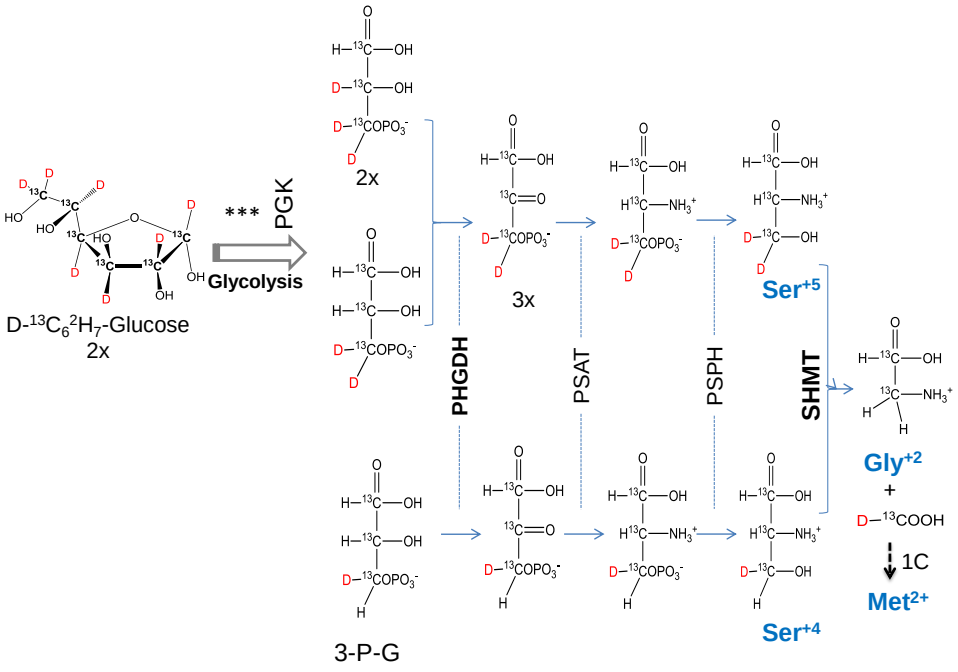
Protocol for the analysis of amino acids derived from glucose in the one-carbon metabolic pathway

Background

Serine biosynthesis undergoes a glycolysis branched out pathway that starts from the conversion of the glycolic intermediate, 3-phospho-glycerate, into serine, through the rate-limited enzyme PHGDH followed by PSAT and PSPH. Further, serine is converted into glycine by SHMT before entering the 1C metabolic pathways, providing one methyl group to homocysteine to form amino acid methionine. Therefore, serine, glycine, and methionine are the three major amino acids that can be derived from glucose. Here we describe a GC/MS method to measure these three amino acids by isotope tracking of $^{13}C_6{}^2H_7$-glucose metabolites.



Isotope tracking of amino acids formed by serine biosynthesis through D-$^{13}C_6{}^2H_7$-glucose metabolism

Preparation

1) Have the following materials ready

    a. PBS precooled at 4 °C

    b. 90% methanol precooled at 4 °C (45 mL 100% MeOH + 5 mL ddH_2O in 50 mL centrifuging tube)

    c. A foam bucket with ice

    d. A bucket with dry ice

2) Rinse the centrifugal filter vials

    a. Place 500 µL of ddH_2O into centrifuge filter vials (Amicon ultra-0.5 centrifugal filter unit with ultracel-3 membrane, MilliporeSigma, Ca# UFC500396)

    b. Spin at 10,000 rpm for 30 minutes

    c. Repeat one more time

3) Amino acid (AA) internal standard (IS) mixture (2×10^5 M in 50% MeOH)

    a. Preparation of 10 mM AA stock solution

        I. Weigh 0.89 mg of Met+3 (Cambridge Isotope Laboratories, L-Methionine (Methyl-D_3, 98%), Ca#: DLM-431-1, MW: 323, 1 grams) and dissolve it in 276 µl of ddH_2O to make 10 mM Met+3 stock solution

        II. Weigh 1.43 mg of Ser+7 (Cambridge Isotope Laboratories, L-Serine ($^{13}C_3$, 97-99%, D_3, 97-99%, ^{15}N, 97-99%, Ca#: CDNLM-6813-0.25, MW: 397, 250 mg) and dissolve it in 361 µl of ddH_2O to make 10 mM Ser+7 stock solution

