Quantitative and Qualitative Metabolomics for the Investigation of Intracellular Metabolism

Targeted Analysis on the QTRAP® 5500 System and Reverse-Phase Ion-Pairing Chromatography

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Liquid chromatography-mass spectrometry (LC-MS) provides a powerful analytical tool for understanding and monitoring intracellular metabolism by measuring the metabolome. The study of intracellular metabolism of model organisms, such as *E. coli*, is vital to further our biochemical knowledge[1], to develop new pharmaceuticals that target harmful pathogens[2], and to improve industrial applications that aim to metabolically engineer bacteria in order to produce commodity chemicals from renewable resources[3]. Paramount to these endeavors is the ability to reliably and accurately measure the intracellular metabolome. For microorganisms, the compounds of most interest comprise intermediates of high flux pathways such as glycolysis, the pentose phosphate pathway, the citric acid cycle, amino acid metabolism, as well as energy and redox cofactors such as ATP and NADH (Figure 1). By measuring the absolute metabolite levels of such compounds, one is able to calculate reaction and pathway thermodynamics[4] and infer *in vivo* enzyme kinetics[5]. In addition, when microorganisms are grown on a specifically chosen labeled substrate (e.g. 1-^{13}C glucose) during a metabolic labeling experiment, the isotopomer distribution of intracellular compounds can be used to calculate the absolute flux through specific reactions of interest[6].

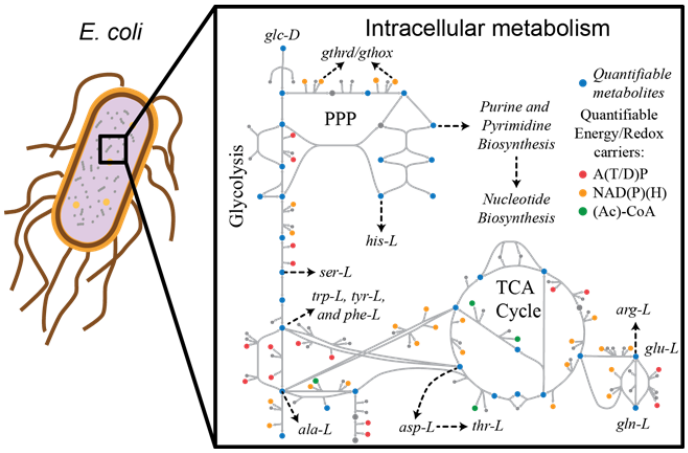

Figure 1. Metabolomics of Intracellular Pathways for Investigations into the Biochemistry of Microorganisms. High flux pathways such as those shown are key to generating a biological picture and targeted metabolomics strategies provide a robust quantitative strategy for monitoring changes.

In this work, the QTRAP® 5500 system (a hybrid triple quadrupole linear ion trap mass spectrometer) was used to implement both quantitative and qualitative workflows aimed at measuring anionic and polar compounds of intracellular metabolism.

Key Features of the QTRAP® 5500 System for Qualitative and Quantitative Metabolomics

- The hybrid triple quadrupole linear ion trap QTRAP system coupled with powerful IDA workflows allow for
  - Confidence in compound identification for quantitative workflows
  - Broad compound coverage and structural information for qualitative workflows
- A qualitative workflow was used to confirm the uniform labeling of intracellular compounds from a metabolic labeling experiment where *E. coli* was grown on uniformly labeled ^{13}C glucose for use as internal standards.
- Using MRM mode analysis and isotope dilution mass spectrometry (IDMS)[7], the absolute levels of intracellular metabolites in *E. coli* grown in two different media conditions were measured.
- Improved duty cycle using the *Scheduled* MRM™ Pro Algorithm allows for data for more compounds to be acquired in a single run

Material and Methods

Sample Preparation: Samples of *E. coli* K12 MG1655 (ATCC 700926), obtained from the American Type Culture Collection (Manassas, VA), were grown in 4 g/L glucose or complex M9 minimal media [8] with trace elements [9] and sampled from an anaerobic chamber[4]. Samples were taken and extracted using a fast filtration approach using 0.45 µM filters (PVDF, Millipore) and serially extracted using 40:40:20 acetonitrile + 0.1% formic acid:methanol:water pre-cooled to -40°C.

Chromatography: A XSELECT HSS XP 150 mm × 2.1 mm × 2.5 µm (Waters) with an UFLC XR HPLC (Shimadzu) was used for chromatographic separation. Mobile phase A was composed of 10 mM tributylamine (TBA), 10 mM acetic acid (pH 6.86), 5% methanol, and 2% 2-propanol; mobile phase B was 2-propanol. Oven temperature was 40°C. The optimized chromatographic gradient is described in Table 1. The autosampler temperature was 10°C and the injection volume was 10 µL with full loop injection.

Mass Spectrometry: A QTRAP® 5500 System (SCIEX) operating in negative mode was used for targeted profiling using the Multiple Reaction Monitoring (MRM) approach. Electrospray ionization parameters were optimized for 0.4mL/min flow rate, and are as follows: electrospray voltage of -4500 V, temperature of 500 °C, curtain gas of 40, CAD gas of 12, and gas 1 and 2 of 50 and 50 psi, respectively. Analyzer parameters were optimized for each compound using manual tuning. The instrument was mass calibrated with a mixture of polypropylene glycol (PPG) standards.

Table 1. Gradient Used for Chromatography.

Time (min)	% Eluent B	Flow Rate (mL/min)
0	0	0.4
5	0	*0.4*
9	*2*	*0.4*
9.5	*6*	*0.4*
11.5	*6*	*0.4*
12	*11*	*0.4*
13.5	*11*	*0.4*
15.5	*28*	*0.4*
16.5	*53*	*.15*
22.5	*53*	*0.15*
23	*0*	*0.15*
27	*0*	*0.4*
33	*0*	*0.4*

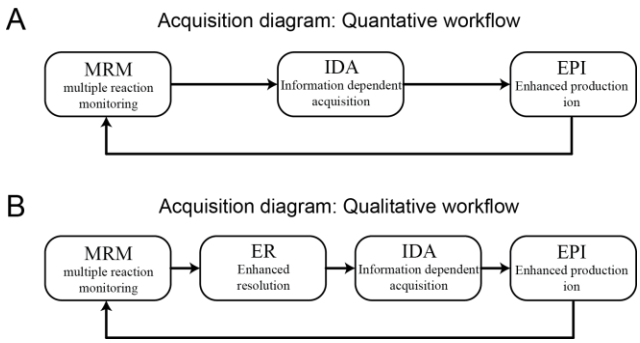

Figure 2. Metabolomics Workflows on QTRAP® 5500 system. The IDA workflow depicted in method A was the main workflow used for quantitation. MRM data for quantitation is acquired along with Enhanced Product Ion (EPI) data for qualitative confirmation of the detected metabolite. The IDA workflow depicted in method B was used to elucidate the isotope distribution of metabolites. Enhanced resolution (ER) data of the target compound was collected along with EPI data of the detected metabolite. The ER data provides information on the isotope distribution, while the EPI data provides information on the location of the heavy labels.

