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PII: S0308-8146(97)00056-3

Food Chemistry, Vol. 61, No. 3, pp. 373–380, 1998

0308-8146/98 $19.00+0.00

Microassay for the quantitation of protein precipitable polyphenols: use of bovine serum albumin–benzidine conjugate as a protein probe

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(Received 16 November 1996; accepted 26 February 1997)

A simple, sensitive and indirect spectrophotometric method for the determination of protein precipitable polyphenols (tannins) has been developed, based on the ability of the polyphenols to precipitate the synthetic, brown coloured azo-protein, bovine serum albumin–benzidine conjugate (BSA–benzidine, mole ratio 1:7), which shows an absorption maxima at 405 nm. The amount of unprecipitated BSA–benzidine is measured directly at 405 nm, which is inversely related to the polyphenol concentration. Tannic acid was used as a reference standard. The microassay was performed in citrate/phosphate buffer (0.1 M), pH 4.8. The method was found to be linear in the range of 5–150 μg (3–88 nmol) of tannic acid ($y = 1.0 + (-0.007)x$; $r = -0.989$). Spiking studies carried out with various levels of tannic acid (0.01, 0.1 and 1.0%) indicated a recovery in the range of 94–101% and 94–98% in rice and sorghum samples, respectively. Free phenolics, when added in the range of 50–150 μg (catechin, chlorogenic acid, ferulic acid, caffeic acid and *p*-coumaric acid) had no influence on the protein precipitation in the microassay. Also spectral analysis of free phenolics and acid-methanolic sorghum extracts showed no interference in the present method. The conjugate was found to be stable over a period of 24 weeks in a freeze-dried condition and at 4°C, with $< 5\%$ deterioration in aqueous condition. The microassay method developed has been used for the quantitation of protein precipitable polyphenols in various sorghum (*Sorghum bicolor* L. Moench) genotypes and compared with the widely used Folin–Denis chemical method of analysis.

INTRODUCTION

Polyphenolic and phenolic compounds are synthesized as secondary metabolites by plants and are widely distributed in the plant kingdom. In plants, these compounds are known to provide defence against viral, bacterial and fungal attack, which is akin to animal immune system (Friend, 1985; Deshpande *et al.*, 1986; Harborne, 1990; Leinmuller *et al.*, 1991). Nutritionally, polyphenols of dietary origin have been implicated to have adverse effects on human health (Salunkhe *et al.*, 1982). Contrary to these studies, polyphenols also have been shown to inhibit tumour formation and growth (Yang & Wang, 1993). Important sources of dietary polyphenols and free phenolics, include pigmented cereals and legumes which contain 2–4% polyphenols (Mabbayad & Tipton, 1975). Sorghum (*Sorghum bicolor* L. Moench) is one of the important coarse food grain consumed as staple by millions of people in semi-arid tropics of Asia and Africa (Murthy & Subramanian, 1981). The polyphenol content of grain sorghum has been reported to range from 0.1 to as high as 8% (Harris & Burns, 1970).

Various analytical methods have been reported for the quantitation of polyphenols (both condensed and hydrolysable tannins) and total phenolics in a wide range of agricultural commodities. These analytical procedures include chemical methods of analysis as well as protein precipitation methods, wherein the property of polyphenols to form insoluble complexes with soluble proteins has been successfully exploited (Deshpande *et al.*, 1986; Leinmuller *et al.*, 1991). Both condensed and hydrolysable tannins have been found to precipitate BSA (Hagerman & Butler, 1978; Dawra *et al.*, 1988). Newer modified analytical methods to measure polyphenols include the use of ^{125}I-iodoalbumin,

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haemoglobin, blue dye and enzymes as protein probes (Hagerman & Butler, 1978, 1980; Schultz *et al.*, 1981; Asquith & Butler, 1985; Deshpande *et al.*, 1986; Dawra *et al.*, 1988; Ittah, 1991). Chemical methods, such as Prussian blue, Folin–Denis, vanillin assay and Folin–Ciocalteu procedures measure phenolic acids, flavonoids and tannins, based on the reductive ability of phenolic hydroxyl groups (Folin & Denis, 1912; Burns, 1971; Price & Butler, 1977; Deshpande *et al.*, 1986). However, these chemical methods are not very specific and detect all phenolic compounds (phenolic acids, flavonoids and polyphenols) with varying degrees of sensitivity and do not distinguish between polyphenols and low molecular weight phenolic compounds that co-occur naturally in foods of plant origin (Maxson & Rooney, 1972; Deshpande *et al.*, 1986). However, most of these methods are found to be limiting in their application to quantitate biologically active polyphenols in biological samples, with respect to simplicity, sensitivity and accuracy of the analytical method.

