

Folin-Ciocalteu Reagent for Polyphenolic Assay

Review Article

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Abstract

The chemistry of the Folin-Ciocalteu is described and two Folin assays (single and dual reagent) are described for the assay of phenols and polyphenols with respect to experimental detail and critically evaluated for pure compounds and for mixtures (plant extracts). The single reagent method was found to be more precise and sensitive. The problem of interferences in the Folin assay was evaluated for both methods. Interferences for the dual reagent methodology can be eliminated by a solid phase removal of phenols using a commercial resin (Oasys HLB) or polyvinylpyrrolidone resin (Polyclar AT). A new basic/acid hydrolysis combined with the Polyclar AT was used to measure the total phenols in a sample as previous methods measured only the phenolic groups not bound as ether or ester groups. A semi-automated method, microplate reader, is described as to the experimental procedure and applicability. Miscellaneous uses of the Folin assay including flow injection, urine analysis and a mixed standard are briefly described.

Keywords: Phenol, Folin-Ciocalteu reagent, solid phase extraction

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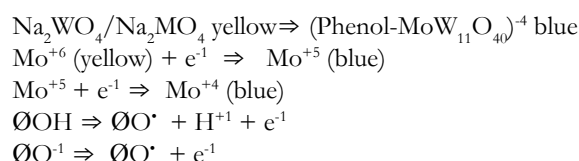
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Description of Folin Ciocalteu Reagent

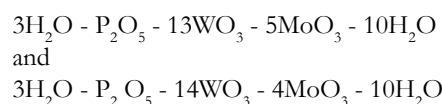
The Folin-Ciocalteu (F-C) method of assay is the simplest method available for the measurement of phenolic content in products. It is a development of Folin Denis reagent used in the early 19th century for the determination of tyrosine in proteins [1]. F-C reagent can be prepared by dissolving 100 g sodium tungstate (VI) dihydrate and 25 g sodium molybdate (VI) dihydrate with 700 ml distilled water, 100 ml concentrated hydrochloric acid, and 50 ml of 85% phosphoric acid to which is added 150 g of lithium sulphate hydrate. This reagent is very stable if protected from reductants and even when diluted if protected from light. For many years now, the F-C method of assay has been in use as a measure of polyphenol in natural products, and the basic mechanism is an oxidation/ reduction reaction with the phenolic group being oxidized and the metal ion reduced.

Chemistry controlling the reaction with Folin-Ciocalteu reagent

Folin-Ciocalteu phenol reagent consists of a mixture of the heteropoly acids, phosphomolybdic and phosphotungstic acids in which the molybdenum and the tungsten are in the 6+ oxidation state. On reaction with a reductant, the molybdenum blue and the tungsten blue are formed and the mean oxidation state of the metals is between 5 and 6.



The above reaction is slow at acidic pH and faster when basic. It is very sensitive, precise but lack specificity. Singleton and Rossi [2] further improved the method with a molybdotungstophosphoric heteropolyanion reagent that reduced polyphenols more specifically with the λ max for the product at 765 nm.



To achieve meaningful, reliable and predictable results, some conditions such as proper volume ratio, optimal reaction time, and temperature for colour development, standard optical density, and use of a particular reference-standard polyphenol are required.

The improved method F-C reagent can be used by two principal procedures for the measurement of polyphenolic content i.e. as dual reagent [2,3] and as single reagent [4,5].

Summary of Manual Dual Reagent Procedure [2]

Up to 100 μL of properly diluted sample in an organic water-miscible solvent with at least 6 mL of water + 0.5 mL of F-C reagent (Sigma); wait between 1 - 8 min, add 1.5 mL of Na_2CO_3 ; mix and bring to 10 mL total volume with water; then measure absorbance 0.5 - 2 hr later at 760 nm against a reagent blank and standards.

The volume can be scaled down to conserve reagents.

Summary of Single reagent Procedure [4,5]

To 10-100 μL of sample, add to a total volume of 1000 μL 10-fold diluted F-C reagent (Sigma) and read 10-20 min later at 750 nm vs. a reagent blank and standards.

Specific antioxidant classes and compounds detected by the F-C reagent

There are over 4000 citations of Folin in Chemical Abstracts and over 500 in PubMed as of December, 2008. Both the dual and the single Folin methods are good for the detection of a wide range of antioxidant compounds in a large variety of plants and plant-derived foods and beverages. The single reagent has been used for phenolic antioxidants from fruits [4,6,7], vegetables [8,9], cereals [7], fruit juices [10,11,12], caffeinated beverages [13,14], alcoholic beverages [15,16] chocolate [17], herbs and spices [18,19] and plant extracts [20] by our group and other investigators. The major classification of antioxidant compounds: flavonols, flavones, flavanones, flavanols, proanthocyanidins, isoflavones, anthocyanins, phenolic acids are detected by the Folin methods.

Procedure for the dual and single reagent assay methods for comparison

Antioxidant compounds representing the different classes of polyphenols listed above were analyzed using both the dual and single reagent methods.

Dual reagent assay

The Singleton and Rossi [2] original method has been modified to suit different laboratory needs. The procedure was applied in our laboratory.

Standard Preparation

Preparation of catechin standard: Dissolve 2.9 mg of catechin powder in 10ml of methanol, therefore resulting in a 1000 μM

solution. Keep in refrigerator when not in use. Prepare a new solution monthly.

