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Supporting Information

Positional Enrichment by Proton Analysis (PEPA): A One-Dimensional ^{1}H-NMR Approach for ^{13}C Stable Isotope Tracer Studies in Metabolomics

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1 Materials and Methods

1.1 Cell Cultures

U2OS-cells human osteosarcoma cell line were cultured in Dulbeccos modified Eagle Medium (DMEM, Invitrogen 41966) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma F7524-500ML) and 100U/ml penicillin/streptomycin (Reactiva 01030311B000). For [U-^{13}C]-Glucose labeling experiments, cells (in duplicated batches) were cultured in a humidified atmosphere at 5% CO2 , 37°C and grown for 8-9 days in polypropylene bottles; the medium was changed once daily initially and twice daily near the end of the growth period. Then, U2OS-cells were seeded on 15-cm tissue culture plates in triplicates. During 6 hours, cells were cultures using DMEM powder without glucose. This DMEM was supplemented with either 5 mM glucose in one batch or 5 mM [U-^{13}C]-Glucose (Cambridge Isotope Laboratories) in the other batch. After these 6 hours, culture medium was removed and cells were collected by trypsinization and 10^6 cells were pelleted and snap-frozen for further metabolites extraction. For [U-^{13}C]-Glutamine labeling experiments, the same procedure was repeated using using DMEM without glutamine and supplementing it with either 2mM L-glutamine or [U-^{13}C]-Glutamine.

1.2 Metabolites Extraction

Metabolites were extracted into the extraction solvent by adding 2 mL of a cold mixture of chloroform/ methanol (2:1 v/v). The resulting suspension was bath-sonicated for 3 minutes, and 2 mL of cold water was added. Then, 1 mL of chloroform/methanol (2:1 v/v) was added to the samples and bath-sonicated for 3 minutes. Cell lysates were centrifuged (5000 g, 15 min at 4°C) and the aqueous phase was carefully transferred into a new tube. The sample was frozen, lyophilized and stored at 80°C until further NMR analysis.

1.3 NMR analysis

For NMR measurements, the hydrophilic extracts were reconstituted in 600 μl of D_2O phosphate buffer (PBS 0.05 mM, pH 7.4, 99.5 % D_2O) containing 0.73 mM of deuterared trisilylpropionic acid (d_6-TSP). Samples were then vortexed, homogenized for 5 min, and centrifuged for 15 min at 14000 g at 4°C. Finally, clear redissolved samples were tranferred into 5 mm NMR tubes. NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz and a carbon frequency of 150.93 MHz using a 5 mm CPTCI triple resonance (^{1}H, ^{13}C, ^{31}P) gradient cryoprobe. One-dimensional $_1$H pulse experiments were performed using the nuclear Overhauser Effect Spectroscopy (NOESY) presaturation sequence (RD-90°-t_1-90°-t_m-90°-ACQ) to suppress the residual water peak with mixing time (t_m) of 100 ms. Solvent presaturation with irradiaton power of 50 Hz was applied during recycling delay (RD = 5 s) and mixing time . The 90° pulse length was calibrated for each sample and varied from 8.06 to 10.26 μs. The spectral width was 12 kHz (20 ppm), and a total of 256 transients were collected into 64 k data points for each ^{1}H spectrum. The exponential line broadening applied before Fourier transformation was 0.3 Hz. One-dimensional ^{13}C-NMR spectra were recorded using Inverse Gate decoupled ^{13}C pulse experiment and WALTZ-16 scheme proton presaturation and a relaxation delay of 10 s. The 90° pulse length was 14.75 μs. The spectral width was 36 kHz (240 ppm), and a total of 6144 transients were collected into 64 k data points for each ^{13}C spectrum. The exponential line broadening applied before Fourier transformation was 1 Hz. Two-dimensional TOCSY spectra were recorded using MLEV17 spin lock with 63 ms of duration, B_1 field strength of 8,33 kHz, and acquisition times of 0.28 s in t_2 and 0.071 s in t_1. The recycling delay was set to 5s, and 64 scans for each transient were collected into 2k of data points in t_2 and 1024 transients were acquired in t_1. Data were zero-filled to 4k points, apodized with a shifted Gaussian and a 1 Hz line broadening exponential function in both dimensions prior to Fourier transformation.Two-dimensional gradient ^{1}H-^{13}C-HSQC spectra were recorded using an acquisition time of 0.128 s in t_2 and 0.0084 s in t_1 and a recycle time of 1.5 s, with the ^{13}C GARP decoupling set to 100 ppm, and the evolution delay set for 145 Hz corresponding approximately to an average value of JCH coupling constants. The data were apodized with an unshifted Gaussian and a 1 Hz line broadening exponential in both dimensions prior to Fourier transformation.

1.4 NMR metabolite identification

^{1}H-NMR resonances were assigned using different database search engines, including BBioref AMIX database from Bruker, Chenomx, HMDB[1] and Complex Mixture Analysis by NMR (COLMAR)[2].

Both heteronuclear 2D-^{1}H-^{13}C-HSQC, ^{1}H-^{13}C-HMBC and homonuclear 2D ^{1}H-^{1}H-COSY,^{1}H-^{1}H-TOCSY were used for structural confirmation. Metabolites identified in U2OS hydrophilic extracts are listed in Table S1

1.5 NMR metabolite profiling. Data Analysis

The frequency domain spectra were phased, baseline-corrected and referenced to TSP signal (δ= 0 ppm) using TopSpin software (version 2.1, Bruker). Dolphin 1D[3] was used to profile metabolites identified in the ^{1}H-NMR spectra. Dolphin is a spectral profiling approach based on decomposition of the NMR spectrum as linear combination of a set of individual pure reference spectra obtained from known compounds in a reference library (Figure S1). Poorly resolved and partially overlapped resonances are resolved using line shape fitting and consequently, their area can be properly quantified. Profiled areas were row-wise normalized using ERETIC [4] signal. Area of each profiled metabolite in unlabeled experiments were compared with the same area in experiments using isotopically enriched substrates using t-test ($p<0.05$ were considered for statistical significance). Figure S2 depicts a comparison of unlabeled and [^{13}C-Glc]-labeled ^{1}H-NMR spectra deconvolution for those resonances in which isotopically enrichment was detected using PEPA. Data analysis was performed using R version 3.2.0. Interactive Pathway Explorer 2 (iPath2.0) to visualize and analyze of cellular pathways[5]. MetPa[6] was used to perform pathway enrichment and topological analysis (Figure S4).

2 Spectral data: Availability and Reproducibility

2.1 Metabolights Repository

Raw spectral data used to develop and valiadate PEPA and Dolphin-derived area quantifications are available at Metaboligths, an open-access general-purpose repository for metabolomics studies and associated meta-data[7]. Click the following link http://www.ebi.ac.uk/metabolights/MTBLS247 to access the data. Data directory organization follows Bruker format. Each sample is placed in a main directory named as such sample. This main directory contains different subfolders corresponding to different experimental numbers (expno). These expno contain the different NMR experiments performed on the same sample, i.e., 11: noesypr1d (^{1}H-NMR); 22:zpg(^{13}C-NMR) and so on. Bruker processed data files are stored in a folder named pdata and they are titled 1r or 2rr. Details of the expno used to develop and validate PEPA can be found below:

2.1.1 PEPA: 1D-^{1}H-NMR profiling

- [U-^{13}C]-Glc
  Sh00_AQ_13C_22/11/pdata/1/1r
  Sh00_AQ_13C_23/11/pdata/1/1r
  Sh00_AQ_13C_24/11/pdata/1/1r

- Unlabelled Glc
  Sh00_AQ_12C_10/11/pdata/1/1r
  Sh00_AQ_12C_11/11/pdata/1/1r
  Sh00_AQ_12C_12/11/pdata/1/1r

- [U-^{13}C]-Gln
  Sh00_AQ_13CGln_122/11/pdata/1/1r
  Sh00_AQ_13CGln_123/11/pdata/1/1r
  Sh00_AQ_13CGln_124/11/pdata/1/1r

- Unlabelled Gln
  Sh00_AQ_12CGln_107/11/pdata/1/1r
  Sh00_AQ_12CGln_108/11/pdata/1/1r
  Sh00_AQ_12CGln_109/11/pdata/1/1r

2.1.2 PEPA validation: ^{13}C-NMR; ^{1}H-^{1}H-TOCSY; ^{1}H-^{13}C-HSQC

- [U-^{13}C]-Glc
  Sh00_AQ_13C_23/22/pdata/1/1r
  Sh00_AQ_13C_22/22/pdata/1/1r
  Sh00_AQ_13C_24/22/pdata/1/1r

- Unlabelled Glc
  Sh00_AQ_12C_11/22/pdata/1/1r
  Sh00_AQ_12C_10/22/pdata/1/1r

- [U-^{13}C]-Gln
  Sh00_AQ_13CGln_122/12/pdata/1/1r
  Sh00_AQ_13CGln_123/12/pdata/1/1r

