2021

Modifying an Ergosterol Extraction Protocol to Quantify Fungal Biomass at the University of Minnesota-Morris

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Undergraduate Research Opportunities Program (UROP) Spring 2021

Abstract

In aquatic ecosystems, fungi play an essential role in decomposing leaves and making nutrients available to other organisms such as invertebrates and detritivores. Therefore, it is useful to quantify the amount of fungal biomass growing on submerged leaves, and this quantity can be estimated by measuring the chemical ergosterol, which is found only in fungi. Gessner (2005) developed an ergosterol extraction protocol using lipid extraction, purification with solid-phase extraction (SPE), and analysis with high-performance liquid chromatography (HPLC). However, not all of the equipment mentioned in the protocol was available at the University of Minnesota-Morris. Therefore, the goal of this project was to adapt the Gessner (2005) ergosterol extraction protocol for use at the University of Minnesota-Morris using available materials.

Background

In the fall, the leaves that drop from the trees into streams and rivers are one of the main sources of allochthonous carbon and energy for the aquatic ecosystem (Freund et al., 2013). Initially, however, the fresh leaves are not easily digestible by aquatic invertebrates. The leaves are first colonized by bacteria and aquatic fungi, known as hyphomycetes, which break down the compounds in the leaves and make the nutrients more accessible to invertebrates and detritivores (Suberkropp et al., 1983). Although bacteria contribute to decomposition, fungi are more dominant and can comprise over 90 percent of the total microbial biomass (Gulis et al., 2009).

These fungi are essential for freeing nutrients that can subsequently be used by organisms throughout a stream ecosystem (Freund et al., 2013).

To measure the rates of leaf decomposition, one method is to quantify the biomass of the fungus on the leaves. However, it is difficult to measure fungal mycelium directly because the hyphae grow throughout the leaf, so a common method is to measure the chemical ergosterol, a compound found uniquely in the cell membranes of fungus (Gessner, 2005). Ergosterol is a suitable measure of fungal biomass because it is not found in other organisms such as bacteria and plants that could be included in samples (Toh Choon et al., 2012).

In order to quantify ergosterol, Gessner (2005) developed an extraction protocol that measured the ergosterol in decomposing leaves using lipid extraction, purification with solid-phase extraction (SPE), and analysis with high-performance liquid chromatography (HPLC). Numerous other researchers have utilized this method for fungal decomposition studies (e.g. Martins et al., 2017). Since the University of Minnesota-Morris did not have all the equipment suggested in the protocol, the goal of this project was to adapt the ergosterol extraction methods developed by Gessner (2005) for use at the University of Minnesota-Morris.

For this project, a vacuum manifold was assembled using available materials, and the HPLC conditions were adjusted for a different HPLC column (Pinnacle II C18, 5 μm, 250 x 4.6 mm). The methods were tested on ergosterol stock solutions, leaf samples collected from a hardwater stream, and aquatic fungi grown in petri dishes on MS medium. The following protocol was modified from Gessner (2005) and can be used at the University of Minnesota-Morris to quantify ergosterol and fungal biomass on decaying leaves from an aquatic ecosystem.

Equipment:

- Glass vials (20 ml) with plastic lids
- Hole punch (7 mm diameter)
- Heat block and thermometer
- Heat/stir plate and stir bars
- Rubber septa
- Disposable syringe needles
- Vacuum gas manifold
- Bunsen burner base
- Plastic tubing
- Vacuum pump (Welch DryFast Ultra Diaphragm Pump)
- Solid-phase extraction cartridges (Waters Sep-Pak, Vac RC, tC18, 500 mg sorbent)
- HPLC for isocratic operation (Buck Scientific BLC-20 HPLC) and blunt syringe
- HPLC column (Pinnacle II C18, 5 µm, 250 x 4.6 mm)
- Glassware, pipettes, and pipette tips

Chemicals:

- Methanol (analytical grade)
- Isopropanol (HPLC or analytical grade)
- Ergosterol standard (98% purity, ACROS Organics, Fisher Scientific)
- KOH (pellets, analytical grade)
- HCl (analytical grade)

Solutions:

