Minimizing Ergosterol Loss during Preanalytical Handling and Shipping of Samples of Plant Litter†

STEVEN Y. NEWELL*

Marine Institute, University of Georgia, Sapelo Island, Georgia 31327

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Common preliminary treatments of samples of decaying material can involve changes in water content (e.g., via storage in relatively dry air or rinsing) that could conceivably result in loss or gain of fungal membranes and, consequently, ergosterol. A related problem is that collecting of ergosterol content data from widely distributed locales by shipment of samples ideally requires an inexpensive, safe alternative to submerging the samples in methanol for prevention of ergosterol loss. Experimental testing showed that fungal occupants of decaying salt marsh grass leaves did not exhibit loss or gain of ergosterol during air drying (to a water potential of <-8 MPa) or rewetting (to -0.8 MPa). Wet leaves of one grass species (*Juncus roemerianus*, black needlerush) could be fixed and dried for shipment by microwaving, or by fully drying after alcoholic or pentane fixation, without ergosterol loss, but those of smooth cordgrass (*Spartina alterniflora*) lost about 40% of their ergosterol content by all three of these drying methods. Ergosterol content of wet leaves of cordgrass could be maintained by alcoholic fixation and subsequent drying down to a thin film of alcohol.

Ergosterol analysis has become a prominent means of measuring living (membrane-containing) fungal mass that is contained in samples of solid substrates (e.g., see references 17 and 32). The strong interest in ergosterol analyses has spurred research into potential inaccuracies that could result from inferior choices of methodology in the basic processing of natural samples (e.g., see references 2, 4, 6, 18, and 19). One potential cause of ergosterol loss that has not been examined thoroughly is drying that could take place while samples await processing for extraction. West et al. (33) found that when soils were air dried at 20°C, 70 to 88% of soil ergosterol content was lost. Leaves of decaying plants can lose water rapidly at normal room temperature and relative humidity; e.g., Newell et al. (20) found that decaying leaf blades of a salt marsh grass (presoaked in tap water) exhibited a drop in water potential from -0.8 to <-8.0 MPa in less than 1 h of air drying at about 23°C and 58% relative humidity (1 MPa = 10 bars). Water potentials of <-8 MPa prohibit activity by all but the most xerophilic microorganisms (20), and the stress of very low water availability might conceivably cause reductions in fungal mass (26).

By the same token, decaying plant material collected in the dry state could begin to support rapid fungal growth (or lysis of fungal mass killed during prior drying [31]) upon wetting due to preanalytical processing (e.g., rinsing to remove adherent sediment). Newell et al. (20) found that the water potential of dry (water potential, <-150 MPa) leaves of a salt marsh grass was raised to -1.5 MPa after 5 min of contact with tap water. The corresponding water content ($\approx 40\%$ of fresh mass) is well above the minimum (about 15% water) permitting fungal metabolic activity (23). If leaves remained fully wetted for several hours and fungi were growing at high specific rates, detectable change in ergosterol content could occur (e.g., 6 h at a growth rate of 0.8 day^{-1} [22] would yield a 20% increase).

Now that the application of the ergosterol method is permitting the collection of diverse data sets for dynamics of

living-fungal standing crop (e.g., see references 7, 26, 34), an important question that will arise is, “How consistent are the fungal patterns over large geographical areas?” (16). In determining ergosterol concentrations in samples from widespread sites, preservation and shipping are likely to be involved. Newell et al. (19) identified direct storage in methanol extractant as the best of several options (including drying at 50°C, freezing at -20°C , and lyophilization) tested for optimizing preservation of original ergosterol contents of decaying-leaf samples. However, for shipping purposes, storage in methanol is not an ideal method, since methanol is flammable and toxic. Newell et al. (19) found only small ergosterol losses (20%) when decaying leaves of salt marsh grass were lyophilized, and they suggested that instant freezing might reduce these losses, a point subsequently confirmed by Davis and Lamar (4) for soil samples. Gessner et al. (6) found no difference between direct submergence in methanol and lyophilization, with respect to ergosterol preservation, for decaying leaves from a freshwater stream. Therefore, for at least some types of sample lyophilization is an effective means of preserving fungal samples. But if one wishes to obtain samples from many distant points, it is likely that at some of those outposts, lyophilization (especially with instant-freezing capability) equipment will not be available.

