Liquid Chromotography Protocol

Method based off of Wang et al. 2014 paper (Hazen Method).

Column: 150 by 2 mm, 3 μm particle, Silica (2) 100 A from Phenomenex; Catalog #00F-41620-B0

Solvents: A: Water, 0.1% Acetic Acid – use A2 line B: Methanol, 0.1% Acetic Acid – use B2 line

Flow Rate: 0.25 mL/min

LC Method:

Time (min)	A%	B%
0	98	2
5	85	15
6.25 *	0	100
8.00 *	0	100
8.25	98	2
10	98	2

*Rinse time to remove stuff stuck on column so that it doesn't carry over into readings for next sample

<u>Analyte</u>	<u>RT (min)</u>
TMAO	3.3
Choline	4.3
Betaine	3.4
Carnitine	3.4
Methionine	2.3
Phosphocholine	3.4
GPC	3.5
DMG	2.6
CUDA	7.7

Prepare solvents

Solvents A and B

A solvent is the aqueous solvent and should be prepared fresh before each run in case there are bacteria growing in it. 0.1% acetic acid brings pH down to 4 for both solvents, though A solvent should be more polar than B solvent. Old solvent A can be poured down the sink. Prepare both solvents in the fume hood.

- 1. Remove the end of the tubing for A2 and/or B2 from bottles and store them in the cleaning bottle (70% isopropanol, 30% water). Bring solvent bottles back to lab.
- 2. If making both solvents, make <u>solvent B</u> first:
 - Measure out 500 mL 100% **Optima LC/MS grade** methanol in a graduated cylinder and pour it into the solvent B bottle. (Scale down if necessary.)
 - Pipet 500 uL of acetic acid (Optima stock) into solvent B bottle, mix thoroughly.
- 3. Make <u>solvent A</u>:
 - Fill graduated cylinder with 500 mL **Optima LC/MS grade** HPLC water and pour it into the solvent A bottle.
 - Pipet 500 uL of acetic acid (Optima stock) into solvent A bottle, mix thoroughly.
- 4. Rinse out cylinder with a little bit of 100% methanol, allow it to evaporate, then cover the cylinder with foil to prevent dust accumulation.

Weak wash and strong wash

Keep an eye on the weak and strong washes located in bottles behind solvents A and B on the HPLC and make sure that they don't run out. The weak wash usually runs out faster than the strong wash. Running out of these washes will stop the run. If necessary, make more strong and weak wash using HPLC-grade reagents. All bottles (weak wash, strong wash, solvents A & B) should be covered with foil to prevent dust and/or tiny bug exposure.

- Weak wash: 70% water, 20% methanol, 10% 2-propanol
- Strong wash: 50% methanol, 50% acetonitrile

ACQUITY Console Software Protocol

Set up profile project in "Analyst Software" – Only need to do this once at the beginning of a study. For the CC Diet Pre-diet samples, we did the following:

- 1. Create a new folder specific to the project in <u>D:/Analyst Data/Projects</u>/2018 Pre CC Diet Plasma
- 2. Copy all protocols and files from *D:/Analyst Data/Projects/2018 TMAO CC Diet* to *D:/Analyst Data/Projects/2018 Pre CC Diet Plasma*
- 3. Use the MRM scan type, set acquisition method to LC150 by 2mm 10 minute with MRM
- 4. Other MS parameters that affect how molecules react:
 - -Decoupling potential (DP) affects Q1

-Collision energy (CE) – affects Q3; Too much CE breaks everything, so you can't see what you're looking for. If there's too little CE then the molecule of interest can't be detected.

Prepare HPLC for sample run:

ACQUITY Console → Control LC, go to Binary solvent manager

 Go to "Control" in menu bar, then "Prime A/B solvents" for 1 minute to get rid of bubbles in the tubing. Make sure that black wire/tube is pumping solvents A and B to the <u>waste (not the mass spec)</u> and be sure to pick A2 and B2 for the input in the software!

