Extraction for Hormone Measurement (USDA-ARS MU 2017)

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This is the extraction of samples for ABA and GA (and GA precursor) measurement in Columbia, Missouri (USDA-ARS in Oliver Lab) using LCMS. Based on the protocol from RIKEN 2013, but with substitutions for tools that we do not have here. The 2016 version of this protocol used 15 mL tubes, but it has now been further modified to use ***5 mL polypropylene round-bottomed tubes (Falcon, product: 352063)*** *for most steps. This allows speed-vac steps to accommodate 40 tubes at once with our new rotor for 5 mL tubes. For larger samples, refer to 15 mL or 50 mL tube protocols. (Max 400 mg sample : 50 mL tubes.)*

5 mL tubes used: 1

START DAY 0

1. Measure the weight of the freeze-dried samples in 5 mL tube. (desired: 50-100 mg DW)

> a. Do this beforehand. Pre-weigh empty tubes before adding ground tissue and then weigh again after freeze drying. Subtract the first from the second to get the total dry weight. Be sure to use the sensitive scale and record all 4 digits after the decimal for your pre-weights.

5 mL tubes used: 0

START DAY 1

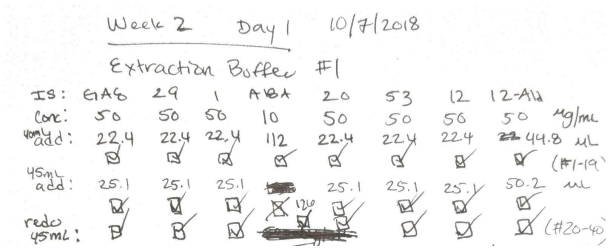
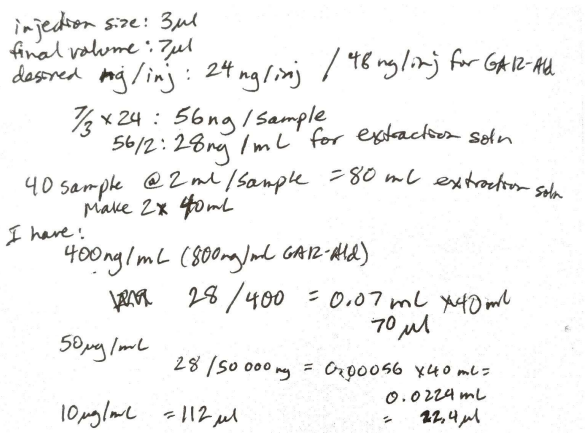
↧ ON BENCH OR IN HOOD

2. Add 2 mL of the 1st extraction solution to each samples

> a. For all extraction solutions, use HPLC quality H2O. Don't store extraction solution as it contains internal standards (ISs) which can degrade over time, make fresh.
> b. 1st extraction solution: 80% Acetone containing 1% AcOH and IS-MIX solution
> c. Add IS (GA and ABA internal standards) to extraction solution "master mix" so that each sample will get the desired final ng per sample. **Record the per-sample ng of IS added.**
> d. **How much IS-MIX to add?** Decide based on amount of endogenous GA/ABA expected and peaks within the linear range of detection in test LCMS runs of ISs.
> e. **Example:** If 7 µl final volume (per sample) and injecting 3 µl. If you desire 24 ng/injection, then there should be 56 ng/sample (24 × 7/3). However we are adding 2mL per samples, therefore 56/2 = 28 ng/1mL.

.

1st Extraction Solution	Chemical	Concntration	40mL	45mL	unit
	Acetone	80%	32	36	mL
	AcOH	1%	400	450	µL
	Internal Standard Mix	24ng/inj & 48ng/inj	291.2	326.8	µL
	H2O	-	7.6	8.55	mL

A	B
	

3. After mixing by vortex, incubate the tube for 6 hours (or ON) at 4°C. (1st incubation)

5 mL tubes used: 1

START DAY 2

4. After 6 hours, remove caps, centrifuge samples at 4000 rpm (3399 g) for 10 min.

> a. Large centrifuge, max 5100 rpm. Settings: S.51 and set PLA/BUC to BUC (buckets).
> b. While spinning 1) label new tubes (tube set #2) 1-40 if you havent already done so, 2) make 2nd Extraction solution (see step 6), and 3) Turn on speedvac with vapor trap (it takes 90min to reach temperature; see step 10).

5. Collect the supernatant in labeled 5 mL tubes (save the supernatent tube set #2 until step 9).

> a. Use a 1mL pipette with fresh tips very time.
> b. The supernatent tube set #2 can be left on the bench (or in a drawer to keep IS from degrading in the dark) or at 4C if left for a longer period of time.