- **Solution 1**: Methanol
- **Solution 2**: Storage and extraction solvent: KOH in methanol: 8 g/l; e.g. 4 g in 0.5 l are sufficient for >30 samples
- **Solution 3**: 0.65 M HCL (ca. 100 mL for 30 samples)
- **Solution 4**: Conditioning solution: methanol (1 volumetric part) + KOH in methanol (5 parts) + 0.65 M HCl (1 part); e.g. 35 ml Solution 1 + 175 ml Solution 2 + 35 ml Solution 3; sufficient for > 30 samples; check before use whether pH is < 3.
- **Solution 5**: Washing solution: 0.4 M KOH in methanol:H20 (6:4; vol:vol); e.g. 1.8 g KOH + 48 ml methanol + 32 ml H_2O; sufficient for > 30 samples
- **Solution 6**: Isopropanol
- **Solution 7**: Ergosterol standard in isopropanol: weigh ca. 10 mg ergosterol to nearest 0.1 mg in volumetric flask (50 ml), dissolve in isopropanol under stirring, adjust volume, and transfer to tightly closing glass bottle; store in refrigerator (4 °C), where the solution is stable for several months. Ergosterol is slow to dissolve fully.
- **Solution 8**: Ergosterol standard in KOH/methanol (ca. 200 mg/l). Dissolve ergosterol under stirring and gentle heating (50 °C) in volumetric flask, let cool, remove magnetic stirrer, adjust volume, and store at 4 °C.

Methods:

I. Sample Preparation

1. Collect decomposing leaves from stream and transport to laboratory on ice.
2. Ergosterol degrades into vitamin D_2 in the presence of light, so samples should be kept out of direct sunlight at all times.
3. Cut sets of leaf discs with hole punch and blot lightly on Kimwipe.
4. From each leaf, half the discs will be used for the extraction and half will be dried (Fig. 1).
5. Place leaf disks (≥ 30 disks per sample) in glass vial with 10 ml Solution 2 (KOH/methanol), and store in refrigerator overnight.
6. If the samples will not be analyzed immediately, freeze disks at -20 °C in KOH/methanol solution (Solution 2) (M. Gessner, personal comm., Jan. 11, 2021).
7. Pre-weigh and dry glass vials to remove adsorbed water, then add the replicate sets of leaf samples and dry overnight at 104 °C. Weigh to the nearest 0.1 mg in order to determine sample dry mass (20-50 mg dry weight per sample) (M. Gessner, personal comm., Jan. 11, 2021).

II. Lipid Extraction and Saponification (50 min + time to preheat heat block)

1. Add a stir bar to the leaf samples in KOH/methanol.
2. Close vials tightly and heat to 80 °C for 30 min in the heat block. The temperature can be monitored with a thermometer in an open vial of water (Fig. 2).
3. Let extracts cool down (ca. 20 min).
4. To test the protocol and estimate recovery rates, include in each extraction series 1 leaf sample known to contain no ergosterol (e.g. dried leaf disks) but spiked with 250 µl of Solution 8 (ergosterol standard in KOH/methanol).

III. Conditioning of SPE Cartridges (20 min once apparatus is set up – can be done while samples are cooling)

1. Assemble SPE apparatus (Fig. 3-4). Connect vacuum gas manifold to vacuum pump with tubing, and add a Bunsen burner attachment to regulate the pressure. Each collecting vial is capped with a rubber septum and connected to the vacuum gas manifold via a tubing and a syringe needle. Attach a syringe needle to the SPE column and insert it through the rubber septum. A five-port vacuum gas manifold can run four samples at a time; the fifth stopcock is used to regulate the pressure.
2. Add 7.5 ml of methanol to each SPE cartridge.
3. Open fifth stopcock (left open until drying step), turn on the vacuum pump, then open the other stopcocks.
4. Close stopcocks when about 5 mm of methanol remain above the sorbent bed.
5. Add 7.5 ml of Solution 4 (conditioning solvent).
6. Open stopcocks to suck solvent through cartridge, but leave about 5 mm above the sorbent bed.
7. Close stopcocks. Never let cartridge fall dry during conditioning. Restart from beginning if this happens accidentally.
8. Replace the collecting vial before loading the sample.

IV. Loading of lipid extract onto SPE cartridge (50 min)

1. Transfer lipid extract sample to cartridge.
2. Rinse vial with 2 ml methanol using a micropipette.
3. Add 2 ml Solution 3 (0.65 M HCl) and mix by pipetting up and down.
4. Test solution with pH paper to ensure that pH is 2-3. If the pH is too high, add additional Solution 3 (ca. 200 µl) (Gessner, personal comm., Jan. 11, 2021).
5. Open stopcocks to apply vacuum.
6. Set flow rate to <1 ml/min (lowest setting on vacuum pump).