I report herein the results of experiments designed to reveal (i) whether allowing samples of decaying, originally wet leaves to dry (or vice versa) for short periods permits ergosterol contents to change and (ii) whether a simple, inexpensive method of preserving decaying leaves, feasible for shipping purposes, would maintain ergosterol concentrations of samples. Two experiments were performed to determine the efficacy of the preservation method, one involving decaying leaves of standard wetness, in which fixation by quick-drying (in a microwave oven) was tested against submergence in alcohol or pentane followed by full evaporation of the solvent, and one in which initially dry or wetted leaves were submerged in alcohol and then either fully dried or left in a thin film of alcohol. For these preservation experiments, the drying treatments were compared with immediate storage in 5 ml of alcohol, the standard preservation method of Newell et al. (19). In the experiment using initially dry or wetted leaves, ethanol was used

* Phone: (912) 485-2290. Fax: (912) 485-2133. Electronic mail address: newell@uga.cc.uga.edu.

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instead of methanol because of its lower toxicity; it is presumed for present purposes that methanol and ethanol have equivalent capacities for preservation of ergosterol.

Experiment 1: laboratory drying and rewetting of originally wet samples. Leaf blades of smooth cordgrass (*Spartina alterniflora* Loisel.) were used. Leaves were collected from a Sapelo Island marsh (the nearly shredder-snail-free site of reference [21]) on the morning (11 November 1994, when shoots were entirely above tidal level) after a hard nighttime rain (7.2 cm of rainfall; light drizzle occurring during collection, air/water temperature = 14/20°C). Leaves collected were the second or third standing-dead leaf below the currently lowest yellow-green (senescent) leaf on a shoot (25). Because leaves usually die in succession from low to high on shoots (barring insect-borer attack), the second and third leaves in November are relatively high on shoots (ligules about 35 cm above the sediment for the present collection). I excluded leaves that contained the largest black patches of the ascomycete *Buergenerula spartinae* Kohlmeyer et Gessner because of the potential of these patches to increase analytical noise in an ergosterol assay (21).

Leaf blades were cut from sheaths at the ligule; next, a 3-cm length was cut from each leaf at a point approximately 15 cm distal to the ligule end. A 1-cm portion of the 3-cm length was immediately (in the field) submerged in 5 ml of methanol in a screw-cap 20-ml glass vial. The remaining 2-cm portion was cut into two 1-cm lengths, each of which was placed in a separate 20-ml vial. One of these vials was labeled to indicate that its contents would subsequently receive the drying treatment, and the other was labeled to indicate that its contents would receive the drying-rewetting treatment. Enough leaves were cut to yield four replicates of each type of sample; each replicate vial contained 1-cm lengths of blade from each of five leaves. For each numbered set of replicate vials (i.e., replicates 1 for each treatment, etc.), the five leaves used were the same, to reduce the potential impact of interleaf variation (19). Replicate vials of leaf pieces were also obtained for measurement of leaf water and organic content.

Upon return to the laboratory, the vials containing leaf pieces placed directly into methanol in the field were stored at 4°C in darkness. These samples would later provide values for ergosterol content at the time of sampling, prior to any drying treatments. The replicate pieces selected for the drying and drying-rewetting treatments were arrayed on paper toweling at 25°C and 50% relative humidity for 2.5 h. The drying-only replicate sets of pieces were then submerged in 5 ml of methanol and stored at 4°C in darkness. The drying-rewetting pieces were placed in dishes of tap water for 1 h at 25°C, drained for 10 s on paper toweling, and stored in methanol as described above. Replicate leaf pieces for determination of water and ash content (for this set of samples and those described below) were weighed fresh immediately upon return to the laboratory (or removal from water for samples described below), dried in a microwave oven (20), immediately reweighed, ashed (1), and weighed again.

Ergosterol extractions and liquid-chromatographic analyses for the above samples and all those described below were done by the method of Newell (18) for total ergosterol (free and esterified), with leaf pieces removed prior to the saponification.