- [U-^{13}C]-Glc
  Sh00_AQ_13C_23_TOCSY/33

- Unlabelled Glc
  Sh00_AQ_12C_10_TOCSY/12

- [U-^{13}C]-Gln
  Sh00_AQ_13CGln_TOCSY/12

- Unlabelled Gln
  Sh00_AQ_12CGln_109_TOCSY/12

- [U-^{13}C]-Glc
  Sh00_AQ_13C_23_HSQC/13

- [U-^{13}C]-Gln
  Sh00_AQ_13CGln_122_HSQC/13

2.2 Reproduccible Research Code

The following **R** code contains all steps necessary to reproduce Figure 2. Input datafiles `Dolphin_Glc_metabolights.csv` and `Dolphin_Gln_metabolights.csv` are available at http://www.ebi.ac.uk/metabolights/MTBLS247

```
## ---Data dependencies:

#"Dolphin_Glc_metabolights.csv"
#"Dolphin_Gln_metabolights.csv"
#available at http://www.ebi.ac.uk/metabolights/MTBLS247

## ---packages

library(reshape)
library(ggplot2)
library(scales)    # Need the scales package for scientific notation

## ----functions

fc.test<-function(X, classvector){
```

```
  means<-apply(X, 2, function(x) tapply(x, classvector, mean))
  means <- t(means)
  case <- means[,"case"]; control <- means[,"control"]
  logFC <- log2(case/control)
  FC <- case/control;
  FC2 <- -control/case
  FC[FC<1] <- FC2[FC<1]
  fc.res <- cbind(FC, logFC)
  return(fc.res)
}
isotope.fraction<-function(D, cl){
  #Get meanintensites for unlabeled samples
  meanintensities<-apply(D, 2, function(x) tapply(x, cl, mean))
  C12mean <- meanintensities[grep("C-12", rownames(meanintensities)),]
  #Calculate fractional enrichment at site of interest
  resta <- t(apply(D,1, function(x) (C12mean-x)))
  F <- t(apply(resta,1,function(x) (x/C12mean)))
  return(F)
}

myFunc.errorbars = function(x) {
  result = c(mean(x) - sd(x), mean(x) + sd(x))
  names(result) = c("ymin", "ymax")
  result
}


## ---- Read Glucose experiments: Dolphin Integration

file.Glc <- "Dolphin_Glc_metabolights.csv"
Glc <- read.csv(file.Glc, header = TRUE, row.names=1,stringsAsFactors=F)
ppm <- as.character(as.numeric(Glc["ppm",]))
mult <- as.character(Glc["multiplicity",])
substrate <-rep("Glc", times=nrow(Glc))
tot <- paste(ppm,colnames(Glc), mult,substrate, sep="_")
Glc <- Glc[-c(1,2),]
colnames(Glc) <- tot
Glc.f <-data.frame(apply(Glc,2,as.numeric))
rownames(Glc.f) <- rownames(Glc)
pos.eretic <- grep("Eretic", colnames(Glc))

#Row-wise normalization to ERETIC
Xeretic <- data.frame(apply(Glc.f, 2, function(x) ((x/Glc.f[,pos.eretic])*1000)));
Xeretic <- Xeretic[,-c(pos.eretic)]

#Declare factors
sh <- rep("shCTR", times=nrow(Xeretic))
isotope <- rep("C12", times=nrow(Xeretic))
isotope[grep("13C",rownames(Xeretic))] <- "C13"; isotope <- as.factor(isotope)
substrate <- rep("Glc", times=nrow(Xeretic))

#Statistical test to assess significant decays in central peaks
X <- Xeretic
cl <- as.factor(paste(substrate, isotope, sep="_"))
D <- data.frame(X, cl)
RES <- t(sapply(D[,-ncol(D)], function(x)
unlist(t.test(x~cl)[c("estimate","p.value","statistic","conf.int")])))
classvector <- c("control","control","control","case","case","case")
```

```
fc.res <- fc.test(D[,-ncol(D)],classvector)
glc.exp <- cbind(round(RES[,"p.value"],2),fc.res[,"FC"])[fc.res[,"FC"]<0,];
colnames(glc.exp) <- c("p.val","FC")
glc.sig <- colnames(D[,-ncol(D)])[fc.res[,"FC"]<0 & round(RES[,"p.value"],2)<=0.05]
Dglc <- D[,glc.sig]

## ----Read Glutamine experiments: Dolphin Integration

file.Gln <-  "Dolphin_Gln_metabolights.csv"
Gln <- read.csv(file.Gln, header = TRUE, row.names=1,stringsAsFactors=F)
ppm <- as.character(as.numeric(Gln["ppm",]))
mult <- as.character(Gln["multiplicity",])
substrate <-rep("Gln", times=nrow(Gln))
tot <- paste(ppm,colnames(Gln), mult,substrate, sep="_")

Gln <- Gln[-c(1,2),]
colnames(Gln) <- tot
Gln.f <-data.frame(apply(Gln,2,as.numeric))
rownames(Gln.f) <- rownames(Gln)
pos.eretic <- grep("Eretic", colnames(Gln))

#Row-wise normalization to ERETIC
Xeretic <- data.frame(apply(Gln.f, 2, function(x) ((x/Gln.f[,pos.eretic])*1000)));
Xeretic <- Xeretic[,-c(pos.eretic)]


#Declare factors
sh <- rep("shCTR", times=nrow(Xeretic))
isotope <- rep("C12", times=nrow(Xeretic))
isotope[grep("13C",rownames(Xeretic))] <- "C13"; isotope <- as.factor(isotope)
substrate <- rep("Gln", times=nrow(Xeretic))
substrate <- as.factor(substrate)

cl <- as.factor(paste(substrate, isotope, sep="_"))
#Statistical test to assess significant decays in central peaks
D <- data.frame(Xeretic,cl)
RES <- t(sapply(D[,-ncol(D)], function(x)
unlist(t.test(x~cl)[c("estimate","p.value","statistic","conf.int")])))

classvector <- c("control","control","control","case","case","case")
fc.res <- fc.test(D[,-ncol(D)],classvector)
gln.exp <- cbind(round(RES[,"p.value"],2),fc.res[,"FC"])[fc.res[,"FC"]<0,];
colnames(gln.exp) <- c("p.val","FC")
gln.sig <- colnames(D[,-ncol(D)])[fc.res[,"FC"]<0 & round(RES[,"p.value"],2)<=0.05]
Dgln <- D[,gln.sig]

## ----Figure2: FRACTIONAL ENRICHMENTS AT SITE OF INTEREST

M <- data.frame(rbind(glc.exp[glc.sig,],gln.exp[gln.sig,]))
cl <- c("C-12","C-12","C-12","C-13","C-13","C-13")
Fglc <- isotope.fraction(Dglc,cl)
Fgln <- isotope.fraction(Dgln,cl)
Fr <- data.frame(cbind(Fgln, Fglc))
res <- data.frame(rbind(glc.exp[glc.sig,],gln.exp[gln.sig,]))
M.ord <- res[order(res$FC, decreasing = TRUE),]
Fr <- Fr[,rownames(M.ord)]

X2plot <- Fr[grep("C-13",cl),];
```

```
X2plot.m <- melt(X2plot)
t <- rep("Glc",times=nrow(X2plot.m))
t[grep("Gln",X2plot.m$variable)] <- "Gln"
X2plot.m$grp <- t


Colorsv <- c(Glc = "red" , Gln ="royalblue")
Fig2 <- ggplot(X2plot.m, aes(x=variable, y=value, fill=grp)) +
  geom_point(size=5, pch=21, alpha=0.30,colour="gray47")+
  stat_summary(data= X2plot.m,
               fun.data = "myFunc.errorbars",
               geom = "errorbar", width = 0.3,
               color="gray47",size = 0.5)+
  theme(axis.line = element_line(colour = "black"),
        panel.grid.major = element_blank(),
        panel.grid.minor = element_blank(),
        panel.border = element_blank(),
        panel.background = element_blank())+
  labs(y="", x = "", title = "Fractional enrichments (F)")+
  scale_y_continuous(labels=scales::percent,
                                          limits = c(0, 1.2), breaks= pretty_breaks(3))+
  scale_fill_manual(values=Colorsv)+
  coord_flip()
Fig2
```

3 PEPA limitations

Next we discuss two limitations in the determination of fractional enrichment as calculated by PEPA in Eq 2:

1. **Overestimation of fractional enrichments:** PEPA focuses on the central peak decay of the 1D $_1$H-NMR resonance directly bound to the labelled carbon. However the contribution of neighboring labeled isotopologues to the central peak decay can not be ruled out. To illustrate this situation consider Figure S6 depicting all possible isotopologue combinations for alanine together with their theoretical ^{1}H-NMR methyl resonance at δ 1.46 ppm and its corresponding TOCSY. In the presence of these isotopologues, PEPA would not disciminate isotopologue 2 from isotopologues 3, 4 and 5 (Figure S6A) leading to a higher fractional enrichment for a given positional carbon (ratio of isotopologues 2+3+4+5 to 1 in Figure S6). This is not an intrinsic limitation of our methodology but one that affects other indirect methods to observe ^{13}C through ^{1}H such as HSQC and cross-peak patterns in TOCSY[8]. In fact, cross-peaks patterns in 2D TOCSY reported in isotopomers studies may also result from a mixture of different isotopologues (Figure S6B: 2+4 for -CH_3 (blue), 6+7 for CH (red) and 3+5 (purple) for C-CH_3 and CH). Note that the fractional enrichment is calculated from the mixture of non-labeled and labeled carbonylic groups (atom COOH, central signal 1+8 (green)) that can not be discriminated in the central cross-peak. On the other hand, the possible overestimation of fractional enrichments should not affect the relative comparison of one carbon position across samples and between different biological conditions, which is ultimately the main reason for untargeted metabolomic studies.

