Stabilization of the Prussian Blue Color in the Determination of Polyphenols

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A stabilized reagent for the determination of total polyphenols by the Prussian blue method was developed. H_3PO_4 complexed with excess Fe^{3+} in the mixture, stopping the reaction. Gum acacia, a protective colloid, prevented precipitation by impeding the coalescence of the colloidal particles formed. Efficiency of chelating agents tried was H_3PO_4 (A) > Na_4EDTA (B) > sodium hexametaphosphate (C). Effectiveness of protective colloids was gum acacia (D) = Reten (an anionic polyacrylamide) (E) > Methocel 60 HG (F) > phosphomannan 2448 (G) > starch (H) > polysaccharide B 1828 (I). Both the chelating agent and the protective colloid must be added to effect stabilization. Effectiveness of combinations was (A + D or E) > (B + D) > (A + F) > (A + G) > all other combinations. (A + D) prevented change in color density and prevented precipitation for up to 60 h with catechin as the polyphenol.

INTRODUCTION

Price and Butler (1977) proposed the use of a mixture of $K_3Fe(CN)_6$ and $FeCl_3$ in 0.1 N HCl for the colorimetric determination of total polyphenols in plant materials. Polyphenols react with the mixture to give a color whose density is proportional to the amount of polyphenols present. The reaction is carried out in the presence of an excess of Fe^{3+}. In 1980, Budini et al. modified the method. This redox reaction, popularly known as the Prussian blue method, is facile, rapid, economical, and used universally.

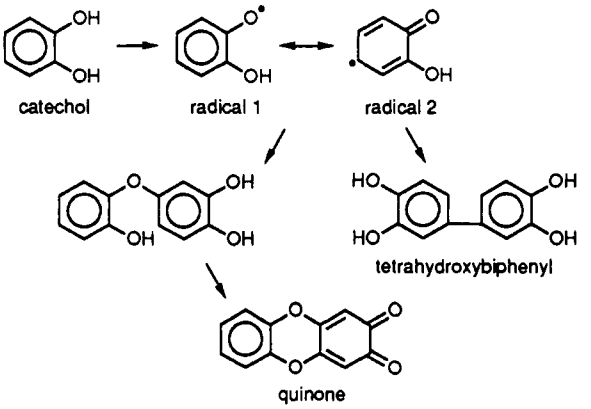
The polyphenol (PP) reacts with the ferricyanide ion $(Fe(CN)_6)^{3-}$ and is oxidized while the $Fe(CN)_6^{3-}$ is reduced to the ferrocyanide ion $Fe(CN)_6^{4-}$. $Fe(CN)_6^{4-}$ then reacts with the ferric ion (Fe^{3+}) to form ferric ferrocyanide ($Fe_4[Fe(CN)_6]_3$), commonly known as Prussian blue. The coupled oxidation–reduction reaction may be summarized as follows (Curtman, 1931, Brown, 1987):

$$\underset{\text{ferricyanide ion}}{PP + 2Fe(CN)_6^{3+}} \longrightarrow \underset{\text{oxidized}}{PP} + \underset{\text{ferrocyanide ion}}{2Fe(CN)_6^{4-}}$$

$$\underset{\text{ferrocyanide ion}}{3Fe(CN)_6^{4-}} + \underset{\text{ferric ion}}{4Fe^{3+}} \longrightarrow \underset{\text{ferric ferrocyanide (Prussian blue)}}{Fe_4[Fe(CN)_6]_3}$$

Oxidation of the polyphenol involves complex chemical reactions, as pointed out by Thompson (1964), Mihailovic and Cekovic (1971), Deshpande et al. (1986), and Harborne (1989). In this oxidation ferric chloride acts as a one-electron-transfer oxidant. Removal of a hydrogen atom from the phenolic hydroxyl group is thought to be the first step in the reaction. This results in the formation of mesomeric phenoxyl radicals $(ArO)^{\bullet}$ which may then dimerize or may react with other radicals to form, in decreasing order of importance, new C–C, C–O, or O–O bonds. Through the formation of intermediate radicals, a mixture containing several tetrahydroxybiphenyls and a quinone results, as outlined for catechol (Thompson, 1964; Deshpande et al. (1986).