Data Processing: Samples were acquired using the *Scheduled* MRM™ Pro Algorithm in Analyst® Software 1.6.2. The information dependent acquisition (IDA) acquisition method consisted of a multiple reaction monitoring (MRM) survey scan coupled with an enhanced product ion (EPI) scan for compound identity confirmation. Samples were quantified using IDMS[7, 10] with metabolically labeled internal standards from *E. coli* and processed using MultiQuant® Software 2.1.1. Linear regressions for compound quantitation were based on peak height ratios and the logarithm of the concentration of calibrator concentrations from a minimum of four consecutive concentration ranges that showed minimal bias. A peak height greater than $1e^3$ ion counts and signal to noise greater than 20 were used to define the lower limit of quantitation (LLOQ). Quality controls and carry-over checks were included with each batch.

Statistical Analysis: All statistical and correlation analyses were done using R[11] (R Development Core Team, 2011) or MetaboAnalyst[12].

Single Method for Quantitative Detection and Confirmation

The quantitative workflow uses the *Scheduled* MRM™ Pro Algorithm to maximize method efficiency. Here, the MRMs for each analyte are monitored using a narrow retention time window around the expected eluting time, such that the maximum number of MRM scans can be acquired in a given run while ensuring sufficient dwell time for reproducible acquisition. When a signal is detected in the MRM scans, acquisition of a full scan MS/MS spectrum (EPI) is triggered via information dependent acquisition (IDA) (Figure 2). The acquired MS/MS spectra can then be searched against a library of compound spectra from pure standards to provide greater confidence in compound detection. This is particularly beneficial when the use of ion ratios for compound identification is problematic. In short, the method can simultaneously quantitate 100 compounds, corresponding to 302 MRM transitions and provide qualitative confirmation of identity for each compound (Table 2).

An example of the output from the method is described in Figure 3. Here, primary and secondary MRM transitions (blue and green respectively) for L-glutamate (glu-L) were monitored in an *E. coli* cell lysate. An EPI scan is triggered when these transitions reach pre-defined thresholds. This MS/MS spectrum matched to the standard reference spectra for glutamate with a greater than 90% match, providing added confidence in the quantitative data. In addition, a uniformly heavy carbon labeled analog is monitored per compound by MRM (red) in order to provide absolute quantitation by IDMS.

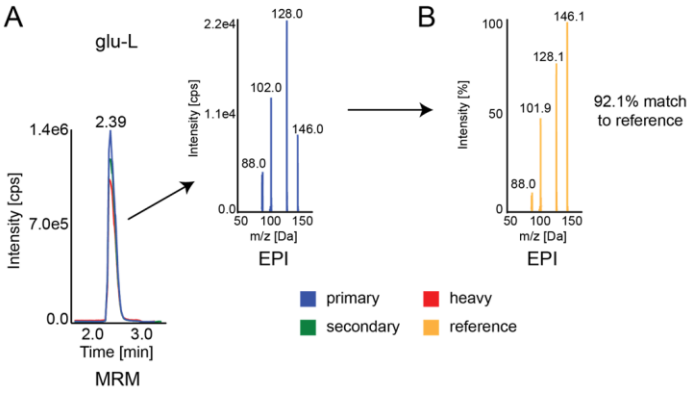

Figure 3. Simultaneous Quantitative Analysis with Qualitative Confirmation of L-Glutamate using the QTRAP® 5500 System. A) The primary and secondary transitions (blue and green) for glu-L are monitored, along with the uniformly heavy carbon labeled analog (red). A full scan MS/MS spectrum (EPI scan) is triggered when the primary and secondary transitions reach a predefined threshold. B) To confirm the identity of the MRM signal, the MS/MS was matched with a greater than 90% match to the reference spectrum (taken for pure standards for glutamate).

Using Heavy Carbon Labeling for Quantitative Analysis

The compounds targeted in this workflow are involved in pathways that include central carbohydrate metabolism (i.e., glycolysis, the pentose phosphate pathway, and the citric acid cycle), amino acid metabolism, and nucleotide and cofactor metabolism (Figure 1). Heavy analogs (e.g. $U^{13}C$) of intracellular metabolites for use as internal standards for absolute quantitation are prohibitively expensive for most labs or commercially unavailable. An efficient and cost-effective means to generate internal standards for quantitation is through metabolic labeling[10]. Metabolic labeling involves growing a micro-organism (e.g. E. coli) on a uniformly labeled carbon source. The resulting fully labeled biomass can be harvested, extracted and subsequently used as internal standards.

In addition to coupling MRM and EPI scans, the qualitative workflow can also include an Enhanced Resolution (ER) scan (Figure 2) which provides a higher resolution MS scan of each analyte with accurate isotope ratios (Figure 4). From the ER scan, the isotope distribution of metabolically labeled compounds can be determined in the labeling experiments. The EPI scan which is also triggered provides additional structural information on the location of the heavy labels (Figure 4). This capability means that individual MRMs to measure each of the potential isotopomers and their fragments do not have to be developed which allows one to conserve dwell time and measure the isotopomer distribution of more compounds.

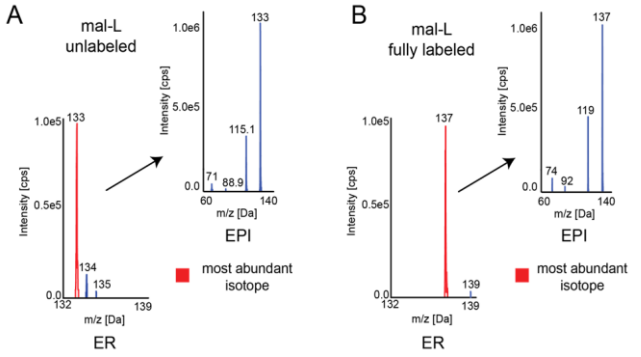

Figure 4. Qualitative Method for Characterizing the Isotopomers, Enabled by the QTRAP 5500® System. The advantage of the qualitative method is highlighted here in this example measuring unlabeled and fully labeled malic acid (mal-L) in E. *coli*. Several transitions corresponding to the isotopomer distribution are monitored per compound. An Enhanced Resolution (ER) scan is triggered when one of the isotopomer transitions reaches a predefined threshold and provides MS and isotope ratio information. An EPI scan is also triggered and provides information regarding the location of the heavy label (if present).

