CHAPTER 13

TOTAL LIPIDS

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1. INTRODUCTION

Lipids are a major class of chemical compounds in plant tissues that have rarely been considered in litter decomposition studies and assessments of litter nutritional quality for aquatic detritivores. This neglect partly reflects the fact that emphasis in the chemical characterization of decomposing litter has been placed on nutrients, particularly in terms of nitrogen (Enriquez et al. 1993), and refractory litter constituents such as lignin (e.g. Gessner & Chauvet 1994, Palm & Rowland 1997). The currently available data show, however, that lipids can be a sizeable fraction of plant litter (Table 13.1), suggesting that information on lipid content may be useful when modelling decomposition as a function of chemical litter composition (e.g. Moorhead et al. 1999).

There is evidence, moreover, that lipids can provide critical cues to detritivore feeding (Anderson & Cargill 1987). For example, the sequence of food preference

Table 13.1. Total average lipid content of undecomposed leaf litter of various tree species. AFDM = ash-free dry mass.

Leaf species	*Common name*	*Lipids (% AFDM)*	*Reference*
Acacia melanoxylon	Blackwood acacia	1.9[a]	1
Carya glabra	Hickory	5.2	2
Eucalyptus viminalis	Manna eucalyptus	12[a]	1
Liquidamber styraciflua	Sweetgum	1.7[a,b]	3
Pomaderris aspera	Hazel pomaderris	3.8[a]	1
Quercus alba	White oak	4.9	2
Quercus nigra	Water oak	1.0[a,b]	3

[a]AFDM assumed to be 90% of dry mass. [b]Based on fatty acid content of saponified methanol extracts. 1 = Campbell et al. (1992); 2 = Suberkropp & Klug (1976); 3 = Mills et al. (2001)

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of two detritivores (*Gammarus tigrinus* and *Pycnopsyche guttifer*) for certain combinations of fungal species grown on leaves could be reproduced by applying lipid extracts of the fungi to uncolonized leaves (Rong et al. 1995). For holometabolic insects with limited food acquisition during the adult stage, lipid content may be a particularly important litter quality attribute, because late instars of these invertebrates benefit from consuming food with a high energy content to build up energy reserves before emergence. Support for this hypothesis has come from a detritivorous caddisfly, the limnephilid *Clistoronia magnifica* (Hanson et al. 1983, Cargill et al. 1985a, b).

Bulk lipid analyses have classically adopted a gravimetric approach (Suberkropp et al. 1976). Lipids are extracted from the tissue with an apolar solvent, which is then evaporated and the dried residue is weighed. The main limitation of this approach is that relatively large sample sizes are needed for accurate analyses. An attractive alternative is a spectrophotometric assay that allows analysis of small samples. Zöllner & Kirsch (1962) described such a method for analyzing blood lipids. This method, known as the sulphophosphovanillin assay, has later been applied to estimating lipid contents of algae (Rausch 1981, Ahlgren & Merino 1991), aquatic invertebrates (Barnes & Blackstock 1973, Meyer & Walther 1989), and fine-particulate organic matter (Neumann 1995). It is based on the reaction of lipid degradation products with aromatic aldehydes, which results in a red coloration that can be quantified at 528 nm (Zöllner & Kirsch 1962). With particulate organic matter, it is essential to extract the lipids from the bulk sample before performing the sulphophosphovanillin assay. This is because strong interference by nonlipid compounds (e.g. carbohydrates) results in high nonspecific absorbance after heating the sample in sulphuric acid and addition of the vanillin reagent, and thus in unreliable results (Ahlgren & Merino 1991, Neumann 1995).

The protocol described here has been adopted from Neumann (1995). Although developed for fine-particulate organic matter, it has also proved reliable for decomposing leaves from streams (M.O. Gessner, unpubl. data).

