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Membrane Lipid Extraction Methods from Sorghum bicolor

Max Regester
University of Nebraska - Lincoln

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By
Max Regester, BA
Biology
College of Arts & Sciences

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Faculty Mentors:
Rebecca Roston, PhD, Biochemistry
Wayne Riekhof, PhD, Biology

Abstract:

Plants are the basis of the world as we know it. As sessile beings, there are often subject to many environment stressors like drought, extreme temperatures, etc. One way of analyzing how plants respond to these stressors is by monitoring changes in their lipid compositions. Having a better understanding of how plants respond to different stressors can have very important implications when it comes to cultivating crops. When testing lipid composition, it is important to measure levels as they were in the living tissue. The problem is, enzymes known as lipases are active during extraction and can greatly change the lipid composition. We tested three methods for inactivating these lipases and extracting the lipids on *Sorghum bicolor* leaf tissues. Two of the methods use formic acid in organic solvent for different times, while the second uses boiling isopropanol. To analyze lipase activity, we measured the relative abundance of lipid species formed by lipase activity. In the end, we determined each method is relatively comparable to the other.

Keywords: Sorghum, Lipids, Lipases, Extraction, Biology

Background:

Plants form the foundation of the world we live in. They form the base of the food chain, converting energy from the sun, carbon dioxide, and water into sugars and oxygen through the process of photosynthesis. Without these sugars and oxygen, life as we know it would fail to exist. Obviously, plants are sessile beings, so they can't run away from environmental stressors like we can. One way plants respond to these stressors is by changing their lipid compositions. Having the ability to accurately analyze these lipid compositions is critical when trying to understand how these plants respond to certain stressors. Understanding how plants respond to

different stressors has many important implications; being able to develop more resilient, higher yield crops may be the most noteworthy.

Ideally, tissue samples from plants should reflect the exact make-up of that tissue at the time of sampling. The problem is, there are a variety of hard-to-kill enzymes in plants that can change the composition of the sample, namely through the oxidation of lipids. The first task is to inhibit these lipid oxidizing enzymes, called lipases. To do this, the tissue samples are "killed" in order to inactivate the lipases while maintaining lipid classes as they were *in vivo*. There are two common methods for the "killing" process. R. L. Bieleski tested these methods on a class of hard-to-kill enzymes called phosphatases. He tested the phosphatase activity as a measure to indicate each methods ability to kill the tissue. The lower the activity, the more effective the method was. Bieleski tested acidified organic solvent Methanol: Chloroform: Formic Acid, (MCF2, 12/5/3, v/v/v) at low temperatures vs. killing of tissues in boiling solvents including 80% isopropanol. Bieleski determined that phosphatases were about 10 times as active in the boiling solvent when compared to the acid at low temp. We tried to determine if these results translate to lipases as well using methods similar to Bieleski, also seen in Browse. This was done by analyzing lipase products like phosphatidic acid or diacylglycerol. For example, if lipases remain active after a certain killing method, we would expect them to be more active, in turn producing more of their products. A second variant of the acidified organic solvent method, as seen in Wang and Benning, was also tested which uses a variant of the MCF2 solvent, but it doesn't involve 24 hours in -20°C.

In addition to the two types of killing, there are two types of extraction solvents used. The first solvent, originally introduced by Bligh and Dyer, later modified to kill tissues by inclusion of formic acid, as MCF2. This method has been employed by Xu et al., Roston et al., Yang et al., amoung others. When using this extraction solvent, we tested both the kill method described by Bieleski (plant in solvent for 24 hours at -20°C) and the method discussed by Wang and Benning that skips the 24 hours in solvent at -20°C step. The second extraction solvent consists of chloroform: isopropanol: methanol: water (30/25/41.5/3.5, v/v/v/v). This extraction method uses the same boiling solvent kill method as described by Bileski. Other modifications of this solvent have been used by de la Roche and Andrews et al, Ryu and Wang et al., Moore et al., and Shiva et al. The one we decided to use has most recently been shown to have even distribution of lipid classes. Our goal was to compare the effectiveness of each method by analyzing the relative concentrations of lipase products to see if they caused repeatable changes to apparent lipid compositions.