Standard Catechin Sample Preparation and Analysis

Standard concentrations of catechin of 0.5, 0 μM , 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 μM , were prepared from the (1000 μM) standard. Prepare standard curve by taking 40 μl of catechin standard solution in to 6 different 10 ml screw cap tubes, the first tube (blank) 40 μl of nanopure water.

Add 800 μl of a 10 fold diluted Folin-Ciocalteu reagent in to each tube and mix well.

Allow the tubes to stand for 5 minutes.

Then add 800 μl of 7% w/v sodium carbonate aqueous solution to each tube and mix well.

Make up volume in each tube with nanopure water (360 μl) to 2mL, mix well, and allowed the tubes to stand for 2hr.

Read absorbance at 760 nm against the blank using a UV-Visible spectrophotometer.

Standard Curve Determination

The absorbances of the standards were plotted against the standard concentrations. The regression line to this graph (Figure 1) was used to determine the catechin equivalent of the polyphenol concentration in the various samples analyzed.

Folin assay of polyphenolic compounds (see Table 1)

Polyphenolic compounds (1mM) were prepared in methanol. 40 μl of sample was transferred in to a 10 ml screw cap tube.

Add 800 μl of a 10-fold diluted Folin-Ciocalteu reagent in to the tube and mix well.

Allow the tube to stand for 5 minutes.

Then add 800 μl of 7% w/v sodium carbonate aqueous solution

Figure. 1. Standard curve: Concentration of catechin against absorbance for the dual reagent method

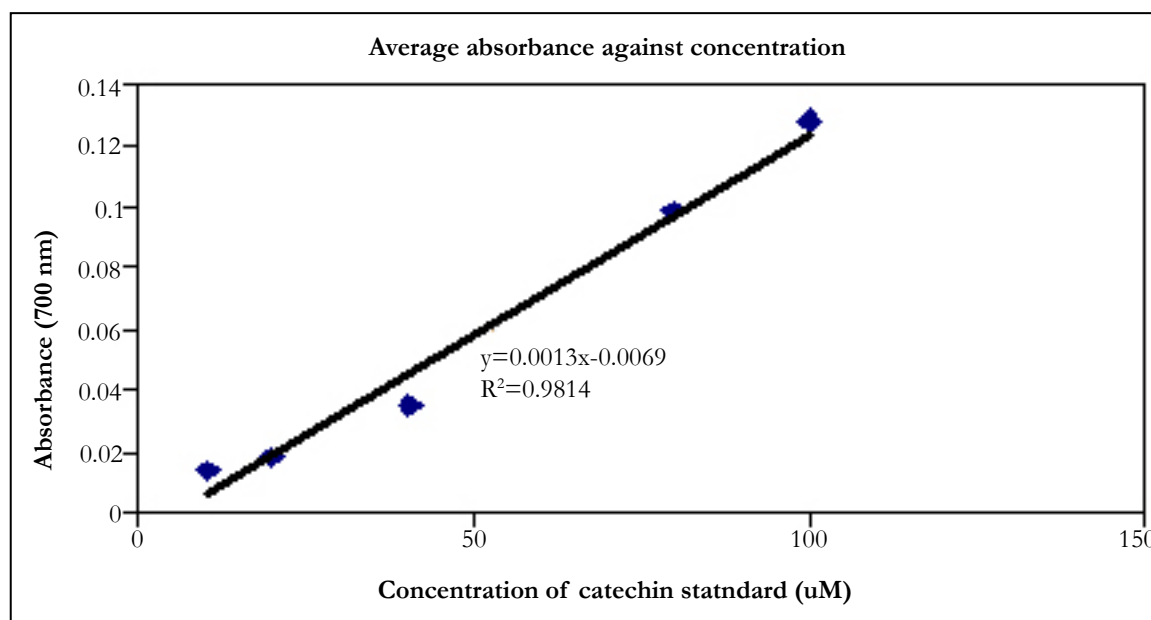


Table 1. Polyphenolic concentration (μM catechin equivalent) of pure compounds and interferences as determined by single and dual reagent method

| pure compounds and interferences | Single reagent Polyphenol (μM) | Single reagent precision (%) | Dual reagent Polyphenol (μM) | Dual reagent precision (%) |
|------------------------------------|---|------------------------------|---|----------------------------|
| Quercetin 1mM | 1132 | 0.7 | 316.1 | 2.07 |
| | ± 7.88 | | ± 6.53 | |
| Kaemferol 1mM | 451 | 2.33 | 164.5 | 2.65 |
| | ± 10.51 | | ± 4.35 | |
| Flavone 1mM | 200 | 1.31 | 82.23 | 6.615 |
| | ± 2.63 | | ± 5.44 | |
| Neohesperidin 1mM | 159.3 | 1.65 | 129.2 | 7.58 |
| | ± 2.65 | | ± 9.79 | |
| Naringin 1mM | 127.8 | 4.11 | 193.4 | 7.59 |
| | ± 5.25 | | ± 14.68 | |
| Taxifolin 1mM | 545.6 | 5.3 | 405.7 | 11.66 |
| | ± 28.89 | | ± 47.32 | |
| EGCG 1mM | 1056 | 1.49 | 591.5 | 7.17 |
| | ± 15.76 | | ± 42.43 | |
| Daidzein 1mM | 139.7 | 4.51 | 98.77 | 12.67 |
| | ± 6.30 | | ± 12.51 | |
| Malvidin 1mM | 380.3 | 13.81 | 195.7 | 18.07 |
| | ± 52.53 | | ± 35.36 | |
| Phloretin 1mM | 148.2 | 1.77 | 236.9 | 18.37 |
| | ± 2.62 | | ± 43.51 | |
| 3-hydroxyanthranilic acid 2mM | 2399 | 0.77 | 350.3 | 5.75 |
| | ± 18.39 | | ± 20.13 | |
| Caffeic acid 2mM | 913.3 | 2.59 | 360.3 | 14.64 |
| | ± 23.64 | | ± 52.76 | |
| Gallic acid 2mM | 709.1 | 1.85 | 577.2 | 0.66 |
| | ± 13.13 | | ± 3.81 | |
| Ferrulic acid 1mM | 203.2 | 2.78 | 252.6 | 12.7 |
| | ± 5.66 | | ± 32.09 | |
| Resveratrol 1mM | 479.9 | 0.93 | 243.4 | 17.21 |
| | ± 4.45 | | ± 41.88 | |
| Vitamin C (10mM) | 2098 | 0.97 | 1135 | 9.15 |
| | ± 20.41 | | ± 103.89 | |
| Tyrosine (10mM) | ND | ND | 218.8 | 2.74 |
| | | | ± 5.98 | |
| Glucose, fructose, sucrose (10 mM) | ND | ND | ND | ND |
| Albumin (5mg/ml) | ND | ND | 493.4 | 0.55 |
| | | | ± 2.72 | |