The present communication reports a simple, sensitive and indirect spectrophotometric method for the determination of protein precipitable polyphenols (tannins). The analytical method is based on the ability of the polyphenols to precipitate the synthetic, brown coloured azo-protein, bovine serum albumin (BSA)–benzidine conjugate. The amount of unprecipitated BSA-benzidine is spectrophotometrically measured directly at 405 nm, which is inversely related to the polyphenol concentration.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (RIA grade, fatty acid and globulin-free), catechin, chlorogenic acid, *p*-coumaric acid, ferulic acid, acrylamide, bis-acrylamide, tris, sodium dodecyl sulphate (SDS), polyvinyl pyrrolidone-40 were obtained from Sigma (USA). Tannic acid was obtained from S.D. Fine Chemicals (India), caffeic acid from Aldrich Chemical Co. (USA), benzidine from E. Merck (India) and spectro grade methanol and *n*-hexane from Spectrochem (India).

Apparatus

UDY cyclone mill (Tecator, Sweden) with 0.4 mm mesh was used to grind the samples. Samples were defatted by Soxhlet extractor (Borosil, India). Precipitated BSA–benzidine conjugate was separated using a TM-12 Microfuge (Beckman, USA). Absorbance measurements were made using a UV-vis spectrophotometer (Beckman, USA) and ELISA Reader (SLT Spectra II, Austria). Purity of the BSA-benzidine conjugate was evaluated and documented by using a slab gel electrophoresis unit, Mighty small II (Hoefer, USA) and a gel documentation system (GDS-5000, Ultra Violet Products, USA), respectively. An ultra free CL-spin filter unit, NMWL 10000 (Millipore, USA) was used to separate the unreacted BSA–benzidine conjugate. Polystyrene ELISA microtitre plates (12×8 wells) were purchased from Nunc, Denmark.

Sample collection

Sorghum samples were collected from the Genetic resources unit, International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, India and also from the National Research Centre for Sorghum (NRCS), Hyderabad, India. Rice variety Ajaya was collected from the Directorate of Rice Research, Hyderabad, India.

Extraction of polyphenols from sorghum samples

Sorghum samples as well as rice samples, were ground on a UDY cyclone mill, to pass through 0.4 mm mesh. The meal was defatted in a Soxhlet extractor with *n*-hexane for 6–8 h. Defatted sorghum flour (200–500 mg) was extracted twice with 3 ml 1% HCl in methanol for 1 h in a mechanical shaker. The extract was centrifuged at 12 000 rpm for 15 min in a microfuge. For tannin estimation, 100 μl of the supernatant was used in the microassay.

Preparation of BSA–benzidine conjugate

Benzidine was conjugated to BSA using the diazotization reaction (Van Regenmortel *et al.*, 1988). The mole ratio of BSA to benzidine used was 1:7. Briefly, 1% sodium nitrite solution was added dropwise with constant stirring at 4°C to 9.71 mg (52.7 μmol) benzidine in 0.1 N HCl. The diazonium salt formed was immediately allowed to react with 500 mg BSA (7.53 μmol) in 0.2 M carbonate buffer, pH 9.0, for 2 h at 4°C with constant stirring. An aliquot was withdrawn from the reaction mixture to assess the efficiency of conjugation. Unreacted benzidine was separated from the reaction mixture using an ultrafiltration unit, spun at 4000 ***g*** for 1 h. The filtrate was analysed spectrally for the presence of free (unreacted) benzidine. Later, the reaction mixture was dialysed for 36 h at room temperature (28°C) against double quartz distilled water containing 0.01% sodium azide and the coloured conjugate was stored at 4°C or lyophilized and kept until further use. Absorption spectra of BSA–benzidine conjugate (in citrate/phosphate buffer 0.1 M, pH 4.8) was analysed using a recording spectrophotometer. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) (7.5%) was run with BSA–benzidine conjugate (14.5 and 29 μg/10 μl) and native BSA at a concentration of 10 μg/10 μl (Frederick *et al.*, 1992). The gel pattern was recorded using a gel documentation system. Percent recovery of the synthesized conjugate was also

determined (Lowry *et al.*, 1951). Spectral analysis of free phenolics and tannic acid, as well as the acid-methanolic extracts of red, white and yellow sorghums were carried out in a recording spectrophotometer.

Optimal conditions for microassay

Studies were carried out to set up the optimal conditions for the microassay, namely, pH and ionic strength of the buffer, concentration of BSA–benzidine conjugate, volume of the solvent used in the extraction of tannins and the incubation temperature for the assay.

Microassay protocol

The microassay was carried out in citrate/phosphate buffer pH 4.8, 0.1 M containing 800 μg (12 nmol) BSA–benzidine in a total volume of 1.3 ml. Reference standard tannic acid in the range of 5–150 μg (3–88 nmol)/100 μl of methanol was added to the assay buffer and was followed by the addition of 100 μl of 1% HCl in methanol and vortexed. In case of samples, 100 μl of sample extract in 1% HCl–methanol was added to the assay buffer, followed by addition of 100 μl of methanol. The microassay was carried out in 1.5 ml microfuge tubes. The total volume of the reaction mixture was 1.5 ml and was incubated for 15 min at room temperature (28°C). The contents were centrifuged in a microfuge, at 10 000 rpm for 10 min. One millilitre aliquots of the supernatant were read at 405 nm in a spectrophotometer or an aliquot of 200 μl per well of microtitre plate was read on an ELISA Reader, using a 405 nm filter. The inhibitory effect of polyvinyl pyrrolidone (PVP-40) at 125 and 250 μg on the interaction of tannic acid (15, 29 and 59 nmol) with BSA–benzidine (12 nmol) was also studied.

