Analysis of Tea Shoot Catechins: Spectrophotometric Quantitation and Selective Visualization on Two-Dimensional Paper Chromatograms Using Diazotized Sulfanilamide†

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A highly specific and sensitive diazotized sulfanilamide reagent is synthesized for determination of tea catechins. The reagent is employed both as spray reagent for selective visualization of tea catechins on two-dimensional paper chromatograms (sensitivity <1 μg of *d*-(+)-catechin) and for their spectrophotometric quantification in the crude extracts of tea polyphenols isolated from fresh or dried tea shoots. The formation of yellow color ($\lambda_{max} = 425$ nm) between catechins and diazotized sulfanilamide was investigated and made the basis of a simple and sensitive spectrophotometric method for estimation of the total and individual catechins in different tea cultivars. At 425 nm, the absorbance was linear ($r = 0.999$) over the (0.4−8.0 μg/mL) concentration range of *d*-(+)-catechin.

Keywords: *Tea; Camellia sinensis; catechin reagent; diazotized sulfanilamide; quantification; seasonal variation*

INTRODUCTION

Tender shoots of tea plant (*Camellia sinensis* L. (O) Kuntze), consisting of an apical bud and the adjoining first two leaves and stalk, contain (on dry weight basis) about 20−25% polyphenols belonging to flavonoid group, the major amount of which (80−85%) are classified as flavan-3-ols, commonly known as the catechin group. This group contains epimers of catechin, gallocatechin, and their gallated esters. In addition to catechins, other major polyphenolic compounds are chlorogenic acids, flavonol and their glycosides, *p*-coumaryl quinic acid, and theogallin (Stahl, 1962; Wikremasinghe, 1978).

The catechin derivatives constitute the natural substrate for polyphenol oxidase (PPO) native to tea shoot, and catechins undergo chemical conversion to highly colored polymeric products theaflavins (TFs) and thearubigins (TRs) mediated by PPO during black tea manufacture. The quality of black tea is central to this process, which is directly linked to the amount and composition of catechins present in tea shoots (Roberts, 1962; Hilton and Ellis, 1972).

A number of methods were developed in the past for the determination of polyphenols in plant extracts like Folin−Denis reagent, vanallin reagent, diazotized amines such as diazotized *p*-nitroaniline and sulfanilic acid, and Folin−Ciocalteau and Price and Butler (ferricyanide−ferric chloride dip reagent) methods, but they are generally nonspecific and cannot distinguish flavonoid classes in general and are best used for determination of total soluble polyphenols (Ribereau-Gayon, 1972; Swain and Goldstein, 1963; Peter and Simon, 1994; Robards and Antolovich, 1997). Likewise other oxidants used volumetrically for the same purpose, such as ceric sulfate and potassium permanganate, are also nonspecific (Swain and Goldstein, 1963).

Direct spectrophotometric measurement in UV region can be employed on separated phenols, and the prior knowledge of extinction coefficients in specific solvent for each phenolic compound is a must for quantification (Peter and Simon, 1994).

The need for the development of simple and economical methods for selective identification and quantification of different classes of flavonoids has long been felt and, in this connection, lately vanillin and recently 4-dimethyl-*p*-cinnamaldehyde have been exploited to quantify flavan-3-ols since both follow the same reaction chemistry (Swain and Goldstein, 1963; Delcor and Janssensde, 1985; Kivits et al., 1996). However, whereas vanillin regent suffers from instability the later chemical is very expensive.

High-performance liquid chromatography (HPLC) could also be cited as a tool basically for separation and identification of flavonoids but is far more expensive and requires special setup.

In the present method the ability of diazotized arylamines to form colored complexes with the A-ring of catechin-type molecules with the exclusion of a host of other polyphenolic compounds present was exploited in the synthesis of stable diazotized sulfanilamide compound, and the reaction conditions were tailored to be suitable for specific and selective detection and quantification of tea shoot catechins. The reaction is one of electrophilic substitution reaction of diazo compound with falvan-3-ol molecules in acid medium preferentially at the activated meta 6 and 8 positions of the phloroglucinol type A ring of the flavan-3-ols (Ribereau-Gayon, 1972).

