SBMRI Acetyl- and Malonyl-CoA Assay

Materials and Instrumentation:

- Acquity UPLC BEH C18 2.1 x 50 mm, 1.7 µm column, Waters Corporation, Cat. No. 186002350
- Oasis HLB 1cc (30 mg) Extraction Cartridges, Waters Corporation, Cat. No. WAT094225
- Acetyl- and Malonyl-CoA standards and heavy isotope-labeled internal standards
- Agilent 1290 Infinity HPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer
    - Software: MassHunter Acquisition (B.04.01); MassHunter Quantitative Analysis (B.05.00)

Sample Preparation:

1. Retrieve study samples (-80°C freezer) and calibrator/internal standard solutions (-20°C freezer) and allow them to thaw on ice.
    - Samples are prepared in 1000 µL of 5% trichloroacetic acid (TCA)

2. Obtain thirteen 1.7-mL Eppendorf tubes and place in a plastic tube rack on ice; label them for the calibration samples, C_1-C_{13}; note that C_{13} will be your high calibrator. Transfer 990 µL of 5% TCA to each tube.

3. Once thawed, vortex the calibrator and IS solutions retrieved from the freezer. Stock calibrator solutions are labeled as "Cals" from Cal 1 to Cal 13, with each Cal corresponding to a single calibration sample (See table below). Transfer 10 µL of a Cal solution to the corresponding calibration tube, and tap lightly to mix.

   The stock calibrator "Cals" are concentrated combinations of acetyl- and malonyl-CoA in 0.1% formic acid. Once diluted as prescribed below, these species are present at the following concentrations:

Cal	Volume Spiked into Cal Tubes (uL)	Calibrator	Conc'n of Ac-/MCoA present (pmol)
13	10	C_{13}	1000
12	10	C_{12}	500
11	10	C_{11}	250
10	10	C_{10}	100
9	10	C_9	50
8	10	C_8	25
7	10	C_7	10
6	10	C_6	5
5	10	C_5	2.5
4	10	C_4	1
3	10	C_3	0.5
2	10	C_2	0.25
1	10	C_1	0.1

4. All tubes (both calibrator and study sample) should now contain 1000 µL. Vortex the thawed internal standard solution and transfer 10 µL to all tubes.

   The stock internal standard solution is a concentrated mix of "heavy" acetyl- and malonyl-CoA in 0.1% formic acid. Once diluted as prescribed below, these ISs are present at the following concentrations:

Solution	Volume Spiked into All Tubes (uL)	Conc'n of ISs present (pmol)
IS Sol'n	10	500

5. Make sure study samples have thawed completely. Then, close the tubes and vortex thoroughly (at least 5 seconds). Centrifuge all tubes at 18,000 x g/5 min/10°C.

6. Prepare the solid-phase extraction (SPE) system by first turning the vacuum on. Open the valve by rotating counter-clockwise a full two turns.

7. Load twenty fresh test tubes into the SPE manifold; discard any tubes that possess cracks.

8. Place the lid onto the manifold and load on as many fresh SPE cartridges as you need (maximum of 20); twist them so that the labels face you.

9. Using the repeater pipettor fitted with a 10-mL Combitip, add 1000 µL of MeOH to each SPE cartridge. Open the manifold chamber to vacuum and allow the MeOH to pass through the cartridges. Once all the MeOH has eluted, close the manifold off and use the ball valve to release the remaining vacuum and restore ambient pressure.
   - Note: The vacuum inside the manifold chamber cannot exceed 22 in. Hg without risking implosion; keep an eye on the vacuum gauge!

10. Repeat Step 9, this time using 1000 µL of dH_2O instead of MeOH.

11. Once your sample tubes finish in the centrifuge, place them back into a plastic tube rack and set in the hood. If you have more than 20 samples, store them in the 4°C fridge while you work with the first 20.

12. For the calibrator samples, load the full volume of liquid (1000 µL) onto the first ten SPE cartridges.
   - Make sure that there is no vacuum in the manifold chamber before you load!
   - Keep track of which cartridges contain which samples; label them if you need to!