Spiking studies

Spiking of sample

Various levels of standard tannic acid (0.01, 0.1, 1.0%) in methanol were spiked to defatted samples of sorghum (SPV-475) and rice (Ajaya variety) flour, which were used as negative controls. The solvent was allowed to evaporate for 10 min in an incubator and then a 200–500 mg sample was extracted in 3 ml 1% HCl in methanol by vortexing extensively for 10 min. The extract was centrifuged at 12 000 *g* for 15 min in a microfuge and the supernatant was used for tannin quantitation via the microassay method.

Spiking of sample extract

Various concentrations of tannic acid (20, 40, 60 μg) were spiked to 100 μl of 1% HCl–methanolic extract of rice and sorghum samples as internal standards. Per cent recoveries of tannic acid were determined by microassay.

Interference of free phenolics

Free phenolics, namely, catechin, caffeic acid, ferulic acid, chlorogenic acid and *p*-coumaric acid at a concentration range of 50, 100 and 150 μg per assay were also tested for their ability to precipitate BSA–benzidine conjugate.

Stability of BSA–benzidine conjugate

Studies were also undertaken to assess the stability of the conjugate synthesized, in water and citrate/phosphate buffer (pH 4.8, 0.1 M), at 4°C, 28°C and under freeze-dried conditions.

Polyphenol (tannin) contents of 19 genotypes of sorghum were analysed by the microassay method and were compared with the Folin–Denis (1912) method.

RESULTS AND DISCUSSION

The inverse relationship between the absorbance (at 405 nm) due to unprecipitated BSA–benzidine with the increasing concentration of tannic acid was found to be linear in the range 5–150 μg (3–88 nmol) of tannic acid. Thus, the assay system provides an indirect method for quantitation of protein precipitable polyphenols (tannins). Figure 1 shows the regression line ($y = A + Bx$) for indirect estimation of tannic acid using BSA–benzidine conjugate, as determined by spectrophotometer

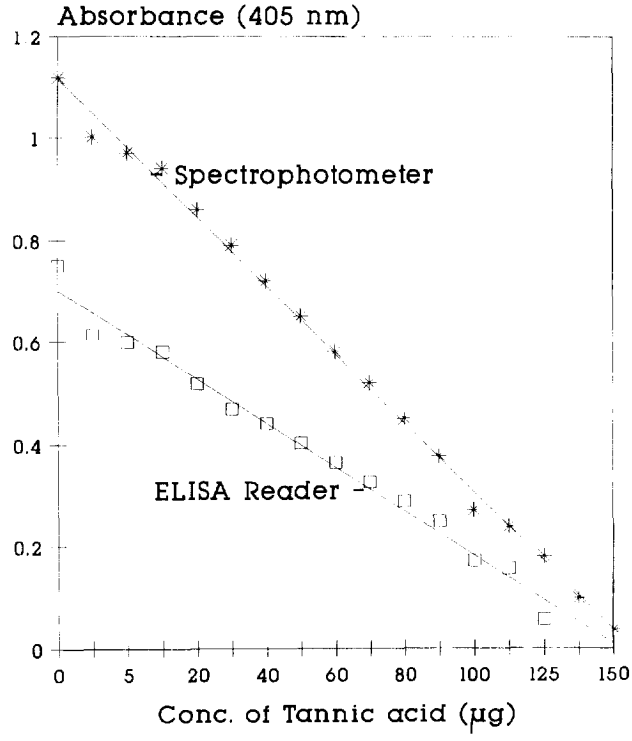

Fig. 1. Regression lines: estimation of tannic acid by microassay method. Spectrophotometer: $y = 1.0 + (-0.007)x$; $r = -0.989$; ELISA Reader: $y = 0.63 + (-0.004)x$; $r = -0.978$.

($y = 1.0 + (-0.007)x$; $r = -0.989$) and an ELISA Reader ($y = 0.63 + (-0.004)x$; $r = -0.978$). The binding of tannic acid to BSA–benzidine shows a typical sigmoid curve, suggesting a positive co-operativity with respect to protein precipitation. Spectral analysis of the brown coloured BSA–benzidine conjugate indicates a broad absorption maxima in the range 405–412 nm (Fig. 2). The mole ratio of BSA to benzidine (1:7) was found to be ideal for the microassay, when the conjugate was used at a concentration of 800 μg (2 nmol)/1.5 ml assay volume resulted in a mean absorbance value of 1.08 at 405 nm (Table 1). The efficiency of conjugation was found to be 100%, as the spectral analysis of the filtrate obtained after ultrafiltration had no detectable benzidine. The electrophoretic (SDS–PAGE) separation pattern of the conjugate was similar to that of the native BSA, suggesting that there was no cross-linking or condensation between BSA molecules during the diazotization reaction (Fig. 3). Table 1 gives the percent recovery and absorbance values of different batches of BSA–benzidine synthesized, with a mean recovery of 77%.