to the tube and mix well.

Make up volume in the tube with nanopure water (360 μL) to 2mL and mix well. In addition, allow to stand for 2hr at room temperature.

Absorbance was read at 760 nm against the blank using a UV-Visible spectrophotometer.

The regression line $y = mx + c$ from the standard curve was used to determine the polyphenolic concentration of the pure com-

pounds.

where y = absorbance, m = gradient and x = concentration (unknown). This shows the different responses of the compounds.

Thus $x = (y + c)/m$

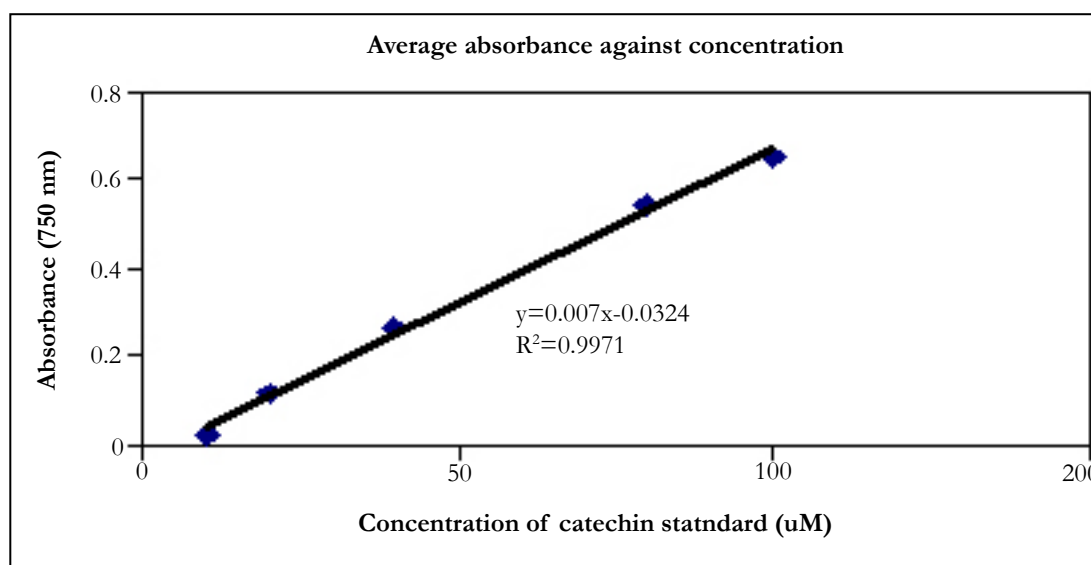
Example: Absorbance of quercetin = 0.410. Based on the standard curve above,

$x = (0.41 + 0.0069)/0.0013 = 321 \mu\text{M}$

Table 2. Free Polyphenolic concentration (mg/g catechin equivalent) of herbs and spices as determined by single and dual reagent method.

| Herbs and spices | Single Reagent Polyphenol (µM) | Single reagent precision (%) | Dual Reagent Polyphenol | Dual reagent precision (%) |
|---------------------------------|--------------------------------|------------------------------|-------------------------|----------------------------|
| P nigrum (leaves) | 7.29 | 4.12 | 22.91 | 7.16 |
| | ±0.30 | | ±1.64 | |
| P nigrum (black pepper, seed) | 7.14 | 1.12 | 22.04 | 1.86 |
| | ±0.08 | | ±0.41 | |
| P umbellatum (leaves) | 10.38 | 1.45 | 39.73 | 2.06 |
| | ±0.15 | | ±0.82 | |
| H cannabinus (leaves) | 5.44 | 1.47 | 12.76 | 3.21 |
| | ±0.08 | | ±0.41 | |
| R. heudelotti (seeds) | 5.86 | 3.92 | 15.08 | 8.16 |
| | ±0.23 | | ±1.23 | |
| S zenkeri (seeds) | 7.97 | 1 | 26.68 | 1.54 |
| | ±0.08 | | ±0.41 | |
| S zenkeri (bark) | 5.38 | 4.65 | 12.47 | 10.75 |
| | ±0.25 | | ±1.34 | |
| A lepidophyllus (seeds) | 6.34 | 5.05 | 17.69 | 13.05 |
| | ±0.32 | | ±2.31 | |
| C mannii (seeds) | 5.7 | 7.89 | 14.21 | 17.31 |
| | ±0.45 | | ±2.46 | |
| I gabonensis (seeds) | 4.69 | 4.9 | 8.7 | 14.14 |
| | ±0.23 | | ±1.23 | |
| T vulgaris (seeds) | 9.05 | 2.54 | 32.48 | 6.87 |
| | ±0.23 | | ±2.23 | |
| G africanum (seeds) | 7.72 | 5.83 | 25.23 | 9.75 |
| | ±0.45 | | ±2.46 | |
| P nigrum (white pepper, seeds)) | 4.32 | 9.95 | 6.67 | 8.1 |
| | ±0.43 | | ±0.54 | |

