CHAPTER 16

ACID BUTANOL ASSAY FOR PROANTHOCYANIDINS (CONDENSED TANNINS)

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1. INTRODUCTION

Tannins are a major class of secondary metabolites that are widespread in plants (Waterman & Mole 1994, Kraus et al. 2003a). They are water-soluble polyphenolics with molecular weights typically ranging from 1000 to 3000 (Swain 1979). By definition, tannins are capable of complexing and subsequently precipitating proteins (cf. Chapter 15), and they can also bind to other macromolecules (Zucker 1983). Two main, chemically distinct groups are commonly distinguished in vascular plants: hydrolysable tannins, which are further divided into the gallotannins and ellagitannins, and condensed tannins, or proanthocyanidins, which cannot be hydrolyzed (Waterman & Mole 1994, Hättenschwiler & Vitousek 2000). Proanthocyanidins are the most widely distributed tannins in woody plants. They are usually also the most abundant group. Their diversity both within and among species is remarkable; however, the polymeric structures of proanthocyanidins can be derived from relatively few building blocks of low-molecular weight compounds. The most important monomers are flavan-3-ols such as catechin, epicatechin, gallocatechin and epigallocatechin; they react with one another in various ways, leading to either linear or branched polymers (Fig. 16.1).

Discussions on the ecological functions of tannins have mainly revolved around their capacity to bind to proteins and precipitate them (Zucker 1983). Both vertebrate and invertebrate herbivores can be affected. Herbivores also tend to prefer diets with low tannin concentrations, suggesting that tannins act as feeding deterrents to these consumers, although evidence supporting this tenet is inconclusive (Ayres et al. 1997). A range of additional general ecological functions at both the organismic and ecosystem level have been proposed (Hättenschwiler & Vitousek 2000, Kraus et al. 2003a). These include the role of tannins as antioxidants, mediators of nutrient availability in soils, and regulating factors of

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litter decomposition. In addition, as Zucker (1983) pointed out more than two decades ago, the chemical structure of tannins suggests that there is tremendous scope for specific chemical interactions of tannins both within organisms and in ecosystems. This view of multiple ecological roles for tannins is now widely accepted but data that would allow assembling a clear overall picture of tannin function are still limited (Hättenschwiler & Vitousek 2000, Kraus et al. 2003a).

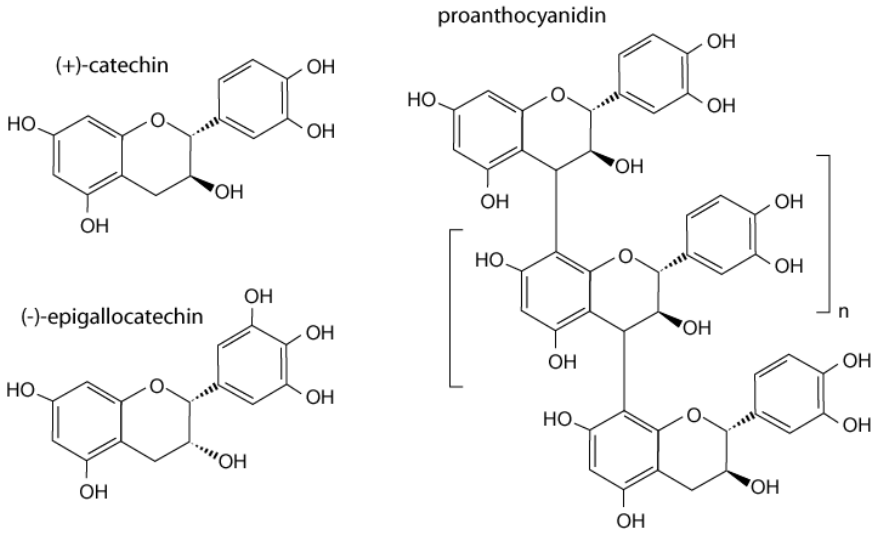

Figure 16.1. Flavan-3-ols (+)-catechin and (-)-epigallocatechin, examples of monomeric precursors that polymerize to form macromolecular products such as linear proanthocyanidins composed of monomeric flavanoid units connected by C4-C8 linkages.

If tannins remain in leaves following abscission (Table 16.1), similar mechanisms as in plant-herbivore interactions would be expected for trophic interactions between leaf litter and detritivores (e.g. Stout 1989, Ostrofsky 1997, Kraus et al. 2003a), with consequent effects on detritivore performance (Zimmer et al. 2002). There is evidence, moreover, that tannins interact with microbial decomposers (Kraus et al. 2003a), indicating that there is significant potential for tannins to affect litter decomposition in both terrestrial (Horner et al. 1988) and aquatic environments (Stout 1989, Ostrofsky 1993, Campbell & Fuchshuber 1995). Tannin concentration thus could be an important indicator of chemical litter quality when addressing a variety of ecological questions relating to litter use and turnover.