The optimal conditions established for the microassay in citrate/phosphate buffer were pH 4.8 (Fig. 4), ionic strength 0.1 M with 200 μl of methanol (Fig. 5) in a total assay volume of 1.5 ml. A pH of 4.8 for the microassay was found to be critical, as the amount of BSA–benzidine precipitated decreased with increase in pH (Fig. 4). Ionic strength of the citrate/phosphate buffer in the range 0.05–0.2 M had no significant effect on protein precipitation. Addition of extracting solvent beyond 300 μl resulted in volume-dependent precipitation of BSA–benzidine (Fig. 5). No significant differences were observed with respect to the protein precipitation, when incubation of the assay was carried out at 28 and 37°C for 15 min. It was observed that addition of PVP-40 at 125 and 250 μg levels resulted in complete reversal of BSA–benzidine precipitation by tannic acid, used at concentrations of 25, 50 and 100 μg (15, 29, 59 nmol)/assay.

The repoducibility of the microassay method was evaluated by spiking studies. Table 2 gives the recoveries of the spiked tannic acid to rice (used as a negative control) and sorghum sample (SPV-475, identified to

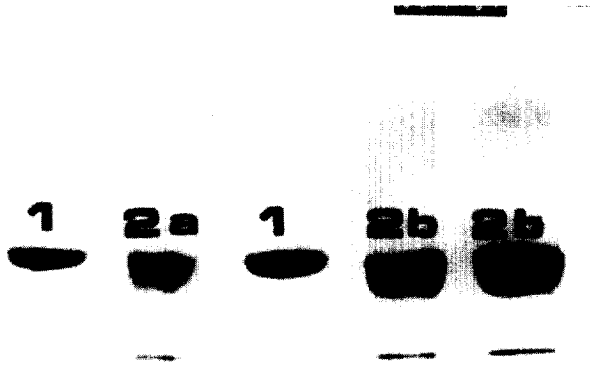

Fig. 3. SDS–PAGE separation profile of [1] Native BSA (10 μg) [2] BSA–benzidine conjugate (a) 14.5 μg; and (b) 29 μg.

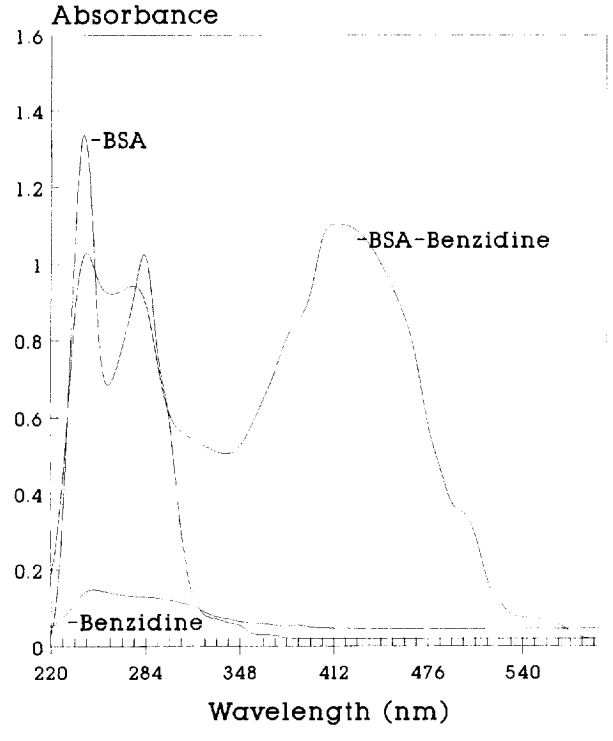

Fig. 2. Spectral analysis of BSA–benzidine (800 μg per 1.5 ml), BSA (1 mg per 1.5 ml), benzidine (100 μg per 1.5 ml), in citrate phosphate buffer, 0.1 M, pH 4.8.

