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Estimation of Tea Polyphenols by Electrochemical Sensors via Screen Printed Electrodes

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Abstract
Tea contains different polyphenols which are antioxidant in nature and therefore those help us to survive from different diseases like cancer, cardio vascular etc. The main aim of the present work is to report a unique electrochemical sensor for detection of polyphenols in tea infusion using different types of screen-printed electrodes (e.g. screen-printed carbon and screen-printed with carbon nano tube). The methodology involved oxidation of alcohol (>C-OH present in polyphenols) to aldehyde or ketone (>C=O present in quinones). Various grades of tea were tested for polyphenols and a very good concentration pattern distinguishing green tea from black and other verities. The results were compared with those obtained by high performance liquid chromatography (HPLC). The method exhibited low limit of detection (0.801-1.151 mg/L), excellent selectivity against interfering compounds. This detection was very fast and required only five minutes because there was no use of chemical substance for immobilization. The method would have potential use in grading tea and fixing cost by the tea manufacturers.

Keywords: *Polyphenols, Screen-printed electrodes, Cyclic Voltammetry, Amperometry*

1 Introduction

Tea is one of the most popular drinks all over the world. It is prepared from two leaves and one leaf bud and internodes of the *Camellia sinensis*. It contains hundreds of compounds which include some polyphenolic compounds. These are the main antioxidants of tea. The antioxidant nature of polyphenols make them scavenge free radicals which are generated by different metabolic pathways in our body and those help to protect us from different diseases like cancer, neurological diseases, cardiovascular diseases etc. [1]. Therefore, for our health benefit it is important to know content of total polyphenols in tea.

Various grades of tea such as white, green, oolong and black tea are available in the market and those are produced depending on physical withering, fermentation condition and time. Green tea contains greater amount of polyphenols, particularly a group of flavan-3-ols commonly known as catechins. Green tea mainly consists of monomeric phenolic compounds like (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechingallate (ECG) and (-)-epicatechin (EC) [2, 3]. But black tea contains theaflavin (TF) and thearubigins (TR) due to oxidation of catechin during fermentation of tea leaves. Due to presence of high amount of polyphenols in tea, the taste of tea is slight astringent and bitter [4]. The quality of tea can be differentiated upon its colour, flavor and taste. Volatile compounds are responsible for flavor whereas color and taste of tea depends upon the different components of liquor like total phenolic compounds (EC, ECG, EGC, EGCG, TF, TR), caffeine etc. [5].

Concentration of polyphenols in tea can be determined by some conventional technique such as gas chromatography [5], high-pressure liquid chromatography (HPLC) [6], spectrophotometry [7] and electrochemical method [8, 9]. But these methods are time consuming and costly. Other methods include biosensors modified with different enzymes like tyrosinase, laccase, peroxidase etc. [10-12]. However, these biosensors using degradable biomaterials are unstable and use is restricted.

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The aim of the present work was the detection of total polyphenols quantitatively in different green and black tea using different electrodes (such as screen-printed carbon and screen-printed with carbon nano tube electrode). The emphasis was given to address the following issues: (i) comparison of electrodes, (ii) limit of detection, (iii) interference with other constituents, (iv) response time.
The sensor would be useful tool to assess the quality of tea for the valuation.

2 Experimental

2.1 Reagents

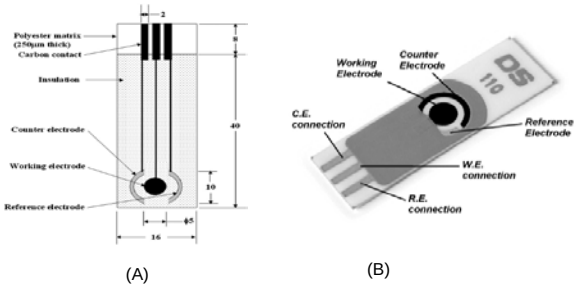
Catechin (≥98%, TLC) was purchased from Sigma, U.K. 0.1(M) of Sodium phosphate buffer (pH-6.8) was prepared using di-sodium hydrogen phosphate, dihydrogen sodium phosphate and potassium chloride purchased from Merck (India). Milli-Q (Millipore India Ltd., India) water was used for preparation of buffer. All other chemicals were of analytical grade and were purchased from Merck (India). Different green and black tea leaves were collected from market. Acetonitrile, acetic acid and H_2O (all are HPLC grade) for HPLC were purchased from Merck (India).