Experiment 2: drying with or without presubmergence in methanol or pentane. Leaves of two salt marsh grasses were used, *Spartina alterniflora* and *Juncus roemerianus* Scheele (black needlerush). Leaf cutting and replicate preparation for blades of the two grasses were as described above, except that (i) cylindric pieces of the leaves of *J. roemerianus* were chosen to be of approximately equal diameter, from the 12- to 16-cm

span above the marsh sediment surface and (ii) leaf pieces for all treatments received standard processing (rinsing and soaking) in the laboratory before being subjected to extraction or drying. Rinsing was for 30 s in running tap water (20°C), with rubbing to remove clay film. Rinsed pieces were stored overnight in air in loosely capped 20-ml vials at 25°C. The following morning, 3 ml of tap water was added to each vial of leaf pieces and vials were incubated for 1 h at 25°C. Next, water was poured off, pieces were drained on paper toweling, vials were dried with lint-free absorbent paper, and pieces were returned to vials. The water in the leaf pieces was very unlikely to have affected ergosterol extraction efficiencies, as it may have in the studies of Davis and Lamar (4) and Anderson et al. (2), who used soils, because the water in the wet cordgrass leaves used in this experiment represented less than 1% of the subsequently added methanol volume.

Two sets of four replicate vials received 5 ml of methanol. One of these sets was immediately stored at 4°C in darkness. A third set of four replicate vials received 5 ml of pentane, and the second and third sets of vials were sealed and stored in darkness at 25°C for 12 h. After the 12-h storage, the vials were opened and the solvent was fully evaporated under streams of air in a heating block set at 65°C in a hood. Vials were removed from the block as soon as solvent disappeared (10 min for pentane and 25 min for methanol), resealed with screw caps, and stored in darkness for 1 week at 25°C to simulate the effects of shipping. After the week of storage, 5 ml of methanol was added to each vial and vials were stored at 4°C.

The leaf pieces in the fourth set of four replicate vials were not exposed to solvent before being dried. They were quick-dried in a microwave oven (20). The dry pieces were stored at 25°C in darkness for 1 week and then stored cold in methanol as described above.

Ascomycetes detected by direct microscopy (25) in the cordgrass leaves used in experiment 2 were, in decreasing order of abundance, *Phaeosphaeria spartinicola* Leuchtman and *Mycosphaerella* sp. II of Kohlmeyer and Kohlmeyer (9). For needlerush leaves, the ascomycete species were a *Massarina* sp. (proposed specific epithet, *ricifera* Kohl., Volkm.-Kohl. et Erikss. [15]); *Anthostomella poecila* Kohl., Volkm.-Kohl. et Erikss.; *Tremateia halophila* Kohl., Volkm.-Kohl. et Erikss.; *Loratospora aestuarii* Kohl. et Volkm.-Kohl.; *Papulosa amerospora* Kohl. et Volkm.-Kohl.; and *Sulcospora junci* Kohl. et Volkm.-Kohl. I used references 9 to 15 in identifying the ascomycetes found.

Experiment 3: full or partial drying, after presubmergence in ethanol, of initially dry or wetted leaves. Leaf blades of *Spartina alterniflora* were used (collection date, 20 December 1994; air/water temperature, 17/14°C), with cutting and allotment as described above for the first experiment, except that leaves were collected during a dry period in the marsh and three replicate 20-ml vials were prepared for each treatment. There were six treatments. For three treatments, leaf pieces were submerged in reagent ethanol in the dry state, immediately after collection. For three other treatments, leaf pieces were submerged in tap water for 1 h in their 20-ml vials, after which the water was poured off but pieces were not drained. Caps were screwed tightly onto the vials and then loosened one-quarter of a turn, so that pieces would not dry out. The wet pieces were incubated at 25°C for 24 h, after which water within vials was removed with lint-free absorbent paper and reagent ethanol was added.