Methods:

Prior to extraction:

1. Obtain plant samples. The plant species we used was *Sorghum bicolor* (Figure 1).
    a. Be sure to take pictures of the date they were planted, the conditions they were grown in (four weeks on a cycle of 28°C for a 12 hour day, and then 22°C for a 12-hour night) and of the plants themselves.
2. Count the number of plants at relatively the same stage of maturation. This will determine how many samples you're able to run.
    a. 2 leaves, ideally of the same size, per sample.

i. Example: If there are only 12 leaves close to the same level of maturation, then you will only be able to do 2 samples for each of the 3 methods (2 leaves per sample x 2 samples per method x 3 methods= 12)

ii. If there are not enough leaves of the same size, then pair 1 leaf from the group of large plants with 1 leaf from the group with small plants.

3. Cut the fourth leaf up on each plant being used.
4. Cut only 4 cm from each leaf using a razor blade and cutting board. You will cut from the tip of the plant inward.
    a. These 4 cm pieces will serve as your leaves to be inserted into each sample.

Isopropanol method:

1. Add 1 volume (in this case, 2 mL) of isopropanol to 3, 13 x 100 mm glass tubes with screw caps each.
2. Set the heater, named "Type 16500 Dri-Bath" to 75°C. Bring the isopropanol in these tubes to a boil and then add 2 sorghum leaves to each tube.
    a. Cut the leaf samples a couple more times before inserting into the tubes to ensure that all of the leaf is submerged in isopropanol.
3. Allow these to boil for 15 minutes.
    a. Be sure to invert the tubes every few minutes to ensure the isopropanol comes in contact with the entire leaf if not all of the leaf is submerged in isopropanol
4. After the 15 minutes, allow them to cool for a couple minutes, and then add 3 volumes (in this case, 6 mL) of chloroform:methanol:water mixture (30:41.5:3.5) to each tube.
5. Allow the tubes to shake in the shaker for a minimum of 24 hours at 37°C and 250 RPM.

6. After 24hrs, put the tubes back in the “Type 16500 Dri-Bath” heater and set it to 75°C.
7. Insert nitrogen distributor into vials.
    a. Attach nitrogen tube, making nitrogen come out just hard enough to create a dimple but not splash.
8. Once solvent has been evaporated off, add 50 mL of chloroform with “needle” looking syringe to each vial.
    a. Wash out syringe 3 times with chloroform in between each insertion of chloroform in to the vials.
9. Add this new mixture to the small vials, store at -20°C.
    a. Don’t forget to remove leaf with glass “hook” made with the Bunsen burner.
    b. Can also decant sample into a new 13 x 100 mm glass tube, leaving the leaf in the old tube.

Organic solvent with 24 hours in -20°C method:

1. Add 1 mL of MCF2 in to 3 vials
    a. Keep vials in ice in between steps
2. Add 2 leaf samples to each vial, store in freezer at -20°Cfor 24 hours.
3. After 24 hours, add bead beater bbs (chrome steel, 3.2 mm, disruption beads) and bead beat the vials for 30 seconds (may need to do another 30 seconds to break up plant material).
    a. Return to ice in between beatings.
4. Add 0.5 mL of ice-cold 1 M KCl/0.2 M H_3PO_4 to each vial.
    a. New tip each time.

5. Shake each vial vigorously using shaker.
6. Centrifuge at max speed for 3 mins.
    a. Return to ice.
7. Remove bottom organic layer and add to smaller tubes.
    a. Add nitrogen to each tube before capping it.
8. Store at -20°C.