Despite its rapidity, simplicity, and economy, the Prussian blue method has two drawbacks, namely, formation of a precipitate after short incubation periods and an increase in color density with time. Gum acacia (Roa and Whitney, 1964) and gelatin (Nicols and Willits, 1934) have been used as protective colloids to stabilize the color developed in the nesslerization method for the determination of nitrogen. Phosphates form fairly stable complexes with ferric ions (Kolthoff and Sandell, 1946). Short-chain polyphosphates, especially pyrophosphates, are good sequestrants of heavy metals such as iron and copper (Ellinger, 1972; Dziezak, 1990), and salts of ethylenediaminetetraacetic acid (EDTA) are known to be powerful chelators of iron (Furia, 1964). Cursory trials indicated that the addition of gum acacia or one of several other hydrocolloids prevented precipitation but not an increase in color density. Addition of H_3PO_4, sodium hexametaphosphate, or EDTA prevented increase in color density but not precipitation. Combinations of hydrocolloids and chelating agents prevented both precipitation and increase in color density for 6–60 h, depending on the combinations. Experiments were designed, therefore, to develop a stabilized reagent and to delineate conditions for using it in the determination of polyphenols by the Prussian blue method.



MATERIALS AND METHODS

Catechin, tannic acid (tannin), gallic acid, and other polyphenols were purchased from the Sigma Chemical Co., St. Louis, MO. Phosphoric acid (85%), ethylenediaminetetraacetic acid, tetrasodium salt (Na_4EDTA), sodium hexametaphosphate, $K_3Fe(CN)_6$, $FeCl_3$, and other chemicals, all of ACS reagent grade, obtained from the Fisher Chemical Co., were used without further purification. Hydrocolloids were gifts from commercial sources.

Preparation of Reagents and Extracts. Unless otherwise stated, distilled water was used in the preparation of the reagents. Aqueous dispersions of different hydrocolloids were used. These were prepared by heating weighed amounts of the materials in distilled water until complete dispersion was achieved and making

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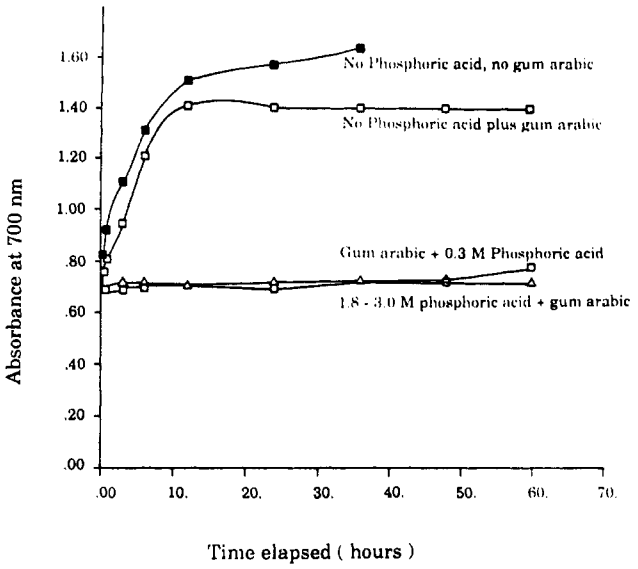

Figure 1. Influence of concentration of H_3PO_4 on color stability using catechin as phenolic substance.

up to the desired volume. These dispersions were refrigerated until needed. Gum acacia was prepared by dispersing it in distilled water, boiling the mixture for 20–30 min, filtering it while still hot over Whatman No. 541 filter paper, making up to volume, and storing it in the refrigerator until needed.

Potassium ferricyanide (0.016 M) was prepared by dissolving 0.2634 g of the salt in about 400 mL of distilled water. The mixture was filtered over Whatman No. 4 filter paper and the paper washed with distilled water. The filtrate and washing were collected in a 500-mL volumetric flask, and the contents were made up to the 500-mL mark, transferred to an amber, glass-stoppered bottle, and stored in the refrigerator until needed.

Ferric chloride (0.02 M) was prepared by dissolving 1.622 g in distilled water and making the final volume up to 500 mL. Filtration and storage were done as described for potassium ferricyanide.

Stock solutions of catechin and tannic acid (50 μg/mL) in distilled water were prepared just prior to use.

The phosphoric acid reagent was prepared by mixing 1 part 85% phosphoric acid with 2 parts distilled water. The mixture was stored at room temperature in a glass-stoppered bottle.