MS/MS Characterization of Metabolite Internal Standards

In this example, the qualitative workflow was used to verify the integrity of our metabolically labeled internal standards prior to use in quantitation. Product ion spectra were generated using the qualitative method on a sample of E. *coli* grown on uniformly labeled ^{13}C glucose generated and analyzed using PeakView® Software (Figure 5). Complete labeling of compounds for use as internal standards for metabolite quantitation were verified by matching the product ion spectra of the unlabeled to uniformly labeled compound. Structural information for each of the compound fragments predicted using PeakView Software was then used to verify that the mass of each of the fragments of the uniformly labeled analyte. The masses were incremented by the number of carbons contained in the fragment as compared to the unlabeled compound.

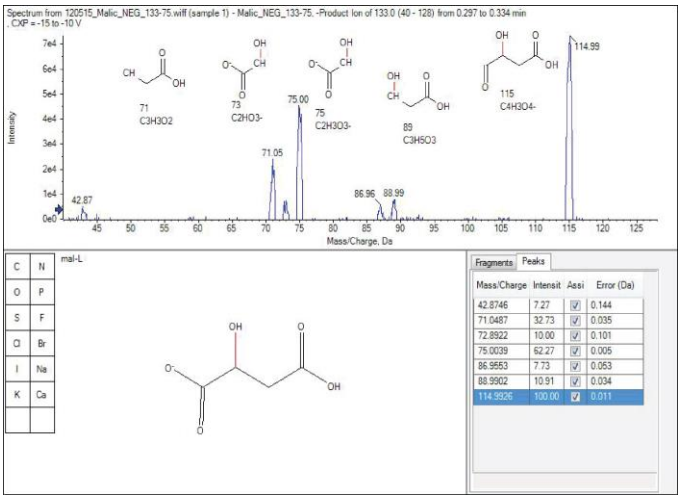

Figure 5. Determination of the Fragment Structures from MS/MS Spectra using PeakView® Software. The formula of the fragments, specifically, the number of carbon atoms, is used to calculate the theoretical nominal mass of the metabolically labeled ^{13}C equivalent. This information is used to verify the complete labeling of the metabolically labeled compound for use as internal standards. Here the structure of malic acid and the predicted fragments is shown.

Comparing Metabolites across Growth Media

With internal standards in hand, the quantitative workflow was used to measure the differences in absolute metabolite levels in *E. coli* grown in two different media conditions. The first media condition was a glucose minimal media (Minimal), and the second was a complex media that included glucose and supplemented amino acids and precursors (Complex). It was found that supplementation with amino acids and their precursors correspondingly increased levels of intracellular amino acids. This finding is consistent with previous studies that found an increase in growth rate for bacteria supplemented with amino acids due to the import of amino acids and their precursors into the cell[13]. This is depicted in the heat map of the top 25 most significant metabolite changes in Figure 6.

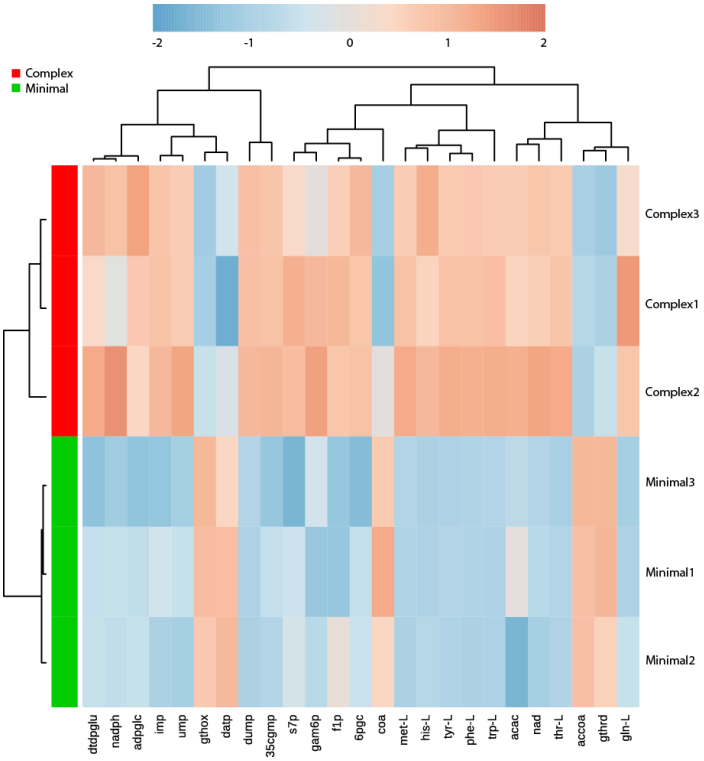

Figure 6. Heat Map of Absolute Metabolite Level Differences Between *E. coli* Grown in Minimal Media and Complex Media. The top 25 most significant metabolites (x-axis) are clustered (Pearson's R, complete linkage) according their significance and fold-change (Students t-test). The media conditions (y-axis) are clustered (Pearson's R, complete linkage) according to similarity in metabolite level of the top 25 most significant metabolite changes.

Conclusions

The work here describes the use of the QTRAP® 5500 System for quantitative and qualitative metabolomics investigations into the intracellular metabolism of microorganisms. Both methods were used to successfully confirm and quantitate 100 metabolites in the metabolome of E. coli grown on two different media sources. The quantitative and qualitative workflows described here can be readily extended to other single cell and multi-cell organisms.