One set of initially dry and one set of initially wet replicates ($n = 3$ each) received 5 ml of ethanol and were stored at 4°C in darkness. The remaining replicates received 2 ml of ethanol (just enough to cover the pieces to a 5-mm depth). The vials

FIG. 1. Mean amounts of ergosterol in samples of leaves of cordgrass (*Spartina alterniflora*) (□) and needlerush (*J. roemerianus*) (■) as affected by preservation treatments. Treatments: MeOH, submergence in methanol (no evaporation); MeOH,dry, submergence in methanol followed by full evaporation of methanol; Pentane,dry, submergence in pentane followed by full evaporation of pentane; Microwave, quick-drying in a 1,000-W microwave oven. Each value is the mean for 4 replicates. Maximum coefficients of variation: cordgrass, 18%; needlerush, 20%. No significant difference was detected among values for needlerush treatments ($P = 0.41$); values obtained with the various drying treatments for cordgrass were not significantly different from one another but were significantly different from those for samples preserved in methanol without subsequent drying ($P = 0.02$).

with 2 ml of ethanol were stored at 25°C for 12 h and then opened for evaporation of the ethanol as in the second experiment described above. One replicate set of vials for initially dry and initially wet pieces ($n = 3$ each) was fully dried (no visible liquid in vials). The ethanol in the final six vials was evaporated until a thin film of liquid remained, not evaporated to full dryness. The dry vials and the vials with a film of ethanol were tightly sealed and stored at 25°C for 1 week, after which they received 5 ml of ethanol and were stored at 4°C in darkness.

Solvents (methanol, reagent ethanol, and pentane) were purchased from Fisher Scientific, and ergosterol was purchased from Calbiochem. The SPSS/PC+ 5.0 statistical package (27) was used in statistical processing of data (analysis of variance and Student-Newman-Keuls least-significant-range testing). Error estimates given below are ± 1 standard deviation, unless otherwise indicated.

Results for experiment 1. The rain-wet leaves used in experiment 1 had an average water content of $62\% \pm 7\%$ of fresh mass and an ash content of $12\% \pm 4\%$ of dry mass. Drying or rewetting had no significant effect upon ergosterol content of leaf pieces ($P = 0.33$). Rain-wet leaves contained 26.8 ± 2.9 µg of ergosterol per sample, air-dried (2.5 h) leaves contained 24.9 ± 2.1 µg of ergosterol, and rewetted (1 h) leaves contained 28.8 ± 4.9 µg of ergosterol. These quantities were equivalent to an average of 432 µg of ergosterol per g (organic mass) of decaying-leaf system.

Results for experiment 2. Tap-water-soaked (1 h) leaves of cordgrass had a water content of $67\% \pm 4\%$ of fresh mass and an ash content of 10.1% of dry mass (replicates were pooled for ashing). Soaked leaves of needlerush contained $38.5\% \pm 1\%$ water and 3.3% ash. All three of the drying treatments (microwaving and dry down after methanol or pentane treatment) caused losses from cordgrass leaves of nearly 40% of the original ergosterol ($P = 0.002$) (Fig. 1). None of the three drying treatments caused significant losses for needlerush leaves ($P = 0.41$) (Fig. 1). The quantities of ergosterol for the samples that were preserved in methanol without subsequent drying were equivalent to 550 µg per g (organic mass) of

FIG. 2. Mean amounts of ergosterol in samples of leaves of initially dry (□) and initially freshwater-soaked (■) cordgrass (*Spartina alterniflora*) as affected by full evaporative drying of extractant (ethanol) or drying down to a thin film of ethanol. Treatments: EtOH, submergence in ethanol (no evaporation); EtOH, film, retention of a thin film of ethanol on leaf pieces after evaporation of the bulk of the ethanol; EtOH,dry, full evaporative drying of leaf pieces. Values indicated by bars bearing the same lowercase letter (directly above bars) are not significantly ($P < 0.05$) different from one another. Each value is the mean for three replicates. The maximum coefficient of variation was 13%.

decaying-leaf system for cordgrass leaves and 385 µg per g for needlerush leaves.

Results for experiment 3. Freshly collected cordgrass leaves used in experiment 3 had a water content of $18\% \pm 2.0\%$ of fresh mass. The leaf pieces that received full evaporation of alcohol showed the lowest ergosterol contents (Fig. 2). For initially dry leaves, fully dried samples had ergosterol contents 90% of the original and values were not significantly different from those obtained for the original ($P > 0.05$), but for initially wet leaves, ergosterol contents were only 62% of original values ($P < 0.05$). In contrast, for the samples in which a thin film of ethanol was retained, there was no detectable difference from original ergosterol content for either initially dry or freshwater-soaked leaf pieces (Fig. 2). The mean ergosterol content per sample for the high group of values that were not significantly different (bars lettered "a" in Fig. 2) corresponds to an ergosterol content of 499 µg per g (organic mass) of decaying-leaf system.