Figure. 2. Standard curve: Concentration of catechin against absorbance for the single reagent method



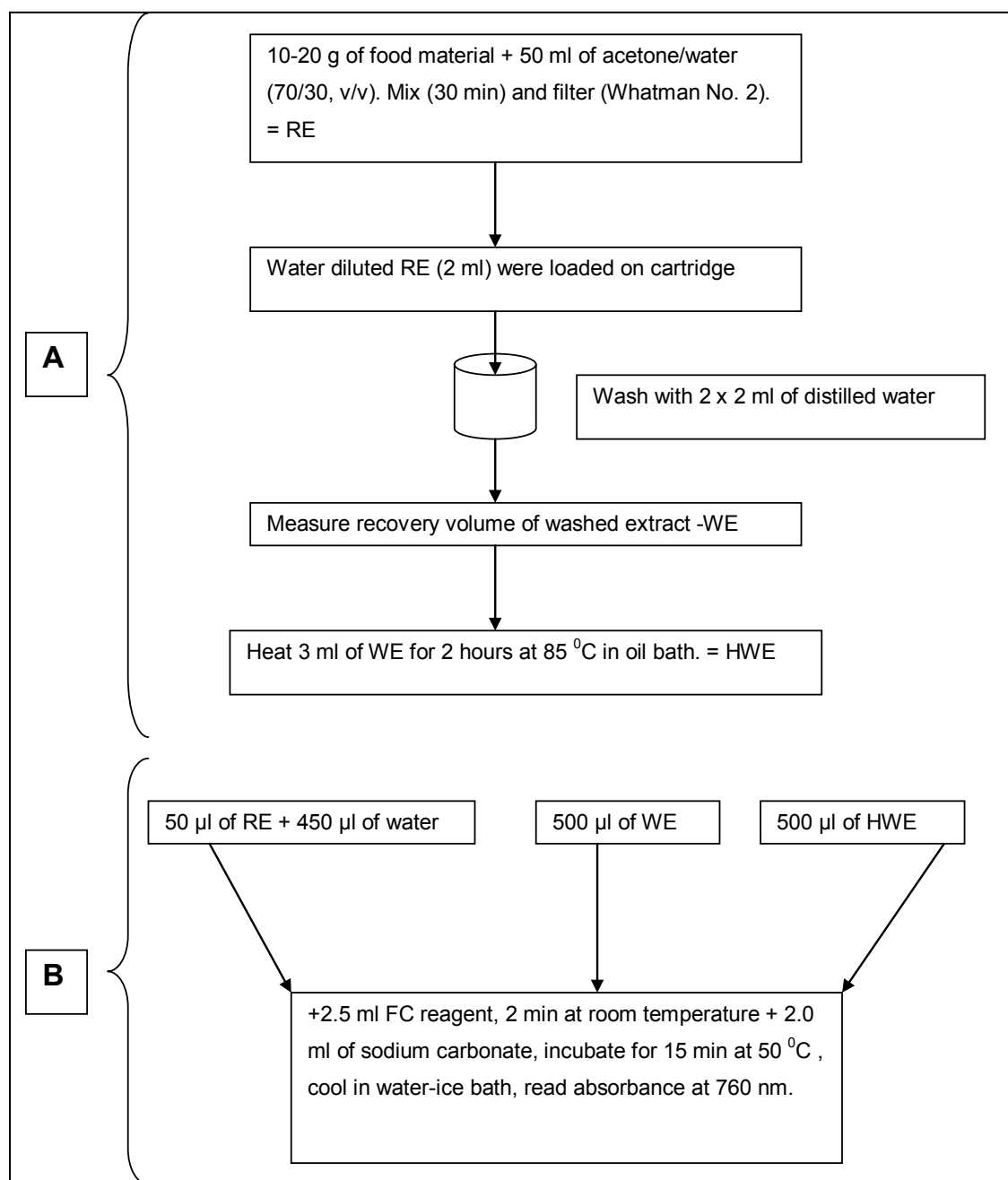
Single reagent assay

Standard Catechin Sample Preparation and Analysis.

Weigh 2.9 mg of catechin powder in 10mL of methanol making a solution of 1mM. This is stable in the refrigerator at 4°C for at least one month.

Prepare standard curve by taking 0, 10, 20, 40, 50, 100 µl of the

Figure 3. Solid phase extraction method to remove interferences prior to Folin assay for free polyphenols



1mM catechin solution to each of 6 micro cuvettes (10 to 100 µM in the cuvette).

