16.

Determination of Total Phenolics

Phenolic compound are ubiquitous in plants and other biological sources, and are the most commonly investigated group of secondary metabolites. Phenolic compounds found in nature include simple phenols, lignans, coumarins, chromones, flavonoids, and different types of tannins. Phenolic hydroxy groups may also be found in alkaloids and less commonly in terpenes. Determination of total phenolics involves quantification of the total concentration of phenolic hydroxy groups present in an extract.

Determination of Total Ploy phenol in Tea

Colorimetric Method Using Folin-Ciocalteu (F-C phenol) Reagent (Based on ISO 14502-1)

Principle Polyphenols are extracted with 70 % methanol from a test portion of finely ground leaf tea at 70°C. Instant teas are dissolved in hot water with 10 % acetonitrile (volume fraction) added to stabilize the extract. The polyphenols in the extract are determined colorimetrically using the Folin-Ciocalteu (F-C) phenol reagent.
The F-C reagent contains phospho-tungstic acids as oxidants, which on reduction by readily oxidized phenolic hydroxy groups yield a blue colour with a broad maximum absorption at 765 nm. This is due to the formation of so-called tungsten and molybdenum blues. The F-C phenol reagent reacts with a wide range of polyphenolic compounds and, although the response can vary with the individual components, selection of gallic acid as a calibration standard enables useful total polyphenol data to be obtained.

Reagents

Methanol/water extraction mixture, 70 % methanol (volume fraction). Add 700 ml of the methanol to a 1 litre volumetric flask. Dilute to the mark with water (4.1) and mix.

Folin-Ciocalteu (F-C) phenol reagent, commercially available ready prepared ..

Dilute F-C phenol reagent, 10 % (volume fraction).
Using a pipette, transfer 20 ml of F-C phenol reagent to a 200 mlvolumetric flask. Dilute to the mark with water and mix.
(Prepare fresh reagent solution daily. To avoid contamination of the concentrated Folin-Ciocalteu reagent, discard any unused dispensed reagent.)

Sodium carbonate solution, 7,5 % (mass concentration).
Weigh (37,50 ± 0,01) g of anhydrous sodium carbonate (Na_2CO_3) into a 500 ml volumetric flask. Add sufficient warm water to half-fill the flask. Swirl to dissolve the sodium carbonate, cool to room temperature, dilute to the mark with water and mix.
NOTE This solution will remain stable at room temperature for up to 1 month.

Gallic acid stock standard solution, corresponding to approximately 1 000 µg/ml of anhydrous gallic acid .
Weigh (0,110 ± 0,001) g of gallic acid monohydrate (M = 188,14) into a 100 ml volumetric flask. Dissolve in water, dilute to the mark and mix. Prepare a fresh standard solution daily.

NOTE Gallic acid monohydrate is preferred over anhydrous, due to its greater solubility, reduced hygroscopic properties and availability of certified reagent grades, If not known, the moisture content (loss in mass at 103 °C) on a portion of the standard material should be determined. The concentration of the stock standard solution on a gallic acid anhydrous basis can then be calculated.

Gallic acid standard solutions A to E Using pipettes, transfer the volumes of gallic acid stock standard solution given in Table 1 to 100-ml one mark volumetric flasks. Dilute to the mark with water and mix. These dilute standard solutions should be prepared on the day of use.

Gallic acid dilute standard solutions

Gallic acid standards Solutions	Volume of gallic acid stock solution Ml	Nominal concentration of the dilute standard µg/ml
A	1.0	10
B	2.0	20
C	3.0	30
D	4.0	40
E	5.0	50

Preparation of test samples
To ensure homogeneity, grind the sample of leaf tea and store samples in well sealed containers protected from light. Grinding of instant tea is only required on samples of a coarse granular structure.

Procedure

General If it is required to check whether the repeatability limit is met, carry out two single determinations
Determination of dry matter content Calculate the dry matter content from the moisture content (loss in mass at 103 °C) - 5g leaf tea or instant in duplicate.
Test portion :Instant tea Weigh (0,500 ± 0,001) g of the test sample into a 50 ml one-mark volumetric flask.
Leaf tea Weigh (0,200 ± 0,001) g of the test sample into an extraction tube

Extraction of Polyphenols

Instant tea Add to the instant tea in the flask approximately 25 ml of hot water (maximum temperature 60 °C). Mix to dissolve the sample and cool to room temperature. Add 5,0 ml of acetonitrile. Dilute to the mark with water and mix.