2. **Detection of small fractional enrichments:** The statistical nature of PEPA makes detection of small fractional enrichments challenging and dependent on the biological variability between (and within) labeled and unlabeled batches of cells. Notwithstanding, detecting small label incorporations have not been experimentally reported using metabolites extracted from batches of cell cultures, but only using kinetic data under unique NMR setups, including customized probes that allow perfusion experiments and monitoring samples continuously [9].

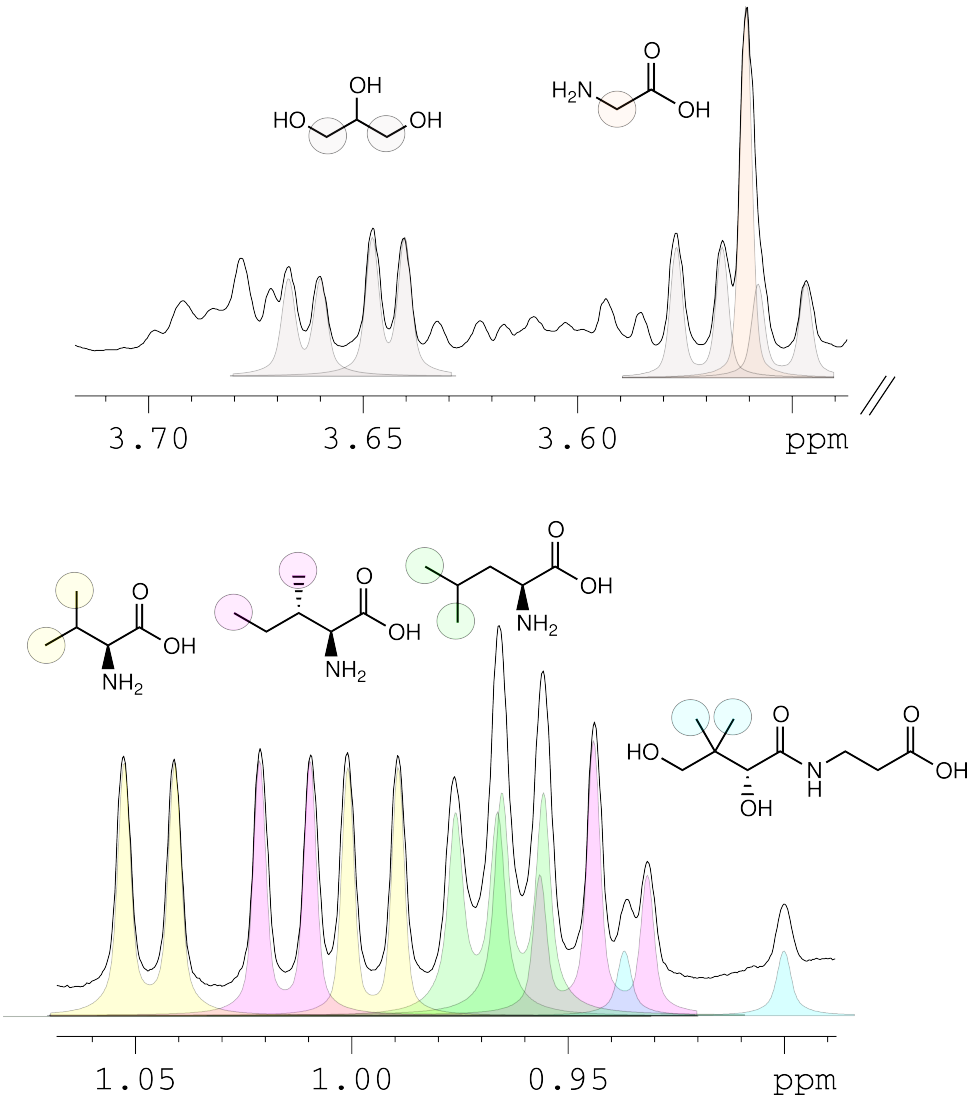
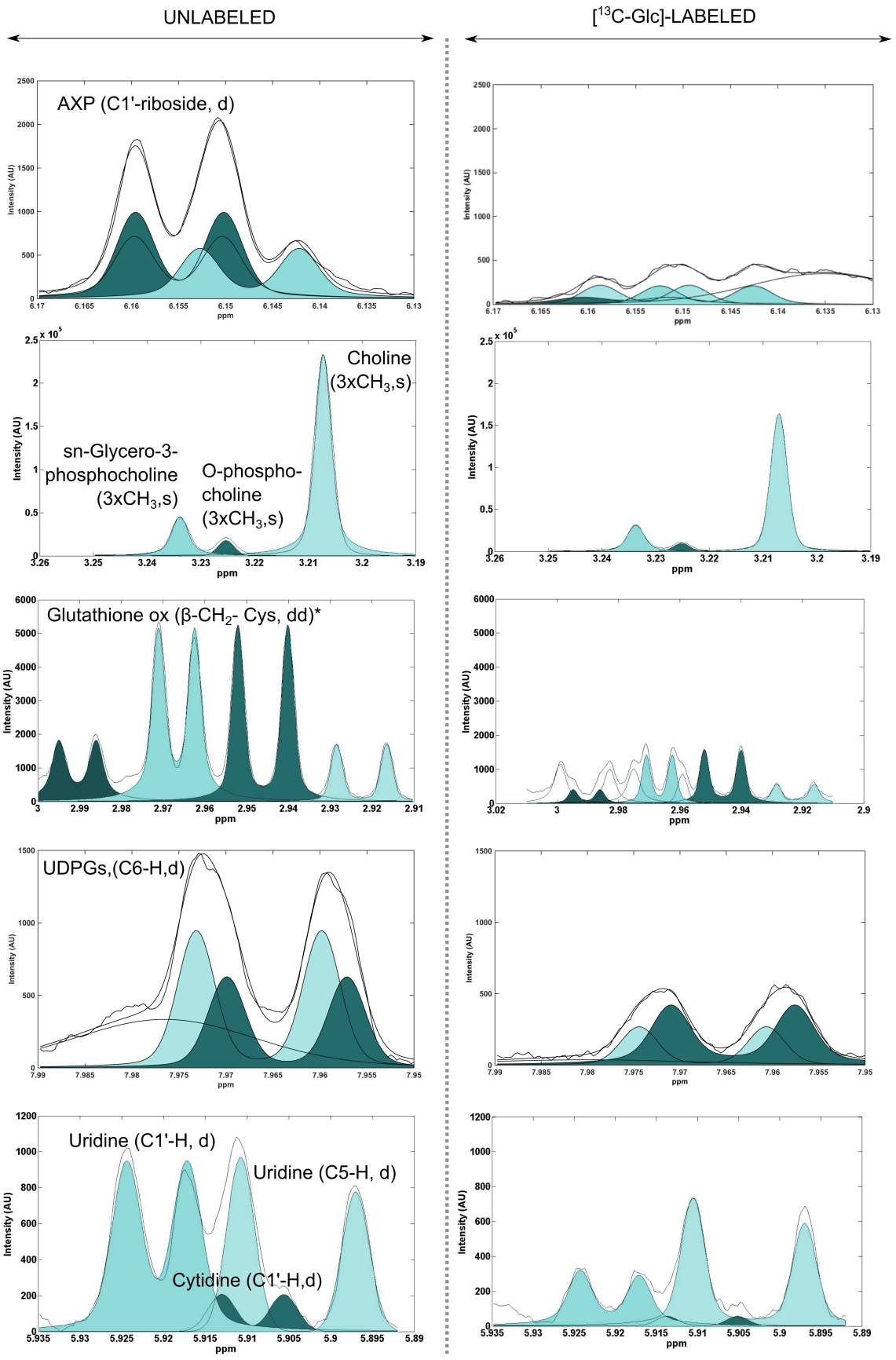
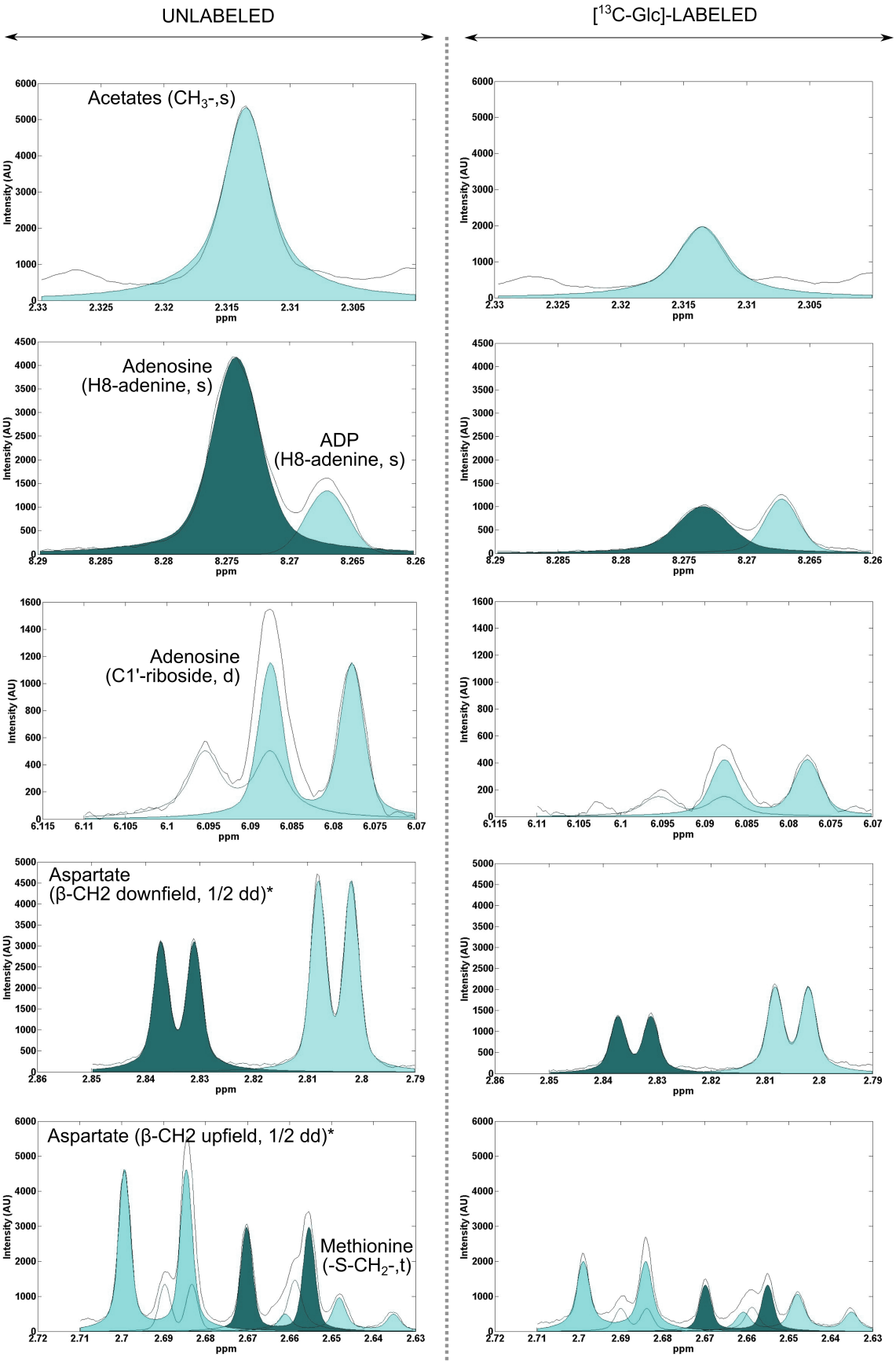