Metabolite	Q1	Q3	RT	DP	EP	CE	CXP	MS Window	Quantifier
L-Arginine-UC13	179	136	0.7	-125.3	-10	-19.1	-6.9	30	1
L-Arginine	173	131	0.7	-125.3	-10	-19.1	-6.9	30	1
L-Ornithine-UC13	136	89	0.7	-61.8	-10	-17.4	-7.9	30	1
L-Ornithine	131	85	0.7	-61.8	-10	-17.4	-7.9	30	1
L-Asparagine-UC13	135	118	0.92	-89.7	-10	-16.1	-7	30	1
L-Asparagine	131	114	0.92	-89.7	-10	-16.1	-7	30	1
L-Asparagine	131	95	0.92	-51	-10	-17.1	-11.3	30	2
L-Serine-UC13	107	76	0.92	-72.8	-10	-15.9	-8.7	30	1
L-Serine	104	74	0.92	-72.8	-10	-15.9	-8.7	30	1
L-Serine	104	42	0.92	-73.9	-10	-18.7	-12.2	30	2
L-Citrulline-UC13	180	136	0.93	-76.4	-10	-19.1	-6.4	30	1
L-Citrulline	174	131	0.93	-76.4	-10	-19.1	-6.4	30	1
L-Glutamine-UC13	150	132	0.93	-19.9	-10	-13.3	-17.7	30	1
L-Glutamine	145	127	0.93	-19.9	-10	-13.3	-17.7	30	1
L-Glutamine	145	101	0.93	-20.2	-10	-14.3	-6.8	30	2
Hexose_Pool_fru_glc-D-UC13	185	61	0.94	-36.2	-10	-23.1	-5.8	30	1
Hexose_Pool_fru_glc-D	179	59	0.94	-36.2	-10	-23.1	-5.8	30	1
Hexose_Pool_fru_glc-D	179	89	0.94	-37.4	-10	-12.9	-6.7	30	2
L-Cystine-UC13	245	123	0.94	-59.7	-10	-17	-14.8	30	1
L-Cystine	239	120	0.94	-59.7	-10	-17	-14.8	30	1
L-Threonine-UC13	122	76	0.94	-46.5	-10	-14.2	-6.7	30	1
L-Threonine	118	74	0.94	-46.5	-10	-14.2	-6.7	30	1
L-Alanine-UC13	91	46	0.95	-14.2	-10	-19.9	-14	30	1
L-Alanine	88	45	0.95	-14.2	-10	-19.9	-14	30	1
L-Alanine	88	42	0.95	-11.2	-10	-19.2	-17	30	2
Cytidine-UC13	251	113	1.15	-100	-4	-16.1	-5.2	30	1
Cytidine	242	109	1.15	-100	-4	-16.1	-5.2	30	1
L-Histidine-UC13	160	143	1.15	-56.7	-10	-18.8	-14.8	30	1
L-Histidine	154	137	1.15	-56.7	-10	-18.8	-14.8	30	1
L-Histidine	154	93	1.15	-57.6	-10	-22.8	-9	30	2
Uracil-UC13	115	43	1.15	-100	-10	-26	-7	30	1
Uracil	111	42	1.15	-100	-10	-26	-7	30	1
5-Aminoimidazole-4-carboxamide riboside-UC13	266	129	1.2	-100	-10	-22	-9	30	1
5-Aminoimidazole-4-carboxamide riboside	257	125	1.2	-100	-10	-22	-9	30	1
L-Methionine-UC13	153	48	1.22	-80.1	-10	-28.2	-6.3	30	1
L-Methionine	148	47	1.22	-80.1	-10	-28.2	-6.3	30	1
Uridine-UC13	252	43	1.27	-110	-10	-52	-7	30	1
Uridine	243	42	1.27	-110	-10	-52	-7	30	1
Uridine	243	110	1.27	-110	-10	-22	-7	30	2
Guanine-UC13	155	138	1.28	-135	-10	-20	-13	30	1
Guanine	150	133	1.28	-135	-10	-20	-13	30	1
Hypoxanthine-UC13	140	96	1.28	-62	-6	-22	-4	30	1
Hypoxanthine	135	92	1.28	-62	-6	-22	-4	30	1
L-Tyrosine-UC13	189	172	1.35	-66.4	-10	-18.1	-9.9	30	1
L-Tyrosine	180	163	1.35	-66.4	-10	-18.1	-9.9	30	1
L-Tyrosine	180	119	1.35	-82.3	-10	-23.2	-10.7	30	2
Xanthine-UC13	156	112	1.41	-25	-10	-24	-9	30	1
Xanthine	151	108	1.41	-25	-10	-24	-9	30	1
Inosine-UC13	277	140	1.47	-20	-10	-30	-11	30	1
Inosine	267	135	1.47	-20	-10	-30	-11	30	1
Inosine	267	108	1.47	-20	-10	-52	-13	30	2
Guanosine-UC13	292	155	1.58	-110	-10	-26	-9	30	1
Guanosine	282	150	1.58	-110	-10	-26	-9	30	1
Guanosine	282	133	1.58	-110	-10	-42	-9	30	2
Thymine-UC13	130	43	1.58	-45	-10	-36	-7	30	1
Thymine	125	42	1.58	-45	-10	-36	-7	30	1