2.2 Instrumentation

Amperometric responses were observed by an Autolab (Ecochemie B.V. The Netherlands) electrochemical analyzer (PGSTAT 12). The terminals of the working (WE), reference (RE) and counter (CE) electrodes of the autolab analyzer were connected to the respective terminals of the transducer via standard connectors and experimental input parameters such as scan rate, potential, time of observation, etc. were given through general purpose electrochemical software (GPES) from a computer interfaced with the Autolab analyzer.

2.3 Sensor fabrication

Two sensing systems i.e. S1, S2 have been constructed for the detection of polyphenols. S1 was disposable screen- printed electrode (SPE) having a carbon working electrode, carbon counter electrode and silver/silver chloride reference electrode. These were printed onto a 250 mm thick polyester sheet (Cadillac Plastic Ltd., Swindon, UK) using a DEK 248 screen-printing machine (DEK Printing Machines Ltd., Waymouth, U.K.). The circular electrocatalytic working electrode was fabricated from a commercially available carbon powder (MCA Services Ltd., Cambs., UK), made into a screen-printable paste by mixing1:3 (w/w) with 2.5% w/v HEC in PBS. The reference electrode ink contained 15% w/w silver chloride in silver paste (MCA, UK) [13]. The counter electrode and basal tracks used to connect the electrodes to the measurement device were fabricated from I45R carbon ink (MCA,UK). The basal tracks were insulated from the measurement solution using an epoxy-based protective coating ink 242-SB (Agmet ESL Ltd., Reading, UK). The electrodes were then heat treated at 125^{o}C for 2h to cure the epoxy resin and to stabilize the electrocatalytic pad to allow prolonged use of the device in aqueous solutions. The details of the configuration of the S1 are given in Figure 1(A).
S2 was also disposable SPE having a carbon working electrode (4 mm diameter) imprinted with carbon nano tube, carbon counter electrode and silver/silver chloride reference electrode with dimensions 3.4 x 1.0 x 0.05 cm (Length x Width x Height). Configuration of the S2 is given in Figure 1(B).



(A) (B)

Fig. 1 Schematic representation of S1transducer (all dimensions are in mm) (A) and S2 transducer (B)

2.4 Methodology

The reaction involves electron transfer between the working electrode and counter electrode when a fixed voltage was applied across reference electrode and thus the response was monitored by amperometry. The reaction on the working electrode is given bellow:
Polyphenols → Quinone (1)

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To construct calibration curves, all the experiments were carried out with pure catechin [dissolved in reverse osmosis (RO) water]. To study interference due to presence of probable interfering substances, each of the electrodes was dipped into phosphate buffer (pH 6.8) and each of interfering substances such as amino acid (L-phenylalanine), carbohydrate (D-fructose), organic acid (oxalic acid) and ascorbic acid was added and amperometric response monitored.

2.4.1 Preparation of Tea extract

Different green and black tea samples (e.g. Green tea: Tetley, Darjeeling Longview,Twinnigs, Japanese, Golden tips, Lipton; black tea: CTC, Tulip bari, BOPL, Twinings) were collected from the market.These were given identification numbers as 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 respectively. The collected tea samples were kept in closed containers. An amount of 0.1 g of each tea was weighed and taken in a beaker. Five mL of boiling water was poured into the beaker and tea infusion was filtered after five minutes of soaking. The supernatant of tea infusion was used as sample for our electrochemical studies.

2.4.2 Measurement by electrochemical sensor

2.4.2.1 Cyclic voltammetric analysis

Cyclic voltammetry was performed using 200 mg/L catechin solution in 0.1(M) phosphate buffer solution (PBS, pH 6.8) at a scan rate 0.001V/s with potential -1V to +1V and step potential was 0.00305V for S1 and S2. Again cyclic voltammetry was also repeated for blank [mixture of 5 mL 0.1(M) phosphate buffer solution and 0.5 mL RO water] with S1 and S2. The optimum oxidation potentials were evaluated the same was used as the working potential for amperometric measurement.