Figure S1: Example of Dolphin library guided line-shape fitting profiling for two ^{1}H-NMR regions of the same U2OS cellular extract highlighting spectral fitting of pantothenate (cyan), leucine (green), valine(yellow), isoleucine (pink), glycine(orange) and glycerol(gray).

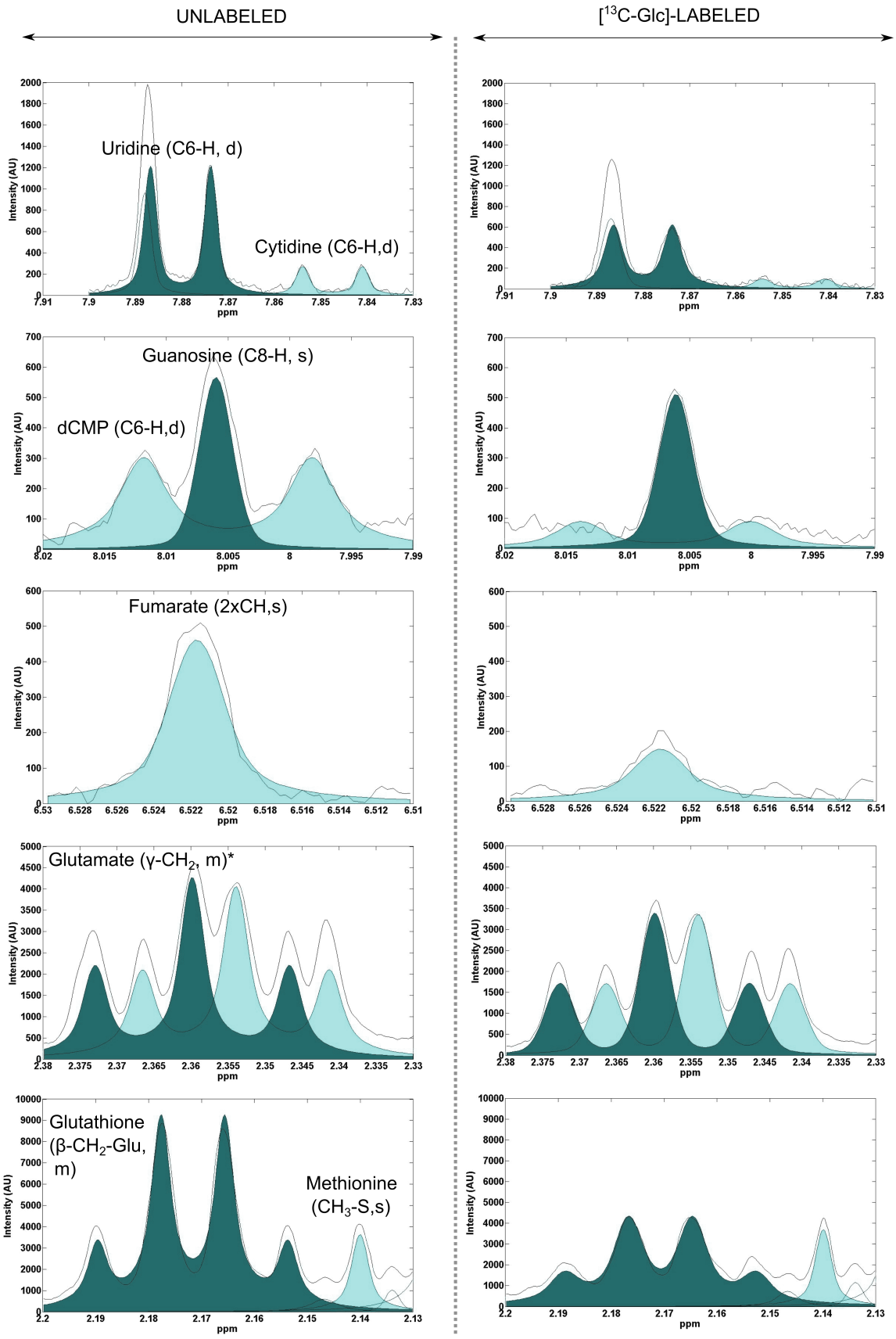


(a) Deconvoluted resonances

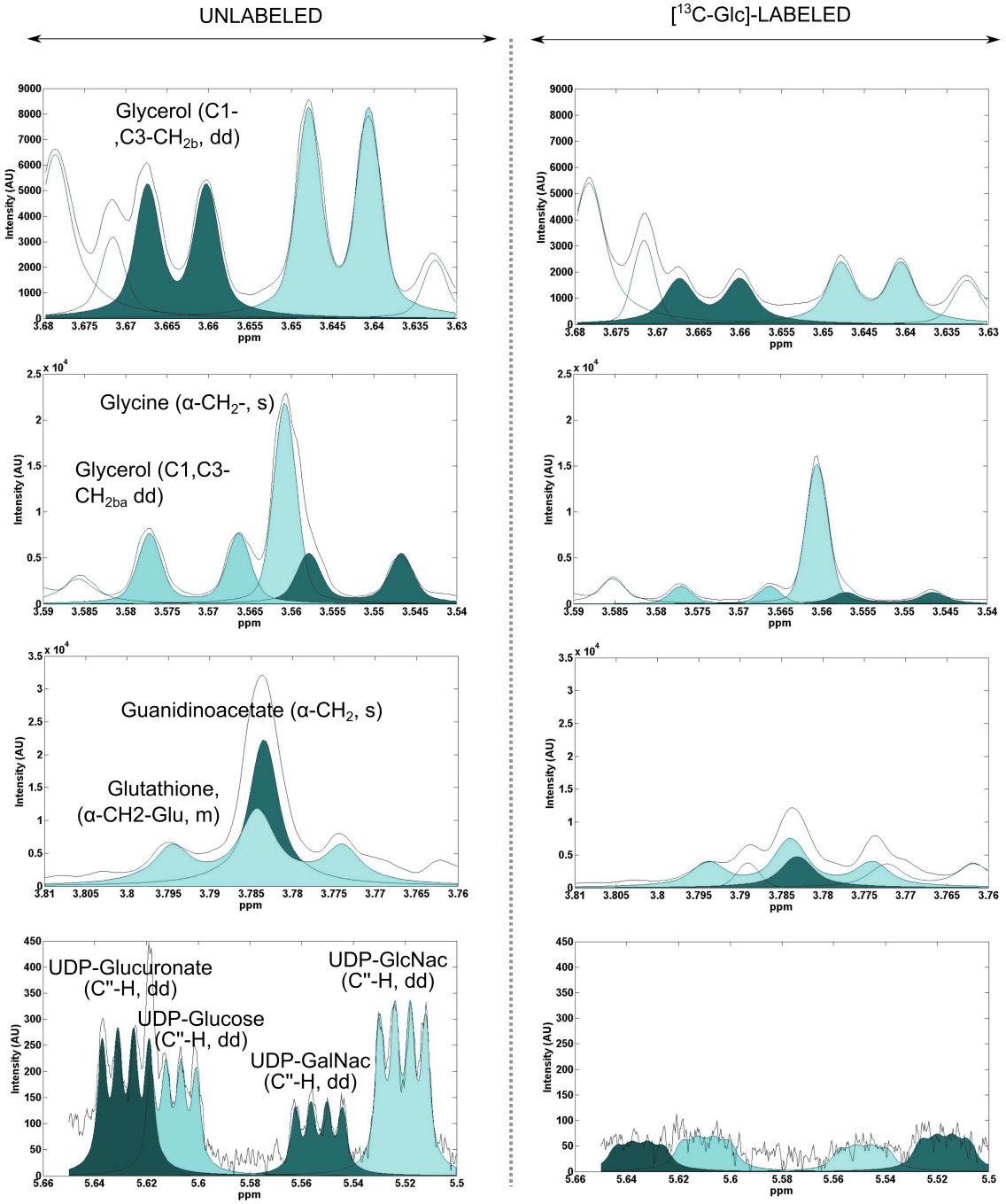
Figure S2: Deconvolution/integration of ^{1}H-NMR spectral resonances from unlabeled (left column) and their corresponding [^{13}C-Glc] labeled samples (right column) showing central peak decays. Multiplicities and resonances assignments are indicated. Refer to Supporting Tables for structural elucidation.