Na_4EDTA (1% in 1 M acetic acid) was prepared by dissolving it in distilled water and adding enough glacial acetic to attain the desired final concentration. Solutions of sodium hexametaphosphate and other chelating agents tried were prepared by dissolving desired amounts of the reagents in distilled water.

Procedure. Three milliliters containing 10–50 μg of catechin or 5–40 μg of tannic acid or an appropriately diluted extract of the substrate was placed in triplicate sets of large (150 × 25 mm) borosilicate test tubes. One milliliter of 0.016 M $K_3Fe(CN)_6$ was added followed immediately by 1 mL of 0.02 M $FeCl_3$ in 0.1 N HCl. The contents were mixed well and left at 24 ± 1 °C for 15 min. Then 3 mL of 6.03 M H_3PO_4 or 0.263 M (1%) Na_4EDTA in 1 M acetic acid was added, and the contents of the tubes were mixed well. After 2 min, 2 mL of 1% gum acacia or other hydrocolloid was added. The contents were mixed well, and the color density was measured at 700 nm against a reagent blank consisting of all of the reagents except the polyphenol. The procedure of Budini et al. (1980) was scaled up to 10 mL to allow rapid measurements usng a Spectronic 20 spectrophotometer.

Preparation of Standard Curves. Ten to 50 μg of catechin or 5–40 μg of tannic acid contained in 3 mL of distilled water was dispensed into 150 × 25 mm borosilicate test tubes. Color development was done as described under Procedure and the absorbance of the resulting colors measured at 700 nm. For reagent blanks tubes containing 3 mL of distilled water instead of catechin or tannic acid were included. All determinations were done in triplicate, and the amount of polyphenol added was plotted against absorbance.

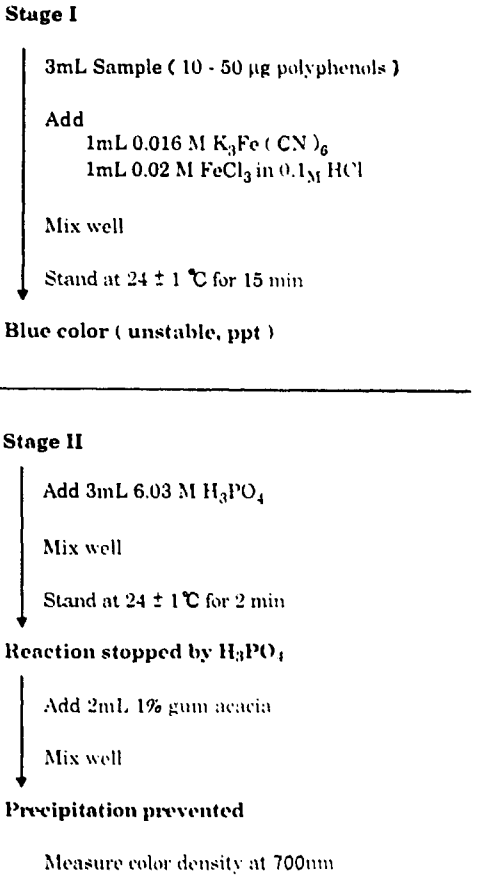

Figure 2. Flow sheet diagram of procedure.

For comparison with the unstabilized reagent [$K_3Fe(CN)_6$ + $FeCl_3$ in 0.1 N HCl], color development was done in the same way except that water was added instead of the H_3PO_4 and gum acacia. To check for any change in the wavelength of maximum absorption, the spectra of the colors developed with both the unstabilized and stabilized reagents were scanned on a Beckman spectrophotometer, Model ACTA C -111, UV–visible, with recorder.

Influence of Various Levels of Chelating Agents, Protective Colloids, and Extractant. Effects of different levels of the chelating agents were assessed by varying the concentrations of H_3PO_4 and EDTA–acetic acid, maintaining all other components of the reaction mixture constant and developing the color as under Procedure. Similarly, the influence of the level of the hydrocolloid was evaluated.

Differences in color density and/or the formation of a precipitate were assessed by varying the time interval between the addition of H_3PO_4 and gum acacia and the measurement of color density. Catechin (50 μg) was used in the above evaluations.