2.4.2.2 Amperometric Analysis

S1 and S2 were connected to the electrochemical analyzer by a standard connector and the sensor assembly was dipped into 5 mL PBS in a 10 mL beaker and amperometry was performed at 0.450V vs. Ag/AgCl reference electrode. This constant oxidation potential was obtained from cyclic voltammetry study (Figure 2). When equilibration was achieved, amperometric study was conducted by adding stipulated amount of catechin solution to measure responses in the range 0-400 mg/L. These were utilized to construct the calibration curve so that the amperometric response for any tea sample could be converted in terms of amount of catechin equivalent per gram of dry tea leaves. This might be noted that due to dilution of the analyte concentration by ten times, actual concentration being measured was ten times less and this might be reflected in the values of limit of detection or sensitivity etc. In case of tea samples, once the equilibration was achieved, 0.5 mL of tea solution was added and responses noted 300 s after the addition of tea solution. Each experiment was repeated three times independently. Tea samples were tested for their polyphenol contents following the same procedure.

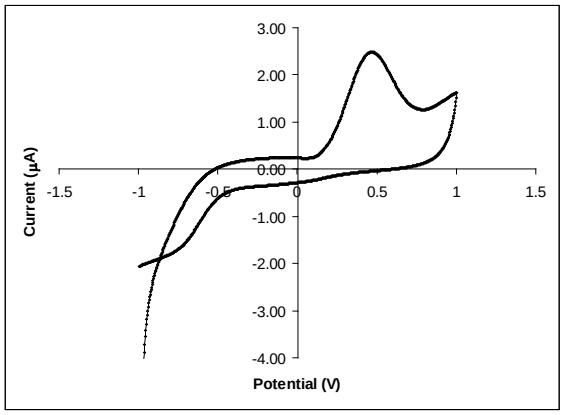

Fig. 2 Cyclic voltammogram for catechin (200 mg/L) using S1

2.4.3 HPLC analysis of tea polyphenols content

Polyphenol standards (gallic acid, epigallocatechin, (+)-catechin, caffeic acid, (-)-epicatechin, catechin-gallate) were used for characterization of the phenolics in tea samples. Stock solutions were diluted with water to get a final concentration of 20µg/mL. The calculations of HPLC were constructed from chromatograms as peak area vs. concentration of standard solutions.

The HPLC apparatus consisted of a Varian LC system (USA) equipped with a ProStar 230 solvent delivery module, and a ProStar 330 PDA detector. The separation of phenolic compounds was performed on an OmniSpher C18 column (25 cm x 4.6 mm, Varian, USA) equipped with

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ChromSep guard-column (1 cm x 3 mm, Varian, USA).

50 mg of tea sample was infused with 10 mL freshly boiled distilled water in a boiling water bath for 10 min. The infusion was filtered through filter paper. Filtrate was treated with equal volume of chloroform. After settling, the lower layer was discarded. This procedure of decaffeination was repeated again two times [14]. The upper level was treated with ethyl acetate. The ethyl acetate layer was dispensed in eppendroff tubes and was evaporated overnight at 45ºC. After the evaporation, it was dissolved in HPLC grade water and injecting into the HPLC. HPLC was carried out according to the method described in a paper [15] and the chromatographic conditions were as follows:

Injection volume: 20 µL

Column: 5µ-Diamonsil^{TM} C18, 4.6 mmx250 mm

Column Temperature: 40^0C

Mobile phase: Solvent A: acetonitrile/acetic acid/water (6:1:193, v);
Solvent B: acetonitrile/acetic acid/water (60:1:139, v)

Gradient: 100% (v) solvent A to 100% (v) solvent B by linear gradient during first 45 min and then 100% (v) solvent B till 60 min.

Flow rate: 1 mL min^{-1}

Detector: Shimadzu SPD ultraviolet detector, 280 nm

2.4.4 Interference

The selectivity of the sensory system should depend on how the response is affected by the presence of interfering substances e.g. amino acid (L-phenylalanine), carbohydrate (D-fructose), organic acid (oxalic acid) and L-ascorbic acid. Thus the responses were observed in presence of 400mg/L concentration of the above substances.

3 Results

To identify the optimum oxidation potentials, cyclic voltammograms were obtained with a scan rate of 0.001V/s where distinct peaks were observed. Figure 2 and 3 show oxidation peak at 0.450V and 0.417V for S1 and S2 respectively. A cyclic voltammogram studies and explanation on summary of the oxidation peaks are given in Table 1. Figure 4 shows the responses of different concentrations of catechin in the range of 0-400 mg/L using S1 and S2. The effect of interfering compounds present in tea has been shown in Figure 5 (for S1).