Experiment 1 (drying-rewetting) revealed that the short-term (a few hours) changes in water content of decaying grass leaves, which can be expected during collection and processing of natural samples, are not likely to bring about changes from the original ergosterol content. This remarkable fungal ability to retain membrane mass through a drying-wetting cycle, reminiscent of the superb adaptation of lichens to highly changeable wet-dry conditions (28), is a finding consistent with the results of Zelles et al. (34) for air drying of several soils (average of 3% increase in ergosterol content for five soils after a 1-month storage of air-dried material at 21°C; 9% increase for organic forest layer [less than the coefficient of variation for the fresh material]) and of Scheu and Parkinson (29) for drying and rewetting of forest soil layers (average loss of 10% [$P > 0.05$] for material air dried at 20°C for 2 weeks; no statistically significant losses for the first 2 days after rewetting). West et al. (33), however, found sharp decreases (70 to 88%) in soil ergosterol content after air drying (20°C) and storage of samples for 1 month, and Nilsson and Bjurman (26) found an approximately 50% decline in ergosterol content of wood that was allowed to dry over a 100-day period. Thus, if one wishes to allow air-dried samples of decaying litter or soil to be stored for periods as long as 1 month, one should test whether the

fungal-membrane material in the samples is susceptible to loss. One potential partial vector for this type of loss, in samples too dry for fungal growth, is microarthropod mycophagy (e.g., see reference 30). Also, it may very well be that decaying leaves from persistently wet environments (e.g., leaves in streams [3]) cannot be air dried without fungal-membrane damage and ergosterol loss (see reference 5).

Although air drying did not detectably reduce ergosterol contents of decaying cordgrass leaves, it is probably not the ideal means of preserving samples for shipping, since the fungal mass remains alive and could grow if moisture content were to increase (see reference 23). A better method would involve killing the fungal mass and fixing its ergosterol content. I tried three potential versions of such a method, using leaves taken through a standard wetting regime, and found that different decay systems reacted quite differently to the various methods (Fig. 1). For needlerush, all three drying treatments were effective preservers of ergosterol content. For cordgrass, all three drying treatments caused about a 40% loss of ergosterol. Both grasses harbor ascomycetes as their most prominent decomposers (16, 11–15), so it is not immediately clear why they would react so differently. One peculiarity of smooth cordgrass that may potentially be involved is its high content of ferulic acid that could be part of condensation reactions during drying (8, 16, 24).

I based the design of experiment 3 on two hypotheses: (i) that if a small amount of alcohol were allowed to remain in the vials of leaf pieces, the loss of ergosterol would not take place; and (ii) that the ergosterol that is lost is material newly formed or altered during the period of leaf wetness and is in a form that is susceptible to loss on dry down. Both hypotheses were supported by the experimental outcome. When a thin film of ethanol was allowed to remain on initially wet or dry leaf pieces, no detectable loss of ergosterol occurred (Fig. 2), whereas if leaves were wet for a lengthy period (24 h) prior to preservation, complete dry down caused an approximate 40% loss of ergosterol. Leaves that were dry at preservation showed no significant loss of ergosterol on alcohol evaporation, perhaps suggesting that fungal membrane material formed or altered during the wet period was at least part of the portion lost at full dry down. However, there was not a statistically significant increase in ergosterol content of leaf pieces during the wet period (Fig. 2), so it may be that the principal factor leading to ergosterol loss is a change in state of existent membrane material during the period of leaf wetness, rather than formation of new, susceptible membranes. At any rate, it is clear that wet decaying leaves suffered losses of ergosterol at dry down that were not experienced by dry leaves, strongly suggesting that decaying plant material collected during wet periods or from persistently submerged environs (e.g., leaves in freshwater streams [6]) should not be dried down after alcohol fixation unless it is known that this drying has no effect on ergosterol content.

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