Leaf tea Place the methanol/water extraction mixture contained in the dispenser in the water
bath set at 70 °C, and allow at least 30 min for the extraction mixture to equilibrate.

Place the extraction tube containing the sample in the water bath set at 70 °C. Dispense 5,0 ml of hot methanol/water extraction mixture into the extraction tube, stopper and mix on the vortex mixer. Continue heating the extraction tube in the water bath for 10 min, mixing on the vortex mixer after 5 min and 10 min. It is important to mix the samples thoroughly to ensure complete extraction. Remove the extraction tube from the water bath and allow it to cool to room temperature. Remove the stopper and place the tube in the centrifuge at 3 500 r/min for 10 min. Carefully decant the supernatant into a graduated tube. Repeat extraction steps. Combine the extracts, make up to 10 ml with cold methanol/ water extraction mixture and mix the contents.

The extract is stable for at least 24 h if stored at 4 °C. Allow the extract to attain room temperature before proceeding with the assay. Re-suspension of the small amount of particulate material that may settle during storage is not necessary.

Pipette 1,0 ml of the sample extract (instant tea extract leaf tea extract) into a 100 ml volumetric flask. Dilute to the mark with water and mix.

Determination

Using a pipette, transfer 1,0 ml of the gallic acid standard solutions A, B, C, D and E in duplicate, into separate plastic or graduated tubes.
NOTE; These correspond to approximately 10 µg, 20 µg, 30 µg, 40 µg and 50 µg of anhydrous gallic acid.

i. Pipette 1,0 ml of water, in duplicate, into separate tubes. These are reagent blanks.

ii Pipette 1,0 ml of diluted sample extract in duplicate, into separate tubes.

iii Pipette 5,0 ml of dilute F-C phenol reagent into each tube and mix. Within 3 min to 8 min after the addition of the dilute F-C phenol reagent, pipette 4,0 ml of sodium carbonate solution into each tube. Stopper and mix. Allow to stand at room temperature for 60 min, and then measure the optical densities in 10-mm path length cells against water on the spectrophotometer set at 765 nm. The reagent blank should have an optical density of < 0,010. Higher values indicate contamination problems from poor quality water, reagents or glassware. It is also important that the sample optical density be within the calibration range. Repeat the colorimetric assay with an increased dilution if the sample optical density is higher than the 50 µg gallic acid standard E.

Calculation

Calculate, to the nearest 0,1 µg, the mass of anhydrous gallic acid, *m*, in the 1,0 ml aliquots of the Standard solutions A, B, C, D and using the formula:

$$m = \frac{m_0 \times V \times {}^{w}DM,std \times 10000}{100 \times 100}$$

Where
M_0 = is the mass of gallic acid monohydrate in grams used to prepare the stock standard solution

V = is the volume of gallic acid stock standard solution in milliliters used to prepare the standard solution

${}^{w}DM$ std = is the dry matter content expressed as a mass fraction in percent of gallic acid

Construct a best fit linear calibration graph from the mass of anhydrous gallic acid standards as calculated from the above formula, against the gallic acid standards optical densities (absorbance) after subtracting the reagent blank optical density.

Obtain the calibration line slope and intercept value. A linear calibration should be obtained. Calculate the calibration line slope value to nearest 0.00001 for use in subsequent calculations. The linear calibration should also have intercept close to the origin.

The total polyphenol content (w_T) expressed as a percentage by mass on a sample dry matter basis is given by the formula.

$$W_T = \frac{(D_{sample} - D_{Intercept}) \times V_{sample} \times d \times 100}{S_{std} \times {}^{m}sample \times 10000 \times {}^{W}DM_{sample}}$$

D_{sample} = is the optical density for the sample test solution,
$D_{ntercept}$ = is the optical density at the point the best fit linear calibration line intercept the y-axis,
S_{std} = is the slope obtained from best fit linear calibration,
m_{sample} = is the mass in grams of the sample test portion
V_{sample} = is the sample extraction volume in milliliters (50 ml for instant tea and 10 ml for leaf tea)
d = is the sample dilution factor used prior to the colorimetric determination (typically 1.0 ml to 100 ml, thus a dilution factor of 100)
$W_{DM\ Sample}$ = is the dry matter content expressed as mass fraction in percent of the test sample determined