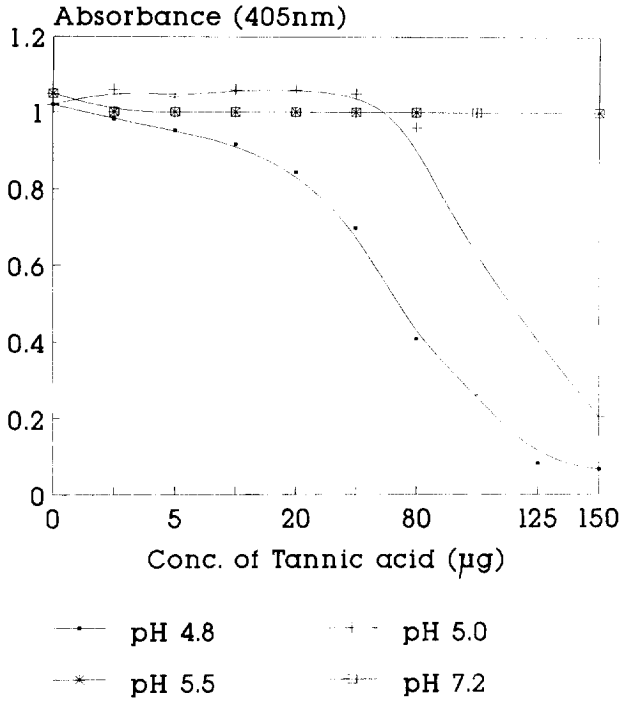

Fig. 4. Effect of buffer pH on the precipitation of BSA–benzidine by tannic acid.

Table 1. BSA–benzidine recovery after diazotization reaction and its subsequent purification

Batch No.	BSA taken (mg)	BSA recovered (mg)	% recovery	$A_{405\,nm}$ per 800 μg of BSA–benzidine
1	500	380	76	1.05
2	500	400	81	1.13
3	400	270	67	1.11
4	400	280	70	1.10
5	500	385	77	1.09

Table 2. Recovery studies of tannic acid spiked at various levels in rice, rice extract and sorghum, sorghum extract

% tannic acid spiked	% recovery[a]	
	Rice	Sorghum
0.01%	101 (2.0)	94 (12.0)
0.10%	97 (2.0)	96 (5.8)
1.0%	94 (4.0)	98 (7.0)
Concentration of tannic acid spiked (μg per assay)	% recovery[a]	
	Rice extract	Sorghum extract
20	100 (4.0)	99 (3.0)
40	95 (4.0)	95 (1.0)
60	93 (3.0)	97 (2.0)

Values based on triplicate analysis.
[a]Mean and (coefficient of variation).

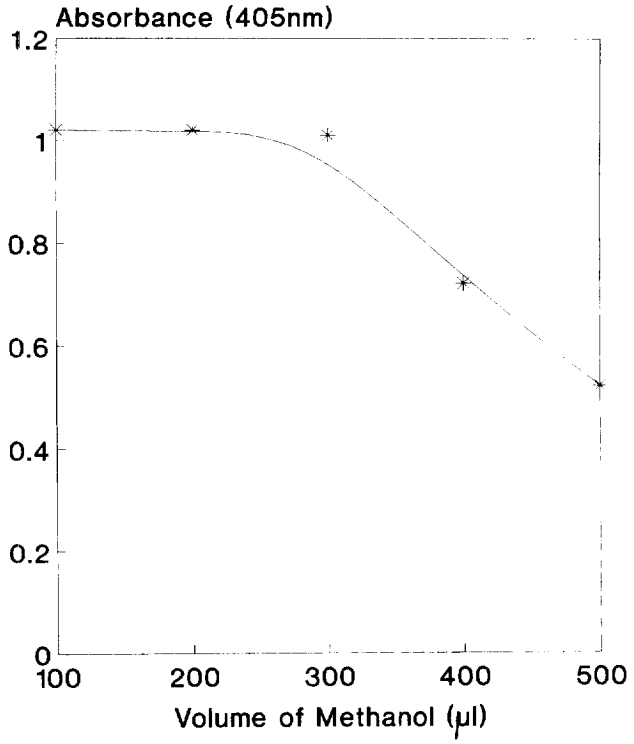

Fig. 5. Influence of methanol on the precipitation of BSA–benzidine.

have low polyphenol content, Table 3) at a concentration of 0.01, 0.1, 1.0%, and the tannic acid recovered was determined by the microassay method. The results indicate that in rice, the percent recovery ranged from 94 to 101%, while in sorghum sample it ranged from 94 to 98%. Table 2 also shows the recoveries of tannic acid (20, 40, 60 μg) spiked to the rice and sorghum sample extracts. The recoveries ranged from 93 to 100% in rice and 95–99% in sorghum samples, respectively. No BSA–benzidine precipitation was observed in the microassay, when the free phenolics were added in the range of 50, 100 and 150 μg per assay.

BSA–benzidine conjugate was found to be stable over a period of 6 months in the lyophilised condition and with an insignificant deterioration (< 5%) at 4°C in water. The conjugate was found to be less stable under acidic buffer conditions (pH 4.8), as compared with water, wherein the per cent deterioration was observed to be 15%, at 4°C, and 30% at 28°C, respectively, at the end of 4 weeks (Fig. 6), thus, bulk preparation can be made and stored.