Adenine-UC13	*139*	*111*	*1.83*	*-90*	*-10*	*-24*	*-13*	*60*	*1*
Adenine	*134*	*107*	*1.83*	*-90*	*-10*	*-24*	*-13*	*60*	*1*
Adenine	*134*	*92*	*1.83*	*-90*	*-10*	*-26*	*-11*	*60*	*2*
D-Glucosamine 6-phosphate-UC13	*264*	*79*	*1.9*	*-54.3*	*-10*	*-63*	*-9.2*	*120*	*1*
D-Glucosamine 6-phosphate	*258*	*79*	*1.9*	*-54.3*	*-10*	*-63*	*-9.2*	*120*	*1*
D-Glucosamine 6-phosphate	*258*	*97*	*1.9*	*-53.9*	*-10*	*-23.4*	*-5.4*	*120*	*2*
Adenosine-UC13	*276*	*139*	*2.3*	*-115*	*-10*	*-30*	*-17*	*60*	*1*
Adenosine	*266*	*134*	*2.3*	*-115*	*-10*	*-30*	*-17*	*60*	*1*
L-Aspartate-UC13	*136*	*91*	*2.3*	*-40.5*	*-10*	*-18.2*	*-9.2*	*120*	*1*
L-Aspartate	*132*	*88*	*2.3*	*-40.5*	*-10*	*-18.2*	*-9.2*	*120*	*1*
L-Aspartate	*132*	*115*	*2.3*	*-65.8*	*-10*	*-16.9*	*-5.9*	*120*	*2*
L-Glutamate-UC13	*151*	*133*	*2.3*	*-62.8*	*-10*	*-14.1*	*-8.6*	*120*	*1*
L-Glutamate	*146*	*128*	*2.3*	*-62.8*	*-10*	*-14.1*	*-8.6*	*120*	*1*
L-Glutamate	*146*	*102*	*2.3*	*-70.3*	*-10*	*-20*	*-7.4*	*120*	*2*
L-Phenylalanine-UC13	*173*	*156*	*2.4*	*-124.7*	*-10*	*-16.2*	*-10.1*	*30*	*1*
L-Phenylalanine	*164*	*147*	*2.4*	*-124.7*	*-10*	*-16.2*	*-10.1*	*30*	*1*
L-Phenylalanine	*164*	*103*	*2.4*	*-126.1*	*-10*	*-22.3*	*-12*	*30*	*2*
Shikimate-UC13	*180*	*99*	*2.7*	*-40*	*-10*	*-26.2*	*-10.85*	*60*	*1*
Shikimate	*173*	*93*	*2.7*	*-40*	*-10*	*-26.2*	*-10.85*	*60*	*1*
Shikimate	*173*	*73*	*2.7*	*-43.8*	*-10*	*-19.8*	*-10.88*	*60*	*2*
Glyoxylate-UC13	*75*	*46*	*2.9*	*-140*	*-10*	*-12*	*-7*	*120*	*1*
Glyoxylate	*73*	*45*	*2.9*	*-140*	*-10*	*-12*	*-7*	*120*	*1*
Glycolate-UC13	*77*	*48*	*2.9*	*-63*	*-6.2*	*-14*	*-4.5*	*120*	*1*
Glycolate	*75*	*47*	*2.9*	*-63*	*-6.2*	*-14*	*-4.5*	*120*	*1*
Glycolate	*75*	*45*	*2.9*	*-56*	*-4*	*-15*	*-5*	*120*	*2*
L-Tryptophan-UC13	*214*	*124*	*3.2*	*-57.9*	*-10*	*-20.9*	*-4.9*	*120*	*1*
L-Tryptophan	*203*	*116*	*3.2*	*-57.9*	*-10*	*-20.9*	*-4.9*	*120*	*1*
L-Tryptophan	*203*	*74*	*3.2*	*-57.7*	*-10*	*-22*	*-12.7*	*120*	*2*
5-Oxoproline-UC13	*133*	*86*	*3.3*	*-61.3*	*-10*	*-16.8*	*-10.2*	*120*	*1*
5-Oxoproline	*128*	*82*	*3.3*	*-61.3*	*-10*	*-16.8*	*-10.2*	*120*	*1*
5-Oxoproline	*128*	*84*	*3.3*	*-64*	*-10*	*-15.7*	*-8.8*	*120*	*2*
L-Lactate-UC13	*92*	*45*	*3.3*	*-53.9*	*-10*	*-16*	*-5.3*	*120*	*1*
L-Lactate	*89*	*43*	*3.3*	*-53.9*	*-10*	*-16*	*-5.3*	*120*	*1*
L-Lactate	*89*	*45*	*3.3*	*-48.2*	*-10*	*-14.1*	*-5.1*	*120*	*2*
Urate-UC13	*172*	*128*	*3.5*	*-75*	*-10*	*-20*	*-9*	*240*	*1*
Urate	*167*	*124*	*3.5*	*-75*	*-10*	*-20*	*-9*	*240*	*1*
Urate	*167*	*42*	*3.5*	*-70*	*-10*	*-48*	*-7*	*240*	*2*
Reduced glutathione-UC13	*316*	*148*	*3.6*	*-43.6*	*-10*	*-24.9*	*-8.6*	*240*	*1*
Reduced glutathione	*306*	*143*	*3.6*	*-43.6*	*-10*	*-24.9*	*-8.6*	*240*	*1*
Reduced glutathione	*306*	*272*	*3.6*	*-40.7*	*-10*	*-18*	*-19.6*	*240*	*2*
Nicotinamide adenine dinucleotide-UC13	*683*	*555*	*4.3*	*-42.1*	*-10*	*-26.7*	*-11.9*	*120*	*1*
Nicotinamide adenine dinucleotide	*662*	*540*	*4.3*	*-42.1*	*-10*	*-26.7*	*-11.9*	*120*	*1*
Nicotinamide adenine dinucleotide	*662*	*79*	*4.3*	*-44.1*	*-10*	*-121.9*	*-9*	*120*	*2*
Pyruvate-UC13	*90*	*45*	*4.4*	*-36*	*-10*	*-12*	*-7*	*120*	*1*
Pyruvate	*87*	*43*	*4.4*	*-36*	*-10*	*-12*	*-7*	*120*	*1*
Pyruvate	*87*	*32*	*4.4*	*-46*	*-8*	*-14*	*-15*	*120*	*2*
D-Fructose 6-phosphate-UC13	*265*	*172*	*6*	*-36.4*	*-10*	*-16*	*-9.7*	*240*	*1*
D-Fructose 6-phosphate	*259*	*169*	*6*	*-36.4*	*-10*	*-16*	*-9.7*	*240*	*1*
D-Fructose 6-phosphate	*259*	*79*	*6*	*-45.7*	*-10*	*-66.3*	*-9.7*	*240*	*2*
Glyceraldehyde 3-phosphate-UC13	*172*	*97*	*6*	*-36*	*-11*	*-15*	*-5.8*	*240*	*1*
Glyceraldehyde 3-phosphate	*169*	*97*	*6*	*-36*	*-11*	*-15*	*-5.8*	*240*	*1*
Glyceraldehyde 3-phosphate	*169*	*79*	*6*	*-35*	*-11*	*-38*	*-9*	*240*	*2*
D-Glucose 6-phosphate-UC13	*265*	*203*	*6*	*-58*	*-10*	*-15.7*	*-12*	*240*	*1*
D-Glucose 6-phosphate	*259*	*199*	*6*	*-58*	*-10*	*-15.7*	*-12*	*240*	*1*
D-Glucose 6-phosphate-UC13	*265*	*79*	*6*	*-52.8*	*-10*	*-69.8*	*-9.2*	*240*	*1*
D-Glucose 6-phosphate	*259*	*79*	*6*	*-52.8*	*-10*	*-69.8*	*-9.2*	*240*	*2*
alpha-D-Ribose 5-phosphate-UC13	*234*	*97*	*6*	*-53.1*	*-10*	*-18.9*	*-5.3*	*240*	*1*
alpha-D-Ribose 5-phosphate	*229*	*97*	*6*	*-53.1*	*-10*	*-18.9*	*-5.3*	*240*	*1*

