

# *Thiolysis procedure for proanthocyanidin*

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## *Introduction*

This method is taken from Meth Enz 2001 335: 57-70 (Guyot et al.) and Scioneaux et al, *J. Chem. Ecol.* 2011 (in press).

**Caution: Toluene- $\alpha$ -thiol is very volatile and smells bad. Like all thiols it damages the liver. Every step of the thiol reaction must be carried out in a working fume hood. All reaction mixtures, vials, pipette tips, HPLC vials should be soaked in a dilute Clorox solution before discarding in the trash. Add a little Clorox to the HPLC waste bottle to keep the odor minimized in the HPLC room.**

## *Reagents*

- 32% HCl in Methanol (32 mL conc HCl up to 100 mL with methanol)
- 5.0% toluene- $\alpha$ -thiol in Methanol (2.5 mL T $\alpha$ T up to 50 mL with methanol)

## *Procedure*

1. Dissolve a sample of tannin in 200  $\mu$ L of methanol in a small microfuge tube. Transfer 100  $\mu$ L to a 2<sup>nd</sup> tube. One sample will be used to measure flavan-3-ol monomers, the other sample for the reaction to measure thiolysis products.
2. To the flavan-3-ol sample, add 10  $\mu$ L of acidic methanol and 24  $\mu$ L of methanol. Mix. These can be filtered and run on the HPLC while the reaction is run on the thiolysis samples.
3. To the thiolysis samples add 10  $\mu$ L of acidic methanol and 24  $\mu$ L of T $\alpha$ T reagent. Mix and incubate at 40 $^{\circ}$  for 30 min. Immediately transfer to the freezer for 5 min. Samples are now ready to filter and run on the HPLC.
4. We use cellulose membrane centrifugal filters for all samples on the HPLC. Filter the samples (1 min at 10 K rpm in the hood), transfer to vials, and run. Detect at 220 nm. Currently using on the 1090 HPLC LI6\_01.M with TFA-modified acetonitrile and water.

It is hard to guess how large the peaks will be, so you may need to run several concentrations of a given sample to get a nice-looking chromatogram.

## *Data analysis*

Use catechin, epicatechin, and any other flavan-3-ols that are available as standards. Use a proanthocyanidin of known composition, such as sorghum PC, to identify epicatechin thiol.

The flavan-3-ol sample will have essentially no defined peaks for polymeric PA, just a large unresolved set of late peaks representing polymers. In crude plant extracts or unpurified tannin, flavan-3-ols that are present as monomers, dimers and trimers are identifiable in the extract.

In order to quantitate, run standard curves of area vs. mass for each compound and convert peak areas to mass. You can purify the thiol adducts by Sephadex chromatography, and use them as the standards for quantification of extender units. Quantification allows calculation of average chain length, as number of extender units per terminal (flavan-3-ol) unit. Be sure to correct the latter for any unreacted flavan-3-ol in the sample.