxide. Gradient conditions were: linear gradient from 20% to 80% B; 20–20.5 min: from 80% to 20% B; 20.5–28 min: hold at 20% B. The column oven and autosampler tray were held at 25 °C and 4 °C, respectively.

**Accucore-HILIC chromatography**: One ml of reconstituted sample was injected into a Thermo Fisher Scientific™ Accucore™ 150 Amide HILIC (150x3 mm, 2.6 mm particle size; Thermo Fisher Scientific) operated on a Vanquish™ Flex UHPLC Systems (Thermo Fisher Scientific, San Jose, CA). Chromatographic separation was achieved using the following conditions: buffer A was acetonitrile; buffer B was 20 mM ammonium carbonate, 0.1% ammonium hydroxide. Gradient conditions were: linear gradient from 20% to 80% B; 20–20.5 min: from 80% to 20% B; 20.5–28 min: hold at 20% B. The column oven and autosampler tray were held at 35 °C and 4 °C, respectively.

**LUNA-NH2 chromatography**: One  ml of reconstituted sample was injected into a Luna® 3 µm NH2 100 Å, LC Column (150x2 mm, 3 µm particle size; Phenomenex, 00F-4377-B0) operated on a Vanquish™ Flex UHPLC Systems (Thermo Fisher Scientific, San Jose, CA). Chromatographic separation was achieved using the following conditions: buffer A was acetonitrile; buffer B was 5 mM ammonium acetate and 0.2% ammonium hydroxide. Gradient conditions were: 20 min linear gradient from 10% to 90% B; 20-25 min hold at 90% B; 25–26 min from 90% to 10% B; 26–34 min hold at 10% B. The column oven and autosampler tray were held at 30 °C and 4 °C, respectively

*Orbitrap conditions for targeted analysis of polar metabolites*

MS data acquisition was performed using a QExactive benchtop orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe (Thermo Fisher Scientific, San Jose, CA) and was performed in postive and negative ionization mode in a range of m/z= 70–1000, with the resolution set at 70,000, the AGC target at 1x106, and the maximum injection time (Max IT) at 20 msec. For tSIM scans, the resolution was set at 70,000, the AGC target was 1x105, and the max IT was 100 msec. for PRM scans, the resolution was set at 17,500, the AGC target was 1x105, and the max IT was 20 msec. The following inclusion list and energies were used:

**Table 1**.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Name** | **m/z** | **Polarity** | **NCE** | **RT range (min)** |
| GSH-Ellman’s | 503.0552 | negative | 20, 40, 60, 80 | 8-12 |
| GSH-13C2-15N-Ellman’s | 506.0580 | negative | 20, 40, 60, 80 | 8-12 |
| GSH | 308.0911 | positive | 20, 40, 60, 80 | 8-12 |
| GSH-13C2-15N | 309.0802 | positive | 20, 40, 60, 80 | 8-12 |
| GSSG | 613.1592 | positive | 20, 40, 60, 80 | 10-14 |
| NADPH | 746.0984 | positive | 20, 40, 60, 80 | 9-14 |
| NADP | 744.0827 | positive | 20, 40, 60, 80 | 8-13 |
| NADH | 666.1320 | positive | 20, 40, 60, 80 | 6-11 |
| NAD | 664.1164 | positive | 20, 40, 60, 80 | 6-11 |

Relative quantitation of polar metabolites was performed with TraceFinder 4.1 (Thermo Fisher Scientific, Waltham, MA) using a 5 ppm mass tolerance and referencing an in-house library of chemical standards (submitted separately). Pooled samples and fractional dilutions were prepared as quality controls and only those metabolites were taken for further analysis, for which the correlation between the dilution factor and the peak area was >0.95 (high confidence metabolites) and for which the coefficient of variation (CV) was below 30%. Normalization for biological material amounts was based on the total integrated peak area values of high-confidence metabolites within an experimental batch after normalizing to the averaged factor from all mean-centered chromatographic peak areas of isotopically labeled amino acids internal standards. Where indicated, data was control mean centered, otherwise data were Log transformed and Pareto scaled within the MetaboAnalyst-based statistical analysis platform (Xia et al, Nucleai Acid research, 2015). All heatmap, PCA, or PLSDA analysis were performed using the MetaboAnalyst online platform. Individual one-way Anova and t-tests were performed in Prism software.