Changes in color density due to increasing levels of the commonly used extractants, HCl, NaCl, and methanol, were assessed by adding varying amounts to test tubes containing 50 μg of catechin and developing the color as outlined under Procedure.

Influence of Time and Temperature on Color Development. To study these, 150 × 25 mm borosilicate test tubes containing 50 μg of catechin or 20 μg of tannic acid in 3 mL of water were preincubated at 15, 20, 25, 30, and 40 °C. Then the reagents for colorimetry, as outlined under Procedure, were added. The color developed at each temperature, after 5, 10, 15, 20, 25, 30, and 45 min, was measured at 700 nm. A reagent blank containing no polyphenol and treated in the same way as the other tubes was included for each time interval at each temperature. All determinations were done in duplicate.

Response of Various Compounds to the Test. Five to 10 mg of substances of different chemical classes were placed in 150 × 25 mm borosilicate test tubes. A "control" or "blank" tube containing no added material was included. The reagents for colorimetry, outlined under Procedure, were added, and each tube was observed visually. Any substance which gave color

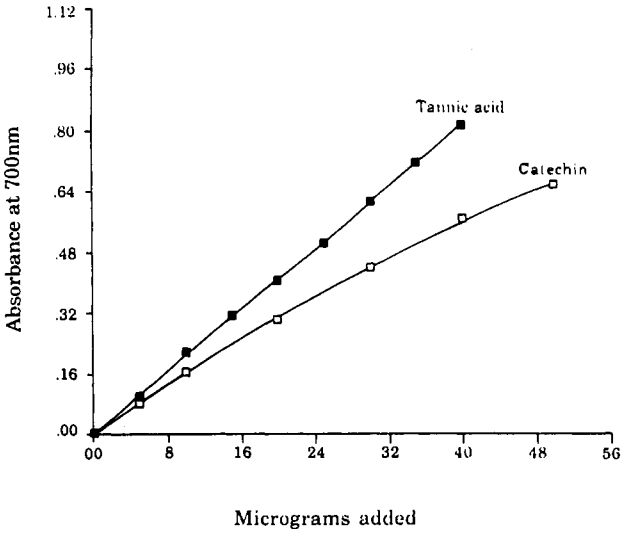

Figure 3. Standard curves for catechin and tannic acid.

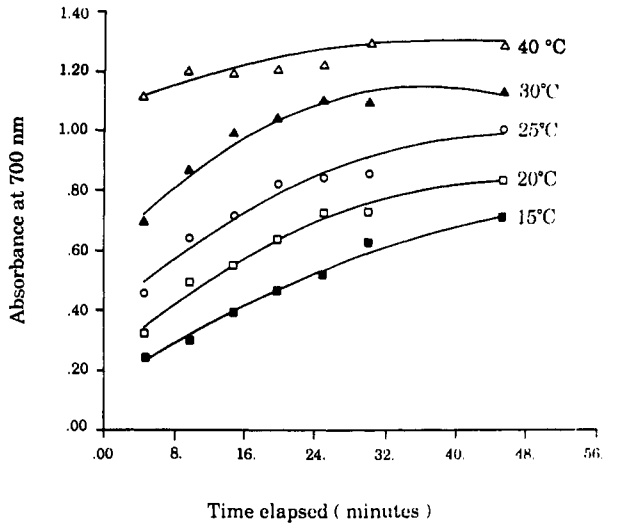

Figure 4. Effect of time and temperature on density of color developed using catechin as phenolic substance.

density in excess of that produced by the control was labeled "positive" and was further probed quantitatively.

Determination of Total Polyphenols Extracted from Breadfruit and Mabi Bark Using the Unstabilized and Stabilized Reagents. Polyphenols in the breadfruit pulp were extracted following the method used by Budini et al. (1980). Ten grams of the pulp was blended in 200 mL of 2 N HCl and heated for 30 min in a boiling water bath, and the hot contents were filtered through a coffee-filtering bag. The bag was washed with five 40-mL aliquots of boiling distilled water. The filtrate and washings were collected in a 500-mL volumetric flask and the contents cooled to 24 ± 2 °C and made up to the 500-mL mark with distilled water. An aliquot of this preparation was passed over Whatman No. 4 filter paper and the filtrate used for polyphenol determination.