Organic solvent without 24 hours in -20°C method:

1. Add 1 mL of MCF2 in to 3 vials along with bead beater bbs.
    a. Keep vials in ice in between steps.
2. Add 2 leaf samples to each vial.
3. Bead beat the vials for 30 seconds (may need to do another 30 seconds to break up plant material).
    a. Return to ice in between beatings.
4. Add 0.5 mL of ice-cold 1 M KCl/0.2 M H_3PO_4 to each vial.
    a. New tip each time.
5. Shake each vial vigorously using shaker.
6. Centrifuge at max speed for 3 mins.
    a. Return to ice.
7. Remove the bottom organic layer and add to smaller tubes.
    a. Add nitrogen to each tube before capping it.
8. Store at -20°C.

TLC:

1. Cut NH_4SO_4-included plates to desired size, put in oven for 60 mins.
    a. This can be longer, up to 3 hours. Can also bake plates for another day, then put them in for only 30 mins on the day you are going to use them.
2. Place samples in order you plan to put on TLC plate in vial holder.
    a. Should include Oil PA and Arabidopsis standard on outside and your lipid samples in the middle.
3. Dry these tubes with nitrogen for 15 mins (or as needed).
    a. Can add 75mL of acetone to each tube to ensure water evaporates if taking a while.
4. Make solvent mixture in Erlenmeyer flask, put in chamber along with trifold paper on each side.
    a. When adding solvent, poor it over the trifold paper to dampen it.
    b. First solvent- 85 mL chloroform: 12.5 mL methanol: 12.5 mL acetic acid: 3 mL water.
    c. Second solvent- 96 mL chloroform: 4 mL acetone: 1 mL acetic acid
5. Remove TLC plate after 30 mins in oven, mark them up using pencil.
    a. Mark 1 cm up (on uncut side) and 1.5 cm over on each side. There will be 17 cm in between. Divide 17 by number of samples you have (i.e. 17/8= about 2.1). So in this case, mark over 1.2 cm for sample and then over another 0.9 cm for space in between samples. Repeat this all the way down.
        i. Don't make sample slot size less than 1 cm.
    b. Mark up the side of the plate 10.5 cm. This will be the stopping point for the first solvent and will then be switched to the second solvent.

6. Resuspend the dried samples each with 40 microliters of chloroform.
    a. Have 2 tubes set up, 1 labeled “load”, 1 labeled “wash”.
        i. Load will be filled with chloroform you use to resuspend the samples.
        ii. Wash will be used to wash syringe (needle like syringe), 5 times each, in between samples.
7. When adding resuspended samples on to plate, add a little bit at a time, drying the samples with nitrogen tube.
    a. Add 4 uL of each sample in a spot at the top of the TLC plate for a “total” sample,
    b. Add 30 uL of each sample at the bottom of the plate. If there is not 30 ul remaining, measure and note the added volume.
        i. When doing OPA standard, only add 2 uL of oil and 2 uL of PA
        ii. When doing Arabidopsis standard, only add 15 uL.
8. Once all the samples are added, put in TLC chamber with first solvent.
    a. If doing 2, place them facing each other.
9. After ~20 mins (run 10.5 cm), pull plate out and dry with nitrogen.
    a. You’ll know if it’s dry when plates are no longer cold.
10. Set the TLC into the second solvent with tri fold paper.
11. After ~ 40 minutes (run to just below the “total” samples), pull plate out and dry with nitrogen tube.
12. Cut standards off plate.
    a. Spray 1 standard in naphthol for about 20 seconds and then bake for about 5 mins.
    b. Put 1 standard in iodine chamber until bands appear yellow.
13. Scan standards.

a. Epson scan, preview, highlight area you want to scan, click scan, cahnge date to current date, start number 1, then scan.

14. When scraping, use the standards as a guide for the rest of the samples.
    a. If there is a slant in one of the samples, dye the middle samples to correctly identify their location.
        i. This will lower quality of samples, so don't do it unless it's necessary.
15. If you need to stop at this point, store the TLC plate in a plastic tote and fill with nitrogen. Store the plate at -20°C.