V. Washing and drying of sorbent in SPE cartridge (75 min)

1. After complete loading of sample onto cartridge (takes ca. 30 min), wash sorbent bed with 2.5 ml Solution 5 (washing solution).
2. Replace the collecting vial before drying.
3. Turn vacuum on all the way; close fifth stopcock and Bunsen burner valve. Dry sorbent for 60 min under stream of air.
4. SPE column should be kept cool; wrap columns in a damp paper for evaporative cooling.

VI. Elution of Ergosterol (10 min)

1. Replace collecting vial with a clean vial (pre-weighed to the nearest 0.1 mg).
2. Open fifth stopcock and Bunsen burner valve, apply gentle vacuum (lowest setting).
3. Elute ergosterol with 4 x 400 µl of isopropanol.
4. Stop pump, remove vials, close lids tightly, and weigh to the nearest 0.1 mg.
5. Calculate fluid volume in vial (given a density of isopropanol of 0.786 g/ml at 25 °C).

VII. HPLC Analysis (30 min per sample + conditioning time)

1. HPLC analysis should be performed within 24 hours since the sample begins to degrade (Beni et al., 2014).
2. Set chromatograph to the following conditions; retention time of ergosterol should be about 27 min:
   - **Mobile phase**: 100% methanol
   - **Flow rate**: 1.40 ml/min (adjust on Solvent Prg tab of BuckChrom software)
   - **Conditioning time**: 1 min
   - **Separation time**: 30 min (ergosterol peak at 27 min)
   - **Detection wavelength**: 282 nm
3. Run standards on HPLC and prepare standard curve (concentrations of 0, 5, 10, 20, 40, and 80 µg/ml) from Solution 7 (ergosterol stock solution in isopropanol) (Fig. 5).
4. Inject samples (each sample 2x).
5. Check identity of putative ergosterol peaks by co-injection of the ergosterol standard with sample extract.
6. Measure area and/or height of ergosterol peaks (Fig. 6).
7. Calculate ergosterol concentration in extract and leaves, based on concentration in final extract, total sample volume, and sample dry mass.
8. Calculate fungal biomass per gram of leaf dry mass using a conversion factor (5.5 mg ergosterol per gram of fugal biomass is a common conversion factor).

Figure 1. Leaf disk procedure. Leaf disks were cut using a hole punch, avoiding major leaf veins. The disks from half the leaf were used for the extraction and the replicate samples from the other half of the leaf were dried to determine leaf dry mass.

Figure 2. Lipid extraction of ergosterol. Samples were boiled with stirring for 30 min at 80 °C. The temperature was monitored with a thermometer in water.

Figure 3. Solid phase extraction (SPE) apparatus. The vacuum was connected to the samples via a five-port vacuum gas manifold. Four samples could be run at once since the fifth stopcock was used to control vacuum pressure.

Figure 4. SPE column apparatus. The SPE column was connected to a syringe needle, which was inserted through a rubber septum on the collecting vial. The tube on the right connects the system to the vacuum through another syringe needle.