Add to a total volume of 1000 µL a 5-fold diluted Folin-Ciocalteu reagent (FCR) and mix well. Allow the tubes to stand for 15 minutes and read absorbance at 750 nm. The colour is stable for 30 minutes.

Standard Curve Determination

The absorbance was plotted against the standard volumes (µl). The regression line to this graph is used to determine the catechin equivalence in the various samples to be analyzed.

Folin assay of polyphenolic compounds

Polyphenolic compounds (1mM) were prepared in methanol. 40 µl of the sample was transfer in to a micro cuvette.

1 ml of a 5-fold diluted Folin-Ciocalteu reagent was added and mixed by inversion.

The cuvette was allowed to stand for 15 minutes and the absorbance read at 750 nm in a UV spectrophotometer against a reagent blank.

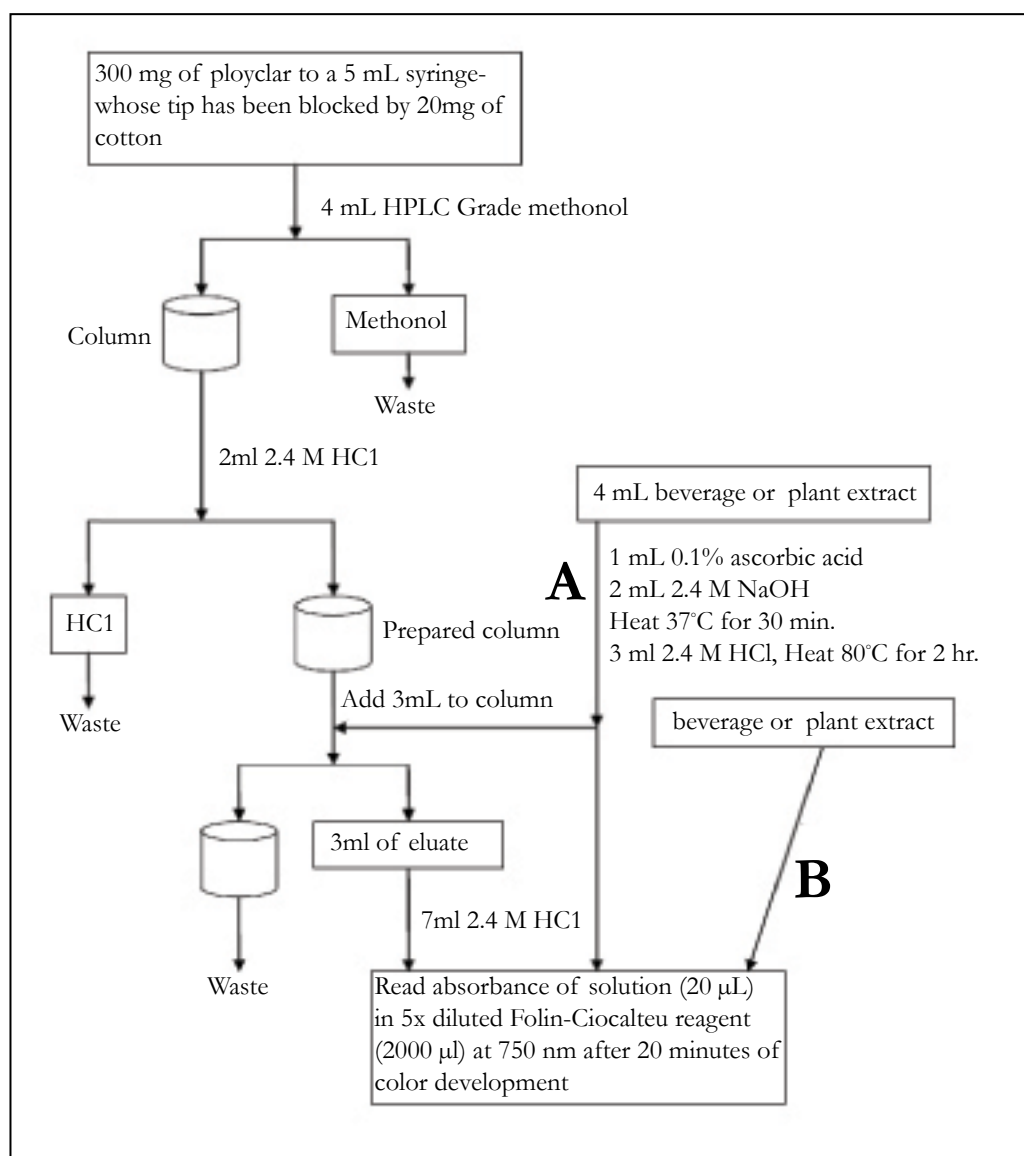
The regression line of the standard curve ($y=mx+c$) was used to determine the polyphenolic concentration (µM) in the various samples analyzed.

y = absorbance, m =gradient, x is the concentration to be determined.

Thus Polyphenolic concentration (x) = $(y+c)/m$

Example using quercetin (1mM), Volume of Folin reagent 1000 µl, volume of quercetin = 40µl, total volume of the reaction mixture = 1040 µl, Absorbance = 0.271. Then concentration of quercetin = $[(0.271+0.0324)/0.007]x (1040/40) = 1127$ µM catechin equivalent (since catechin was used as the standard).