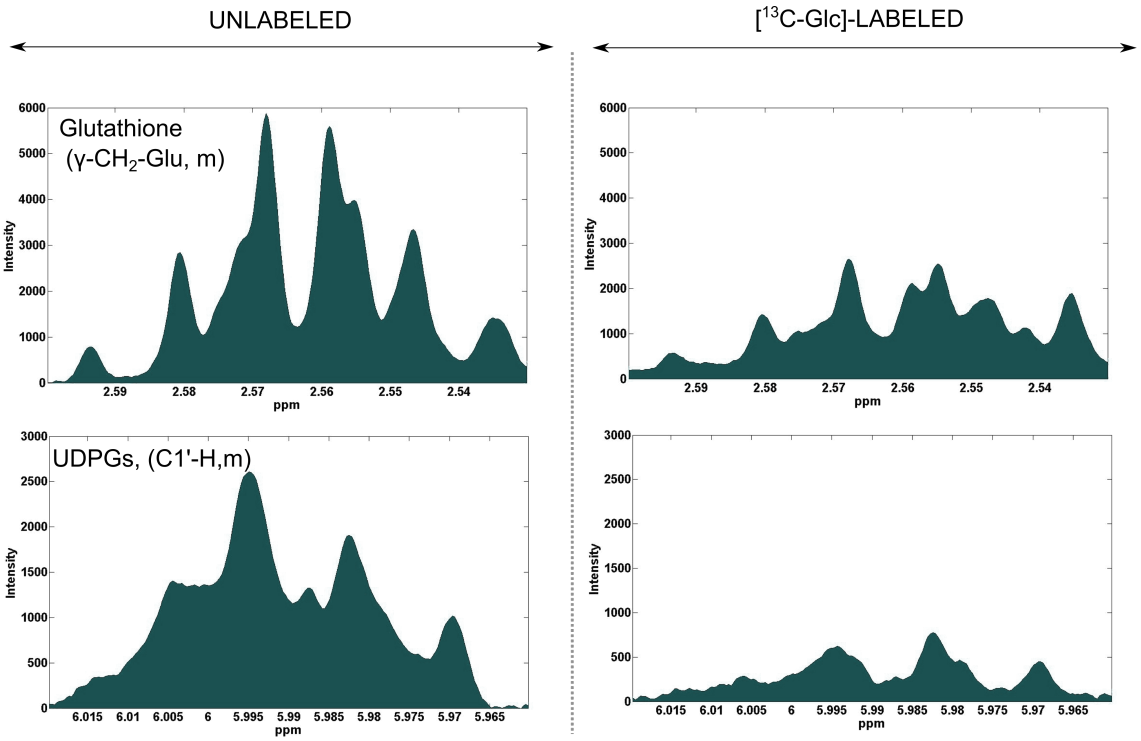
(b) continue from previous page



(c) continue from previous page



(d) continue from previous page



(e) Integrated resonances

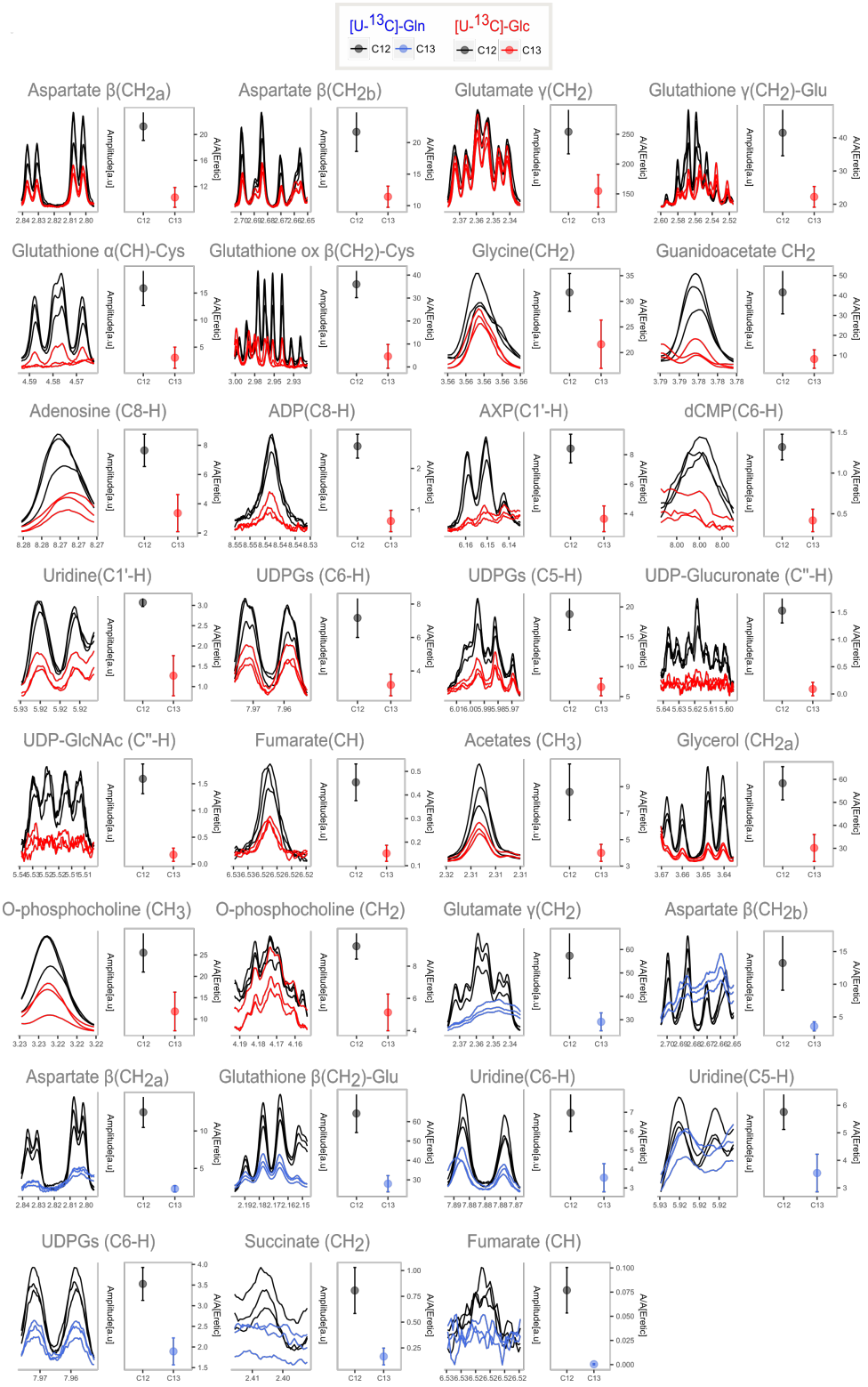


Figure S3: Raw ^{1}H-NMR resonances (left spectra) and integrated area using Dolphin software (right plots). The red, blue and black dots and lines represent [U-^{13}C-Glc], [U-^{13}C-Gln] and controls respectively. Error bars represent the standard deviation.

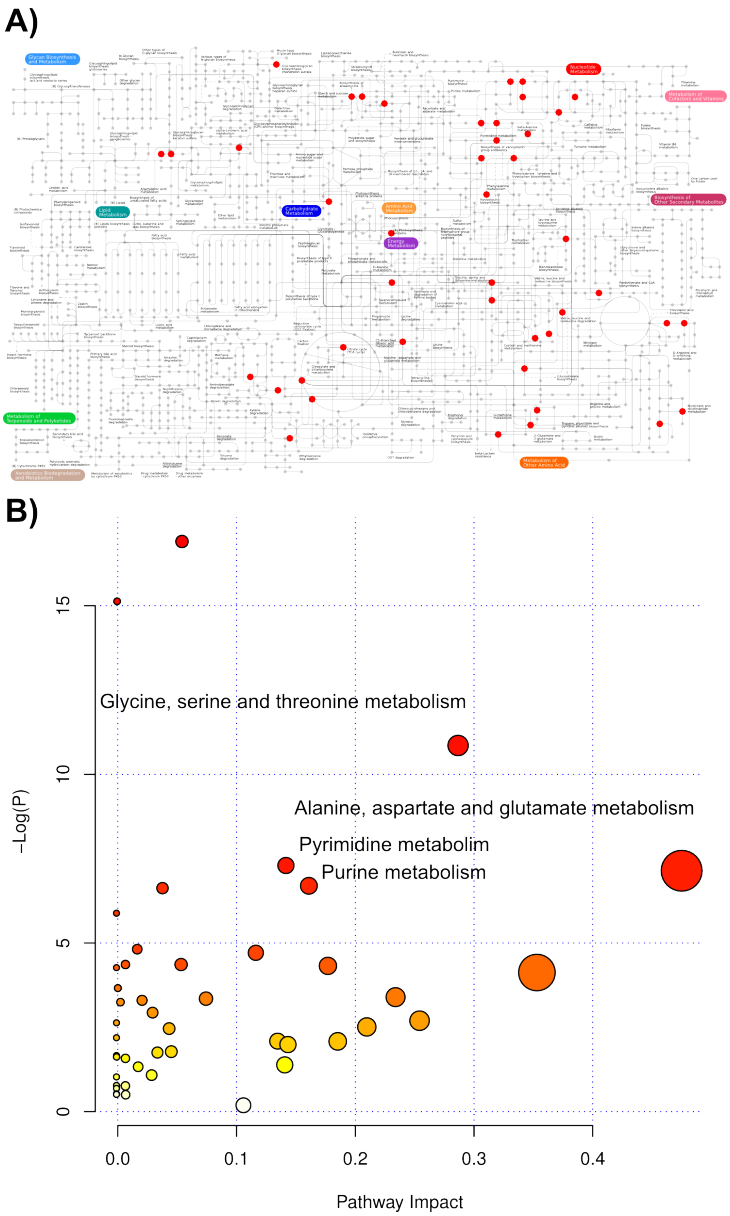


Figure S4: (A) 1D-^{1}H-NMR profiled metabolites are highlighted in the entire metabolic flow chart. Red dots represent 45 (out of the 46 detected) metabolites systematically detected in the 1D-^{1}H-NMR spectra. These include purine and pyrimidine nucleotides as well as aminosugar and nucleotide sugar metabolites. Polar-lipid related compounds and a wide range of amino acids are also represented in this