alpha-D-Ribose 5-phosphate	229	139	6	-46.2	-10	-19.4	-7.3	240	2
Sedoheptulose 7-phosphate-UC13	296	97	6.4	-54.3	-10	-26.9	-11.5	240	1
Sedoheptulose 7-phosphate	289	97	6.4	-54.3	-10	-26.9	-11.5	240	1
Sedoheptulose 7-phosphate	289	79	6.4	-60	-10	-66.3	-9.2	240	2
D-Arabinose 5-phosphate-UC13	234	97	6.5	-53.1	-10	-18.9	-5.3	240	1
D-Arabinose 5-phosphate	229	97	6.5	-53.1	-10	-18.9	-5.3	240	1
D-Arabinose 5-phosphate	229	139	6.5	-46.2	-10	-19.4	-7.3	240	2
Glycerol 3-phosphate-UC13	174	79	6.5	-38.3	-10	-29.9	-9.4	240	1
Glycerol 3-phosphate	171	79	6.5	-38.3	-10	-29.9	-9.4	240	1
Glycerol 3-phosphate	171	63	6.5	-54.1	-10	-94.4	-7.1	240	2
D-Glucose 1-phosphate-UC13	265	247	7	-62.1	-10	-17.7	-14.2	240	1
D-Glucose 1-phosphate	259	241	7	-62.1	-10	-17.7	-14.2	240	1
D-Glucose 1-phosphate	259	79	7	-53.8	-10	-63.9	-9.12	240	2
CMP-UC13	331	97	7.6	-46.1	-10	-27.7	-8	240	1
CMP	322	97	7.6	-46.1	-10	-27.7	-8	240	1
CMP	322	278	7.6	-25.8	-10	-33.8	-13.8	240	2
D-Ribulose 5-phosphate-UC13	234	97	7.8	-41.3	-12	-17	-6	240	1
D-Ribulose 5-phosphate	229	97	7.8	-41.3	-12	-17	-6	240	1
D-Ribulose 5-phosphate	229	139	7.8	-46.2	-10	-19.4	-7.3	240	2
2-Oxobutanoate-UC13	105	75	8.4	-32.1	-10	-10.44	-9.8	240	1
2-Oxobutanoate	101	73	8.4	-32.1	-10	-10.44	-9.8	240	1
2-Oxobutanoate	101	32	8.4	-33.9	-10	-11.7	-15.7	240	2
D-Fructose 1-phosphate-UC13	265	79	8.8	-44	-10	-70	-9.2	240	1
D-Fructose 1-phosphate	259	79	8.8	-44	-10	-70	-9.2	240	1
D-Fructose 1-phosphate	259	97	8.8	-56	-10	-20	-5.3	240	2
D-Xylulose 5-phosphate-UC13	234	97	8.8	-41.3	-12	-17	-6	240	1
D-Xylulose 5-phosphate	229	97	8.8	-41.3	-12	-17	-6	240	1
D-Xylulose 5-phosphate	229	139	8.8	-46.2	-10	-19.4	-7.3	240	2
dCMP-UC13	315	200	8.9	-49.4	-10	-23.4	-12.9	240	1
dCMP	306	195	8.9	-49.4	-10	-23.4	-12.9	240	1
dCMP	306	79	8.9	-48	-10	-77.2	-9.1	240	2
UMP-UC13	332	79	9	-44.6	-10	-76.7	-9.1	240	1
UMP	323	79	9	-44.6	-10	-76.7	-9.1	240	1
UMP	323	97	9	-21.8	-10	-28.1	-5.7	240	2
Dihydroxyacetone phosphate-UC13	172	97	9.5	-36	-11	-15	-5.8	240	1
Dihydroxyacetone phosphate	169	97	9.5	-36	-11	-15	-5.8	240	1
Dihydroxyacetone phosphate	169	79	9.5	-35	-11	-38	-9	240	2
3',5'-Cyclic GMP-UC13	354	155	10	-24	-11	-34	-9.4	120	1
3',5'-Cyclic GMP	344	150	10	-24	-11	-34	-9.4	120	1
3',5'-Cyclic GMP	344	79	10	-42	-9	-78	-9	120	2
dUMP-UC13	316	79	10.4	-40.5	-10	-78	-9.1	240	1
dUMP	307	79	10.4	-40.5	-10	-78	-9.1	240	1
dUMP	307	195	10.4	-52.5	-10	-22.7	-12.9	240	2
dIMP-UC13	341	140	11	-29.9	-10	-30.9	-7.1	120	1
dIMP	331	135	11	-29.9	-10	-30.9	-7.1	120	1
dIMP	331	79	11	-20	-10	-65	-9	120	2
dGMP-UC13	356	79	11.23	-22	-10	-76.9	-9.1	120	1
dGMP	346	79	11.23	-22	-10	-76.9	-9.1	120	1
dGMP	346	133	11.23	-53.7	-10	-58.7	-3.9	120	2
dTMP-UC13	331	200	11.3	-47.5	-10	-23.6	-12.9	120	1
dTMP	321	195	11.3	-47.5	-10	-23.6	-12.9	120	1
Riboflavin-UC13	392	268	11.4	-54.1	-10	-24.6	-15.3	120	1
Riboflavin	375	255	11.4	-54.1	-10	-24.6	-15.3	120	1
AMP-UC13	356	139	11.6	-31.4	-10	-43.9	-15.8	120	1
AMP	346	134	11.6	-31.4	-10	-43.9	-15.8	120	1
AMP	346	79	11.6	-20	-10	-71.6	-12.9	120	2
GMP-UC13	372	79	11.6	-26.2	-10	-77.9	-10.9	120	1
GMP	362	79	11.6	-26.2	-10	-77.9	-10.9	120	1

GMP	362	97	11.6	-40.2	-10	-42.1	-4.9	120	2
IMP-UC13	357	140	11.6	-37.9	-10	-41.6	-7.2	120	1
IMP	347	135	11.6	-37.9	-10	-41.6	-7.2	120	1
IMP	347	97	11.6	-29.4	-10	-26.8	-11.1	120	2
cAMP-UC13	338	139	11.7	-50	-7.2	-32.8	-9	60	1
cAMP	328	134	11.7	-50	-7.2	-32.8	-9	60	1
cAMP	328	79	11.7	-27	-4	-75	-9.2	60	2
Oxidized glutathione-UC13	631	316	11.7	-30	-10	-33.1	-10.1	60	1
Oxidized glutathione	611	306	11.7	-30	-10	-33.1	-10.1	60	1
Oxidized glutathione	611	272	11.7	-41.9	-10	-39.2	-16	60	2
Biotin-UC13	253	43	11.8	-57.7	-10	-55.3	-6.8	60	1
Biotin	243	42	11.8	-57.7	-10	-55.3	-6.8	60	1
Biotin	243	200	11.8	-67.5	-10	-21.6	-12	60	2
Methylmalonate-UC13	121	58	11.8	-34	-13	-31.6	-7	60	1
Methylmalonate	117	55	11.8	-34	-13	-31.6	-7	60	1
Methylmalonate	117	73	11.8	-19.9	-12.3	-12	-7.7	60	2
Succinate-UC13	121	103	11.8	-42	-10	-14	-5	60	1
Succinate	117	99	11.8	-42	-10	-14	-5	60	1
Succinate	117	73	11.8	-32	-10	-14	-5	60	2
L-Malate-UC13	137	119	12	-55	-4	-15	-5.4	60	1
L-Malate	133	115	12	-55	-4	-15	-5.4	60	1
Glutaconate-UC13	134	89	12.1	-37.9	-10	-11.3	-4.6	60	1
Glutaconate	129	85	12.1	-37.9	-10	-11.3	-4.6	60	1
Fumarate-UC13	119	74	12.18	-80.2	-3	-13	-4	60	1
Fumarate	115	71	12.18	-80.2	-3	-13	-4	60	1
UDPglucose-UC13	580	247	12.2	-30	-10	-42.73	-12.3	60	1
UDPglucose	565	241	12.2	-30	-10	-42.73	-12.3	60	1
UDPglucose	565	323	12.2	-30	-10	-33.76	-20	60	2
dAMP-UC13	340	79	12.3	-41.8	-10	-73.4	-9.1	60	1
dAMP	330	79	12.3	-41.8	-10	-73.4	-9.1	60	1
dAMP	330	134	12.3	-39.2	-10	-39	-7.4	60	2
Oxalate-UC13	91	63	12.4	-20.6	-10	-11.9	-6.82	60	1
Oxalate	89	61	12.4	-20.6	-10	-11.9	-6.82	60	1
Oxalate	89	45	12.4	-39.5	-10	-12.8	-5.1	60	2
Acetyl phosphate-UC13	141	63	12.5	-12.4	-10	-75.8	-7.2	60	1
Acetyl phosphate	139	63	12.5	-12.4	-10	-75.8	-7.2	60	1
Acetyl phosphate	139	79	12.5	-147	-10	-30.8	-9.2	60	2
2-Oxoglutarate-UC13	150	105	12.5	-24	-12	-13.8	-5.93	60	1
2-Oxoglutarate	145	101	12.5	-24	-12	-13.8	-5.93	60	1
Oxaloacetate-UC13	135	90	13	-47	-9	-12.8	-13	60	1
Oxaloacetate	131	87	13	-47	-9	-12.8	-13	60	1
dTDPglucose-UC13	579	331	13.2	-20	-10	-33.6	-23.1	60	1
dTDPglucose	563	321	13.2	-20	-10	-33.6	-23.1	60	1
dTDPglucose	563	79	13.2	-20	-10	-117.3	-9.17	60	2
Nicotinamide adenine dinucleotide - reduced-UC13	685	418	13.2	-20	-10	-44.3	-16.3	120	1
Nicotinamide adenine dinucleotide - reduced	664	408	13.2	-20	-10	-44.3	-16.3	120	1
Nicotinamide adenine dinucleotide - reduced	664	79	13.2	-30	-10	-132.8	-12.7	120	2
ADPglucose-UC13	604	356	13.3	-15.66	-10	-35.45	-14.26	60	1
ADPglucose	588	346	13.3	-15.66	-10	-35.45	-14.26	60	1
ADPglucose	588	79	13.3	-16.3	-10	-110.65	-7.21	60	2
chorismate-UC13	235	97	13.6	-42.08	-10	-19.96	-11.3	120	1
chorismate	225	91	13.6	-42.08	-10	-19.96	-11.3	120	1
Folate-UC13	459	325	13.6	-52	-10	-30	-19.5	60	1
Folate	440	311	13.6	-52	-10	-30	-19.5	60	1
Folate	440	175	13.6	-88	-10	-48.7	-12.1	60	2
dGDP-UC13	436	79	13.77	-42.4	-10	-86	-8.9	120	1
dGDP	426	79	13.77	-42.4	-10	-86	-8.9	120	1
dGDP	426	275	13.77	-35	-10	-27.7	-14.1	120	2