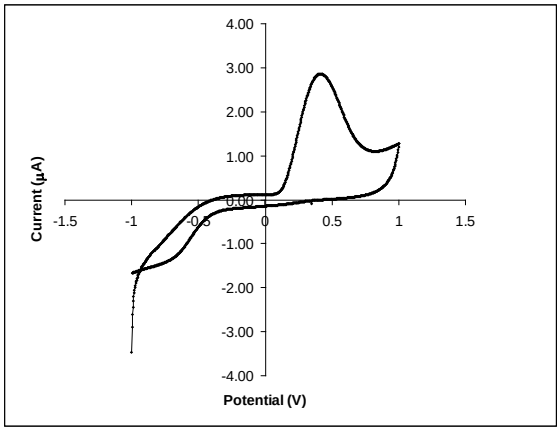

Fig. 3 Cyclic voltammogram for catechin (200 mg/L) using S2

Table 1: Comparison of peak voltages obtained from cyclic voltammograms using different electrodes (S1 and S2)

Electrode Details	Figure no.	Analyte	Oxidation peak (V)	Remarks for oxidation peak
Screen-printed (S1)	2	Polyphenols	0.450	Oxidation of polyphenols to quinone
Screen-printed (with carbon nano tube) (S2)	3	Polyphenols	0.417	Oxidation of polyphenols to quinone

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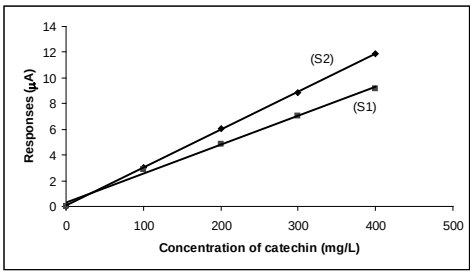

Fig. 4 Comparison between calibration curves for catechin using different electrodes (S1 and S2): (S1) y = 0.0225x + 0.2959; R^2= 0.9954; (S2) y = 0.0295x + 0.057; R^2 = 0.9998)

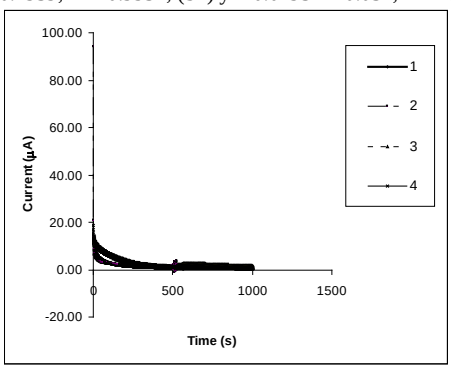

Fig. 5 Effect of presence of probable interfering substances on response given by screen printed electrode (S1). (1)Amino acid (D-phenylalanine), (2)Ascorbic acid (3)Carbohydrate (D-fructose) (4)Organic acid (oxalic acid)

3.1 Limit of detection, limit of quantification and sensitivity

The limits of detection (LOD) [16], limit of quantification (LOQ) [16], and sensitivity [17] of different electrodes are given Table 2. These parameters were calculated following the given expressions:

LOD = 3S/m (2)
LOQ = 10S/m (3)

Where, m represents the slope of the graph or sensitivity and S is the standard deviation.
The response due to addition of tea samples changed with time until 5 minutes. Hence a time of 5 minutes was chosen as sensing time.

Table 2: Limit of Detection, Limit of Quantification and Sensitivity values for S1 and S2 electrodes

Name of the electrodes	Limit of Detection (mg/L)	Limit of Quantification (mg/L)	Sensitivity nA(mg/L)$^{-1}$
S1	1.151	3.838	2.248
S2	0.801	2.671	0.453

3.2 HPLC measurements

The TP contents of investigated tea were determined by comparing with peak areas given by standard solutions of a number of polyphenols in the catechin family. The individual peak area given by HPLC chromatograms at 280 nm corresponding to the individual members of catechin family was calculated and summed up to get TP [18]. The HPLC chromatogram of standard (gallic acid and epicatechin), one green tea (sample 4) and one black tea (sample 7) were represented in Figure 6 (a), (b), (c) and (d) respectively.