profile. Finally, the TCA cycle intermediates fumarate, succinate and the glycolysis end-product lactate are detected as well. (B) Pathway enrichment analysis resulting from 1D-^{1}H-NMR profiled metabolites: x-axis represents Pathway Impact calculated from topological analysis based on the centrality measures of a metabolite in a given metabolic network. The higher the dots the more impact the metabolites hold. Y-axis represents FDR p-values resulting from hypergeometric test. Overrepresented pathways with higher impact in U2OS cell extracts ^{1}H-NMR profilig are highlighted.

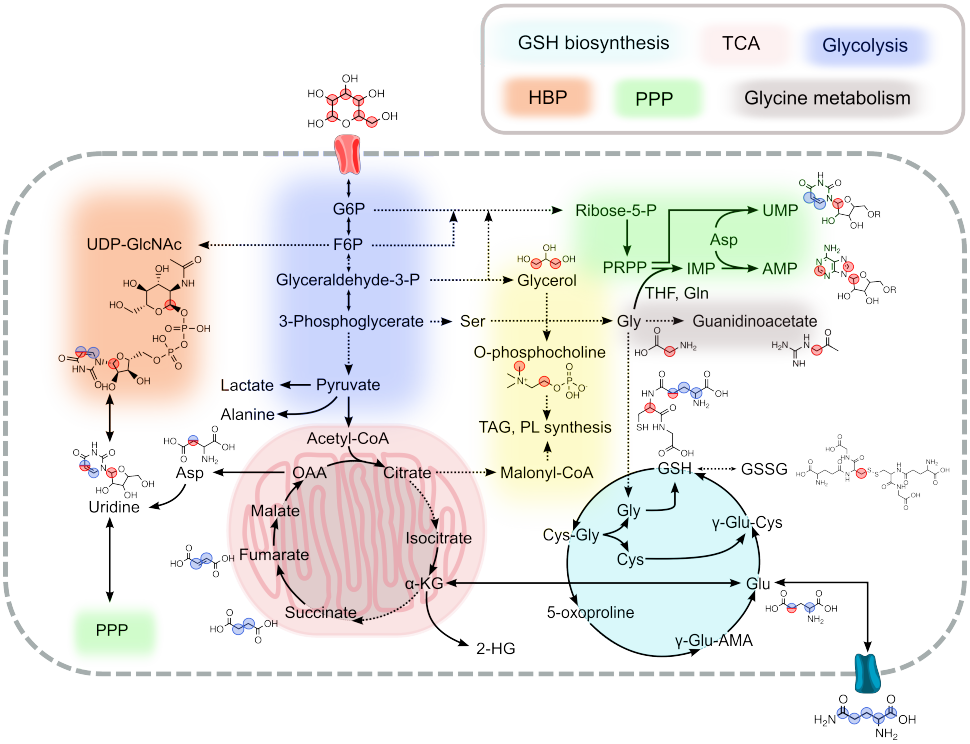


Figure S5: Metabolic chart showing active pathways in U2OS cell lines as revealed by PEPA. Chemical structures depicted with blue and red dots indicate metabolic fates of [U-^{13}C]-Gln and [U-^{13}C]-Glc, respectively. HBP: Hexosamine Biosynthesis Pathway; TCA: Tricarboxylic Acid Cycle; PPP: Pentose Phosphate Pathway.