GDP-UC13	452	79	13.77	-35.4	-10	-75.9	-13.4	120	1
GDP	442	79	13.77	-35.4	-10	-75.9	-13.4	120	1
GDP	442	344	13.77	-33.4	-10	-26.7	-18.8	120	2
6-Phospho-D-gluconate-UC13	281	97	13.8	-56.4	-10	-22	-5.24	120	1
6-Phospho-D-gluconate	275	97	13.8	-56.4	-10	-22	-5.24	120	1
Pool_2pg_3pg-UC13	188	97	13.8	-34.1	-10	-20.9	-5.8	240	2
Pool_2pg_3pg	185	79	13.8	-35.8	-10	-50	-9.05	240	1
Pool_2pg_3pg	185	97	13.8	-34.1	-10	-20.9	-5.8	240	2
dCDP-UC13	395	159	13.9	-34.3	-10	-30.8	-10.6	240	1
dCDP	386	159	13.9	-34.3	-10	-30.8	-10.6	240	1
dCDP	386	79	13.9	-20	-10	-78.9	-9.2	240	2
ADP-UC13	436	139	13.98	-22.4	-10	-31.7	-6.3	120	1
ADP	426	134	13.98	-22.4	-10	-31.7	-6.3	120	1
ADP	426	79	13.98	-23.7	-10	-92	-12	120	2
2-Hydroxybutane-1,2,3-tricarboxylate-UC13	212	130	14	-52.5	-10	-19	-3.6	120	1
2-Hydroxybutane-1,2,3-tricarboxylate	205	125	14	-52.5	-10	-19	-3.6	120	1
2-Hydroxybutane-1,2,3-tricarboxylate	205	87	14	-33.9	-10	-27	-5	120	2
UDP-UC13	412	115	14	-18	-10	-27.7	-13.1	120	1
UDP	403	79	14	-18	-10	-86.8	-8.8	120	1
UDP	403	159	14	-18	-10	-35.1	-10.7	120	2
cis-Aconitate-UC13	179	134	14.03	-20.5	-8.3	-11.5	-12	60	1
cis-Aconitate	173	129	14.03	-20.5	-8.3	-11.5	-12	60	1
cis-Aconitate	173	85	14.03	-16	-10	-16.8	-7.1	60	2
Nicotinamide adenine dinucleotide phosphate-UC13	763	635	14.2	-32.5	-10	-25.8	-11	120	1
Nicotinamide adenine dinucleotide phosphate	742	620	14.2	-32.5	-10	-25.8	-11	120	1
Nicotinamide adenine dinucleotide phosphate	742	79	14.2	-30.4	-10	-134.3	-7.2	120	2
Phosphoenolpyruvate-UC13	170	79	14.2	-33.8	-10	-25.1	-9.2	60	1
Phosphoenolpyruvate	167	79	14.2	-33.8	-10	-25.1	-9.2	60	1
Phosphoenolpyruvate	167	63	14.2	-25.3	-10	-90.1	-7.1	60	2
dADP-UC13	420	79	14.3	-54	-10	-85.1	-9	120	1
dADP	410	79	14.3	-54	-10	-85.1	-9	120	1
dADP	410	159	14.3	-24.1	-10	-30.4	-10.6	120	2
Citrate-UC13	197	90	14.5	-74	-5	-27	-5	120	1
Citrate	191	111	14.5	-47.7	-10	-19.1	-6.8	120	1
Citrate	191	87	14.5	-74	-5	-27	-5	120	2
Pool_cit_icit-UC13	197	116	14.6	-47.7	-10	-19.1	-6.8	120	1
Isocitrate-UC13	197	76	14.7	-44	-10	-29.4	-8.4	120	1
Pool_cit_icit	191	111	14.7	-47.7	-10	-19.1	-6.8	120	1
Isocitrate	191	73	14.7	-44	-10	-29.4	-8.4	120	2
FAD-UC13	811	454	15	-20	-10	-41.3	-9	60	1
FAD	784	437	15	-20	-10	-41.3	-9	60	1
FAD	784	79	15	-20	-10	-128.2	-9	60	2
D-Fructose 1,6-bisphosphate-UC13	345	247	15	-42.6	-10	-21.8	-12.1	120	1
D-Fructose 1,6-bisphosphate	339	79	15	-37.8	-10	-73.6	-6.5	120	1
D-Fructose 1,6-bisphosphate	339	241	15	-42.6	-10	-21.8	-12.1	120	2
UDP-D-glucuronate-UC13	594	412	15	-30	-10	-34.6	-24	60	1
UDP-D-glucuronate	579	403	15	-30	-10	-34.6	-24	60	1
UDP-D-glucuronate	579	323	15	-30	-10	-31.77	-16.9	60	2
dGTP-UC13	516	159	15.6	-20	-10	-34.2	-10.9	60	1
dGTP	506	159	15.6	-20	-10	-34.2	-10.9	60	1
CTP-UC13	491	159	15.8	-47.6	-10	-33.9	-21	120	1
CTP	482	159	15.8	-47.6	-10	-33.9	-21	120	1
CTP	482	79	15.8	-34	-10	-130.1	-9.4	120	2
dCTP-UC13	475	79	15.8	-45.9	-10	-110	-9.1	120	1
dCTP	466	79	15.8	-45.9	-10	-110	-9.1	120	1
dCTP	466	368	15.8	-143	-10	-34.6	-7	120	2
dITP-UC13	501	79	15.8	-24.1	-10	-104	-9.1	120	1
dITP	491	79	15.8	-24.1	-10	-104	-9.1	120	1