MATERIALS AND METHODS

Chemicals and Plant Materials. *d*-(+)-Catechin, EC, and sulfanilamide were from Sigma Chemical Co. Sodium nitrite,

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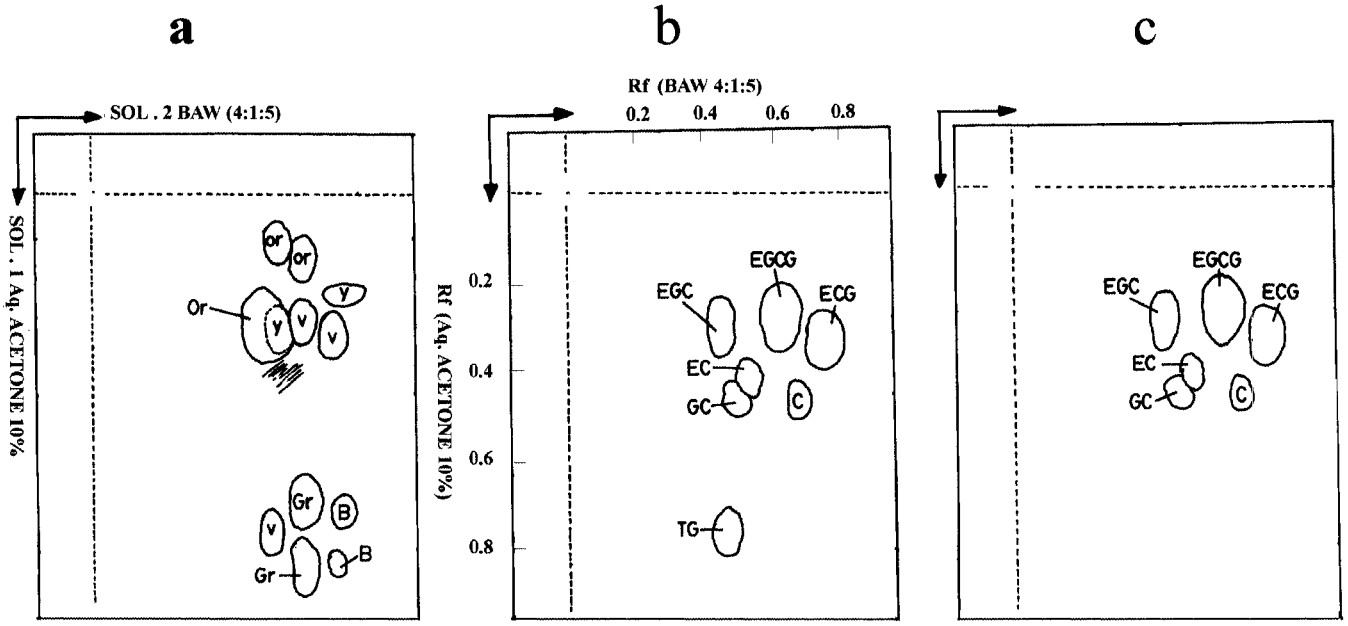

Figure 1. Detection of tea polyphenols by 2D-PC [solvent 1, aqueous acetone (10% v/v); solvent 2, butanol−acetic acid−water (4:1:5 v/v, organic phase)]. (a) Fluorescent spots in UV after exposure to ammonia vapors; V (violet) spots, galic acid, theogallin, and catechin gallates; B (blue) spots, *p*-coumaryl quinic acid; Gr (green) spots, chlorogenic acids; Or-Y (orange yellow) spots, flavonols and their glycosides; (b) dip reagent (acid ferric chloride−ferricyanide) revealed spots; TG, theogallin, gallic acid, and catechin groups; (c) sulfanilamide reagent revealed spots of the catechins alone.

acetone, hydrochloric acid (sp.gr. 1.18), and petroleum benzine (60−80 °C) of analytical reagent (AR) grade were from E. Merck Ltd. (India). Double-distilled water was used in the preparation of all reagents and solutions throughout the experiments. Tea shoots from different tea clones/cultivars were obtained from Banoori tea experimental farm of the Institute.