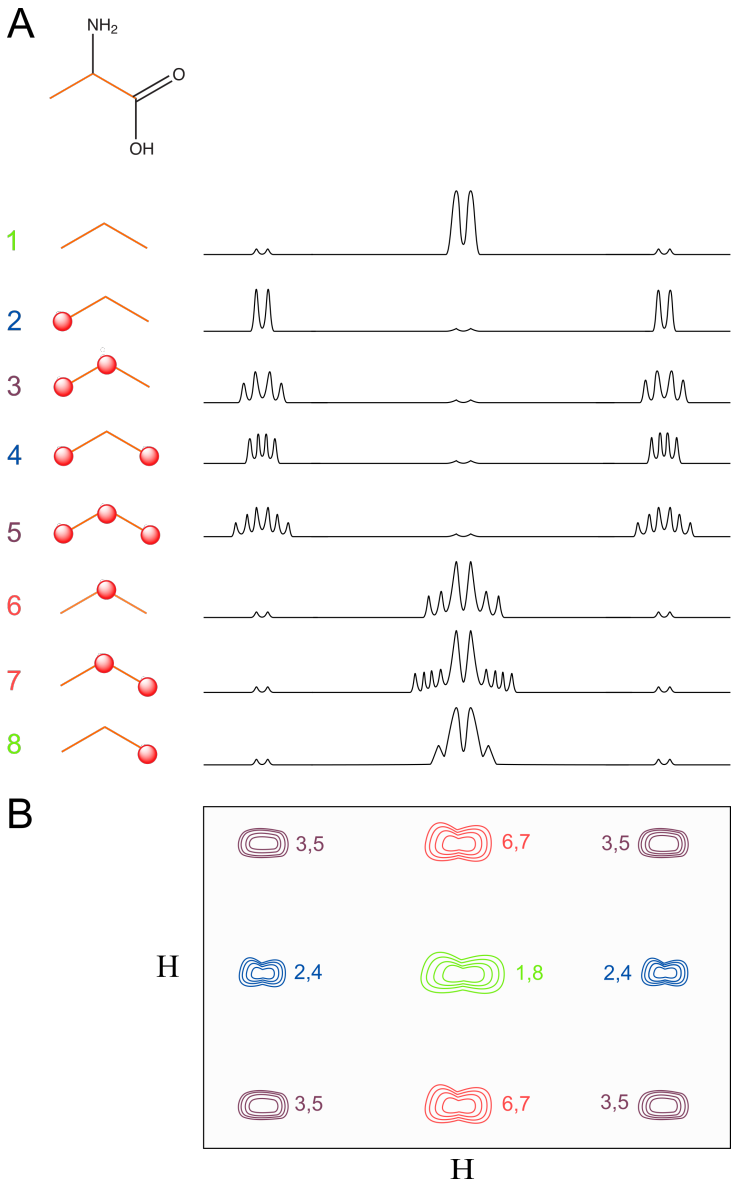


Figure S6: Theoretical spectra of alanine for all possible isotopologues and isotopomers. A) 1D H-NMR. B) 2D TOCSY pattern. Red circles in the carbon skeleton of alanine indicate ^{13}C labeled positions.

Table S1: 1D-^{1}H-NMR profiling used to determine metabolites isotope enrichment. ^{1}H chemical shifts, multiplicities and assignments are indicated.

Class	**Metabolite**	**Moieties Assignment**	$\delta(^1\mathbf{H})$
Energy metabolism	Glucose	C-α+C-β	5.23(d)+ 4.64(d)
	Glycogen	C1-H	5.41(m)
	Lactate	CH_3-	1.33(d)
		-CH-	4.11(q)
	Succinate	2×(CH_2)	2.41(s)
	Fumarate	2×(CH)	6.52(s)
	Pantothenate	CH_3-	0.90(s)
		CH_3-	0.93(s)
	Acetates	CH_3-	2.31(s)
	Formate	H-COOH	8.46(s)
Lipid metabolism	Glycerol	C1, C3 (CH_{2a})	3.56 (dd)
		C1, C3 (CH_{2b})	3.65 (dd)
	Choline	$(CH_3)_3$-N-	3.20(s)
		-N-CH_2	3.52(m)
		-CH_2-OH	4.07(m)
	O-Phosphocholine	$(CH_3)_3$-N-	3.22(s)
		-CH_2-O-P	4.17(m)
	sn-Glycero-3-phosphocholine	$(CH_3)_3$-N-	3.23(s)
Nitrogen metabolism	Aspartate	$\beta(CH_2)$ downfield	2.82(dd)
		$\beta(CH_2)$ upfield	2.68(dd)
	Glutamine	$\gamma(CH_2)$	2.46(m)
	Glutamate	$\gamma(CH_2)$	2.36(m)
		α(CH)	3.76(m)
	Glutathione	$\beta(CH_2)$-Glu	2.17(m)
		$\gamma(CH_2)$-Glu	2.56(m)
		α(CH)-Glu,Cys	3.79(m)
		α(CH)-Cys	4.58(m)
	Glutathione ox	$\beta(CH_2)$-Cys	2.95(dd)
	Glycine	$\alpha(CH_2)$	3.56(s)
	Methionine	-S-CH_2-	2.64(t)
		CH_3-S	2.14(s)
	Threonine	$\beta(CH_3)$	1.33(d)
		α(CH)	3.59(d)
	Leucine	2×$\gamma(CH_3)$	0.96(d), 0.97(d)
	Isoleucine	$\beta(CH_3)$	1.01(d)
		$\gamma(CH_3)$	0.94(t)
	Valine	$\beta(CH_3)$	0.99(d)
		$\beta(CH_3)$	1.05(d)
	Phenylalanine	(C2-C6)-H ring	7.38(m)
	Tyrosine	C3-H, C5-H ring	6.90(d)
		C2-H, C6-H ring	7.20(d)
	Tryptophan	C4-H indole	7.74(d)
		C7-H indole	7.54(d)
		C6-H indole	7.28(t)
		C5-H indole	7.20(t)
	Alanine	$\alpha(CH_3)$	1.46(d)
	Creatine	N-(CH_3)	3.04(s)
		$\alpha(CH_2)$	3.89(s)
	Guanidoacetate	$\alpha(CH_2)$	3.78(s)
	Anserine	C5-H ring histidine	7.10(s)
		C2-H ring histidine	8.24(s)
Pyridine, purine nucleotides	Adenosine	C1'-riboside	6.08(d)
		H8-adenine	8.27(s)

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Table S1 – *Continued from previous page*

Class	**Metabolite**	**Moieties Assignment**	$\delta(^1\mathbf{H})$
		H2-adenine	8.35(s)
	AMP	H2-adenine	8.61(s)
		C3'-riboside	4.51(m)
	ADP	H8-adenine	8.26(s)
		H2-adenine	8.54(s)
	AXP	C1'-riboside	6.15(d)
	NAD+	H2-nicotinamide	9.34(s)
		H6-nicotinamide	9.15(d)
		H4-nicotinamide	8.84(d)
		H8-adenine	8.44(s)
		H2-adenine	8.18(s)
		C1'-riboside(adenine)	6.04(d)
	1-methylnicotinamide	C2-H nicotinamide	9.29(s)
		C4-H nicotinamide	8.97(d)
		H6-nicotinamide	8.90(d)
		CH_3-	4.48(d)
	Xanthine	C8-H ring	7.68(s)
	Guanosine	C8-H guanine	8.01(s)
	Cytidine	C6-H cytosine	7.85(d)
		C1'-riboside	5.91(d)
	dCMP	C6-H cytosine	8.01(d)
	Uridine	C6-H uracil	7.88(d)
		C5-H uracil	5.90(d)
		C1'-riboside	5.92(d)
	Uridine 5'-monophosphate	C6-H uracil	8.11(d)
	UDPGs	C6-H uracil	7.96(d)
		C1'-H riboside	5.99(m)
	UDP-Glucuronate	C1"-H	5.61(dd)
	UDP-Glucose	C1"-H	5.59(dd)
	UDP-GlcNac	C1"-H	5.52(dd)
	UDP-GalNac	C1"-H	5.55(dd)

AMP: Adenosine monophosphate; ADP: Adenosine diphosphate; AXP: Adenine nucleotides: ATP,ADP,AMP; NAD+: Nicotinamide adenine dinucleotide; dCMP:Deoxycytidine monophosphate; UDPG: Uridine diphosphaten nucleotides; UDP-GlcNac: Uridine diphosphate N-acetylglucosamine; UDP-GalNac:Uridine diphosphate N-acetylgalactosamine. s: singlet; d: doublet; t: triplet, dd: double doublet; m: multiplet.