13. For study samples, load the full volume of liquid (~1000 µL) onto the remaining SPE cartridges, making sure to keep track of which samples were added to which cartridges. Avoid pipetting tissue pellet.

14. **Slowly** open the manifold chamber to vacuum and allow the liquid to **slowly** pass through the cartridges. Aim for a flow rate of 0.5 mL/min, or a total time of 2 minutes per 1000 µL.
    - Different samples will flow through the cartridges at different rates. Adjust the vacuum such that the "fastest" sample flows at a rate of 0.5 mL/min, even if that means that other samples will move more slowly, or not at all. After the fastest sample finishes, increase the vacuum to allow other samples to flow at 0.5 mL/min

15. Once all samples have passed through the cartridge, restore the vacuum to ambient conditions. Load 1000 µL of dH_2O onto each cartridge and allow the liquid to pass through at a flow rate of 1 mL/min. Note that this flow is twice as fast as the initial "Load" step in step 16. Adjust the vacuum to account for variable flow through different cartridges.

16. Repeat step 17 twice, once with 1000 µL of dH_2O, once with 500 µL of dH_2O. This amounts to a total wash volume of 2500 µL.

17. Once the chamber is restored to ambient pressure, remove the manifold cover. Transfer the contents of all test tubes to a 250-mL glass beaker, and place the empty test tubes into a plastic waste beaker. Transfer the liquid waste to the "Acid Waste" bottle in the fumehood, and dispose of the test tubes in the broken glass box.

18. Replace all test tubes and return the cover to the manifold. Load 1000 µL of MeOH to each cartridge, and **slowly** open the manifold chamber to vacuum. Allow the liquid to **slowly** pass through the cartridges. Aim for a flow rate of 0.5 mL/min, or a total time of 2 minutes per 1000 µL. Adjust the vacuum to account for variable flow through different cartridges.

19. Once the chamber is restored to ambient pressure, discard of the SPE cartridges in chemical waste. Remove the manifold cover and transfer the contents (1000 µL) of each test tube to a 96-well plate (see scheme below), keeping track of where each sample is placed. Discard of the empty test tubes in the broken glass box.

	1	2	3	4	5	6	7	8	9	10	11	12
A	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}
B	C_{13}	1	2	3	4	5	6	7	8	9	10	11
C	12	13	14	15	16	17	18	19	20	21	22	23
D	24	25	26	27	28	29	30	31	32	33	34	35
E	36	37	38	39	40	41	42	43	44	45	46	47
F	48	49	50	51	52	53	54	55	56	57	58	59
G	60	61	62	63	64	65	66	67	68	69	70	71
H	72	73	74	75	76	77	78	79	80	81	82	83

*Note: Calibrators should progress from C_1 (low cal) to C_{13} (high cal)

20. Dry the samples under N_2 at 30°C. Once complete, reconstitute the samples in 100 µL of 10 mM Ammonium carbonate, pH 9.5. Seal the plate and vortex for 1 minute using the plate vortexer; then place the sealed plate into the autosampler.
    - If you are running more than 20 total samples, begin to dry down the first batch while you continue to prep the second batch on the SPE manifold
    - Drying will take 30-45 minutes at 30°C. Do not attempt to speed up drying by increasing the temperature! These metabolites will degrade at temperatures above 30°C.
    - The stability of Acetyl- and Malonyl-CoA in the reconstitution buffer is low, even when placed in a refrigerated autosampler. Be prepared to run your samples immediately upon reconstitution.