6. Add 2 mL of the 2nd extraction solution to the pellet (1st tube set) of each sample. with repeat pipettor, reusable tips undr the hood, 1mL x2.

> a. 2nd extraction solution: 80% Acetone containing 1% AcOH. Make this with the units on the falcon tube).
> b. Make 3x 50mL falcon tubes, the solution can sit over time. c. re-cap tubes for next step.

2nd Extraction Solution	Chemical	Concntration	50mL	unit
	Acetone	80%	40	mL
	AcOH	1%	500	µL
	H2O	-	fill to 50mL	mL

7. After mixing by vortex, incubate the tubes for 10 min at 4ºC. (2nd incubation) Votex time: count to 10
8. Centrifuge samples without caps at 4000 rpm (3399 g) for 10 min.
9. Collect the supernatant (tube set #1) and add to the same 5 mL tube (tube set #2) from step 5 (discard pellet)

> a. Use a 1mL pipette with fresh tips very time.

10. Evaporate extraction solution using speedvac in lab (new 40 × 5 mL tube rotor)

> a. 43-45°C, vacuum level 20.0, heat time 4hrs, time required: ≈3 hours (it's ok if ≈0.5 mL or less remaining)
> b. To turn speed vac on: switch speedvac switch on, vapor trap swtich on, pump switch on (if it didnt turn on with the vapor trap)
> c. Change the rotor 0-12 that fits 40 5mL falcon tubes.
> d. The vapor trap needs to reach the temperature -104C (takes ~90min)

LUNCH

↧ **IN HOOD**
11. Add 2 mL of Hexane to the dried down sample.

> We are using the Hexane to pull out un-wanted junk

12. Add 2 mL of 50% Acetonitrile (MeCN) and vortex.

> a. Solution will form two layers or can (but not necessary) be centrifuged at 8000 rpm for 5 min

13. (3×) Remove the upper layer (Hexane) and discard, add 2 mL Hexane.

> a. 3× = do this a total of 3 times, check the first box and repeat two more times: ☐ ☐ ☐
> b. *Do not add Hexane on the 3rd time and move on to step 14*
> c. In layman's terms: 1) remove Hexane layer, discard, 2) add Hexane, vortex, remove Hexane layer, discard and 3) add Hexane, vortex, remove Hexane layer, discard. Proceed to Step 14

14. Evaporate extraction solution using speedvac (no heat, run overnight OR 43°C, ≈3-4 hr).

> a. For the next day: Make PVP solution (50/50 of PVP and HPLC-grade water) if needed. b. we want as little as possible liquid
> c. The yellow tint is likely from the poly-phenols in the seeds that stayed in the extraction

PREP FOR TOMORROW: Make 0.5M K2HPO4 soln (see step 15 notes); Label 2 sets of tubes; Make 1% AcOHaq

5 mL tubes used: 2

START DAY 3
↧ **ON BENCH**
15. Add 0.5 mL 0.5M K2HPO4 to the dried down sample, vortex, and allow to dissolve in the dark drawer.

> a. Add 4.355 g to total of 50 mL of water; MW = 174.18 (Or 43.55g to 500mL) Can use millipore water if need be
> b. While waiting for samples to dissolve, prepare and condition the PVP columns (step 16)

16. Conditioning the PVP column (PVP = Poly(vinylpolypyrrolidone), ex: Sigma prod. 77627)

> a. Fill Varian Reservoir Frits 1 mL columns (Agilent Bond Elut 12131013) to 1/2 volume with PVP
>
> > i. To do this, add 1 mL of 50/50 mix of PVP and HPLC-grade water to column. Reuse one tip.
> > ii. Use a syringe (in pen drawer) with column adapter to gently push through some water to start the gravity-flow of liquid through the column. You should see one droplet at the bottome of the column.
> > iii. Check pH of flow through with lithmus/pH paper once most of all the liquid has flowed-through; it is probably about pH 7
> > iv. Add ≈500µl each time of 0.5M K2HPO4 up to 2 mL (4 times) or until the pH gets to about pH8~9 (Check flow-through with litmus/pH paper each time) ☐ ☐ ☐ ☐
> > *We are just getting the PVP/column to the pH as K2HOP4 You cant go too high since you will only level out to K2HOP4s pH.*

17. Collect flow-through: Once dissolved load the 0.5 mL sample in the PVP column, add another 0.5 mL 0.5M K2HPO4 to the sample tube and add this to the column. ☐ ☐