FAME:

1. Remove silica surrounding identified lipid spots from the TLC plate with a razor blade. Scrape the lipid containing silica and transfer the silica powder using a funnel into a glass tube with a teflon-lined screw cap.
    a. Turn on water bath to 80°C before scraping. This will save you time.
2. Add 50 uL of 50 ug/mL pentadecanoic acid (15:0) (in bottom shelf in door of freezer) using 200 uL pipette and 200 uL yellow plastic tip to each sample. Keep a tube with only pentadecanoic acid in methanolic HCl as a control.
3. Add 1 mL 1 N HCl in anhydrous methanol (in right side of fridge in large plastic container) to each sample by glass pipette. Add nitrogen gas, then close glass tubes tightly.
4. Incubate glass tubes in an 80°C water bath for 25 mins. Make sure the tubes are sealed well so that the solvent does not evaporate.
    a. Check that the solvent is not boiling after 5 mins. If necessary, add more solvent and tighten the cap.

5. After tubes have cooled down, add 1 mL 0.9% sodium chloride followed by 1 mL hexane and vortex vigorously. Centrifuge samples at 1000xg for 3 minutes.
    a. If you need to stop, samples can be stored at this point at -20°C.
    b. The whole process from step 1 to step 6 takes about 2 ½ hours, and you can't stop in the middle. Plan accordingly.
6. In the fume hood, remove the hexane/upper layer of the sample with Pasteur pipette (the disposable glass ones) and place it into a new 13x100 mm glass tube. The pipette should be discarded after each sample.
7. Evaporate hexane under a slow stream of nitrogen gas until dried completely.
8. After the hexane has been evaporated, pipette 70 microliters of hexane in to each tube to resuspend. Pipette up and down a few times to mix.
9. Transfer the resuspended fatty acyl methyl esters in to autosampler vials.
    a. Fill the tubes with nitrogen and then cap them.
    b. Label each tube with a number and describe what each number represents in the lab notebook.
10. Store in box at -80°C.

Results

All *Sorghum bicolor* plants grown under same conditions

Plants at different stages of maturation have different membrane lipid compositions. When growing *Sorghum bicolor*, we made a great effort to make sure there was as little plant to plant variation as possible. We grew the plants under the same conditions, for the same amount of time, and used the same methods for collecting plant tissue samples (see "prior to extraction" in

methods). We were fairly successful in growing plants of the same size and collecting tissues at the same time. It's almost impossible to do this perfectly, so there was likely some variance that could've affected the data. The key here is to minimize this variation as much as possible.