Figure 4. Polyclar solid phase extraction and basic hydrolysis prior to Folin assay for total polyphenols



Free Polyphenolic concentration of herbs and spices by single and dual reagent (Table 2)

In order to analyze for polyphenol in plant material or natural products, there has to be extraction.

Extraction procedure: Samples were cleaned with tap water, air dried at room temperature, or at low temperature (40°C) in an oven.

Blend samples in a blender then lyophilized to constant weight (48 hours).

The samples in duplicate is then extracted with methanol by dissolving 100 mg of plant material in methanol and make final volume to 10 ml. Heat at 90°C for two hours with intermittent shaking. Allow to cool, then centrifuge at 3000 rpm and filter. The filtrate is then analyzed by either the single or dual reagent method for polyphenolic assay. Extracts can be stored at -20°C until required for subsequent polyphenolic assay.

Comparison of the two methods for pure polyphenols and samples

The two methods (single and dual reagent) may be compared using four criteria; precision, analysis time, sensitivity and selectivity (interferences). The single step blank reads 0.000 and the two step blank reads 0.009, both vs. water as 0.000. Four standards were measured in quadruplicate and the simple Folin precision was 3% and the two-step Folin 5%. The time for analysis is 25 minutes for the single step and 1.6 hours for two-step method for a preparation of a standard curve. For precision of pure compounds, the single reagent averaged 3.1% and the dual 9.7%. The dual had a significantly poorer precision, $p < 0.001$. For the herbs the single reagent average precision was 5.2% and the dual 8.3%, $p < 0.05$. So the single reagent with fewer steps and pipetings had significantly better precision. The slope of the standard curve is a measure of sensitivity. The slope was about 2.5 times higher (accounting for 2000 uL total volume in the dual method and 1000 uL for the single procedure) for the single reagent procedure indicating a greater sensitivity. There was no interference for glucose, tyrosine and albumin with the single reagent while tyrosine and albumin gave a positive Folin response with the dual reagent. The average

for the herbs was almost 3 times higher for the dual reagent, 17.7 mg/g vs. 6.7 for the single reagent ($p < 0.001$). This is probably indicative of the dual reagent method being more prone to amino acid and protein interference.

Other Dual Reagent Applications

Microplate reader for polyphenols [21]

Instrument

- Multi-detection microplate reader (Synergy HT, Bio-Tek) (see Equipment Setup)
- Mixer mill disruptor with adaptor sets for 2 ml tubes (Qiagen tissue lyser) or mortar and pestle

Reagent Setup

Methanol Prepare solution of 95% (vol/vol) methanol in water.
Sodium carbonate Prepare solution of 700 mM Na_2CO_3 in water.

Gallic acid Prepare solutions of 50 mM–2.5 mM gallic acid in 95% (vol/vol) methanol.

Equipment Setup

Microplate reader Set up the microplate reader to run an end-point absorbance read at 765 nm (at room temperature: $\sim 20^\circ\text{C}$).

Procedure

F–C extraction and assay

- 1) Harvest plant material (approximately 20 mg) in screw-capped tubes and freeze immediately in liquid nitrogen and store at -80°C . Samples can be stored at -80°C for 1–2 months. Extract with methanol and collect the supernatant in a fresh 2 ml micro-tube.
- 2) To homogenize the tissue, place three tungsten carbide leads and 2 ml of ice-cold 95 % (v/v) methanol in each sample tube and insert samples into pre-cooled Teflon adaptors. Homogenize tissue for 5 minutes at 30 Hz. If a mixer mill is not available, tissue can be homogenized in an ice-cold mortar and pestle.
- 3) Remove tungsten carbide leads with magnet and incubate the samples at room temperature for 48 hours in the dark.
- 4) Centrifuge the samples (13,000g for 5 minutes at room temperature) and collect the supernatant in a fresh 2 ml microtube.
- 5) Add 100 ml of each sample supernatant, standard or 95% (vol/vol) methanol blank to duplicate 2 ml microtubes.
- 6) Add 200 ml 10% (vol/vol) F–C reagent and vortex thoroughly. The F–C reagent should be added before the alkali to avoid the air-oxidation of phenols.
- 7) Add 800 ml 700mM Na_2CO_3 into each tube and incubate the assay tubes at room temperature for 2 h.
- 8) Transfer 200 ml sample, standard or blank from the assay tube to a clear 96-well microplate and read the absorbance of each well at 765 nm.

Free phenolics calculation

9) Calculate a standard curve from the blank-corrected absorbance at 765nm of the gallic acid standards. Calculate phenolics as gallic acid equivalents using the regression equation between gallic acid standards and absorbance at 765 nm.

Solid Phase Extraction Method to remove interferences [22]

Sample Preparation.

All of the food products (apple purees and juices) were purchased at a local market and stored at 4°C until analysis (Figure 3).

Material and Solvent Standards.

All solvents were of the highest analytical grade. Reference compounds were from Extrasynthèse (Genay, France) and reagents and solvents from Sigma-Aldrich Chimie (Saint Quentin Fallavier, France).

Cartridges. The SPE cartridge was an Oasis HLB from Waters (Milford, MA). HLB is an acronym for hydrophilic-lipophilic balance, the solid-phase being a copolymer [poly(divinylbenzene-co-N-vinylpyrrolidone)]. After any analysis, the cartridge was conditioned with 4 mL of pure methanol and rinsed with 2 X 4 mL of water.

Preparation of Raw Extracts.