Preparation of Diazotized Sulfanilamide. Diazotized sulfanilamide was prepared by the general diazotization reaction carried out in an ice cooled (<10 °C) solution in the presence of sodium nitrite ($NaNO_2$) and HCl.

Small-Scale Synthesis. The following method was used: (1) Sulfanilamide (1.0 g) was dissolved in double-distilled water (50 mL) by warming and made acidic by adding concentrated HCl (0.8 mL). The solution was cooled to below 10 °C in an ice bath before carrying out diazotization reaction by adding $NaNO_2$ (1 mL of 1% $NaNO_2$ (0.145M) in water). As a result of diazotization the solid diazotized derivative of sulfanilamide was precipitated immediately in the solution as a pale lemon yellow salt.

(2) The precipitate was filtered immediately through Whatman no. 1 filter paper under ice-cooled conditions and washed free of chloride ions (as judged by $AgNO_3$ precipitation test of washings) with ice-cooled double-distilled water and dried under cool conditions.

The dried diazotized sulfanilamide (pale yellow powder, yield approximately 60%) is stable at room temperature and can be safely stored in desiccator for future use.

CAUTION: All steps of the operation during diazotization reaction should strictly be carried out at or below 10 °C in an ice bath.

Preparation of Tea Shoot Polyphenol Extracts. *From Fresh Tea Shoots.* Standard tea shoots were steamed (1−2 min) before processing to inactivate oxidizing enzymes of the shoots. Shoots were then finely ground with acetone in a mortar and pestle and macerate filtered through sintered glass funnel (porosity G3) under mild suction. The residue was washed with two aliquots of acetone first and then again with two aliquots of 60% acetone to remove all the water-soluble polyphenols of the macerated tea shoots. The combined extract so obtained was partitioned between twice the volume of petroleum benzine (v/v). The lower aqueous acetone layer, containing phenolic constituents, was removed to a small glass crucible. The acetone from the layer was removed by placing

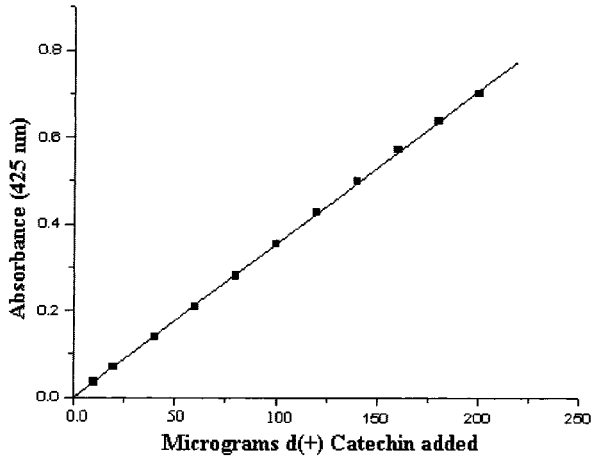

Figure 2. Standard curve for catechin with sulfanilamide reagent.

the crucible over the boiling water bath. The extract so obtained was cooled and brought to a suitable volume by addition of water, for estimation of catechins.

From Steamed Dried Tea Shoots. The steamed dried shoots were finely ground, and ground material (100 mg) was taken in a glass-stoppered 25-mL conical flask. Next 5 mL of 60% acetone was added to the flask and stoppered firmly. The material extracted for 1 h over a mechanical shaker and then filtered through sintered glass funnel (porosity grade G3). The clear extract so obtained was used for catechin estimation.

Qualitative Separation and Detection of Tea Catechins. Descending two-dimensional (2D) paper chromatography (PC) of prepared tea shoot extracts was done on Whatman no. 1 chromatographic paper (three sets) on one eighth the size of original paper (46 × 57 cm) essentially as described by Roberts and Wood (1953) with slight modification to effect better separation. An aliquot (5 μL of each extract was spotted on one corner of the paper, and 2D chromatography was carried out using 10% acetone as the first solvent and *n*-butanol/acetic acid/water (4:1:5 v/v) upper organic phase as the second solvent. The chromatograms were air-dried and polyphenolic constituents of the extract so separated were detected, in one set by exposing to ammonia vapor and viewing