> a. (optional) If sample is dirty, can first pass through and empty 3mL Varian Reservoir Frits column (0.5 mL × 2) to remove larger debris, then add that 1 mL to the PVP columns**
> b. lift rack to see if liquid went through & tap to let last drop fall.
> c. PVP/column can be discarded in regular trash

18. Collect flow-through: add 2 mL 0.5M K2HPO4 (4 × 500µl) to elute. ☐ ☐ ☐ ☐

> a. Total volume of flow-through is 3 mL

LUNCH *suggested stopping point, but any break above works too. I tend to eat during step 18 while I wait*

↧ IN HOOD

Turn on speed vac + vapor trap (90min prior to step 24)

19. Add 6N HCl 100 - 300 µl to the sample until the pH is 2 - 3, check by litmus/pH paper.

> a. add 100uL to all, spin uncapped, check multiple tube pH's. b. add 100uL to the #1 tube, check pH, add 100uL more if you need more to get 2-3 pH.
> c. then add 200uL to all of the tubes (or 100uL if you only needed 100uL more instead of 200uL).
> d. spin uncapped, check pH, recap, and set in the dark drawer, and procees to step 20

20. Conditioning the 3 mL HLB column (Oasis HLB 3cc Vac Cartridge, Waters WAT094226)

> a. Add 3 mL of MeCN and discard flow-through
> b. Then 3 mL of MeOH and discard flow-through
> c. Then 3 mL of 1% AcOHaq and discard flow-through
>
> > Columns are snug in the column holder, dont need to push all the way down.
> > Constantly remove the waste solution into properly labeled `Waste Container`

Chemical	Conc.	Amount	unit
AcOH	1%	1	mL
LCMS H2O	-	99	mL

21. Discard flow-through: Load the extracts to HLB (ABA/GA is retained in column).
22. Discard flow-through: Add 2 mL of 1% AcOHaq twice (2× wash step). ☐ ☐

> a. Near the end, you can use the syringe to push the column solution all the way through, to get that extra last bit out. However push slowly and steady.

23. Collect flow-through: Move columns above new 5 mL tubes, add 2 mL 80% MeCN containing 1% AcOH twice (2× elution step). ☐ ☐

> 4mL/sample * 40 samples = 160mL

Chemical	Concntration	160mL	unit
MeCH	80%	128	mL
AcOH	1%	1.6	mL
H2O	-	30.4	mL

24. Evaporate extraction solution using speedvac (43°C, run overnight OR 43°C, ≈4-5 hr). no liquid left

5 mL tubes used: 2

START DAY 4
↧ IN HOOD
25. Add 0.5mL MeOH to each of the dried down samples, pellet will dissolve easily, vortex briefly to mix.

DO NOT ALOW COLUMNS TODAY TO DRY OUT

26. Conditioning the 1 mL DEA columns (Bond Elut DEA cartridge, Agilent 14102016)

> a. Add 1 mL MeOH and discard flow-through

27. Discard flow-through: Load the extracts into the DEA columns (ABA/GA is retained).
28. Discard flow-through: Add 1 mL MeOH three times (3× wash). ☐ ☐ ☐
29. Collect flow-through: Move columns above new 5 mL tubes: Add 1 mL MeOH containing 0.5% AcOH three times (3× elution step). ☐ ☐ ☐

Chemical	Concntration	150mL	unit
MeOH	-	150	mL
AcOH	0.5%	750	µL

30. Evaporate extraction solution using speedvac (43°C, ≈2 hr).

> a. Place at 4°C if dry before lunch.

LUNCH

31. Add 0.5 mL of 50% Chloroform 50% ethylacetate containing 0.1% AcOH to the dried down samples, vortex briefly.
32. Conditioning 1 mL Sep-Pac Silica columns (Sep-Pac Silica 1cc, Waters WAT023595)

> a. Add 3 × 1 mL of 50% Chloroform 50% ethylacetate 0.1% AcOH (discard) ☐ ☐ ☐

Chemical	Concntration	440mL	unit
Chloroform	50%	220	mL
Ethylacetate	50%	220	mL
AcOH	0.1%	440	µL

33. Collect flow-through: Load extracts to Silica and collect in pre-labeled 5 mL tubes.
34. Collect flow-through: Add 1 mL 50% Chloroform 50% ethylacetate containing 0.1% AcOH twice (2× elution step; total 2.5 mL in tube) ☐ ☐
35. Evaporate extraction solution using speedvac (43°C, ≈1 hr).