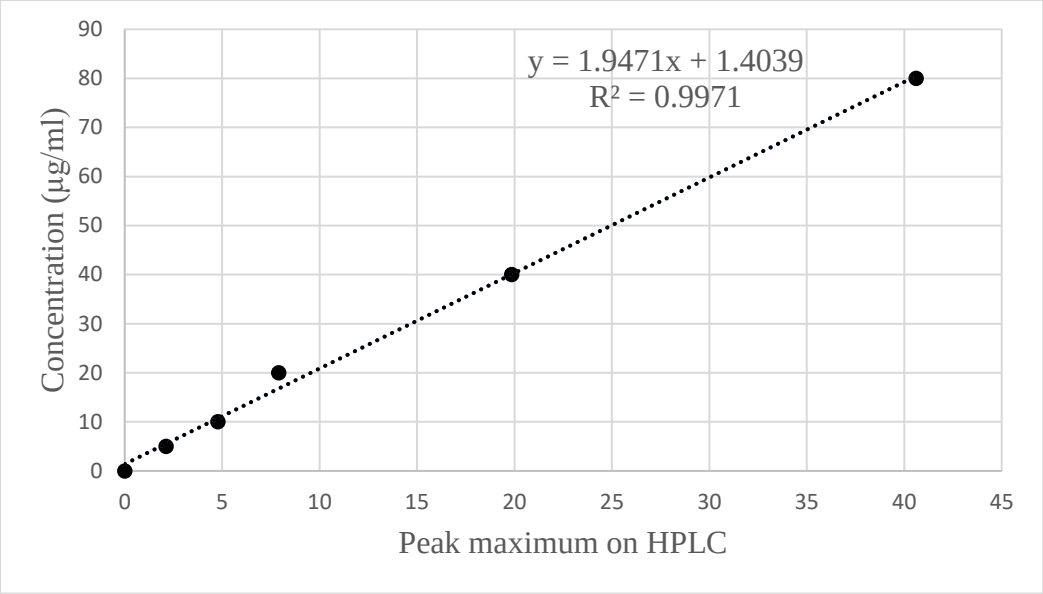
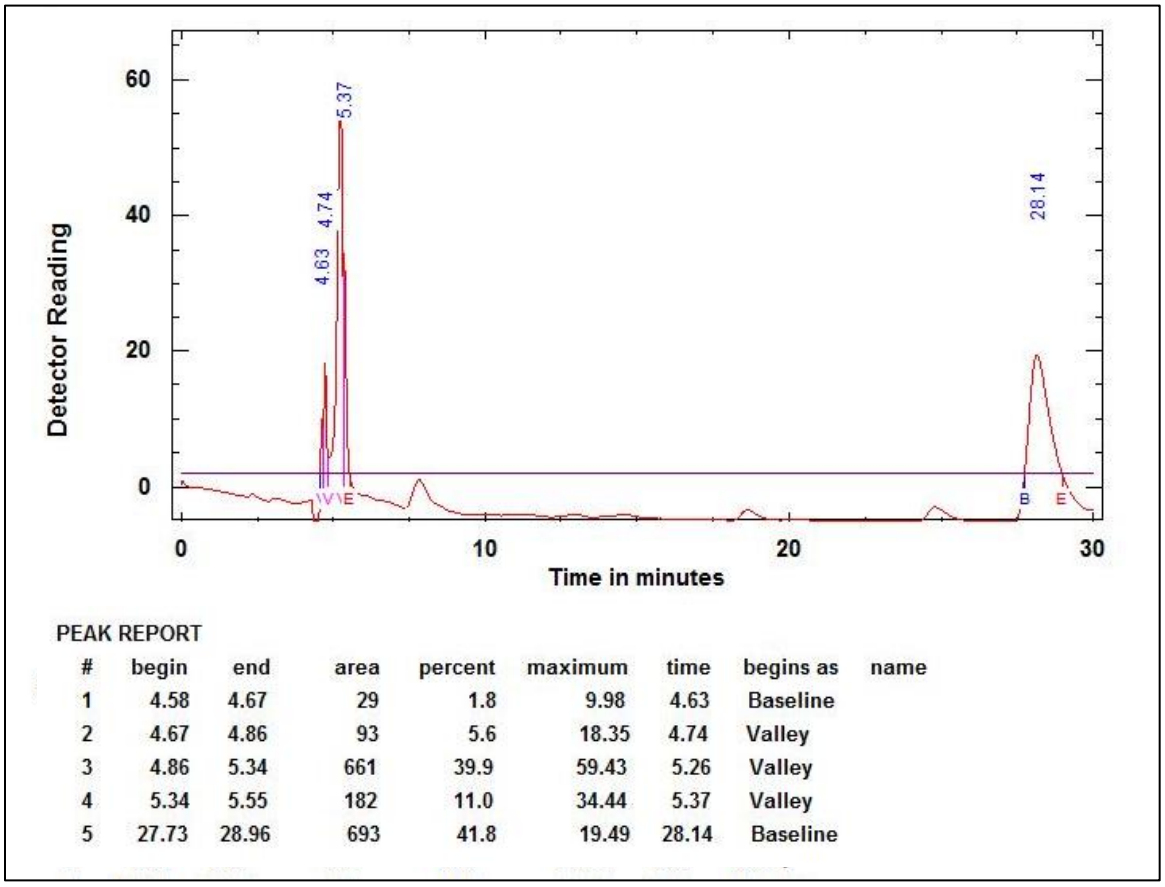


Figure 5. Standard curve with concentrations of 0, 5, 10, 20, 40, and 80 µg/ml. The sample concentration of ergosterol in isopropanol can be calculated using the standard curve.