Table 1. Analysis of Standard Addition of *d*-(+)-Catechin Solution of Known Concentration to an Unknown Solution of Tea Polyphenols[a]

standard added (μg/mL)	standard found (μg/mL)	recovery (%)
0.0	0.0	0.0
0.4	0.4	100
0.8	0.8	100
1.2	1.19	99.4
2.0	2.03	101.5
2.4	2.39	99.9
3.2	3.18	99.4

[a] Average of three determinations. At 0.05 CL the means are not significantly different.

in short wave UV (Figure 1a), in another by the dip reagent (Figure 1b), and in the third set by spraying the chromatograms first with reagent A (stock diazotized sulfanilamide 1% w/v in acetone) followed by reagent B (concentrated hydrochloric acid (35.4% of Sp.gr. 1.18) diluted (30:100 mL v/v) with double-distilled water) in a covered fuming hood. Only catechins showed up as bright yellow spots on the white background in the third set in about 15 min time. (Figure 1c). The identification of the catechin spots so revealed were done according to their R_f values in the two solvents as reported earlier. (Roberts, 1955; Roberts et al., 1956).

CAUTION: The spray of chromatograms must always be done in covered fuming chamber to avoid inhalation of harmful aerosols of acid hydrochloride and organic diazotized salt in acetone.

Diazotized Sulfanilamide Reagent (Reagent A). Diazotized sulfanilamide stock solution (1% w/v) was prepared in acetone and stored, well stoppered, in dark.

Dilute Hydrochloric Acid (Reagent B). Concentrated hydrochloric acid (35.4% of Sp.gr. 1.18) was diluted (30:100 v/v) with double-distilled water to make reagent B.

Stock Catechin Solution (1 mg/mL) was prepared just before use in double-distilled water.

Procedure. *Estimation of Total Catechins.* A known aliquot (5 μL) of the prepared extract of polyphenols of tea shoots were placed in triplicate sets of 25 mL volumetric flasks. One milliliter of reagent A (1% diazotized sulfanilamide in acetone, w/v) followed by 1 mL of reagent B (30% hydrochloric acid v/v) was subsequently added to the extracts and allowed to react at room temperature for 1 h. The reagent blank minus tea extract was included. At the end of incubation period the volumes were brought up to the mark with water and mixed well, and the absorbance was measured at 425 nm in Hitachi model 150-20 spectrophotometer against reagent blank. The concentration of catechins in the sample extract was estimated from standard curve of *d*-(+)-catechin in the concentration range of 0.4−8.0 μg/mL.

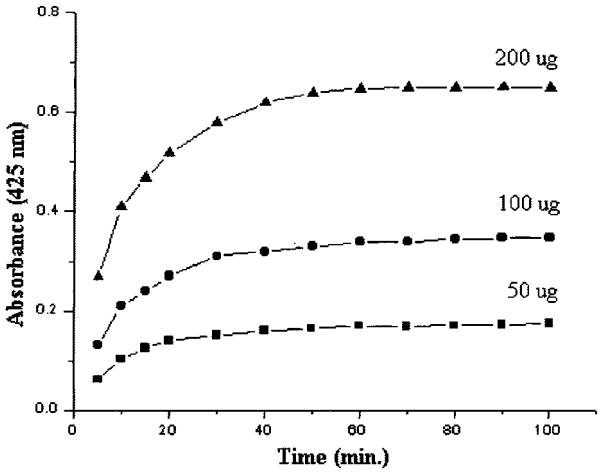

Figure 3. Time course of color development with sulfanilamide reagent at three catechin concentrations.

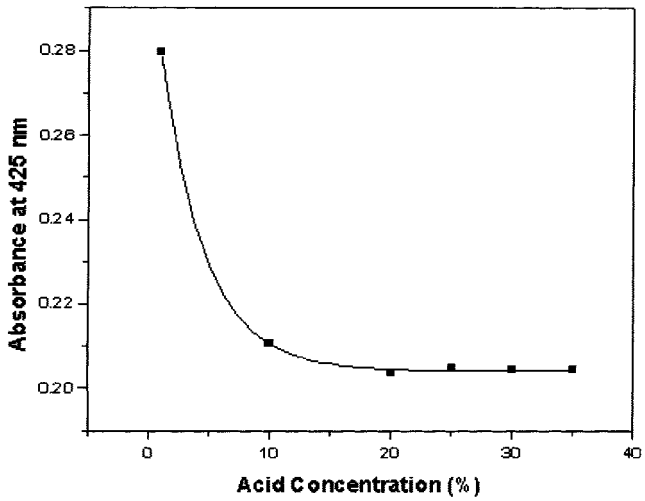

Figure 4. Effect of hydrochloric acid concentration (%) on color development of catechin solution with the reagent.