The structural diversity of proanthocyanidins provides challenges for accurate quantitative analyses. Chromatographic characterization of cleavage products is therefore increasingly being used (Waterman & Mole 1994, Hernes & Hedges 2000), especially when specific functions of tannins are to be elucidated. Nevertheless, two simple methods for determining total proanthocyanidins, are

considered to give ecologically meaningful information; these are known as the vanillin and the acid butanol assay, respectively (Hagerman & Butler 1989, Waterman & Mole 1994, Kraus et al. 2003b). Since oxidative cleavage of proanthocyanidins in alcohols yields anthocyanidins under strongly acidic conditions and the cleavage products (mainly cyanidin and delphinidin) absorb light in the visible range, proanthocyanidins can be quantified spectrophotometrically following depolymerization. The acid butanol assay recommended by Hagerman & Butler (1989) and Waterman & Mole (1994) for determining total proanthocyanidins is based on this reaction.

Table 16.1. Range of relative condensed tannin contents of undecomposed leaf litter from woody plant species.

Leaf material	*Tannin concentration*	*Reference*
5 *Acer* species	0.015—0.128[a]	1
6 *Quercus* species	0.017—0.107[a]	1
37 other woody plant species	0.003—0.276[a]	1
6 tropical *Eucalyptus* species	9—25[b]	2
6 nontropical *Eucalyptus* species	8—25[b]	2
6 non-*Eucalyptus* species	1—21[b]	2
4 Populus species or hybrids	0—53.3[c]	3

[a] Values are optical densities per mg of extracted dry leaf material; [b] Values are arbitrary relative numbers; [c] Values are given in mg g^{-1} leaf dry mass with tannin extracted from *Populus angustifolia* used as standard; 1 = Ostrofsky (1993); 2 = Campbell & Fuchshuber (1995); 3 = Driebe & Whitham (2000).

Before tannins can be analyzed, they need to be extracted from the sample matrix. Various extractants and extraction procedures have been described. Their relative efficiency depends on the analyzed material, due to differences in both tannin structure and the sample matrix (Waterman & Mole 1994, Yu & Dahlgren 2000), making compromises unavoidable when analyzing a range of different plant materials in comparative studies. One of the most common and frequently recommended extraction solvents is 50% methanol (Hagerman 1988, Waterman & Mole 1994); it is used in the procedure described below. The exact extraction procedure presented here has not been previously published, whereas the proposed protocol of the acid butanol assay has been adopted from Porter et al. (1986) and is also described in the comprehensive review by Waterman & Mole (1994).

2. EQUIPMENT, CHEMICALS AND SOLUTIONS

2.1. Equipment and Material

- Freeze-dryer
- Mill

- Analytical balance
- Glass tubes (10 ml, pressure resistant, with Teflon-lined screw-caps)
- Multiple-position magnetic stirrer (e.g. Variomag Telesystem HP 15 or Poly 15, or IKAMAG RO 15 Power, all with 15 stirring points)
- Disposable syringes (5 ml)
- Custom-made rack holding syringes upright on the magnetic stirrer
- Glass fibre filters (e.g. GF/F, Whatman)
- Cork borer (well sharpened; size matching the inner diameter of syringes)
- Stop cocks with Luer lock fitting the syringe tips
- Magnetic stirring bars (5 mm length)
- Volumetric flasks (100, 500, 1000 ml)
- Pipettes (e.g. Eppendorf Multipette and/or Varipette; 100—500 µl and 7 ml)
- Glass vials (e.g. 1.6-ml HPLC vials, with Teflon-lined caps), individually weighed to the nearest 0.1 mg
- Test tubes (10 ml)
- Vortex
- Water or dry bath (95 °C)
- Spectrophotometer (set at 550 nm)

2.2. Chemicals

- Methanol, reagent grade
- Deionized water (e.g. Nanopure®)
- $FeSO_4 \cdot 7\ H_2O$
- n-Butanol, reagent grade
- Concentrated HCl (37%)
- Quebracho tannin (preferably purified; see http://www.users.muohio.edu/hagermae), optional

2.3. Solutions

- Solution 1: 50% methanol:H_20 (v/v).
- Solution 2: Dissolve 700 mg $FeSO_4 \cdot 7\ H_2O$ in 50 ml conc. HCl and adjust volume to 1000 ml with n-butanol.
- Solution 3: Stock solution of quebracho tannin standard (10—100 mg l^{-1}, depending on purity of tannin): weigh out 10—100 mg of (purified) quebracho tannin to the nearest 0.1 mg and dissolve in 100 ml of Solution 1, then dilute 10 fold with Solution 1.
- Standards: Use Solutions 1 (50% methanol) and 3 to prepare quebracho tannin standard solutions in the range 0—2.0 mg ml^{-1} or lower depending on purity of standard used.