> Can prep the LC/MS vials in for step 36

A time you can store @ RT for 1-2hr OR @4C >2hrs

36. Resuspend in 200µl MeOH, transfer to LC/MS vial insert, dry by speedvac (43°C, 30 m).

> For steps 36 and 37, you can use a speedvac without a vapor trap.

37. Repeat step 36: Add 200µl MeOH, transfer from 5 mL tube to vial insert, and dry down.

> a. Store in this form and resuspend for run.

38. For small injection sizes (eg 3µl per inj), resuspend in X µl of 99% MeCN 1% AcOH.
(X = inject vol × # of inj + extra; ex: 7µl; higher MeCN is needed for GA12/12-Ald peaks)
For larger injections (eg 20µl per inj), resuspend in X µl of 15% MeCN 1% AcOHaq.

PROCEED TO RUNNING SAMPLES ON LCMS

LCMS Sequence Parameters

Example Parameters to keep record of:

Run Pameters

Folder	FileName				Method	Injection
NameDate	Date_01_1				SV_PHENYL150X3MM3UM_ABA_GA_SIM4_Shantel.M	3uL

Sample Sequence

Tube	pos	reps	sample
ACN	91	1	MeCN
43	51	3	sample_001
42	52	3	sample_002
41	53	3	sample_003
ACN	91	2	MeCN

Updating Software Sequence Info

1. `Sequence` `Sequence Parameters` Subdirectory: `NameDate` Click Prefix cell (will ask to create, `yes`).
   Prefix should be the file name `Date_01_` and the counter will start with `001`
   Check post seq command: `STANDBY` For a single run. If I wanted to run multiple runs, and whaat to run right after one another. You can change WHILE your sequence is running. Uncheck it if I dont want the LCMS to go to standby. `OK`
2. `Sequence` `Sequence Table` Click a cell and you can edit the cell. Input the pos into `vial` column. Highlight a row to cut it out. Insert to add more rows. Edit `Sample Name`, `Inj/Vial`, and `Method Name` needs to be my prefered method (all samples HAVE TO BE the same method). Once done, click `OK`

> a. This can be done ahead of time during the extraction method.

3. Once the dilution samples are dried down, re-suspend to 7uL in 1% AcOH in MeCN (Assuming your sample is to be diluted to a final volume of 7uL, this may change on your preference).
4. Place the samples in the LCMS tray and record the position in the `sample sequence table` above.
5. Double check `Sequqence Parameters`, and `Sequencec Table`

LCMS Machine Run & Configuration

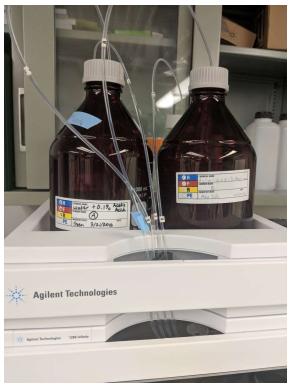
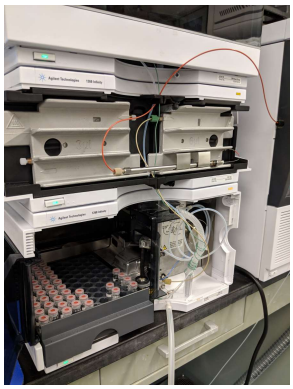
The basis of the LC : sample/hormone will flow based on both the chemistry in the column AND the size.
And the change of ratios of the solvents, you can push/pull out as slow or fast. Because if you have a low % solv B, things come out slower, but close peaks together seperate (more resolution of peaks close). But if you have a high % solvent B, things will come out faster. The same things with the disposal columns, but we arent keeping all, getting rid of things, this time we what to spread the hormones out, so when it gets shot out to the mass spec, it reads the masses.

1. LCMS Chemicals
First, Do we have enough solvents to run the LCMS *Use HPLC graduate cylinder (in the hood). Rinse the cylinder with HPLC grade water before use* When switching jars while on, turn the tube knob to waste

	water + 0.1% Acetic Acid	Acetonitrile + 0.1% Acetic Acid		
total	1700mL	1700mL		
Acetic Acid	1.7mL	1.7mL		
H2O/MeCN	Fill to total	Fill to total		
		Must mix very well or it will effect your results		

Turn knob back to column.
MOST IMPORTANT THING TO REMEMBER WHEN CHANGING SOLVENTS When/if you change the solvents, you need to tel lthe computer how much is in the bottles. The machine will stop the run before it gets to 0mL.