The mabi bark extract was prepared by boiling 10 g of the bark in tap water and making the final volume up to 100 mL (Graham and Zengotita, 1980). Filtration and washing were done as described for the breadfruit extract (except for volumes of wash water). To obtain material for polyphenol determination in seeds of *Canavalia maritima*, dry seeds were ground to pass a 20-mesh sieve and 1 g of the meal was extracted for 1 min with MeOH following the method of Price and Butler (1977). Filtration and washing were done as described for breadfruit pulp (except for volume of wash water), and the final volume was made up to 25 mL. Appropriate standard curves for catechin and tannic acid were prepared using the unstabilized reagent to make comparative calculations.

Table I. Survey for Interfering Substances

substance tested	interference: positive[a]	interference: negative
carbohydrates	0	39
sugars (16)	0	16
sugar alcohols (6)	0	6
uronic acids (3)	0	3
polysaccharides (14)	0	14
proteins (12)	0	12
amino acids (30)	3[b]	27
vitamins (12)	3[c]	9
auxins (9)	8	1
indole compounds (10)	10	0
others (28)	0	28

[a] When 5 μg gave an absorbance of 0.2 or higher at 700 nm, measured against distilled water. [b] Cysteine, homocysteine, tryptophan. [c] Ascorbic acid, pyridoxine, pyridoxamine.

RESULTS AND DISCUSSION

Increase in color density was prevented by the following chelating agents for up to 60 h at the indicated final concentrations (total volume 10 mL): H_3PO_4 (A) 1.8 M; EDTA (B) (0.05%); sodium hexametaphosphate (C) (0.2%). Citric, oxalic, formic, and tartaric acids, sodium lauryl sulfate, and sodium potassium tartrate were ineffective. Of the chelating agents experimented with, H_3PO_4 was the most suitable since it readily formed soluble complexes with excess Fe^{3+} in the mixture. As a result, no more Fe^{3+} was available to react with the ferrocyanide ion leading to the formation of the colored Prussian blue. Ready formation of soluble H_3PO_4–Fe^{3+} complex(es) also inhibited the possible formation of quinone(s). H_3PO_4 also lowered the pH to 2.3. When the final pH was above 2.6, stabilization of the reaction was less effective. For this reason when Na_4EDTA and sodium metaphosphate were used, the pH of these mixtures had to be lowered by the addition of acetic acid and H_3PO_4, respectively. Therefore, for simplicity and effectiveness, H_3PO_4 was chosen. A final level of 1.81 M was sufficient to effect stabilization at levels of catechin and tannic acid of 10–50 and 5–30 μg/10 mL of reaction mixture, respectively. Figure 1 shows the trend for catechin. Tannic acid behaved similarly.

Of 25 potential protective colloids tried (22 polysaccharides or their derivatives, 2 proteins, and 1 polyacrylamide derivative), 7 that prevented precipitation were selected for more detailed studies. As criterion, prevention of precipitation for a minimum of 10 h, using catechin and tannic acid as the polyphenolic substances, was selected. The order of efficiency was gum arabic (D) = Reten (E) > Methocel 60HG (F) > phosphomannan 2448 (G) > pregelatinized starch (H). Potato starch and the starch phosphates were much less effective than pregelatinized starch.

As seen from Figure 1, with the unstabilized reagent where neither H_3PO_4 nor gum arabic (0.2%) was added color density continued increasing with time and precipitation was noticed after about 20 min. Addition of gum arabic but no H_3PO_4 did not prevent increase in color density, although the absorbance was slightly lower than when neither additive was included in the mixture. Addition of 1.3–3.0 M H_3PO_4 plus gum arabic resulted in stabilization of the color density developed after 15 min of reaction time. In addition, precipitation was prevented for up to 60 h.

Precipitation was prevented by the hydrocolloids for the following periods, at the indicated final concentrations (final volume 10 mL): D, 72 h (0.2%); E, 72+ h (0.01%); F, 36 h (0.01%); G, 36 h (0.01%); H and I, 10–12 h (0.01%).