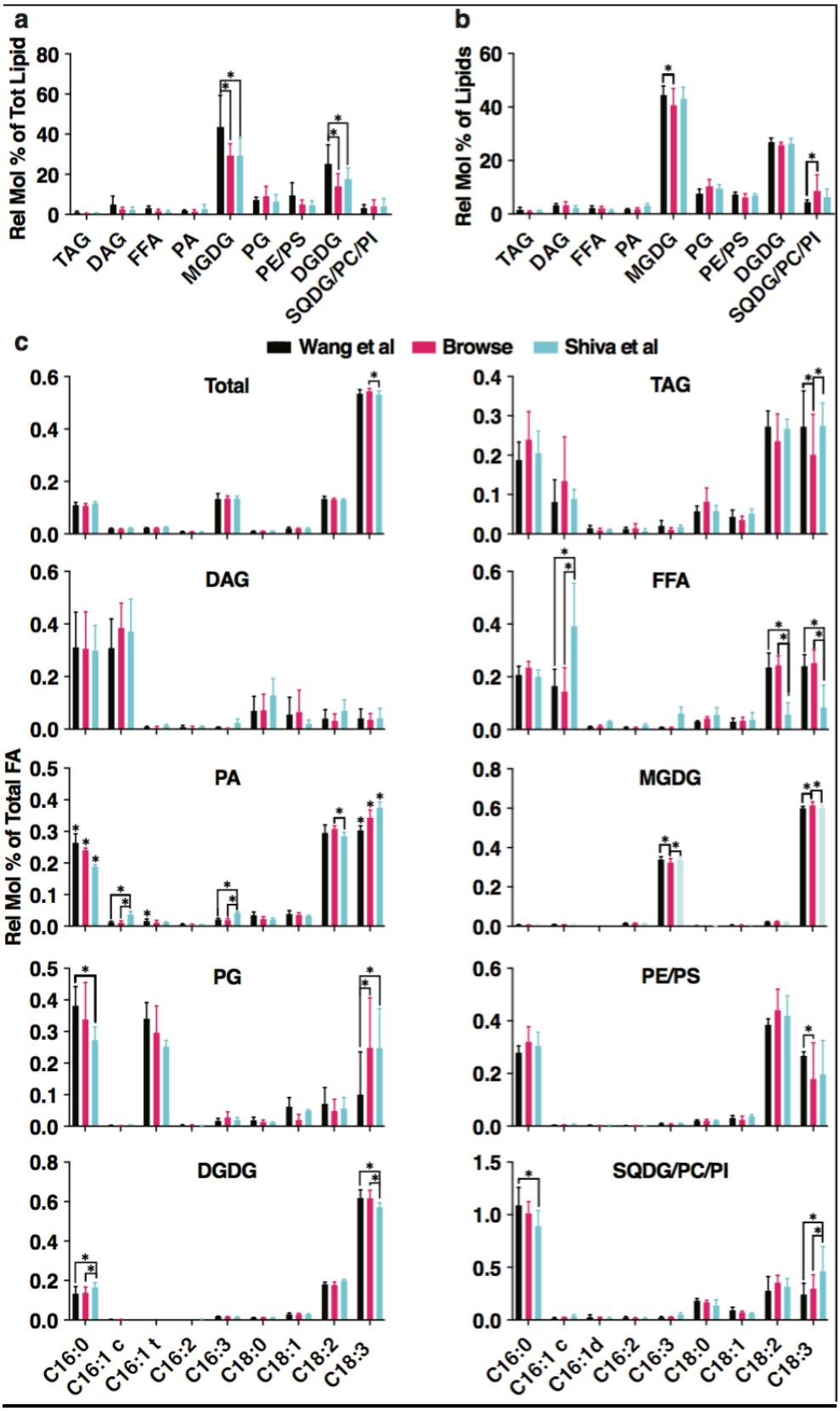
Figure 1: *Sorghum bicolor* example. Each plant was grown for four weeks on a cycle of 28°C for a 12 hour day, and then 22°C for a 12-hour night. Plant samples were also collected at about the same time of day to prevent introducing noise from lipid responses that may change throughout the day.

Three extraction methods of *Sorghum bicolor* lipid produced similar results

We look at plant lipid profiles in order to determine the relative activity of lipases. Examining DAG, FFA, and PA, in particular, can serve as a great metric for determining lipase activity because, when lipases are active on lipids, they produce these products. As a result, we would expect to see an increase in the products the more active lipases are. We also analyzed other significant membrane lipids bands. This is because if we see an increase in DAG, FFA, or PA, we would expect to see a decrease in another membrane lipid band because the lipase has to be "attacking" one of these bands to produce its increased products. We analyzed the overall total amount of lipids for each band in (a) and the relative amount of each lipid band when compared to the total amount of lipids in (b). You can see that the Wang et al method yielded an overall larger amount of total lipids in (a), but, as seen in (b), the distributions of the abundances of each lipid band are fairly similar across all three methods. This suggests that the Wang et al method may have been more effective at extracting a larger overall amount of lipids, but it doesn't seem to change the lipid profile distribution, and it doesn't suggest it's any better than the other methods at inhibiting lipases.