        III. Weigh 1.06 mg of Gly+5 (Cambridge Isotope Laboratories, L-Serine ($^{13}C_2$, 97-99%, 2, 2-D_2, 97-99%, ^{15}N, 97-99%, Ca#: CDNLM-6799-0.25, MW: 251, 250 mg) and dissolve it in 424 µl of ddH_2O to make 10 mM Gly+5 stock solution

    b. Dilution of 10 mM stock solution into 0.2 mM (2×10^{-4} M) AA solution

        Pipet out 20 µl of 10 mM stock AA solution and add it to 980 µl of 50% MeOH

    c. Dilution of 0.2 mM AA solution into 2×10^{-5} AA working solution (IS)

d. Pipet out 100 µl of each 0.2 mM AA solution (Met+3, Ser+7, and Gly+5) and add it to 700 µl of 50% MeOH

4) Amino acid (AA) mixture standard solution (2 x 10^5 M in 50% MeOH)

   Use the same procedure as preparation for IS mixtures

Cell Culture

1) Thaw frozen A375 and A375 PHGDH over-expressing (A375+PHGDH) cells
2) Grow cells in a 6-well plate with 2 mL SILAC DMEM medium until ^{10}R fully-incorporated and froze some ^{10}R-cells for future use
3) Split cells in a new 6-well plate with 2 mL glucose, serine, glycine-free DMEM medium (US Biological, Ca#: D9800-16) and added 3.5 g/L $^{13}C_6{}^2H_7$-glucose (Cambridge Isotope Laboratories, D-Glucose-$^{13}C_6$,1,2,3,4,5,6,6-d_7, Ca#: CDLM-3813-1) for three days
4) Split cells in a 12-well plate for amino acid analysis and a 6-well plate for protein analysis
5) Add PHGDHi (0.5 µM & 10 µM) and SHMTi (0.5 µM & 10 µM) to the growing media for inhibitory cells and the same amount of DMSO to the controls, then grow cells for 24 hours (one day)

Cell harvest

1) Pipet out the media and wash the cells with cold PBS (1 mL) three times
2) In the wells of 12-well plate, add 0.5 mL 90% MeOH and scrape the plate with a rubber policeman. The cell pallets with MeOH are collected into a 2.0 mL microcentrifuge tube and washed once with 0.1 mL 90% MeOH (Note: This extraction cell procedure must be performed on the top of an experimental bench other than inside the cell culture hood.)

Extraction of AA with solication

1) Lysate cells in 0.6 mL 90% MeOH as described in **Cell harvest** using solication
   a. Clean sonicator needle with ethanol and then water
   b. Sonicate lysate in tube for 30 seconds at 30 amps
   c. Repeat 3x and wait 3 minutes between each sonication (to reduce chance of heating lysate)

2) Centrifuge lysate at 6,600 rpm for 5 minutes

3) Transfer supernatant lysate into new 2.0 mL micro centrifuge tubes

4) Store cell pellets in -80 °C freezer for further analysis or extract proteins from them by lysis (Protein concentrations may be used to normalize the AA concentration measured among different samples)

AA sample preparation

1) Filter above-mentioned lysate by the centrifugal filter vials

    a. Spin at 10,000 rpm for 30 minutes

    b. The maximum volume you can filter at a time is 400 µL

2) Transfer filtered lysate to new micro centrifuge vials

3) Place 100 µL of filtered lysate into a GC vial and add 20 µL of AA IS mixture

4) Prepare one sample with only standard amino acids (Gly, Met, and Ser) from 2 $x10^{-5}$ M stock standard solution

5) Prepare one sample with only IS from the stock 2 $x10^{-5}$ M IS solution

6) Dry completely the solution by a SpeedVac

Methoximation and derivatization

1) Place 100 µL of methoxamine solution (40 mg/mL) and 2 µL of 1M KOH in GC vials with samples and heat at 60 ^{0}C for 40 minutes with vial cap securely tight

2) Uncap the vials and dry completely the samples by a SpeedVac (it takes two hour or longer)