dITP	*491*	*393*	*15.8*	*-28*	*-10*	*-32.2*	*-13.9*	*120*	*2*
dUTP-UC13	*476*	*159*	*15.8*	*-31.7*	*-10*	*-104.3*	*-9.9*	*120*	*1*
dUTP	*467*	*159*	*15.8*	*-31.7*	*-10*	*-104.3*	*-9.9*	*120*	*1*
dUTP	*467*	*177*	*15.8*	*-26*	*-10*	*-68.9*	*-9.3*	*120*	*2*
ITP-UC13	*517*	*419*	*15.8*	*-47*	*-10*	*-29.7*	*-12.4*	*120*	*1*
ITP	*507*	*409*	*15.8*	*-47*	*-10*	*-29.7*	*-12.4*	*120*	*1*
ITP	*507*	*79*	*15.8*	*-46*	*-10*	*-106*	*-9.1*	*120*	*2*
UTP-UC13	*492*	*394*	*15.8*	*-22.5*	*-10*	*-30.1*	*-8*	*120*	*1*
UTP	*483*	*385*	*15.8*	*-22.5*	*-10*	*-30.1*	*-8*	*120*	*1*
UTP	*483*	*79*	*15.8*	*-32*	*-10*	*-130.2*	*-7*	*120*	*2*
3-Phospho-D-glyceroyl phosphate-UC13	*268*	*170*	*16*	*-48.2*	*-10*	*-18.32*	*-11.23*	*120*	*1*
3-Phospho-D-glyceroyl phosphate	*265*	*167*	*16*	*-48.2*	*-10*	*-18.32*	*-11.23*	*120*	*1*
3-Phospho-D-glyceroyl phosphate	*265*	*177*	*16*	*-47.1*	*-10*	*-23.17*	*-9.71*	*120*	*2*
ATP-UC13	*516*	*159*	*16*	*-20*	*-10*	*-34.2*	*-10.9*	*120*	*1*
ATP	*506*	*408*	*16*	*-133.8*	*-14*	*-39*	*-42*	*120*	*1*
ATP	*506*	*159*	*16*	*-183*	*-14*	*-56*	*-49*	*120*	*2*
dATP-UC13	*500*	*159*	*16*	*-25.5*	*-10*	*-34.3*	*-9.2*	*120*	*1*
dATP	*490*	*159*	*16*	*-25.5*	*-10*	*-34.3*	*-9.2*	*120*	*1*
dATP	*490*	*79*	*16*	*-40.2*	*-10*	*-94*	*-9.2*	*120*	*2*
dTTP-UC13	*491*	*79*	*16*	*-28*	*-10*	*-104*	*-9.1*	*120*	*1*
dTTP	*481*	*79*	*16*	*-28*	*-10*	*-104*	*-9.1*	*120*	*1*
dTTP	*481*	*383*	*16*	*-26.5*	*-10*	*-29.6*	*-10.2*	*120*	*2*
GTP-UC13	*532*	*79*	*16*	*-23.5*	*-10*	*-138.1*	*-33.3*	*120*	*1*
GTP	*522*	*79*	*16*	*-23.5*	*-10*	*-138.1*	*-33.3*	*120*	*1*
GTP	*522*	*424*	*16*	*-32*	*-10*	*-33.8*	*-4.1*	*120*	*2*
5-Phospho-alpha-D-ribose 1-diphosphate-UC13	*394*	*177*	*16*	*-21.6*	*-10*	*-25.72*	*-15.91*	*120*	*1*
5-Phospho-alpha-D-ribose 1-diphosphate	*389*	*177*	*16*	*-21.6*	*-10*	*-25.72*	*-15.91*	*120*	*1*
5-Phospho-alpha-D-ribose 1-diphosphate	*389*	*291*	*16*	*-21.5*	*-10*	*-20.21*	*-9.94*	*120*	*2*
Phenylpyruvate-UC13	*172*	*98*	*16.2*	*-20*	*-10*	*-14.3*	*-11*	*120*	*1*
Phenylpyruvate	*163*	*91*	*16.2*	*-20*	*-10*	*-14.3*	*-11*	*120*	*1*
Phenylpyruvate	*163*	*119*	*16.2*	*-26*	*-13*	*-19*	*-8*	*120*	*2*
Coenzyme A-UC13	*787*	*79*	*16.4*	*-20*	*-10*	*-141.5*	*-9.1*	*120*	*1*
Coenzyme A	*766*	*79*	*16.4*	*-20*	*-10*	*-141.5*	*-9.1*	*120*	*1*
Acetyl-CoA-UC13	*831*	*79*	*16.6*	*-18.1*	*-10*	*-136.9*	*-9.7*	*120*	*1*
Acetyl-CoA	*808*	*79*	*16.6*	*-18.1*	*-10*	*-136.9*	*-9.7*	*120*	*1*
Malonyl-CoA-UC13	*877*	*79*	*16.6*	*-18.1*	*-10*	*-136.9*	*-9.7*	*60*	*1*
Malonyl-CoA	*853*	*79*	*16.6*	*-18.1*	*-10*	*-136.9*	*-9.7*	*60*	*1*
Succinyl-CoA-UC13	*891*	*79*	*16.6*	*-16.1*	*-10*	*-136.8*	*-9.1*	*60*	*1*
Succinyl-CoA	*866*	*79*	*16.6*	*-16.1*	*-10*	*-136.8*	*-9.1*	*60*	*1*
Nicotinamide adenine dinucleotide phosphate - reduced-UC13	*765*	*418*	*17.2*	*-20*	*-10*	*-44.3*	*-16.3*	*120*	*1*
Nicotinamide adenine dinucleotide phosphate - reduced	*744*	*408*	*17.2*	*-20*	*-10*	*-44.3*	*-16.3*	*120*	*1*
Nicotinamide adenine dinucleotide phosphate - reduced	*744*	*79*	*17.2*	*-20*	*-10*	*-138.9*	*-8.45*	*120*	*2*

Table 2: Table of MRM and Retention Times for All Metabolites and Labeled Metabolites Measured. Quantifier 1 denotes the transition used for quantitation and Quantifier 2 denotes transition used to confirm identification for quantitation.

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