PHENOLIC AND ANTIOXIDANT ASSAYS

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A number of spectrophotometric methods for quantification of phenolic compounds in plant materials have been developed. Based on different principles, these assays are used to determine various structural groups present in phenolic compounds. For many applications, a simple measure of the total amounts of phenolics is often desired. The most common method available for this purpose is a spectroscopic method based on the Folin-Ciocalteau reagent.
Folin-Ciocalteu Assay

- Reduction of phosphomolybdic-phosphotungstic acid (Folin) reagent to a blue-colored complex in an alkaline solution occurs in the presence of phenolic compounds.

- The Folin-Ciocalteu reagent is not specific and detects all phenolic groups found in extracts, including those found in the extractable proteins.

- A disadvantage of this assay is the interference of reducing substances such as ascorbic acid with the determinations.

- Therefore, the FC assay was recently proposed for the measurement of total reducing capacity of samples.
Folin-Ciocalteu Assay

- The total phenolics are assayed colorimetrically as modified by Singleton and Rossi (1965) and Hoff and Singleton (1977):

  2.5 mL of 10-fold diluted Folin-Ciocalteu reagent,
  +
  2 mL of a 7.5% solution of sodium carbonate, and
  +
  0.5 mL of phenolics solution are mixed well.

- The absorbance is measured at 765 nm after a 15-min heating at 45°C; a mixture of water and reagents is used as a blank.
The results are usually expressed as gallic acid equivalents (GAEs), however, this choice is somewhat arbitrary, and other reference standards have been used depending upon the composition of the sample and the purpose of the investigation.

It should be remembered that the method measures the number of potentially oxidizable phenolic groups.

The number of phenolic groups per molecule will vary greatly both within and among different phenolic compound classes.

This procedure still provides a very useful index for phenolic content, but it would not be expected to correlate with the actual weight of phenolics present.

A number of investigations, however, have shown high correlation between total phenolics measured by this method and antioxidant activity as measured by various procedures.
FC assay

- is operationally simple,
- reproducible and
- convenient for assessment of dietary antioxidant capacity since the reagent is commercially available,
- the procedure is rather standardized,
- and the absorption of the product at a long-wavelength minimizes interferences from the sample matrix.
- But, it is performed in aqueous phase, thus it is not applicable for lipophilic compounds/matrices.
Antioxidant Capacity

- Antioxidants have thus become a topic of increasing interest recently.
- A literature search revealed that the number of publications on antioxidants and oxidative stress has nearly quadrupled in the past decade (1684 in 1993; 6510 in 2003)
Antioxidant

- The dictionary definition of antioxidant is:
- “a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides, many of these substances being used as preservatives in various products”
- A more biologically relevant definition of antioxidants is:
- “synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air”
On the basis of the chemical reactions involved, major antioxidant capacity assays can be roughly divided into two categories:

(1) hydrogen atom transfer (HAT) reaction based assays
(2) single electron transfer (ET) reaction based assays

- The ET-based assays involve one redox reaction with the oxidant (also as the probe for monitoring the reaction) as an indicator of the reaction endpoint.
- Most HAT-based assays monitor competitive reaction kinetics, and the quantization is derived from the kinetic curves.
- HAT-based methods generally are composed of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant.
- HAT- and ET-based assays are intended to measure the radical (or oxidant) scavenging capacity, instead of the preventive antioxidant capacity of a sample.
Scavenging of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (ABTS•⁺) or Trolox equivalent antioxidant capacity (TEAC) assay

- The TEAC assay involves the generation of the long-lived radical cation chromophore 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS•⁺) which has absorption maxima at 414, 645, 734, and 815 nm.

ET Based

probe (oxidant) + e (from antioxidant) → 
reduced probe + oxidized antioxidant
In terms of assay conditions, different strategies have been implemented for ABTS•+ generation, reaction time applied, detection wavelength used for monitoring the reaction, and the reference antioxidant chosen.

ABTS•+ radical cation can be generated by chemical reaction using manganese dioxide, AAPH, or potassium persulfate, by enzymatic reaction using metmyoglobin or horseradish peroxidase, or by electrochemical generation.

Reaction times ranging from 1 to 30 min have been adopted throughout the protocols described in the literature.

Concerning the wavelength of detection, the determination at 734nm is preferred because the interference from other absorbing components and from sample turbidity is minimized.
In terms of quantification, the absorbance value, proportional to the remaining ABTS•+ concentration, is measured after a fixed reaction time.

Results are expressed as Trolox equivalents, that is, the concentration of Trolox solution (mM) with an antioxidant capacity equivalent.
This spectrophotometric assay is technically simple, which accounts for its application for screening and routine determinations.

The ABTS•+ scavenging can be evaluated over a wide pH range, which is useful to study the effect of pH on antioxidant mechanisms.

The ABTS•+ radical is soluble in water and organic solvents, enabling the determination of antioxidant capacity of both hydrophilic and lipophilic compounds/samples.

However, as the results obtained for samples are related to an antioxidant standard compound that shows different kinetic behaviour, the results provided by this assay are dependent of time of analysis.

Moreover, this assay has been criticized as the ABTS•+ radical is not representative of biomolecules and not even found in any biological system.

Thermodynamically, any compound that has a redox potential lower than that of ABTS•+ may react with the radical.
Procedure (Re et al., 1999)

- 9.7 mg ABTS + 2.5 mL water
- 37.5 mg potassium persulfate + 1 mL water
- Shake
- 44 µL of this solution is added to solution of ABTS (all solution)
- Allow the mixture to stand in the dark at room temperature for 12-16 h before use.
- (The radical was stable in this form for more than two days when stored in the dark at room temperature)
For the study of phenolic compounds and food extracts, the ABTS solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C.

Working Solution

ABTS:%50 ETOH = 1:88
100 µL sample + 1 mL ABTS

After 4 min

Read absorbance at 734 nm.
The antioxidant capacity was expressed as mmol of Trolox equivalent antioxidant capacity (TEAC) per kg of sample by means of a dose-response curve.