Purees or juices (10–20 g) were homogenized with the same extraction solution (acetone/water, 7/3, v/v) for 30 min. Mixture supernatants were then recovered by filtration (Whatman, England) and reconstituted the raw extracts (REs).

Separation of Polyphenol and Other Water-Soluble Components by Solid-Phase Extraction.

REs were added with distilled water to reduce the proportion of acetone to 7%. Diluted REs (2 mL) were settled on an Oasis cartridge (Waters). Interfering water-soluble components (reducing sugars, ascorbic acid) were recovered with 2×2 mL of distilled water. The recovered volume of the washing extract (WE) was carefully measured.

Elimination of Vitamin C from WE.

Heating was carried out on the washing extract (3 mL) for 2 h at 85°C (Fisons Haake N2 oil bath) and led to the heated washing extract (HWE).

Folin-Ciocalteu Assay.

All extracts (RE, WE, and HWE) were submitted to the Folin-Ciocalteu method (Singleton and Rossi, 1965), adapted, and optimized: A 2.5 mL sample of water-diluted Folin-Ciocalteu reagent (1/10) was added to the different extracts. The mixture was incubated for 2 min at room temperature, and 2 mL of sodium carbonate (75 gL^{-1}) was added. The mixture was incubated for 15 min at 50°C and finally cooled in a water-ice bath. The specific absorbance at 760 nm was immediately measured.

Determination of Polyphenols and Vitamin C and Expression of the Results.

Total polyphenols, determined by subtracting gallic acid equivalent from RE from that of WE, were expressed as mg of gallic acid/100 g of product (slope = 0.012, $R^2 = 0.99$). Linearity was obtained between 50 and 500 mg/L corresponding to absorbance values between 0.1 and 0.6. Vitamin C, determined by subtracting ascorbic acid equivalent from WE from that of HWE, was expressed as mg/100 g of product (slope = 0.008, $R^2 = 0.99$). Linearity was obtained between 50 and 1000 mg/L corresponding to absorbance values between 0.1 and 0.6.

This procedure is designed to separate free polyphenols from Folin interferences. The average recovery of 16 polyphenols following methanol elution of the solid phase column was $86 \pm 16\%$. Ascorbic acid was completely eliminated by the heating step of the water wash. With the dual reagent a maximum of 7% of organic solvent (acetone or methanol) was allowed without interference with the Folin reaction.

Acid hydrolysis of herbs, fruits and vegetables for total polyphenol analysis [5]

In the plant polyphenols are often bound to sugars with an ether linkage or to carboxylic acids with an ester bond. Some or all of these bonds could be broken during digestion in the gastrointestinal tract and thus be available for absorption into the body. Thus almost all of the published literatures on polyphenol compound concentrations in foods and beverages and also antioxidant activity assays with Folin and other methods have underestimated the amount of phenols and often mislabeling their assay as total polyphenols. We used a modification of procedure first published in 1992 [23].

Procedure

A freeze dried sample of fruit or vegetable (50 to 500 mg) or an aliquot of a fruit or vegetable-derived beverage is added to 8 ml of 50% methanol/water containing 1.2 M hydrochloric acid in a screw-capped plastic tube and heated for 2 to 3 hours at 95°C with vortexing every 30 minutes for solid samples. The solution was then allowed to cool to room temperature and then quantitatively transferred and diluted to 10 ml with water in a volumetric flask. This solution is then used to determine total polyphenols in the sample by the single reagent Folin procedure. Free polyphenols can be done by the same procedure except that no HCl was used in the analysis.

Polyclar procedure to remove interferences and measure free and total polyphenols

One of the faults of the acid hydrolysis procedure for total polyphenols is that it is not optimized for phenolic acids which are a class of highly prevalent non-flavonoid polyphenols in plants primarily in an ester form. Natella published an alkaline hydrolysis method which liberates caffeic acid from chlorogenic acid (ester form) in yields of $97 \pm 3\%$ in coffee [24]. This hydrolysis procedure gave equivalent results to the commonly used but expensive glucuronidase/sulfatase enzyme method. Our procedure incorporates a basic hydrolysis with ascorbate present to stabilize the polyphenols and an acidification and heating to destroy the ascorbate and hydrolyze any leftover phenol groups still with an ether or ester linkage [22]. Free phenolic groups can be assayed by Folin prior to hydrolysis and interferences determined after polyphenol treatment described in the following procedure. Solid

samples are extracted with 50% methanol as in our herbs, vegetables and fruits procedure previously described. The solution that is added to polyclar must contain no more than 10% methanol so 50% extracts need to be diluted 5X with water. We tested 1000 μ M solutions of the following pure compounds representative of 6 classes of polyphenols: Ferulic acid (phenolic acid), Quercetin (flavonol), (+)-Catechin (flavanol), Naringenin (flavanone), Taxifolin (flavonone), Genistein (isoflavone), and Malvidin 3-O-glucopyranoside (anthocyanin). All were 100% absorbed by the polyclar.