Previously, various analytical methods have been developed for the quantitation of polyphenols and free phenolics in a variety of agricultural commodities. These methods include chemical means, as well as interaction of polyphenols with proteins resulting in the formation of an insoluble complex (Deshpande *et al.*, 1986: Leinmuller *et al.*, 1991. Newer improved methods based on detection of polyphenol–protein complex by Ponceau's dye (Dawra *et al.*, 1988), alkaline hydrolysis of polyphenol bound BSA and its estimation by ninhydrin (Makkar *et al.*, 1987), use of dye-labelled protein to precipitate tannin (Asquith & Butler, 1985) and use of the enzyme alkaline phosphatase as a probe for protein bound polyphenol (Ittah, 1991) have been reported for the analysis of protein precipitable polyphenols.

The present microassay, based on BSA–benzidine conjugate, has several advantages with respect to its simplicity, sensitivity and application to the analysis of polyphenols in biological samples, as compared with the earlier reported methods of protein precipitation assays by Hagerman and Butler (1978, 1980). The microassay method indirectly estimates the protein precipitable tannins by virtue of its absorption due to unprecipitated BSA–benzidine at 405 nm. The need for SDS solubilisation of the precipitated tannin and its subsequent chemical analysis via the Prussian blue method as reported by Hagerman and Butler (1978) can be completely eliminated. The minimum detection limit of tannic acid reported for this method was 200 μg as compared with 5 μg for the present assay. Later, Hagerman and Butler (1980) reported a modified method based on the use of ^{125}I-radiolabelled BSA, with a sensitivity in the range of 0.5–1.5 mg of BSA, for the detection of protein precipitable tannin using radioiodinated BSA. The amount of protein used in the microassay was 800 μg per assay, while the method reported by Hagerman and Butler (1978) requires 2 mg BSA per assay.

Table 3. Comparison of microassay and Folin–Denis methods for the analysis of polyphenols (tannin) in various sorghum genotypes

Sample No.	Genotype	Microassay[a] (mg g^{-1})[b]	Folin–Denis[a] (mg g^{-1})[b]
	Red sorghum		
1	IS 14384	1.84 (0.14)	3.80 (0.03)
2	IS 715	5.49 (0.07)	8.31 (0.06)
3	IS 19451	2.15 (0.19)	1.74 (0.06)
4	IS 18175	10.40 (0.10)	9.46 (0.90)
5	IS 7155	6.76 (0.15)	7.11 (0.18)
6	AON 486	1.58 (0.05)	2.92 (0.01)
7	R 22163	0.88 (0.05)	2.25 (0.19)
	White sorghum		
8	IS 18422	2.27 (0.04)	1.94 (0.07)
9	IS 22328	1.49 (0.16)	1.11 (0.04)
10	IS 22289	1.58 (0.02)	1.03 (0.01)
11	IS 22291	0.80 (0.11)	0.85 (0.03)
12	IS 3541	1.00 (0.01)	1.63 (0.08)
13	CSH-1	1.36 (0.07)	0.52 (0.01)
14	CSH-5	0.80 (0.14)	ND
15	SPV-475	0.17 (0.01)	ND
	Yellow sorghum		
16	IS 17788	0.75 (0.04)	5.45 (0.74)
17	IS 17777	0.23 (0.01)	ND
18	IS 17780	0.69 (0.08)	ND
19	IS 9744	0.75 (0.18)	ND

ND, not detected.
[a]Milligram tannic acid equivalents per gram of defatted grain flour. Value in parentheses is ± SD.
[b]Values based on triplicate analysis.

More recent modifications based on the use of haemoglobin as the precipitable protein by polyphenols have been developed by Bate-Smith (1973) and Schultz *et al.* (1981). This method was found to be limiting with respect to the requirement of fresh blood and the interference of saponins and plant metabolites, and thus inconvenient for routine analyses (Bate-Smith, 1977). In comparison with the present method the haemoglobin precipitation method is less sensitive with a minimum detection limit of 200 μg, while the microassay is more accurate and versatile with respect to routine analysis of polyphenols. The high sensitivity of the microassay as compared with earlier protein precipitable methods can be attributed to molecular interactions of BSA–benzidine conjugate with tannic acid. The enzymatic methods developed for polyphenol assays suffer from a high degree of variability, even though they have comparable sensitivity to that of the microassay. The major limitations of the enzymatic methods include accuracy and reproducibility with respect to the analysis of biological samples (Goldstein & Swain, 1965). The alkaline phosphatase method (Ittah, 1991) is equally sensitive but it has the limitation of having prolonged incubation periods and repeated washings. Methods developed by Dawra *et al.* (1988) and Makkar *et al.* (1987) are time-consuming and laborious, as they involve elaborate chromatographic procedures and chemical hydrolysis of precipitated protein, as compared with the simplicity and rapidity of the microassay methods. The total assay time required for the analysis was found to be less than 45 min using a spectrophotometer, while it was less than 35 min with an ELISA Reader.