3. EXPERIMENTAL PROCEDURES

3.1. Tannin Extraction

1. Dry leaves and grind to powder that passes through a 0.5-mm mesh screen.
2. Cut discs from glass fibre filters with a well-sharpened cork borer and place inside the disposable syringes.
3. Connect syringes to stop cocks with valves closed.
4. Add 50 mg sample material (weighed to the nearest 0.1 mg) to the syringes.
5. Place a small stirring bar in each syringe.
6. Place syringes on custom-built rack on the magnetic stirrer. The Luer ends of the syringes may have to be slightly shortened to minimize the vertical distance between the surface of the magnetic stirrer and the stirring bars in the syringes, so as to ensure continuous movement of the bars during extraction.
7. Add 400 µl of 50% methanol (Solution 1).
8. Connect plungers to the top of syringe barrels.
9. Extract tannins for 30 min with stirring at room temperature.
10. Filter extract directly into tared HPLC vials by slowly pushing plunger into the syringe barrel.
11. Repeat extraction three more times with 350 µl of Solution 1 (50% methanol) each time.
12. Rinse the stop cock with 50 µl methanol (50%) after the first two extraction steps.
13. Cap vials and reweigh them to the nearest 0.1 mg.
14. Calculate the volume of the extract, assuming a density of 0.9266 g ml^{-1} for 50% methanol.

3.2. Spectrophotometric Analysis

1. Pipette exact volume of 100—500 µl sample extract in test tube.
2. Add appropriate volume of deionized water to adjust total volume (i.e. sample extract plus water) to 500 µl.
3. Add 7 ml of Solution 2 ($FeSO_4 \cdot 7\ H_2O$) and vortex.
4. Measure absorbance at 550 nm (control to correct for colour of extract).
5. Place tube in water bath at 95 °C and incubate for exactly 50 min.
6. Let cool to room temperature before measuring absorbance again at 550 nm.
7. Calculate absorbance due to the acid butanol reaction by subtracting the absorbance before heating from that after heating.
8. If (purified) quebracho tannin is available, proceed in the same way with the standard tannin solutions to establish a standard curve.
9. Express results in relative units or, preferably, in (purified) quebracho tannin equivalents based on absorbance readings and the standard curve.

4. FINAL REMARKS

Acetone interferes with the acid butanol assay. Consequently, the acetone-water mixtures commonly used for extracting tannins (e.g. Chapter 15) cannot be used unless the extract is completely evaporated and the residue redissolved in a solvent compatible with the assay.

The assay is very sensitive to varying amounts of water. It is essential to ensure, therefore, that the volumetric ratio of Solution 1 and 2 is always 1:14 (e.g. 500 µl of Solution 1 plus 7 ml of Solution 2). The water content is then 6.8%, which is close to the water content found by Porter et al. (1986) to yield the highest colour yield.

Waterman & Mole (1994) suggested not using an unheated reagent-sample mixture because some substances in plant tissue may yield red coloration even without heating. However, in our experience with a wide range of leaf litter from deciduous trees and shrubs, this potential problem is not generally encountered. Conversely, the substitution of HCl by H_2O as recommended by Waterman & Mole (1994) can result in precipitates.

A proanthocyanidin standard of sufficient purity is not commercially available, limiting quantitative comparisons among studies. To improve this situation, the use of purified quebracho tannin has been recommended; a protocol for purification – along with a wealth of useful information on tannin structural chemistry, other purification methods, biological activities and biosynthesis – can be downloaded from http://www.users.muohio.edu/hagermae, maintained by A.E. Hagerman. Alternatively, commercial cyanidin can be used as a relative standard (Hagerman & Butler 1989), keeping in mind that its colour yield differs from that of delphinidin. Procyanidin and prodelphinidin are also commercially available.

The standard curve may be discontinuous, the reason for which is unknown (Waterman & Mole 1994). One possibility to circumvent this effect may be to dilute sample extracts and use 5-cm or 10-cm cuvettes instead of standard 1-cm cuvettes for spectrophotometric measurements.

5. REFERENCES

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