

# ***Metmyoglobin Assay***

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## ***Introduction***

The metmyoglobin assay is a rapid method which provides the degree of antioxidant protection possessed by an individual species. Original descriptions of the method can be found in References 1-3, and our application to tannins in Reference 4.

The reagents for the metmyoglobin method can be purchased as a kit from Randox. It is much less expensive to prepare them yourself as follows.

## ***Reagents***

- PBS Buffer (0.005 M Phosphate, 0.145 M NaCl)
- A: Dissolve 0.68 g KH<sub>2</sub>PO<sub>4</sub> and 8.5 g NaCl in 1 L of distilled water
- B: Dissolve 1.14 g K<sub>2</sub>HPO<sub>4</sub> x H<sub>2</sub>O and 8.5 g NaCl in 1 L of distilled water
- Mix the two solutions together to give a pH of 7.4 (approximately 77 mL of A and 200 mL of B). (Final concentration in assay is 5 mM).
- Myoglobin (initial concentration between 1 mg myoglobin/mL PBS to 5 mg/mL). The initial concentration doesn't matter since the solution will be diluted as it runs through the column. The myoglobin is purchased from Sigma (# M-0630). K<sub>3</sub>Fe(CN)<sub>6</sub> (0.24 mg K<sub>3</sub>Fe(CN)<sub>6</sub> in 1 mL water)

- 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (0.8 mg ABTS in 1 mL PBS buffer). The ABTS is purchased from Sigma (# 1888). (Final concentration in the assay is 610  $\mu$ M).
- Trolox Standard (0.1564 g Trolox in 250 mL PBS buffer). The Trolox is purchased from Aldrich (# 23,881-3). If the solid does not dissolve, sonicate gently. (Final concentration in the assay is 2.5 mM).
- Tannin Sample (1 mg tannin/mL of water). Make sure the actual concentration used is recorded in the laboratory notebook for the calculations. Prepare 1:10 dilutions.
- Hydrogen Peroxide Dilute the 30 % w/v stock solution as follows: #1 1 part (H<sub>2</sub>O<sub>2</sub>) plus 9 parts water #2 1 part (#1) plus 9 parts water #3 1 part (#2) plus 9 parts water #4 1.7 parts (#3) plus 8.3 parts PBS buffer (Final concentration in the assay is 250  $\mu$ M).
- Sephadex G-10-120 column (Height of 20 cm and Diameter of 1 cm). Equilibrate the column with the PBS buffer before using. This column can be prepared and reused indefinitely, stored at room temperature.
- Metmyoglobin. Mix equal volumes of myoglobin and K<sub>3</sub>Fe(CN)<sub>6</sub> solutions. Run sample through column and collect the second fraction where the brown color starts to come off the column. The first fraction is just buffer and the third fraction is yellow containing the K<sub>3</sub>Fe(CN)<sub>6</sub>. Read the Abs @ 490 nm of the second fraction. Adjust the solution with buffer to give an absorbance reading of 0.147 so that the final concentration of metmyoglobin in the assay will be 6.1  $\mu$ M). The equations used to calculate the amounts of the various forms of the myoglobin are found in Reference #3.

## ***Procedure***

1. Set up an appropriate number of 0.65 mL microfuge tubes to run each sample in duplicate or triplicate.
2. Add the 20  $\mu$ L of the sample (water, standard, or tannin), 250  $\mu$ L of metmyoglobin and 250  $\mu$ L of ABTS to the tubes. Vortex the tubes.
3. Using a microcuvette (1 mL), blank the spectrophotometer at 600 nm with water.
4. Read the absorbance of each sample and record as Abs1.
5. Add 100  $\mu$ L of the hydrogen peroxide substrate to the tubes.
6. After exactly 3 minutes, read the absorbance and record as Abs2. (Hint: Add the substrate to four tubes at 30 second intervals. This provides enough time to read each sample at exactly three minutes).
7. Subtract Abs1 from Abs2 for each sample.
8. The typical change in absorbance for the control (water as sample) is 0.296.

## ***Calculations***

The amount of putative antioxidant required to suppress absorbance of the ABTS radical cation by 50% is compared to the amount of Trolox required for 50% suppression in order to compare potency of various antioxidants. A Trolox standard curve is run with each set of samples because there is substantial day-to-day variability in the assay.

## ***Chemical preparation of ABTS radical cation***

Described by Re et al. (Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C Antioxidant activity applying an improved ABTS radical cation decolorization assay *Free Rad Biol Med* 1999; 26(9-10):1231-7). ABTS is prepared in the desired buffer at 3.84 mg/mL (7.01 mM). That solution (15 mL) is mixed with 1 mL of  $K_2S_2O_8$  (10.6 mg/mL, 39.2 mM) prepared in the same buffer. The mixture is incubated at room temperature in the dark for 16 hours, and is then diluted with buffer to obtain the working solution of radical cation. This method has fewer side reactions and is much simpler than the metmyoglobin method.

The  $\lambda_{max}$  of the ABTS radical cation is 734 nm, and there is a linear relationship between radical cation concentration and absorbance through at least an absorbance of 2.0. The extinction coefficient  $E$  is  $12867 M^{-1}cm^{-1}$  and is independent of pH at pH values 3-7.4.

## ***ABTS radical cation quenching and capacity by tannin-protein complexes***

As described in Riedl, K.M.; Hagerman, A.E. Tannin-protein complexes as radical scavengers and radical sinks. *Journal of Agricultural and Food Chemistry* 2001 49, 4917-4923.

### ***Quenching***

Protein and procyanidin are mixed in 1.5 mL cuvettes by combining 450  $\mu$ L protein solution (0-180  $\mu$ g/mL) with 450  $\mu$ L of PC solution (4-8  $\mu$ g/mL). The solution was inverted to mix, and incubated for 10 min at room temp before zeroing the spectrophotometer at 734 nm. The decolorization reaction was initiated by adding 100  $\mu$ L of 65  $\mu$ M ABTS+•, and immediately mixing by inversion and placing the mixture in the spectrophotometer. Under these conditions, the  $A_{734}$  at the beginning of the reaction was about 0.7.

### ***Capacity***

To maintain a large excess of radical, 163 nmoles (150  $\mu$ L of 1.09 mM ABTS+•) is added to 900  $\mu$ L of solution containing 0.203 nmoles (1  $\mu$ g) of PC at the desired pH. The starting  $A_{734}$  is about 2.0. The absorbances of both tannin-free control, and of PC-containing samples, are monitored during the reaction to account for the spontaneous decolorization of ABTS+•, which increases as pH is increased. The amount of ABTS+• scavenged at any time corresponds to the difference in absorbance between the control and the PC sample. The extinction coefficient of the ABTS+• can be used to calculate the number of moles of radical scavenged per mole of PC.

## ***References***

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