Estimation of Individual Catechins. For the estimation of the individual catechins 5 μL of the tea polyphenols were spotted in duplicate on paper chromatograms, and 2D-PC runs were made identically as described earlier. One chromatogram was sprayed with the reagent for revealing the catechin spots

Table 2. Concentration of Total and Individual Catechins in Shoots of Different Tea Cultivars[a]

tea cultivars	tea shoot catechins [concentration equivalent of *d*-(+)-catechin, mg/(g of fresh shoot)[b]]							
	individual catechins[c]					total[d] (observed)	total[e] (computed)	recovery (%)
	ECG	EGCG	EGC	GC + C	C			
TV-1	4.72 ± 0.03	6.63 ± 0.06	4.64 ± 0.05	6.24 ± 0.04	1.71 ± 0.02	29.52 ± 0.03	23.94	81.1
TV-7	3.01 ± 0.01	9.7 ± 0.001	3.70 ± 0.01	2.51 ± 0.01	1.41 ± 0.02	25.49 ± 0.02	20.33	79.8
TV-2	4.69 ± 0.03	15.2 ± 0.03	10.19 ± 0.02	5.50 ± 0.01	0.96 ± 0.05	43.49 ± 0.03	36.54	84.01
TV-17	4.94 ± 0.04	8.40 ± 0.01	7.61 ± 0.02	6.01 ± 0.02	0.6 ± 0.01	31.01 ± 0.01	27.56	88.9

[a] Average of three determinations. [b] Average moisture content of fresh shoots = 75.6% ± 0.41. [c] Measured after elution from paper chromatograms. [d] Measured from solution extracts. [e] Computed from estimation of individual catechins.

Table 3. Seasonal Variation of Total Catechins in Tea Shoot Components of China Hybrid Tea Plants[a]

shoot components	months (concentration equivalent of *d*-(+)-catechin, g/100 g on dry weight)						
	April	May	June	July	August	September	October
bud	14.06	15.50	15.51	14.51	13.49	13.34	12.68
first leaf	14.50	16.25	17.04	16.01	15.01	16.06	14.02
second leaf	16.02	16.31	16.25	16.60	17.00	15.25	12.95
stem	17.25	16.49	16.01	16.30	16.51	16.71	13.50

[a] Average of three determinations. At 0.05 CL the means are not significantly different.

and with the help of that, individual spots for catechin were marked on the other chromatogram. The marked spots were cut and eluted in 60% acetone (1 mL) in separate test tubes for 15−20 min, and incubated for 1 h for color development with 1 mL each of reagent A and B. The reagent blank was included prepared similarly except for the polyphenol. At the end of the incubation volumes were made to 5 mL each and absorbance read at 425 nm in the spectrophotometer against the reagent blank. The concentration of individual catechins were determined with the help of standard curve and expressed as percentage equivalent amount of *d*-(+)-catechin.

Preparation of Standard Curve of Catechin. Aliquots of catechin stock solution containing 10−200 μg of catechin were dispensed into 25 mL volumetric flasks, and color development was done exactly as described previously. Absorbance of the resulting color was measured at 425 nm. A reagent blank was included which did not contain any catechin solution. All determinations were done in triplicate, and the amount of catechin added was plotted against absorbance. The regression equation was computed for catechin values (x) ranging from 0.4 to 8.0 μg/mL (Figure 2).

Influence of Time on Color Development. Time course of color development of catechin-diazotized adduct in solution assays was followed at 425 nm at regular intervals of 10 min for 90 min at three catechin concentrations of 50, 100, and 200 μg (Figure 3).