Note: It is possible to prime A and B solvents separately. If priming solvent B first while making solvent A, set solvent B to 100% and proceed to steps 2-5a and change flow rate to 0.1 mL/min, gradually increasing flow rate up to final flow rate of 0.25 mL/min. Then proceed to step 5b.

- Go to "Control" in menu bar, then "Prime seal wash" for ~ 2 minute until pressure stabilizes to lubricate column (this also goes to the waste). Manually turn off prime seal wash by unchecking it.
- 3. Go to Column Manager to select the correct column and check that the temperature setting for the column is room temperature. Sample Manager should always be 10° C.

- 4. Wash out the column with 10 column volumes of methanol before running samples:
 - a. Go back to the binary solvent manager and set the flow rate to 0.1 mL/min with 100% solvent B (methanol) for ~ 1 minute or until pressure stabilizes.
 - b. Go back to the binary solvent manager and set the flow rate to 0.2 mL/min with 100% solvent B (methanol) for ~ 1 minute or until pressure stabilizes.
 - c. Go back to the binary solvent manager and set the flow rate to 0.25 mL/min with 100% solvent B (methanol) for ~20-30 minutes until pressure stabilizes.

Notes:

- Washing for 20-30 minutes washes the column with ~10 column volumes of methanol; this cleans the column thoroughly and allows system pressure to equilibrate. Check for leaks in the column during this wash time.
- For solvent B, ~1485 psi is good; 5000 psi is bad! If the pressure is unusually high, it probably means that the column is dirty. If the pressure is unusually low, there might be a pump problem. For solvent A, ~2360 psi is good.
- Record the psi for each solvent at equilibrium in your lab notebook.
- Do not use higher than 0.25 mL/min for the flow rate! If the flow rate is too high, then the pressure will be too high passing through the column which may dislodge the silica.
- 5. Wash out the column with 10 column volumes of solvent A before running samples:
 - Go back to the binary solvent manager and gradually change proportions of A:B solvents from 0 A: 100% B to 98% A: 2% B to avoid big changes in pressure. For example, change solvent B from 100% -> 70% -> 50% -> 30% ->15% -> 2%, waiting ~10 seconds between each change.
 - b. Keep the flow rate at 0.25 mL/min with 98% solvent A for ~20-30 minutes until pressure stabilizes.

Set up sample plate:

Build Acquisition Batch - tells software/HPLC which spot is rack is what sample

1. A. <u>For the first time</u>: Click "Open" (file) icon after selecting the correct folder, select copied Acquisition Batch File pasted from post CC Diet run, rename samples/slots as necessary, save as a new batch file with correct name/date/type of sample (pre-diet).

B. <u>For subsequent batches</u>: Click "Open" (file) icon after selecting the correct folder, select Acquisition Batch File and rename the file to current date and batch number, rename samples/slots as necessary, save as new batch file.

- 2. Click on the View Queue in the upper left hand corner to bring up Queue Manager and set the following in the "Sample" tab:
 - Data file: Pre CC Diet Plasma Samples Batch _ [Date] (e.g. Batch 1 6-19-2018)
 - Injection Volume: 5 uL
 - Data files end it *.dab

- Fill in correct standards, controls, and samples, etc; can use "auto increment" or "fill down" if samples are in numerical order or the same (e.g. data file name).