Table S2: PEPA validation using complementary ^{13}C-NMR and 2D-edited experiments (HSQC, HMBC, TOCSY)

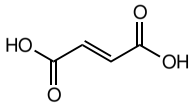
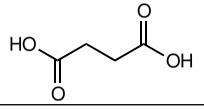
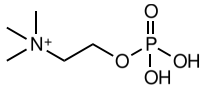
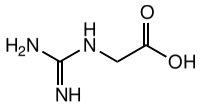
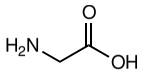
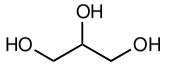
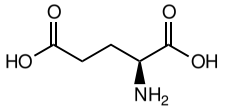
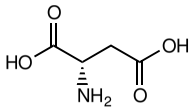
Metabolite	**Structure**	**Moieties**	δ(**^{1}H**)	δ(**^{13}C**)	**Spectra**	**PEPA**
Fumarate		2,3-CH	6.52(s)	138.4	^{1}H, HSQC	$\star$, †
Succinate		2×(CH_2)	2.41(s)	36.8	^{1}H, HSQC	†
Acetates		CH_3-	2.31(s)	32.2	^{1}H, HSQC	$\star$
o-phosphocholine		N-(CH_3)$_3$	3.22(s)	57.0	^{1}H, HSQC	$\star$
		-CH_2-O-P	4.17(m)	60.8	^{1}H, HSQC	$\star$
Guanidoacetate		α(CH_2)	3.78(s)	46.7	^{1}H,HSQC	$\star$
Glycine		α(CH_2)	3.56(s)	44.4[d(Glc)/-]	^{1}H,^{13}C, HSQC	$\star$
Glycerol		C1,3-H_{2a}	3.65(dd)	65.4[m(Glc)/-]	^{1}H, ^{13}C, HSQC	$\star$
		C1,3-H_{2b}	3.56(dd)	65.4[m(Glc)/-]	^{1}H, ^{13}C, HSQC	$\star$
		C2-H	3.78(m)	74.9[t(Glc)/-]	^{1}H, ^{13}C, HSQC	n.q
Glutamate		γ(CH_2)	2.36(m)	36.3[d(Glc),dd(Glc)/dd(Gln)]	^{1}H, ^{13}C, HSQC	$\star$, †
		β(CH_2)	2.07(m)	29.8[t+d(Glc)/t(Gln)]	^{1}H, ^{13}C, HSQC	n.q
		α(CH)	3.76(m)	57.2[-/dd(Gln)]	^{1}H, ^{13}C, HSQC	†
		(C=O)		177.4[-/d(Gln)]	^{13}C, HMBC	-
		δ(C=O)		184.2[d(Glc)/d(Gln)]	^{13}C, HMBC	-
Aspartate		β(CH_{2a})	2.82(dd)	39.5[d+d+dd(Glc)/dd(Gln)]	^{1}H, ^{13}C, HSQC	$\star$, †
		β(CH_{2b})	2.68(dd)	39.5[d+d+dd(Glc)/dd(Gln)]	^{1}H, ^{13}C, HSQC	$\star$, †
		α(CH)	3.90(dd)	55.3[d+d+dd(Glc)/dd(Gln)]	^{1}H, ^{13}C, HSQC	n.q
		γ(C=O)		180.5[d(Glc)/d(Gln)]	^{13}C, HMBC	-
		(C=O)		177.2[d(Glc)/d(Gln)]	^{13}C, HMBC	-

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Table S2 – *Continued from previous page*

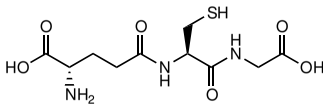
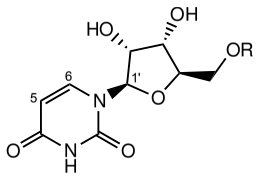
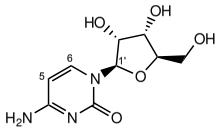
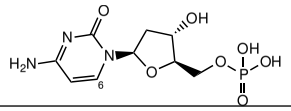
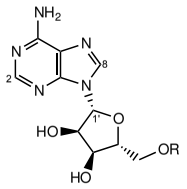
Metabolite	Structure	Moieties	$\delta(^1H)$	$\delta(^{13}C)$	Spectra	PEPA
Glutathione		$\gamma(CH_2)$-Glu	2.56(m)	34.2[d(Glc)/dd(Gln)]	^{1}H, ^{13}C, HSQC	⋆
		$\beta(CH_2)$-Glu	2.17(m)	29.0[-/t(Gln)]	^{1}H, ^{13}C, HSQC	†
		α(CH)-Glu	3.79(m)	57.1[-/d(Gln)]	^{1}H, ^{13}C, HSQC	†
		α(CH)-Cys	4.58(m)	58.4[-/-]	^{1}H, HSQC	⋆
Glutathione ox		$\beta(CH_2)$-Cys	2.98(dd)	41.9[-/-]	^{1}H, HSQC	⋆
Uridine		C6-H	7.88(d)	144.8[d(Glc)/d(Gln)]	^{1}H,^{13}C, HSQC	†
		C5-H	5.90(d)	105.0[m(Glc),t(Gln)]	^{1}H,^{13}C, HSQC	†
		C1'-H	5.92(d)	92.2[d(Glc)/-]	^{1}H,^{13}C, HSQC	⋆
		C2'-H	4.35(t)	76.8[t(Glc)/-]	^{1}H,^{13}C, HSQC	n.q
		C3'-H	4.24(t)	72.2	^{1}H, HSQC	n.q
		C4'-H	4.12(m)	87.0[t(Glc)/-]	^{1}H,^{13}C, HSQC	n.q
		C5'-H_a	3.92(dd)	63.5[dd(Glc)/-]	^{13}C, HSQC	n.q
		C5'-H_b	3.81(dd)	63.5[dd(Glc)/-]	^{13}C, HSQC	n.q
UDPGs		C6-H	7.96(d)	144.3[d(Glc)/d(Gln)]	^{1}H,^{13}C, HSQC	⋆, †
		C5-H	5.99(m)	105.3[m(Glc)/t(Gln)]	^{1}H,^{13}C, HSQC	†
UDP-Glucuronate		C1"-H	5.61(dd)	98.5[d(Glc/-)]	^{1}H,^{13}C, HSQC	⋆
UDP-Glucose		C1"-H	5.59(dd)	98.5[d(Glc/-)]	^{1}H,^{13}C, HSQC	⋆
UDP-GluNAc		C1"-H	5.52(dd)	97.6[d(Glc/-)]	^{1}H,^{13}C, HSQC	⋆
UDP-GalNAc		C1"-H	5.55(dd)	98.5[d(Glc/-)]	^{1}H,^{13}C, HSQC	⋆
Cytidine		C1'-H	5.91(d)	90.5[d(Glc)/-]	^{1}H,^{13}C, HSQC	⋆
dCMP		C6-H	8.01(d)	144	^{1}H, HSQC	⋆
Adenosine		C8-H	8.27(s)	155.6[s(Glc)/-]	^{1}H, ^{13}C, HSQC	⋆
		C2-H	8.35(s)	143.8[s(Glc)/-]	^{1}H, ^{13}C, HSQC	n.s
		C1'-H	6.08(d)	91.07[d(Glc)/-]	^{1}H, ^{13}C, HSQC	n.s
		C2'-H	4.80	76.5[t(Glc)/-]	^{13}C, HSQC	n.q
		C3'-H	4.40	73.5[t(Glc)/-]	^{1}H, ^{13}C, HSQC	n.q
		C4'-H	4.30(m)	88.7[t(Glc)/-]	^{1}H, ^{13}C, HSQC	n.q
		C5'-H_a	3.90(dd)	64.3[dd(Glc)/-]	^{13}C, HSQC	n.q

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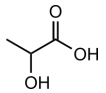
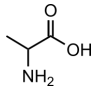
Table S2 – *Continued from previous page*

Metabolite	Structure	Moieties	$\delta(^1H)$	$\delta(^{13}C)$	Spectra	PEPA
		C5'-H_b	3.85(dd)	64.3[dd(Glc)/-]	HSQC, ^{13}C	n.q
AXP		C1'-H	6.15(d)	90.0[m(Glc)/-]	^1H, ^{13}C, HSQC	⋆
ADP		C8-H	8.54(s)		^1H	⋆
Lactate		CH_3-	1.33(d)	22.9[d(Glc)/-]	^1H,^{13}C, HSQC	n.s
		-CH-	4.11(q)	71.4[dd(Glc)/-]	^1H,^{13}C, HSQC	n.s
		-(C=O)	-	185.3[d(Glc)/-]	^{13}C, HMBC	-
Alanine		CH_3-	1.46(d)	18.9[d(Glc)/-]	^1H,^{13}C, HSQC	n.s
		-(C=O)	-	178.7[m(Glc)/-]	^{13}C, HMBC	-

^{13}C and ^1H chemical shifts in either labeled glucose or glutamine experiments. The presence of isotope enrichment is verified by the corresponding carbon chemical shift and multiplicity in either ^{13}C-NMR, HSQC, HMBC or TOCSY spectra (column labeled as "Spectra" lists the NMR experiments detecting the labeled signals. s: singlet; d: doublet; t: triplet, dd: double doublet; m: multiplet). The last column labeled as "PEPA" indicates if the isotope enrichment was detected by PEPA in the glucose (⋆) or glutamine (†) labeled experiments; n.q.: resonance could not be quantified by PEPA due severe overlap in 1D ^1H-NMR; n.s.: resonances that despite showing a central peak decay in 1D ^1H-NMR spectra of labeled experiments as compared to unlabeled controls did not reach the conventional 5% significance level using Students t-test. Note that non-protiated sites carbonyl labeling as detected per ^{13}C-NMR or/and HMBC can not be detected by PEPA.

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