Effect of Concentration of Acid on Color Development. Different concentrations (%) of hydrochloric acid was taken ranging from 2.5% to 30% for color development at fixed catechin concentration by the reagent A to select optimum acid concentration for the work (Figure 4).

Recovery Studies. For recovery studies different aliquots of standard catechin solution containing 10−80 μg of catechin, in 10 μg increments, were added in separate sample flasks containing the same aliquot of tea extract and were compared with stand alone tea sample (same aliquot).

RESULTS AND DISCUSSION

The diazotized sulfanilamide reagent was found to be selectively and specifically coupling under strong acid conditions with flavn-3-ols in aqueous tea shoot extracts and was highly sensitive toward catechins (Figure 1c). The detection limit was less than 1 μg of catechin on 2D-PCs. In this respect, its sensitivity is comparable with dip reagent. This catechin-specific reagent is very useful as a spray reagent for selective detection of tea catechins either on PCs or on thin-layer chromatograms (TLCs).

Under the described procedure, a plot of absorbance versus concentration gave a linear relationship (r = 0.999) over the measurements recorded at 425 nm for 10−200 μg catechin equivalents in 25-mL reaction volume. Regression line has the equation $y = -0.00082 + 0.00355x$, where y denotes absorbance values and x the amount of catechin in micrograms over the selected range of catechin concentration (Figure 2). The yellow color of catechin−diazotized sulfanilamide adduct has λ_{max} at 425 nm in aqueous acidic solutions and this is true with other catechin derivatives as well, as observed independently with isolated catechins from paper chromatograms. The maximum color measured at 425 nm was reached in 1 h, and no significant increase was observed further. The time curve response followed a typical rectangular hyperbola (Figure 3). As the catechin−diazotized sulfanilamide adduct is not very soluble in water, it is advisable to keep the acid concentrations to the optimum, and solutions, after preincubation, are diluted with water to the desired volume only at the time of taking reading.

The exponential decay type curve (Figure 4) of the effect of acid concentration on the color development with the diazo reagent and catechin solution showed that below optimum levels of acid concentration the results could be erratic due to solubility of the product or lower reactivity, whereas higher concentrations had no effect.

The reagent did not show any reaction with flavonols and their glycosides, organic acids, depsides, phenolic acids sugars, amino acids, or purines all of which, being water soluble, come in the acetone extracts of tea leaf. These compounds did not interfere in the detection of catechins with the reagent.

Recovery percent was close to 100% measured in solution extracts for added catechin aliquots in the estimation of tea catechins (Table 1). However, average recoveries of individual catechins from paper cromatograms all added up and, compared with the values measured in solution extracts, were found to vary between 80% to 89% (Table 2) and are in close agreement with the recoveries reported from paper chromatograms in an earlier work on the subject (Bhatia and Ullah, 1968).

The quality of black tea depends on the nature and amount of product formed during processing namely TFs and TRs which in turn are directly dependent not only on the amount of catechins present in the shoot but also on the ratio and amount of each individual catechin. The concentration of individual catechins as estimated by the present method follows a similar trend as reported by Punyasiri et al. (1990). However, values of ECG and GC and EC combined as reported by Bhatia and Ullah (1968) are far below normal. In fresh tea shoot extracts, EGCG was found to be the major catechin derivative and C the minor one.

Variation was observed in total catechins concentration in different parts of the tea shoot. The percentage concentration varied between 17% and 14%, being highest in stem. The values declined as tea bush approaches dormancy with the onset of winter in the month of October in the Kangra region of Himachal Pradesh (Table 3). Such seasonal variations were also noted by Bhatia and Ullah (1968). The concentration of total catechins in different components of tea shoot follow the order stem > second leaf > first leaf > bud.

Thus the reagent is found to be very helpful in the estimation of catechins in different parts of tea shoot for quality assessment following tea manufacture and in breeding programs.

ABBREVIATIONS USED

PPO, polyphenol oxidase; 2D-PC, two-dimensional paper chromatography; TF, theaflavin; TR, thearubigin; TG, theogallin; EC, epicatechin; GC, gallocatechin; C, d(+) catechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; TV, vegetatively propagated tea planting material developed by Tocklai Experimental Station, Jorhat, Assam, India.

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