**1) and 2) methoximation steps are only required for analysis of AA including 2-ketoglutaric acid and 2-ketoglutamine.*

3) Place 20 µL of acetonitrile and 20 µL MTBSTFA + 1% TBDMCS (Sigma, Ca#: M-108-5X1ML) into the GC vials with samples

4) Securely cap the GC vials and heat them at 60 °C for 1 hour

GC/MS analysis of amino acids

1) Instrument set up

   a. GC

   Colum: Agilent J&W DB-5ms GC column (40 m x 250 µm x 0.25 µm including 10 m DuraGuard), **Mobile phase**: 99.999% helium, **Flow rate**: 54.2 mL/min, **Header temperature**: 290 °C, **Initial oven temperature**: 100 (hold time: 1 min) °C, **Ramp 1**: 10 °C/min to 300 °C then hold at 300 °C for additional 15 min, **Total run time**: 36 min.

   b. MS

   I. **Normal (full-mass) scan**: Scan range from 50 to 600 *m/z*, **Source temperature**: 230 °C, **Quad temperature**: 150 °C, **Solvent delay**: 5 min, **Method name**: Amino_Acid_Fast_PulsedSplitless_Scan.

   II. **SIM scan**: Create groups with selected-ion-monitoring (SIM) rather than full scan from 50 to 600 *m/z* as shown in following table; other conditions are the same as **Normal scan**:

Group #	# Ion	Start time (min)	Ion	Dwell (ms)	Resolution	Group name	Targeting ion
1	1	0	246	30	1	Gly	Gly
1	2	0	247	30	1	Gly	Gly^{+1}
1	3	0	248	30	1	Gly	Gly^{+2}
1	4	0	249	30	1	Gly	Gly^{+3}
1	5	0	251	30	1	Gly	Gly^{+5} (IS)
2	6	11	320	30	1	Ser-Met	Met
2	7	11	321	30	1	Ser-Met	Met^{+1}
2	8	11	322	30	1	Ser-Met	Met^{+2}
2	9	11	323	30	1	Ser-Met	Met^{+3} (IS)
2	10	11	390	30	1	Ser-Met	Ser
2	11	11	393	30	1	Ser-Met	Ser^{+3}
2	12	11	394	30	1	Ser-Met	Ser^{+4}
2	13	11	395	30	1	Ser-Met	Ser^{+5}
2	14	11	396	30	1	Ser-Met	Ser^{+6}
2	15	11	397	30	1	Ser-Met	Ser^{+7} (IS)

   Method name: AA SIM

2) Create a blank of 20 µL of acetonitrile and 20 µL MTBSTFA + 1% TBDMCS

3) Make sure both GC and MS are in the ***Ready*** conditions

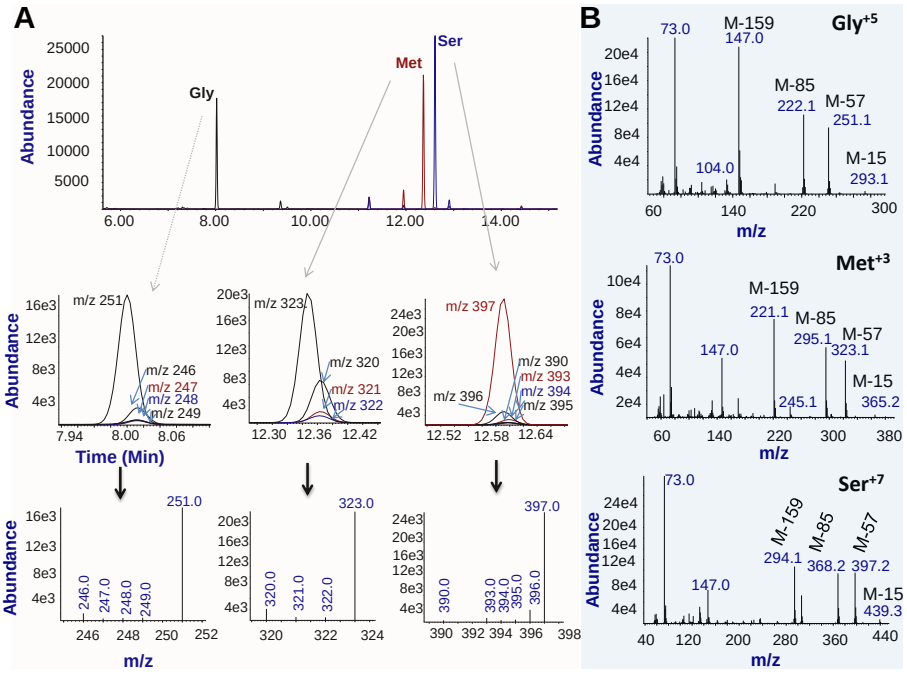
4) Make sure the MS instrument is ***Calibrated*** with the *Atune.u* updated, when needed, recalibrate the instrument by running *Tuning* method