Diving deeper into (c), we can see that there are some significant differences. The one that seems to be the most significant is the large peak in the Shiva et al C16:1 FFA when compared to the other two methods. We do see a dip in the C18:2 and C18:3 for FFA with this method, which is expected after seeing more C16:1 produced. We then look to the other major membrane lipids to see where lipases may have attacked to produce this increased FFA product. The issue is, there is no clear culprit according to this data, so it's difficult to tell which type of lipase was active. The

main takeaway here is there are some significant differences produced by unknown lipase classes, and all of the methods are similarly successful at inhibiting lipases/extracting lipids.


a
Rel Mol % of Tot Lipid
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Wang et al
Browse
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C16:2
C16:3
C18:0
C18:1
C18:2
C18:3
C16:1d

Figure 2: Three extraction methods of *Sorghum bicolor* lipid. Legend Note: Wang et al represents the organic solvent method without 24 hours in -20°C; Browse represents the organic solvent method with 24 hours in -20°C, and Shiva et al represents the isopropanol method. (a) Represents the relative mole percent of total lipids when compared to fatty acid containing lipids per sample. (b) Represents relative mole percent of total lipids when compared to the lipids that were collected for analysis. (c) Total represents the relative mole percent of each lipid chain when compared to the total lipids collected for each sample. For the rest of the bands seen in (c), statistical significance is denoted by (*) meaning p value <0.05. Statistical analysis was done using a 2-way ANOVA test. We analyzed a total of 16 sets of data with outliers being removed using ROUT analysis with a Q value of 10%. 16:1, 16:2 and 16:3 were not used for outlier calculations because their abundances are so low. Some groups contain more than one lipid band, and that's because these groups ran in parallel on TLC plates, so they were collected together as one sample. TAG stands for triglyceride, DAG stands for diglyceride, FFA stands for free fatty acids, PA stands for phosphatidic acid, MGDG stands for monogalactosyldiacylglycerol, PG stands for phosphatidylglycerol, PE stands for phosphatidylethanolamine, PS stands for phosphatidylserine, DGDG stands for digalactosyldiacylglycerol, SQDG stands for sulfoquinovosyl diacylglycerols, PC stands for phosphatidylcholine, and PI stands for Phosphoinositides. Note: the higher error bars in certain bands are a result of their lower overall abundances. TAG, for example, has a fairly low relative abundance, so noise will affect it much more than a band like MGDG, which has a fairly high relative abundance.

Discussion:

After analyzing the data in Figure 1, it seems that the three extraction methods of *Sorghum bicolor* lipids show similar lipid abundances and fatty acid profiles. Some statistically significant differences can be seen in each lipid band except for DAG, but, there doesn't seem to be any patterns to suggest there is a so called "best method." The main take away is that each method is adequate for "killing" lipases and extracting plant lipids; the method you should use depends on the situation you're in.

Our lab has used the organic solvent method without 24 hours in -20°C because we have the equipment to do so, and it seems to be the most efficient in our case. This method is fastest, and we are usually only processing a small number of samples at a time. That said, if we wanted to process a large number of samples, the organic solvent method with 24 hours in -20°C would be optimal. If you're in the field, it may be more feasible to use the boiled isopropanol method instead of hauling around liquid nitrogen to keep samples cooled during the organic solvent methods. Because the results are all so similar, understanding how each method works and the situation you'll be in when collecting samples is they key when deciding which method is best.

As previously stated, we made a great effort to make sure there was as little plant to plant variation as possible because as plants continue to grow, their cell membrane and chloroplast membrane lipid profiles change. By collecting plants of similar size and same stage of maturation, we attempted to limit this variance as much as possible. We also made sure to minimize the time between plant sample harvesting and the application of lipase inactivation solvent. This was very important because the longer it takes to get the tissues in the solvent, the more time lipases have to alter lipid composition of the sample.

Down the road, I would like to see this research replicated in other labs to see if they produce similar results. I would also like to see these methods performed on different plants species to see if that alters the results.

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