Table II. Summary of Influence of Variables on Color Development and Stability

variable	range investigated	reproducible range	selected
protective colloid	25 tried	gum arabic, Reten	gum arabic
chelating agent	H_3PO_4, EDTA, polyphosphates, citric, six other organic acids	H_3PO_4	H_3PO_4
$K_3Fe(CN)_6$ (reagent A), M	0.032–0.080	0.016–0.020	0.016
$FeCl_3$ in 0.1 M HCl (reagent B), M	0.01–0.1	0.02–0.03	0.02
reaction time after addition of A + B, min	5–40	10–15	15
H_3PO_4 (final concn) (reagent P), M	0.03–9	0.3–3.0	1.80
gum arabic (final concn) (reagent G), %	0.05–2.0	0.1–1.0	0.2
time lapse after adding reagent P, min	2–30	2–10	2
time lapse between adding of reagent G and color measurement, h	0.033–72	up to 36–60	0.08–6
reaction temp, °C	15–40	24 ± 1–25 ± 1	24 ± 1
wavelength for color measurement, nm	340–710	690–710	700
interfering substances	140 substances tried	absence of ascorbic acid, cysteine, and indole compounds	
mixing H_3PO_4 and gum arabic	immediately and up to 21 days after	use within 5 days	
extractants	HCl, NaCl, methanol	maxima HCl, 0.1 M NaCl, 0.05 M methanol, 1%	

Reten was as effective as gum acacia and gave a crystal clear dispersion. However, the dispersion was very viscous at a concentration of even 0.1% and was difficult to pipet quantitatively. Moreover, it is no longer available commercially. On the other hand, gum acacia is highly dispersible in H_2O and was effective at a final concentration of 0.05–0.2%. The other hydrocolloids either formed very viscous dispersions at concentrations above 0.2% or were ineffective as protective colloids. For these reasons, gum acacia was chosen. A final concentration of 0.2% was satisfactory when the level of catechin and tannic acid ranged from 10 to 50 and from 5 to 30 μg/10 mL of reagent mixture, respectively.

Both the chelating agent and the protective colloid must be present in the mixture at the same time to effect stabilization. The order of effectiveness of combinations was (A + D or E) > (B + D) > (A + F) > (A + G) > all other combinations. Gum acacia and H_3PO_4 may be combined to give a single reagent (dubbed "the stabilizer") by mixing water, 85% H_3PO_4, and 1% gum acacia in the volume proportions 3:1:1 and adding 5 mL to the reaction tubes. Fresh preparations should be made up weekly.

Figure 2 outlines the procedure. It shows that stabilization (stage II) is an extension of stage I, the conventional procedure using the unstabilized reagent. Maximum absorption of the color produced by catechin and tannic acid occurred at 690–710 nm.

Catechin (Price and Butler, 1977), epicatechin (Budini et al., 1980), and tannic acid (Deshpande and Cheryan, 1987) have been used as standards in the determination of total phenolic substances by the Prussian blue procedure. Tannic acid reportedly had 25% more ability to reduce Fe^{3+} than did catechin (Deshpande and Cheryan, 1987). This increased sensitivity of tannic acid over catechin also held true when the stabilized Prussian blue reagent was used. Tannic acid was claimed to be also more sensitive to the Prussian blue reagent when methanol was used as the extractant (Deshpande and Cheryan, 1987). However, this might have been due to the slight increase in color density caused by the MeOH–Fe^{3+} interaction, as noted by Price and Butler (1977) and observed in this investigation. The standard curve for catechin is linear up to about 2.5 μg of added catechin/10 mL of reaction mixture (Figure 3).

Color density was greatly affected by the time and temperature at which the reaction was carried out (Figure 4). For any specific time period, color density increased as the reaction temperature increased. At 40 °C a very high color density was reached within 5 min and rose only slightly more afterward. At 30 °C increase in color density after 5-min intervals was less drastic than at 40 °C. At 15 and 20 °C, increase in color density between 5 and 20 min was almost linear. Between 15 and 25 °C, color density almost doubled after a reaction time of 5 min. After 15 min at 25 °C, increase in color density slowed down. If the reaction time is extended beyond 15 min, precipitation ensues, due to coalescing of the particles in the Prussian blue complex. In view of this, a 15-min reaction time, prior to the addition of H_3PO_4, at 24 ± 1 °C, was chosen.