PEAK REPORT

#	begin	end	area	percent	maximum	time	begins as	name
1	4.58	4.67	29	1.8	9.98	4.63	Baseline	
2	4.67	4.86	93	5.6	18.35	4.74	Valley	
3	4.86	5.34	661	39.9	59.43	5.26	Valley	
4	5.34	5.55	182	11.0	34.44	5.37	Valley	
5	27.73	28.96	693	41.8	19.49	28.14	Baseline	

Figure 6. Sample HPLC chromatogram report with ergosterol peak at 28.14 min. This sample contained fungus grown from leaf disks on MS medium in a petri dish, so it contained more ergosterol than would be expected for a field sample.

Recovery rates:

Recovery rates were calculated by running the entire extraction procedure on control samples (n = 5) containing 30 dried leaf disks in 10 ml of Solution 2 (KOH/methanol) and spiked with 250 µl of Solution 8 (ergosterol standard in KOH/methanol). For the five control solutions, two did not return any ergosterol. The other three controls had recovery rates of 79%, 58%, and 44%. These highly variable recovery rates were potentially due to differences in sample pH or length of sample loading. One of the challenges with the vacuum manifold apparatus is that the flow rate is often inconsistent across samples, but all SPE cartridges must be fully loaded and washed before drying can begin.

To test the recovery rates for the SPE cartridges, a second set of controls (n = 4) containing 250 µl of Solution 8 (ergosterol standard) in 10 ml of Solution 2 (KOH/methanol) and no leaf disks were run through steps III to VII (controls were not boiled). The recovery rates for these samples were 90%, 89%, 68%, and 66%. These results suggest that high recovery rates are possible using this ergosterol procedure, but further refinement may be needed maintain consistent recovery rates.

Method validation:

The methods were tested on boxelder (*Acer negundo)* and buckthorn (*Rhamnus cathartica)* leaf samples that were incubated in the Pomme de Terre River in Morris, MN for 5, 12, and 19 days during November 2019. Leaf samples were then stored in plastic bags in the freezer for 12 months until sample analysis. Using the modified extraction methods, no ergosterol was isolated from any of the leaf samples. It is likely that the ergosterol levels in the samples were too low to be visualized using these methods. The ergosterol may have degraded during the storage and thawing process, so samples should be frozen in KOH/methanol (Solution

2) instead of plastic bags (M. Gessner, personal comm., Jan. 11, 2021). Each sample contained 30 leaf disks (7 mm diameter), which averaged 36 mg of leaf dry weight. In the future, this could be increased to 40 or 50 disks per sample to increase the initial amount of fungal biomass per sample. In addition, leaf samples were incubated for a maximum of 19 days, so future research could extend the incubation period. In temperate streams, maximum fungal biomass production generally occurs after 20 to 40 days of leaf decomposition (Duarte et al, 2009; Ferreira and Canhoto, 2015). However, one study found maximum biomass production at 57 days (Gessner, 2005), so extending the incubation period could be beneficial since it would increase the amount of ergosterol per sample.

To ensure that ergosterol could be extracted from fungi using the adapted methods, the frozen leaf disks were plated on MS medium for 6 days until there was visible fungal growth. The extraction procedure was then conducted on the amplified fungal samples (n = 2), and ergosterol was successfully isolated in both (Fig. 6). The extract concentrations were estimated to be 39 and 80 μg/ml, respectively. These results indicate that the modified ergosterol extraction methods can be effective if there is sufficient fungus present in the original sample.

Future research:

At the University of Minnesota-Morris, these methods can be applied to analyze ergosterol content, and therefore fungal biomass, on decomposing leaves in aquatic ecosystems. However, further research is needed to refine the methods and improve recovery rates. Future research could apply the methods to compare fungal growth on different types of leaves, such as comparing the decomposition of native tree leaves versus invasive plants like buckthorn.

Acknowledgements:

This project was funded by the Undergraduate Research Opportunities Program (UROP). I would like to sincerely thank my mentor, Dr. Tracey Anderson, for helping develop this project, Dr. Alyssa Pirinelli for providing lab space, equipment, and training with the procedures, and Dr. Steven Deslauriers for assisting with growing fungal cultures.

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