Standard and Internal Standard Preparation:

Below is a list of the metabolite standards used for the construction of the calibration curve, as well as the initial stock concentrations prepared:

Metabolite	Carrier	Cat. No.	Stock Conc'n (nmol/mL)	Diluent
Acetyl-CoA	Sigma	A2056	1000	0.1% formic acid
Malonyl-CoA	Sigma	M4263	1000	0.1% formic acid

Individual stock solutions are then combined to prepare the highest calibrator standard stock solution. The nmol/mL concentration of each amino acid in the combined mixture is given below:

Metabolite	Initial Individual Stock Conc'n (nmol/mL)	Final Combined Stock Conc'n (nmol/mL)
Acetyl-CoA	1000	100
Malonyl-CoA	1000	100

This is the stock solution for the highest calibrator, C_{13}. Serial dilution of C_{13} yields the stock solutions for all ten calibrator samples, C_1-C_{13}:

Calibrator Stock Sol'n	Conc'n of Ac-/MCoA (nmol/mL)
C_{13}	100
C_{12}	50
C_{11}	25
C_{10}	10
C_9	5
C_8	2.5
C_7	1
C_6	0.5
C_5	0.25
C_4	0.1
C_3	0.05
C_2	0.025
C_1	0.01

Based on the protocol above, the calibrator curve ultimately generated during prep will have the following range:

Calibrator Stock Sol'n	Conc'n of Ac-/MCoA (pmol)
C_{13}	1000
C_{12}	500
C_{11}	250
C_{10}	100
C_9	50
C_8	25
C_7	10
C_6	5
C_5	2.5
C_4	1
C_3	0.5
C_2	0.25
C_1	0.1

Below is a list of heavy-isotope-labeled internal standards used for the construction of the calibration curve, as well as the initial stock concentrations prepared:

Internal Standard	Carrier	Cat. No.	Stock Conc'n (nmol/mL)	Diluent
Acetyl-CoA-$^{13}C_2$	Sigma (Isotec)	658650	1000	0.1% formic acid
Malonyl-CoA-$^{13}C_3$	Sigma (Isotec)	655759	1000	0.1% formic acid

Individual stock solutions are then combined to prepare the working internal standard stock solution. The nmol/mL concentration of each amino acid in the combined mixture is given below:

Internal Standard	Initial Individual Stock Conc'n (nmol/mL)	Final Combined Stock Conc'n (nmol/mL)
Acetyl-CoA-$^{13}C_2$	1000	50
Malonyl-CoA-$^{13}C_3$	1000	50

Our limits of quantitation are set by the high and low points of our calibrator curves. Some calibrator curves display quadratic character under our instrument conditions.

Assay Conditions:

- Autosampler
  - Temperature: 10°C
  - Injection Volume: 20 µL
  - Needle Wash Solution: 80/20 Methanol/Water
- Column
  - Temperature: 27°C
  - Maximum Pressure: 1200 bar
- Binary Pump
  - Flow Rate: 0.4 mL/min
  - Solvent A: 20 mM ammonium carbonate, pH 9.5
  - Solvent B: Acetonitrile
  - Gradient Conditions:

Segment	Time (min)	% B	Flow Rate (mL/min)
0 (Start)	0.00	0.0	0.40
1	1.75	20.0	0.40
2	1.85	95.0	0.70
3	2.35	95.0	0.70
4	2.45	0.0	0.75
5	6.50	0.0	0.75
6	6.60	0.0	0.40
Re-Equil.	Addt'l 2.5 min	0.0	0.40

- Mass Spectrometer
    - Gas Temperature: 325˚ C
    - Gas Flow: 11 L/min.
    - Nebulizer: 45 psi
    - Sheath Gas Temperature: 325˚ C
    - Sheath Gas Flow: 10 L/min.
    - Capillary Voltage: 3500 V
    - Nozzle Voltage: 500 V
    - Electrospray ionization: Positive

- MRM Transitions

Metabolite	Precursor Ion (m/z)	Product Ion (m/z)	Dwell	Fragmentor	Collision Energy	Cell Acc. V
Malonyl-CoA-$^{13}C_3$	857.1	350.2	50	380	28	6
Malonyl-CoA	854.1	347.2	50	380	28	6
Acetyl-CoA-$^{13}C_2$	812.1	305.1	50	380	28	6
Acetyl-CoA	810.1	303.2	50	380	28	6