One hundred and forty compounds commonly found in plant and other tissues and which may be extracted along with polyphenols were tested for possible interference (Table I). Carbohydrates and intact proteins did not interfere. Protein hydrolysates responded. Cysteine, homocysteine, and tryptophan interfered strongly, while tyrosine responded weakly. The phenolic group in tyrosine was responsible for its color response. Tryptophan's response was due to the presence of the indole nucleus since all compounds bearing this moiety (indole and auxins) gave strong coloration (Table I). Among the vitamins, ascorbic acid and pyridoxine gave positive tests. Interference by ascorbic acid was reported by Budini et al. (1980). Pyridoxine's response was due to its strong reaction with $FeCl_3$. No coloration was noted when it was mixed with $K_3Fe(CN)_6$. High final concentrations of each of three common extractants caused interference. HCl and NaCl at final concentrations above 0.1 and 0.05 M, respectively, depressed color development, while MeOH at final levels above 1% increased color density due, most likely, to the activity of the hydroxyl group. Results of these and the influence of other variables are summarized in Table II.

Determination of total phenolic substances in three plant products by the conventional as well as the stabilized method showed good correlation (Table III). Appropriate standard curves must be used in each case. Cursory probing confirmed that, as in the conventional method, the stabilized reagent gave the typical Prussian blue color with a myriad of phenolic substances such as phenol, orcinol, gallic acid, resorcinol, pyrogallol, hydroquinone, and cinnamic acid.

Swain and Goldstein (1964) recommended the Folin reagent (phosphomolybdic–phosphotungstic acids in alkaline solution) for determination of total phenols. The titanium chloride method (Eskin et al., 1978) is thought

Table III. Determination of Polyphenols in Plant Materials

material	tannic acid,[a] mg/100 g		catechin,[a] mg/100 g	
	S[b]	US[c]	S[b]	US[c]
breadfruit pulp	12.5 ± 0.62	12 ± 0.73	16.0 ± 0.82	15.5 ± 0.32
mabi bark	76.5 ± 2.4	81.3 ± 3.20	100 ± 1.50	98.4 ± 1.42
C. maritima (seeds)	14.6 ± 0.62	15.0 ± 0.50	18.0 ± 0.24	17.6 ± 0.56

[a] Triplicate determinations. [b] Stabilized reagent. [c] Unstabilized reagent.

to give results comparable to those of the Folin reagent. Budini et al. (1980) modified the Prussian blue method of Price and Butler (1977) and claimed that their modification is at least 3 times as sensitive as the titanium procedure and 20 times as sensitive as the vanillin reaction, which has been recommended for determination of phenolics where catechin and proanthocyanidins are predominant (Swain and Goldstein, 1964). The Prussian blue method, being so simple, rapid, and economical, could gain prominence and recognition equal to that accorded the Folin procedure for the determination of total phenolic substances in plant tissues. However, as pointed out by Harborne (1989), this will have to be established through its performance in repeated usage over a sufficiently long period. Given the drawbacks of the conventional procedure, the stabilized reagent developed here provides users with a simple, rapid, and reliable procedure for the determination of total polyphenols. Usually, it is recommended that measurements of color density be made within 5 (Budini et al., 1980) or 10 min (Price and Butler, 1977), due to precipitation and/or increase in color density. With the stabilized reagent, density readings may be made after any desired time lapse of up to 48–60 h. Availability of such a stabilized reagent will enable persons with minimal technical training and experience to conduct determinations with more confidence and reliability. Less haste and work pressure will result since samples may be left overnight or even longer with only minor differences in color density. More samples can be analyzed simultaneously, and so the procedure is ideally suited for massive, routine quantitative analyses, including field tests.

Other techniques such as thin-layer chromatography (Bandyopadhyay et al., 1990), high-pressure liquid chromatography (Spanos and Wrolstad, 1990), and colorimetric methods using different reagents (Swain and Hills, 1959; Singleton and Rossi, 1965; Broadhurst and Jones, 1978; Eskin et al., 1978) have been used to determine polyphenols. However, the Prussian blue method is easiest, quickest, and economical. It has played a significant role in delineating the role of polyphenols in plant life and food products. Availability of a stabilized reagent will greatly enhance its usefulness.

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Registry No. EDTA, 60-00-4; Na_4, 64-02-8; H_3PO_4, 7664-38-2; catechin, 120-80-9; starch, 9005-25-8; Prussian blue, 12240-15-2; gum arabic, 9000-01-5; Reten, 39379-48-1; Methocel 60HG, 9004-65-3; phosphomannan, 9044-08-0.