- Quant. Pump window click on the bottle letter. Ex: A change to 2.0 liters (full) with a max of 2.0 liters.
- When you click, you can change all of the final volumes in the pop-up window.
- Click Ok. Then the green liquid levels in the graphic will change.

A	B	C	D
			

2. Turn on the pump 30min - 1hr before starting.

- Start with turning on the machine with a lower pressure (0.5) `On`
- Once the temperature has reach around 60C, then change the pressure back to Method pressure: 1.0 (mL/min)

2. G6100 (online)

> Sampler references black needle that will go from tube to tube. Idle box green (on)
> Quant. Pump represents the A, B, C, D large chemicals. The clear tubes have letter labels. Only ever replace these one at a time, so there is no chance at switching tubes in the wrong liquid. Stanby box (grey offline)
> Column Comp: represents the colume, and there are two shown on the screen because you can have 2 seperate columns and adjust the temp. Displays temp. Not ready (yellow) Watch the pressure to make sure its not too low (leak) too high at red bar (400mL/min) If it gets close to 500 it can handle it, but that might be a sign there is a clog or something thats not too normal. Too much pressure you can develope a leak, or cause issue with other tubes. MS: is the quadrapole shown for the mass spec (6120

> Quadrapole MS)
> Signal 1 plot is the sample endogenous form, signal 2 is the IS form.

Every time you run the LCMS, make sure the `Method File icon` has the correct method name

- SV_PHENYL150X3MM3UM_ABA_GA_SIM4_SHANTEL.M (sven_columntype_hormones_methodversionnumber_user.M)
- the methos `scan` will look at anything with all masses (so anything will show up, more noise that I dont want).
- `sim` allows us to specify the molecular weight of what we are looking for (i.e., ABA) and then the mass spec will only scan at that MW. So you can do this on all the hormones you want to look at.

3. Specify or change a `Run Method` (*in our case, we exluded GA19*)
   `Method` `save as` name the method in the folder `c:\chem32\1\methods` `Edit Entire Method` Leave all checked `OK` desciption of method you can edit but is not necessary `OK` ALS `OK`
   We wont change the Solv A and B ratios, Keep the fow rate at 1.000 mL/min. `OK` `OK`
   Temperature fluctuations can effect peaks, so he keeps a LEFT high temp at 60C. And left and right are combined, so they both are the same. Set Up MSD Signal :Setting up the masses and the retention times we want the MS to start looking for our specific mass.

- general tune file: `atunes.tun` ALWAYS put it on this one, no matter what anyone else tells you. It points to the most recent tune file.
- Mode `SIM` keep that mode
- Polarity, ABA/GA are `negative`, if you dont know run one with negative and then again with positive to see where your peaks show best.
- GA19 Group4 `Select` `cut` both lines
- Change Group 6 from 5.6 to 5.5 And do the same for the IS GA19 samples `OK` `OK`
- The retention times for the Internal Standard or "endogenous" standards are differeent than when you run the samples.

Sample Purity: looking to M- no adduct (mass) and M-H (M - 1) in the negative tab since we are looking `OK` `OK` `OK`
Specify Report: G6100 `Calssic Reporting` check `Screen` uncheck `File` and the Quantitation setting is set to certian numbers based on the injection amount we did (24 and 48). And now everytime it runs, it should pop up on the screen. `OK` `OK` `OK` `OK` `OK` `OK`
`Method` `Save Method` MUST BE DONE now, or it will not run with this new updated method, it will run with the old method. Comment if you would like `OK`
Press giant `ON` button, hear a sound, thats the pump working. MS is still offline, when we actually start a run, the MS is online. and the temperatures are coming up to temperature.
Turn `ON` *the MS* Eveything should turn green and be ready. Once everything is ready, The top left tab will be green `ready`

4. Set up Sequence Parameters and Tables (see intructions here).
5. Double check `Sequqence Parameters`, and `Sequencec Table`, `Method`, and then re- `Save Method` just in case.
6. To start the run: `Run Control` `Run Sequence`

> a. If you ever need it to stop, you can press `Pause Sequence` then once it has paused after the current sample, THEN you can press `Stop Run/Inj...` and it will stop at the end of this sample (or the one following) but never in the middle of a sample.

General LCMS Notes Solvent B will go up in volume during the run, but Solvent A will constantly output the same volume, so solvent A always runs out quicker. `Documents` `Shantel` `screenshot_logs`
Label the files `date_columnname_hormone` HP Computer has no internet, since the software needs to be run on window 7 and not upgraded (and USDA has a policy that all computers with a network must have windows 10)
