Metabolite Extraction Procedures, NMR Sample Preparation and Data Processing, and Example Calculations

Metabolite Extraction Procedures

Hydrophilic Extraction

The cell pellets were thawed on ice. Cold methanol (MeOH) (800 µL) and 170 µL of Millipore H_2O were added to each sample test tube and vortexed. The cells were sonicated for 5 min then vortexed. Cold chloroform (800 µL) and 400 µL of cold H_2O were added to each test tube. The samples were vortexed and incubated on ice for 15 min. The test tubes were centrifuged at 3,500 rpms for 15 min. The aqueous layer was transferred to a sterile Eppendorf tube and concentrated on a speed vacuum until dry. The samples were frozen at -80 ℃ (Ammons et al. 2014 and 2015 and references therein).

Acetone Precipitation

The samples were thawed on ice. The pellets were suspended in 250 µL D_2O and 1,250 µL Acetone. The samples were frozen at – 80 ℃ overnight. The samples were thawed on ice. They were cloudy with precipitated proteins once thawed. The samples were centrifuged at 2,000 rpms for 30 min. The protein pellet was discarded. The speed vacuum was used to dry the metabolite samples. The metabolite pellet was frozen at –80 ℃ (Ammons et al. 2014 and 2015 and references therein).

NMR Sample Preparation and Data Processing

Note: All D_2O used was NMR grade.

NMR Buffer Preparation

An imidazole stock solution was made by adding 27.230 g of imidazole to 4.0 mL of D_2O. Monosodium DSS (21.23 mg) was added to 4.0 mL of D_2O to make a DSS stock solution. In 5.0 mL of D_2O, 0.345 g $NaH_2PO_4 \cdot H_2O$, 0.355 g Na_2HPO_4 (anhydrous) and 0.040 g NaN_3 were dissolved to create a $NaPO_4$ buffer stock solution. A 30 mL D_2O buffer solution was made by combining 300 µL of the DSS stock solution, 60 µL of the imidazole stock solution, 1,500 µL of the $NaPO_4$ buffer stock solution, and 28,140 µL of D_2O (Ammons et al. 2014 and 2015 and references therein).

Sample Preparation

The metabolite samples were placed on ice, and 700 µL of the NMR buffer was added. Three of the same metabolite pellet types were combined into one 700 µL NMR buffer solution. The samples were gently vortexed then transferred to a capped NMR Tube.

Data Acquisition

The samples were acquired using a Bruker DRX 600 MHz NMR. ^{1}H NMR spectra were acquired using Topspin as the data acquisition program. The solvent was set as D_2O, and the number of scans per sample was set to 64. The receiver gain and shimming were checked to ensure they were correct. The spectra were Fourier transformed and phased using the H_2O and DSS resonances.

Data Analysis
The spectra were processed and profiled using Chenomx NMR Suite 8.2 software (Chenomx 2016). Later, the same data was chosen at random to profile again to check for errors. The concentrations of the identified metabolites in each sample were standardized to their concentration. XLSTAT (Addinsoft 2019) was used for hierarchical clustering and for principal component analysis (PCA) for each sample type and corresponding standardized metabolite concentrations. The metabolites and their concentration data were transformed into comma delineated form (CSV). The CSV data were uploaded to the MetaboAnalyst website version 4.0 for statistical analysis (Chong et al 2019 and references therein). The data was normalized by sum, the missing values calculation was skipped, and the fold change was set to 1.5 or higher for statistical analyses. A volcano plot of p-value versus fold change and sparse partial least squares discriminate analysis (sPLS-DA), Pattern Hunter and the very important features (VIP) data were used to determine relationships among statistically significant metabolite changes. sPLS-DA was used to eliminate outliers in each sample type. The list of metabolites generated for each sample type was used in the MetaboAnalyst Pathway Analysis tool and using KEGG pathways (Kanehisa and Goto 2000 and references therein) in order to identify altered metabolomic pathways in the mutants in response to the DABCOMD.

Three Example Calculations

(please see table below)

Sample Type	Mutant Stationary								
Sample ID	**Mut_2_2**			**Mut_2_9**			**Mut_2_12**		
Standardization Concentration	4.289			6.959			9.317		
Calculation Example	$\left(\dfrac{Starting}{Standardization\ Concentration}\right) * 100$								
Metabolite Name	**Starting**	**Calculation**	**Final**	**Starting**	**Calculation**	**Final**	**Starting**	**Calculation**	**Final**
Acetate	0.0321	$\left(\dfrac{0.0321}{4.289}\right)*100$	0.6637	0.1149	$\left(\dfrac{0.1149}{6.959}\right)*100$	1.6518	0.0832	$\left(\dfrac{0.0832}{9.317}\right)*100$	0.8928
Acetoacetate	0.0032	Same as above and continues below	0.0660	0.0098	Same as above and continues below	0.1403	0.0111	Same as above and continues below	0.1193
Adenosine	0.0424		0.8789	0.0116		0.1660	0.0635		0.6813
Alanine	0.1352		2.7994	0.3148		4.5237	0.3053		3.2771
AMP	0.0467		0.9676	0.1177		1.6913	0.1040		1.1158
Aspartate	0.2628		5.4425	0.4411		6.3383	0.6041		6.4836
Betaine	0.0371		0.7679	0.0287		0.4121	0.0596		0.6400
Cysteine	0.0780		1.6146	0.1952		2.8050	0.1898		2.0373
Cytosine	0.0294		0.6088	0.0267		0.3839	0.0409		0.4391
Formate	0.0336		0.6949	0.0666		0.9574	0.0630		0.6758
Fucose	0.0408		0.8445	0	*	0	0.0399		0.4285
Fumarate	0.0072		0.1485	0.0104		0.1497	0.0139		0.1489
Glutamate	0.4582		9.4877	1.0870		15.6206	1.1767		12.6293
Glycine	0.0231		0.4779	0.0221		0.3179	0.0405		0.4348
Histidine	0.0647		1.3389	0.0684		0.9829	0.1009		1.0834
Homocysteine	0	*	0	0	*	0	0.0348		0.3735
Isocitrate	0.5427		11.2388	0.4780		6.8681	0.8950		9.6061
Isoleucine	0.3009		6.2312	0.2618		3.7614	0.4641		4.9809
Lactate	0.0400		0.8283	0.0688		0.9892	0.0775		0.8313
Leucine	0.5480		11.3478	0.5466		7.8546	0.8725		9.3647
Lysine	0.4357		9.0226	0.3671		5.2755	0.8718		9.3574
Methionine	0.1045		2.1638	0.1850		2.6589	0.2016		2.1638
N-Acetylglucosamine	0.0194		0.4014	0	*	0	0	*	0
NAD+	0.0922		1.9095	0.1592		2.2877	0.1851		1.9867
Phenylalanine	0.1182		2.4468	0.1936		2.7821	0.2286		2.4538
Proline	0.0715		1.4801	0.0843		1.2115	0.1267		1.3602
Pyroglutamate	0.3408		7.0571	0.3195		4.5910	0.5099		5.4730
Succinate	0.3153		6.5286	0.7631		10.9655	0.6259		6.7174
Tyrosine	0.1585		3.2827	0.2777		3.9899	0.3472		3.7261
UDP-glucose	0.0313		0.6486	0.0840		1.2071	0.0850		0.9128
Valine	0.3393		7.0266	0.4364		6.2709	0.5953		6.3896
*The final value was set to 1×10^{-11} if needed to eliminate the zero-calculation problem.									