	1	2	3	4	5	6	7	8
Α	Pos. 1	Pos. 2	Pos. 3	Pos. 4	Pos. 5	Pos. 6	Pos. 7	Pos. 8
В	Pos. 9	Pos. 10	Pos. 11	Pos. 12	Pos. 13	Pos. 14	Pos. 15	Pos. 16
С	Pos. 17	Pos. 18	Pos. 19	Pos. 20	Pos. 21	Pos. 22	Pos. 23	Pos. 24
D	Pos. 25	Pos. 26	Pos. 27	Pos. 28	Pos. 29	Pos. 30	Pos. 31	Pos. 32
Е	Pos. 33	Pos. 34	Pos. 35	Pos. 36	Pos. 37	Pos. 38	Pos. 39	Pos. 40
F	Pos. 41	Pos. 42	Pos. 43	Pos. 44	Pos. 45	Pos. 46	Pos. 47	Pos. 48

- Vial positions/sample order: 48 samples per plate

Example plate 1 layout

	1	2	3	4	5	6	7	8
Α	Blank	PRM 0 uM	PRM 0.0475	PRM 0.095	PRM 0.19	PRM 0.39	PRM 0.78	PRM 1.56
В	PRM 3.125	PRM 6.25	PRM 12.5	PRM 25	PRM 50	PRM 100 uM	CON 1-1	CON 2-1
С	CON 3-1	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
D	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15
Е	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	CON 1-2	CON 2-2	CON 3-2
F	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28

Example plate 2 layout

	1	2	3	4	5	6	7	8
Α	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36
В	Sample 37	Sample 38	Sample 39	Sample 40	CON 1-3	CON 2-3	CON 3-3	Blank
С								
D								
Ε								
F								



3. Here is a recommended order of submitting samples:

Submit samples for mass spec run

- Put vials into the rack according to the order/position indicated in the acquisition batch file. Check that tubes and samples listed in the acquisition batch are in the correct position/order!!
- 2. Place rack with the "letter" side closest to the back of the sample manager to reduce the chance of the reading needle misaligning with the sample insert in the vials.
- 3. Switch over to the "Submit" tab in the Batch Editor, highlight the blank, and click "Submit".
- Click the Equilibrate icon (looks like a tiny EKG) and confirm method. For the CC Diet project, we will use the "LC150 by 2mm 10 minute with MRM" Acquisition Method.
 Make sure you select the right method before proceeding!! Note: Queue manager must be open for Equilibrate icon to appear/be selectable.
- 5. Move the black line from the waste and insert it into the mass spec, securing it by tightening the fastener.
- 6. After the mass spec has equilibrated, the Start icon (the tiny flask) will be available in the toolbar. Click the Start icon to begin submitting the blank.
- 7. Click "Open Data File" to view ion signals and check that values are good, i.e. no aberrant peaks. If first blank is noisy, submit blank twice:
 - First left click on the graph to select a time point (a thin black line will appear on the graph), then right click to make options appear.

- Click on "Extract Ions" to look at the signals from individual ions. Highlight multiple ions for a side-by-side comparison in one graph. To view them in separate graphs, select only one ion and repeat this process.
- Switch between samples by clicking on the forward or back arrows in the upper left hand corner or by double-clicking "Open Data File" and selecting specific samples from specific projects.
- 8. Submit controls and then open the data file to check the values and quality of peaks for each ion type (see LC Method for expected retention times). If they look okay, submit the rest of the standards, controls, and samples. For example, here is a good control for TMAO (in blue on the left) and choline (in red on the right):



Post-run rinse

After run is done, the HPLC and mass spec will eventually go into stand-by mode. Move the black line from mass spec to the waste flask and run solvent B 100% at 0.25 mL/min for 10-15 minutes by gradually changing the proportion of A:B solvents.

- Go back to the binary solvent manager and gradually change proportions of A:B solvents from 98% A: 2% B to 0% A: 100% B to avoid big changes in pressure. For example, change solvent A from 98% -> 70% -> 50% -> 30% ->15% -> 2%, waiting ~10 seconds between each change.
- Remove sample racks with samples from Sample Manager and bring them back to lab. Replace the blue caps with red caps for long term storage at -20° C in a box labeled with the date, project name, and sample type, e.g. 7/5/18 CC Diet Prediet TMAO.
- 3. Return the sample racks to the sample manager and remember to turn off the solventB (methanol) rinse by setting the flow rate in the binary solvent manager to 0 mL/min.