Previous studies had indicated that the protein precipitation was temperature-dependent (Dawra *et al.*, 1988) while in our study we found that there is no difference with respect to protein precipitation at 28 and 37°C, which substantiates the observations made

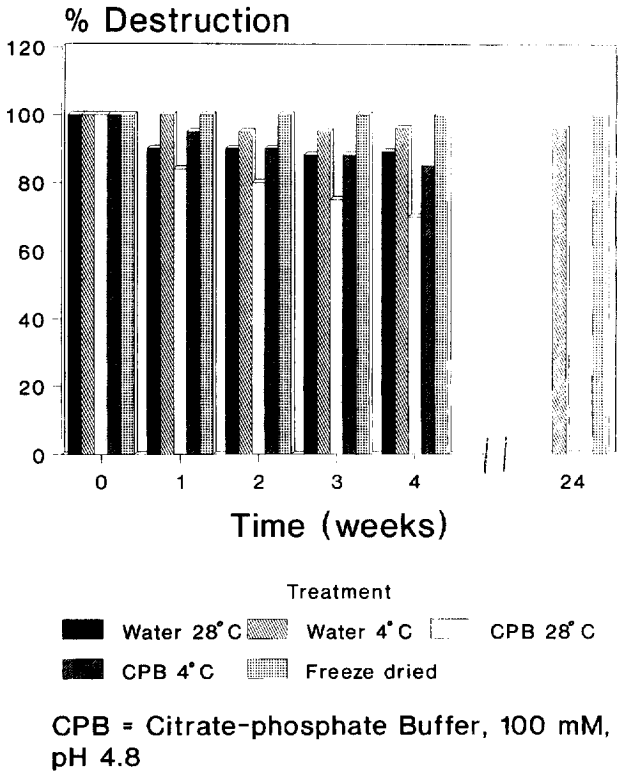

Fig. 6. Stability of BSA-benzidine conjugate.

by Hagerman and Butler (1978). The pH of the buffer in the assay was observed to be critical for protein precipitation, as reported by earlier workers and in the present assay the best results were obtained at pH 4.8. At higher pH (5.5, 7.2) there was no precipitation of BSA–benzidine by tannic acid (Fig. 4), which can be attributed to the ionization of phenolic groups of polyphenols, making them unavailable for hydrogen bonding with protein (Loomis & Battaile, 1966). Addition of PVP-40 to the microassay totally inhibited the precipitation of BSA–benzidine by tannic acid. This reversal can be attributed to the high affinity of tannic acid to heterocyclic vinyl pyrrolidone subunits of PVP-40, as reported by Hagerman and Butler (1981).

Several sorghum genotypes, including red, white and yellow varieties, were analysed by microassay and were compared with the widely used Folin–Denis chemical method (1912), as indicated in Table 3. The estimated values of polyphenols by the Folin–Denis method in the majority of sorghum genotypes were higher as compared with the microassay method (Table 3). This variation can be attributed to the limitation of the chemical method, which also estimates small molecular weight phenols, polyphenols, along with other plant compounds like xanthins, amino acids and proteins (Lowry *et al.*, 1951). Figures 7 and 8 depict the spectral analysis of free phenolics, while Fig. 9 shows the absorption spectra of acid-methanolic extracts of sorghum samples (red, white and yellow genotypes). These results indicate that the interference by free phenolics and sorghum extracts was minimal at 405 nm. This minimal interference was calibrated by using appropriate sample blanks, while analysing the sorghum samples by the microassay method. Further, there was no interference of free

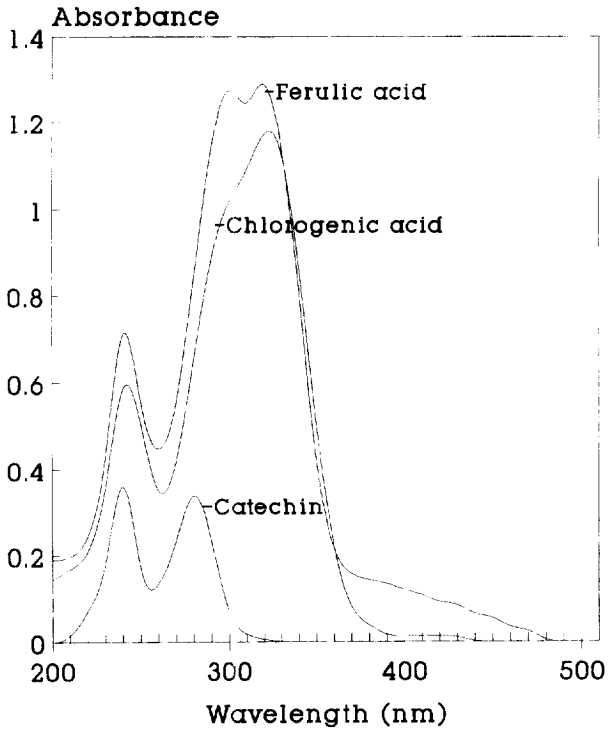

Fig. 7. Spectral analysis of free phenolics: catechin (100 μg per 1.5 ml) chlorogenic acid and ferulic acid (25 μg per 1.5 ml) in citrate phosphate buffer, 0.1 M pH 4.8.