5) Run blank sample before injecting sample using the MS method of *Normal scan*, injection volume: 0.2 µl

6) Run the standard sample using the MS method of *Normal scan*, injection volume: 0.2 µl

7) Run the IS sample using the MS method of *Normal scan*, injection volume: 0.2 µl

8) Determine the retention time of each amino acid by its full mass spectrum and matched isotope distribution profile; shown are examples of MS ion chromatographs and spectra of Gly, Met, and Ser (**Figure GC/MS of amino acids**):



GC/MS of amino acids

A. Top panel, Extracted ion GC/MS chromatography of Ser, Met, and Ser; *Middle panel*, ion chromatography of isotopes, and IS featured with co-elutiton and retention shift; *Bottom panel*, SIM MS spectra.
B. Full-MS spectra of Ser, Met, and Ser; Shown are those of their corresponding IS (Gly^{+5}, Met^{+3}, and Ser^{+7}).

$M^{+\cdot}$: Replacement of an active hydrogen (-NH2, and –OH) with a TBDMS group (*adds 114 to the molecular weight*).

Typical electron impact fragmentation ions:
M^{+}-CH3 (*M-15*), M^{+}-C4H9 (*M-57*), M^{+}-(C4H9 + CO) (*M-85*), and M^{+}-(CO-O-TBDMS) (*M-159*)

9) Generate **Sequence runs** table by starting with *Blank, IS,* and then *Blank*/*Sample/Blank* by turns using the MS method of *AA SIM*

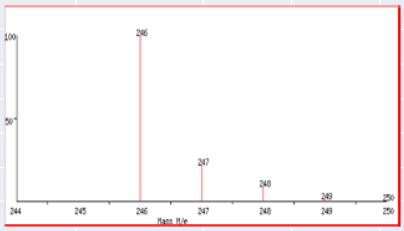
10)Run *Sequence* with injection volume 1.0 µl

11)Use ion extraction method to plot the ion-chromatographs of amino acids together with their IS corresponding to the above-shown Figure and integrate the peak areas for quantitative analysis, the extraction table looks as following:

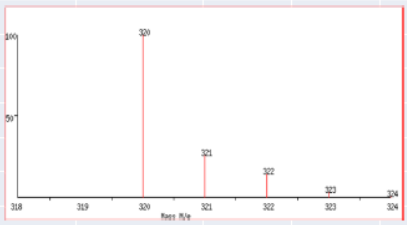
Targeting ion	Ion (*m/z*)	Retention time (min)
Gly	246	8.031
Gly^{+1}	247	8.031
Gly^{+2}	248	8.031
Gly^{+3}	249	8.031
Gly^{+5} (IS)	251	8.020
Met	320	12.383
Met^{+1}	321	12.383
Met^{+2}	322	12.383
Met^{+3} (IS)	323	12.366
Ser	390	12.623
Ser^{+3}	393	12.623
Ser^{+4}	394	12.623
Ser^{+5}	395	12.623
Ser^{+6}	396	12.612
Ser^{+7} (IS)	397	12.612

12)Calculation of AA using peak areas of each peak normalized by areas of IS and corrected by isotopes formed by AA and IS as well (Snapshots from two *Excel* calculation templets are included)

