

# ***Determination of Degree of Polymerization***

*HPLC of phloroglucinol derivatives*

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## ***Introduction***

Modified from Foo and Karchesy, *Phytochemistry* 30, 667-670 (1991); Koupai-Abyazani et al. *J. Chrom.* 594, 117-123 (1992).

Quantitative application for molecular weight determination in Schofield, Hagerman & Harold, *J. Chem. Ecol.* August 1998.

Proanthocyanidins are incubated with phloroglucinol in acidic ethanol. The interflavan bonds break and the extender units react to form the phloroglucinol adduct. The adduct, and unreacted terminal flavanols, are extracted into ethyl acetate and analyzed by reverse phase HPLC. For (4->8) or (4->6) procyanidins the adducts are catechin or epicatechin, substituted at position 4 by phloroglucinol. The terminal unit is unmodified catechin or epicatechin. It is believed that (4->6) interflavan bonds are less easily cleaved than the (4->8) interflavan bonds.

## ***Reagents***

- Acidic ethanol. 1% HCl in ethanol--make 1.0 mL conc HCl up to 100 mL with absolute ethanol.
- Phloroglucinol solution. 5 mg/mL phloroglucinol in the acidic ethanol. Prepare fresh daily.
- 70% aqueous methanol

## ***Reaction with pure proanthocyanidin standards***

1. Pure proanthocyanidin (1 mg) is dissolved in 150  $\mu$ L of the phloroglucinol solution and allowed to react at room temperature overnight.
2. The solvent is then evaporated under nitrogen, and the residue dissolved in 50  $\mu$ L distilled water.
3. This solution is extracted three times with ethyl acetate (150  $\mu$ L per extraction).

4. The three ethyl acetate fractions are combined and evaporated under nitrogen.
5. The residue is dissolved in 100  $\mu$ L of 70% aqueous methanol and then injected onto the HPLC.
6. A parallel sample is run substituting acidic ethanol for the phloroglucinol solution.

Purified catechin, epicatechin and phloroglucinol can be used to identify those peaks on the chromatograms. The usual order of elution is phloroglucinol; epicatechin phloroglucinol, catechin phloroglucinol (as a pair); catechin; epicatechin. A purified proanthocyanidin of known composition is used to confirm the identity of the phloroglucinol adducts.

For example, Sorghum proanthocyanidin is comprised of catechin terminal units and predominantly epicatechin extender units. Purified proanthocyanidin does not have any low molecular weight, ethyl acetate soluble constituents. Because high molecular weight procyanidins are difficult to resolve on HPLC, the samples in which acidic alcohol is substituted for the phloroglucinol should not show any peaks on the HPLC.

Average chain length for the purified proanthocyanidin can be determined by comparing the production of the phloroglucinol-derivitized extenders and of the terminal units. A standard curve must be prepared to convert peak areas to molar concentrations, using pure authentic samples of monomers and adducts.

## ***HPLC system***

- Solvent A: 1% aqueous acetic acid (1.0 mL glacial acetic acid up to 100 mL with distilled & purified water).
- Solvent B: Methanol:Solvent A, 60:40, v/v
- Column: Ultrasphere 5 RP-18 (4.6 mm x 25 cm) (Beckman)
- Guard column: 4 x 4 mm, RP-18 packing
- Runs: Ambient temperature, 1 mL/min, 20  $\mu$ L injection loop, 280 nm detection
- Linear solvent gradient: t = 0, 100% solvent A; t = 60 min, 40% solvent A, 60% solvent B; t = 65 min, 100% solvent B

## ***Assay of crude extracts***

### ***Sample preparation***

1. The aqueous acetone extract from leaves or other plant tissues is evaporated under nitrogen.
2. The residue is dissolved in 150  $\mu$ L of phloroglucinol solution and incubated at room temperature overnight.
3. The sample is evaporated with nitrogen, the residue dissolved in 100  $\mu$ L distilled water, and extracted three times with ethyl acetate (300  $\mu$ L per extraction).
4. The ethyl acetate extracts are combined, evaporated under nitrogen, and dissolved in 75  $\mu$ L of 70% methanol for separation by HPLC.
5. For each extract, a control sample is run, substituting acidic ethanol for the phloroglucinol solution.

The terminal units and phloroglucinol-derivitized extender units are identified by comparison to the standards. The chromatograms are far more complex than for the purified proanthocyanidins, because crude plant extracts contain many ethyl acetate soluble components such as small, nontannin phenolics. The chromatograms of the control samples (not reacted with phloroglucinol) will provide quantitative information about those peaks. Comparison of peak areas can be used as above for determination of an average chain length for the proanthocyanidins.