Polyclar Procedure

- (1) Into a clean, dry 10 mL mailing tube pipette 4 mL of solution to be analyzed
- (2) Into the same tube add 1 mL 0.1% ascorbic acid solution, and 2 mL of 2.4 M NaOH solution.
- (3) Vortex and incubate this tube with cap on at 37 degrees C for 30 min.
- (4) Remove the tube and allow it to cool.
- (5) Add 3 mL of 2.4 M HCl solution (final volume is now 10 mL) vortex or shake.
- (6) Heat at 80 degrees C for 2 hr.
- (7) Remove from heat and let cool.
- (8) Prepare polyclar column:
 - In a clean dry 5 mL syringe
 - Block the tip of the column with 20 mg for cotton
 - On top for the cotton add 300 mg for polyclar (Polyclar VT, ISP Technologies)
 - Prep the column by running 4 mL of HPLC Grade methanol through it
 - Add 2 mL of 2.4 M HCl to equilibrate the column
- (9) Add 3 mL of hydrolyzed sample to the column and allow dripping through, making certain to collect 3 mL of eluate.
- (10) Make the final volume 10 mL by adding 7 mL of 2.4 M HCl
- (11) Dilute Folin-Ciocalteu reagent 5X (this is the working reagent)

To measure free phenols

- (1) Pipette 20 μ L of sample into a clean cuvette and add 2000 μ L of dilute Folin
- (2) Allow the reaction to proceed for 20 min
- (3) After 20 min read absorbance at 750 nm
- (4) Calculate concentration based on standard curve

To measure total phenols

- (1) As before measure the absorbance at 750 nm of the hydro-

lyzed sample plus Folin reaction mixture (before passing through polyclar)

(2) Also read the absorbance of the eluate plus Folin reaction mixture (after passing through polyclar)

(3) Subtract the reading of eluted sample (after polyclar) from the reading of hydrolyzed sample (before polyclar)

(4) Calculate concentration based on standard curve; include dilution factors in the calculation (Figure 2)

This procedure completely eliminates possible sugar interferences, ascorbic acid, any amino acids or proteins, and sulfate. Phenols were found to be > 99% removed by polyclar thus showing the applicability of the methodology. This method removes phenols from solutions ranging from 100% water to 100% methanol. Pure compounds at 100 μM were tested and analyzed after column treatment by HPLC. Classes of polyphenols removed include phenolic acid (test sample ferulic acid), flavonols (quercetin), flavanols (catechin), flavanones (naringenin), flavone (flavone), isoflavones (genestein), anthocyanins (malvidin-3-O-glycoside).

Miscellaneous Methods

Flow Injection Analysis

Rangel et al. [25] published a multi-syringe flow injection analysis in food products using gallic acid as the standard. The sequence used was a single reagent methodology with 12 samples/hour capacity. The precision ranged from 0.34 to 1.33% for gallic acid standards of 40 and 2.5 mg/L. There were no interferences with glucose, citric acid and sodium sulfite.

Development of a mixed polyphenol standard

Luthria and Vinyard [26] at the USDA in Beltsville proposed a 5 compound mixture of polyphenols as a reference standard for evaluating antioxidant activity of food extracts. The mixture is composed of commercially available caffeic acid, hesperetin, morin, catechin and epigallocatechin gallate. They were not combined in equal molar concentrations in the mixture. These compounds were stable in the solid when stored at 4°C for 3 months. They are currently being tested for assay of phenolics by the Folin method.

Urinary analysis of polyphenols

The most innovative use of the Folin assay has been accomplished by a Spanish group who have used it for the urine assay of polyphenol intake [27]. There have been sporadic reports of Folin assays in physiological fluids over the years but they have been largely discounted due to the large protein and small molecules such as ascorbate causing interference. These interferences are larger than the polyphenol concentrations from the diet which are probably less than 10 μM in plasma and 100 μM in urine. The authors used a solid phase extraction (Oasis HLB) to isolate and separate the polyphenols from interferences and then assayed by the two-step Folin (Fig 4). Creatinine was measured by alkaline picrate colorimetry. Recovery of 3 to 30 μM catechin (the standard) was 83 to 107% with a precision averaging 4%. There was an excellent correlation between epicatechin metabolites from human consumption of cocoa powder as measured by Folin and a LC/MS method ($r = 0.83$, $p < 0.001$).

The above method was also used with Gallic acid as the standard for urinary excretion of tomato juice polyphenols using a microplate reader for the Folin assay [28]. The latest methodology published by the same group was the use of the Folin combined with 96-well plate cartridges from Oasys to measure urinary total phenolic compounds as a biomarker of polyphenols intake [29]. This utilized spot urine sampled which positively correlated with polyphenol intake and fruit and vegetable intake. The cartridges reduced the analysis time from 11.5 to only 2 hours.

Other References

There have been a number of excellent articles comparing and criticizing the various colorimetric and antioxidant assays such as Folin, oxygen radical absorbance parameter, total radical trapping antioxidant parameter, trolox equivalent antioxidant capacity among others. They are listed in the reference section [30,31,32,33,34,35]. The results usually show a high degree of correlation between the methods indicating that no particular method is more valid. The Folin method is often criticized as giving higher values for polyphenols compared to the sum of the individual compounds as measured by HPLC as is the case for the flavonols (catechins) in chocolate [36]. However the oligomers and polymers contain multiple phenolic groups and oxidation of them may produce products that are themselves reducing agents thus giving a greater Folin value. This sequence can potentially occur in vivo and thus the Folin measurement may be relevant and it is easier, quicker and less expensive to do the laboratory. Epidemiological studies indicate certain food or class of polyphenol consumption was associated with a significant reduction in risk of chronic diseases such as heart disease, stroke and cancer [37]. No such association has been found for single polyphenol compounds, 20+ of which now have been assayed in market foods and beverages by the USDA. This lack of association is because the polyphenols act in concert when plant foods are consumed thus validating the applicability of the antioxidant assays including the Folin reagent.

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