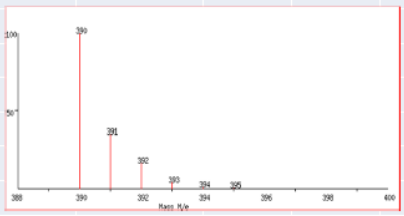
Theoretical isotope distribution



Gly		246	100
	1	247	21.8
	2	248	9
	3	249	1.1
	4	250	0.1



Met		320	100
	1	321	25.9
	2	322	14.5
	3	323	2.6



Ser		390	100
	1	391	34.7
	2	392	16.2
	3	393	3.6
	4	394	0.7
	5	395	0.1

Integration of IS and its impurities

AA	Notes	M+·	Area	isotope corrections					IS correction factors
Gly+0		246	423	4.08645E-09					0.122593416
Gly+1		247	398	3.84493E-09	2.95409E-09				0.088622577
Gly+2	Glucose-derived	248	1407	1.35925E-08	1.32247E-08	1.25807E-08			0.377422133
Gly+3		249	9815	9.48191E-08		9.45532E-08	9.181E-08		2.754318938
Gly+5	IS	251	345043	3.33333E-06					
Met+0		320	285	9.0706E-09					0.272117937
Met+1		321	462	1.47039E-08	1.23546E-08				0.370638952
Met+2	Glucose-derived	322	1191	3.79056E-08	3.65903E-08	3.33905E-08			1.001713947
Met+3	IS	323	104734	3.33333E-06					
Ser+0		390	612	6.2727E-09					0.188180887
Ser+3		393	14078	1.44293E-07	1.44067E-07				4.322000867
Ser+4	Glucose-derived	394	4929	5.05198E-08	5.04759E-08	4.84751E-10			0.01454254
Ser+5	Glucose-derived	395	4224	4.32939E-08		1.99551E-08	1.979E-08		0.593606703
Ser+6		396	33740	3.45818E-07		3.40632E-07	3.406E-07	3.33687E-07	10.01061816
Ser+7	IS	397	325219	3.33333E-06					

				IS conc. in real samples (20µl IS + 100 µl Sample)
	IS	Gly+5	2 x 10^{-5} M	3.33333E-06
		Ser+7	2 x 10^{-5} M	3.33333E-06
		Met+3	2 x 10^{-5} M	3.33333E-06

Calculation of AA concentrations

AA	M^+	Area	Calculated concentration with IS	Isotope Correction +1 (red) and +2 (gray)				IS correction Factors	in 120 µl	In cells
Gly+0	246	117456	2.36183E-06					0.122593	2.35774E-06	1.69757E-05
Gly+1	247	26800	5.38899E-07	2.4E-08				0.088623	2.1067E-08	1.51682E-07
Gly+2	248	36070	7.25302E-07	5.1E-07	5.1E-07			0.377422	4.9492E-07	3.56343E-06
Gly+3	249	12056	2.42424E-07		2.4E-07	1.2963E-07		2.754319	3.78165E-08	2.72279E-07
Gly+5	251	165770	3.33333E-06							
Met+0	320	26739	7.80316E-07					0.272118	7.71245E-07	5.55296E-06
Met+1	321	7275	2.12304E-07	1E-08				0.370639	-2.1524E-09	-1.54972E-08
Met+2	322	5357	1.56332E-07	4.3E-08	4.1E-08			1.001714	7.153E-09	5.15016E-08
Met+3	323	114223	3.33333E-06							
Ser+0	390	7878	1.5433E-07					0.188181	1.48057E-07	1.06601E-06
Ser+3	393	12207	2.39135E-07	2.3E-07				4.322001	8.95123E-08	6.44489E-07
Ser+4	394	13643	2.67266E-07	2.7E-07	1.9E-07			0.014543	1.84649E-07	1.32947E-06
Ser+5	395	23520	4.60756E-07		4.2E-07	3.5868E-07		0.593607	3.38888E-07	2.44E-06
Ser+6	396	24295	4.75939E-07		4.7E-07	4.3754E-07	3.1E-07	10.01062	-2.06095E-08	-1.48388E-07
Ser+7	397	170155	3.33333E-06							

20µl IS + 100 µl Sample 600 µl MeOH Extraction

		IS	Gly+5	2 x 10^{-5} M	3.3E-06	100 µl cells from 6-well dishes	
			Ser+7	2 x 10^{-5} M	3.3E-06	concentration = 600/100*120/100=7.2	
			Met+3	2 x 10^{-5} M	3.3E-06		