2. EQUIPMENT, CHEMICALS AND SOLUTIONS

2.1. Equipment and Material

- Freeze-dryer
- Mill
- Analytical balance
- Centrifuge tubes (12 ml, preferably pressure resistant, with Teflon-lined screw-caps)
- Centrifuge tubes (4 ml)
- Pipettes allowing precise pipetting of solvents with low viscosity (e.g. Eppendorf Multipette® or Varipette®)
- Standard laboratory centrifuge
- Evaporating centrifuge (e.g. SpeedVac concentrator SPD131DDA, Thermo Electron Corp., Woburn, MA, USA)

- Vials (4 ml, with Teflon-lined screw caps)
- Vortex mixer
- Pear-shaped glass bulbs or marbles (only needed if centrifuge tubes do not resist high pressure)
- Water or dry baths (20 and 100 °C)
- Timer
- Spectrophotometer (set at 528 nm)
- Volumetric flasks

2.2. Chemicals

- Chloroform ($CHCl_3$), for residual analysis
- Methanol (CH_3OH), for residual analysis
- Deionized water (e.g. Nanopure®)
- NaCl, reagent grade
- Concentrated sulphuric acid (H_2SO_4, 95—97%), reagent grade
- Concentrated phosphoric acid (H_3PO_4, 85%), reagent grade
- Vanillin
- Cholesterol (5-cholesten-3β-ol)

2.3. Solutions

- 0.9% (w:v) NaCl solution
- Phosphoric acid-vanillin reagent: 20 ml 0.6% (w:v) vanillin solution and 85% H_3PO_4 in a total volume of 100 ml
- Standard solutions: Cholesterol standards in chloroform at concentrations ranging from 10 to 100 mg ml^{-1}.

3. EXPERIMENTAL PROCEDURES

3.1. Lipid Extraction

1. Freeze-dry leaves and grind to powder that passes trough a 0.5-mm mesh screen.
2. Weigh out 25 mg of sample to the nearest 0.1 mg in a 12-ml screw-cap centrifuge tube with a Teflon-lined cap.
3. Add 7 ml chloroform:methanol (2:1, v:v).
4. Shake for 1 min, then let stand for 2 h, with shaking for 1 min every 30 min.
5. Centrifuge for 1 min at about 3000 *g*, rinse tube walls to suspend any adhering particles, then centrifuge for another 10 min to separate particles from the lipid extract.
6. Transfer 5 ml of the lipid extract to a clean tube containing 1 ml of 0.9% NaCl solution.
7. Shake for 1 min, then centrifuge for 10 min at about 3000 *g* to separate phases.

8. Remove and discard upper aqueous phase.
9. Rinse inner walls of the tube twice with 1 ml chloroform:methanol:water (3:48:47, v:v:v).
10. Remove rinsing solution.
11. Evaporate sample to dryness in a SpeedVac concentrator at about 45 °C.
12. Transfer residue with 2 ml chloroform to a clean 4-ml screw-cap vial with Teflon-lined cap.
13. Evaporate sample again to dryness in the SpeedVac concentrator.
14. Redissolve residue in 1 ml chloroform, close vial tightly and run spectrophotometric assay or store at –20 °C until analysis.
15. Run control without sample material in the same way.

3.2. Spectrophotometric Analysis

1. Place 100 µl of the lipid extract in a 12-ml test tube.
2. Evaporate solvent in SpeedVac concentrator at about 45 °C.
3. Add 200 µl of conc. H_2SO_4 and vortex.
4. Close tube tightly or, if not pressure-resistant, cover it with a pear-shaped glass bulb (or marble), and heat for 10 min to 100 °C in a water or dry bath.
5. Let cool for 5 min in a water bath at 20 °C.
6. Add 2.5 ml of H_3PO_4-vanillin reagent and vortex.
7. Measure absorbance after 60—65 min at 528 nm.
8. Run cholesterol standards in the same way.
9. Calculate lipid content as cholesterol equivalents from absorbance reading of sample and standard curve.

4. FINAL REMARKS

The time course of colour development depends strongly on the ratio of sulphuric acid to the H_3PO_4-vanillin solution (Neumann 1995). It is essential, therefore, to keep this ratio strictly constant in a given sample series (e.g., at 1:12.5 as in the protocol described above).

At a 1:12.5 ratio of sulphuric acid to the H_3PO_4-vanillin reagent, absorbance readings must be taken 60—65 min after addition of reagent to the sample. Earlier or later readings result in an underestimation of lipid contents.

Since lipids are a highly heterogeneous class of molecules, choice of an appropriate standard is critical to facilitate accurate quantitative estimates. Neumann (1995) found that cholesterol and a lipid extract from FPOM collected in a stream gave identical responses with the protocol described here. However, use of a specific standard (i.e., a lipid solution from a representative sample, with the lipid content determined gravimetrically) may be preferable when precise information about the absolute magnitude of lipid concentrations is required. For finely ground FPOM, the efficiency of lipid extraction in a single step as described above was 93% compared to three successive extraction steps (Neumann 1995).

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