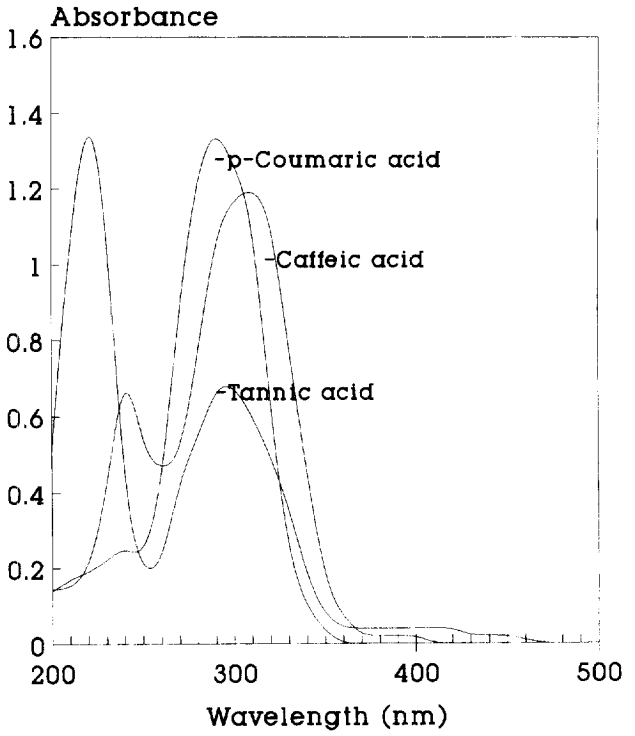

Fig. 8. Spectral analysis of free phenolics: *p*-coumaric acid, caffeic acid and tannic acid (25 μg per 1.5 ml) in citrate phosphate buffer, 0.1 M, pH 4.8.

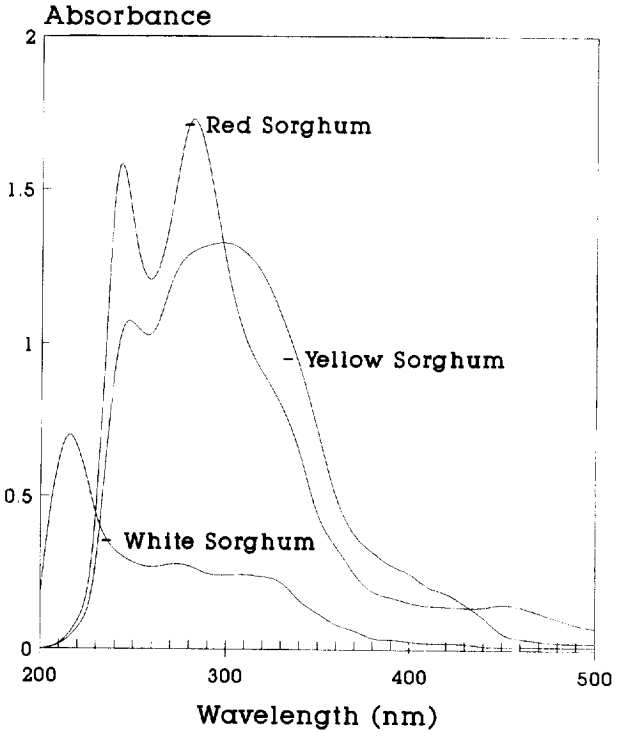

Fig. 9. Absorption spectra of acid-methanolic sorghum extracts (100 μl) in citrate phosphate buffer, 0.1 M, pH 4.8.

phenolics with respect to BSA–benzidine precipitation in the microassay, indicating that the assay was specific to polyphenols. Even though some sorghum genotypes tested negative for the Folin–Denis method (1912), the microassay could estimate protein precipitable polyphenols in the same samples, indicating the sensitivity of the method for biological samples. Possibly, natural distribution of free phenols and polyphenols in the sorghum samples results in the variations observed between the chemical and the microassay methods (Table 3).

The success of any analytical method depends on the simplicity, sensitivity, accuracy and versatility of the method developed. The present microassay method described in this paper has overcome several limitations reported in the earlier methods of polyphenol quantitation by protein precipitation. The microassay developed affords a simple, sensitive, indirect and quick method for the quantitation of biologically active polyphenols, and may find wide application in assessing the biological value of polyphenols containing foods and feeds.

We are currently investigating one of the potential applications of the microassay method to classify various sorghum genotypes on the basis of the ratio of protein precipitable polyphenol content to that of the total phenols, as determined by the chemical method.

ACKNOWLEDGEMENTS

The authors would like to thank the University Grants Commission, New Delhi for providing instrumentational facilities under the COSIST program in the department. C.V.R also wishes to acknowledge the Council of Scientific and Industrial Research, New Delhi, for a Senior Research Fellowship to undertake this research work.

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