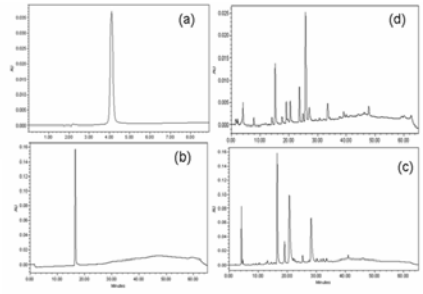

Fig. 6 The HPLC Chromatogram of standard a)gallic acid b)epicatechin c)green tea(sample4) d)black tea (sample7)

4 Discussions

From Table 1, it is clear that the oxidation peak of 0.450V and 0.417V when S1 and S2 were used respectively. Hence amperometry was performed at 0.450V for S1 and 0.417V for S2. With fixed potential values we performed amperommetric study for different green and black teas using S1 and S2 and compare the polyphenols present in different teas with different screen-printed electrodes. The results are encouraging since a pattern of polyphenol content was obtained in all these samples and as expected, green tea and Darjeeling black tea showed higher responses than inferior tea such as CTC (Assam). The results obtained from the study of interference (Figure 5) have been satisfactory since insignificant variations of response were obtained for the interfering substances used. The sensor would be a useful tool to assess the quality of tea by the producers and also by customers. This might be used to estimate polyphenols even in the process of making the tea i.e. fermentation. As the LOD values for S1 and S2 were 1.151 mg/L and 0.801

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mg/L respectively, therefore the sensitivity of the detection was in the order S2>S1. For S1 and S2 currents were measured across carbon–carbon and imprinted carbon nano tube electrodes respectively. Thus the response given by S2 could be the highest due to the least potential drop due to lowest electrode resistance. Therefore S2 was more sensitive for the detection. Due to the porosity and reactivity of the electrodes of S1 might cause a lower response compared to that of S2 where current response was higher with higher sensitivity. S2 is somewhat costly to make whereas S1 is economical and disposable in nature (one time use).

Total polyphenols content in different green and black tea are present in Table 3 (mg polyphenols/gm of tea leaves).

Table 3: Total polyphenols content in different green and black tea samples measured by S1 and S2 electrodes

Sample number	Tea samples	S1 (mg TP/gm of tea leaves)	S2 (mg TP/gm of tea leaves)
1	tetley green tea	8.52±0.3	8.58±0.3
2	darjeeling longview black tea	7.66±0.3	7.85±0.2
3	twinings green tea	7.14±0.2	7.58±0.3
4	japanese green tea	6.29±0.1	6.49±0.2
5	golden tips green tea	6.05±0.1	6.64±0.2
6	lipton green tea	6.02±0.2	6.71±0.2
7	CTC black tea	1.06±0.03	1.32±0.03
8	tulip bari black tea	0.96±0.03	0.78±0.02
9	BOPL black tea	0.81±0.02	0.88±0.02
10	twinings black tea	0.76±0.02	0.63±0.01

Results represent mean values ± standard deviation (SD) of three independent measurements

The HPLC analysis showed that the most abundant polyphenolic compounds in the tea investigated in this study were gallic acid and (-)-epicatechin. Green tea showed the highest TP content due to presence of high amounts of the important tea antioxidants (gallic acid, epigallocatechin, (+)-catechin-hydrate, caffeic acid, (-)-epicatechin) in comparison to the black tea. The results could compare well with results obtained by the proposed sensors. A high correlation coefficient was obtained between different electrochemical sensors and HPLC methods. A correlation matrix represented the comparative study of polyphenols (in respect of catechin) for different methods shown in Table 4. The highest correlation index was obtained for total polyphenols estimation by ES(S2) sensor and HPLC (0.99) whereas lowest correlation showed between ES(S2) sensor with ES(S1) sensor (0.88). Correlation between ES(S1) and HPLC was slightly lower (0.93) than ES(S2) and HPLC (0.99).

Table 4: Correlation matrix obtained from responses due to catechin by the proposed electrochemical sensors and standard HPLC method

	ES(S1)	ES(S2)	HPLC
ES(S1)	1	0.88	0.93
ES(S2)	0.88	1	0.99
HPLC	0.93	0.99	1

5. Conclusions

In this paper, a very simple technique for estimation of polyphenols in different tea has been reported. The procedures were applied to estimate qualitatively as well as quantitatively the presence of polyphenols in tea samples collected from market. The time of response is only a few minutes and measurement is very useful due to highly sensitive, reproducible and selective. The proposed electrochemical technique will be an attractive alternative procedure due to minimal sample preparation, high selectivity, low cost per analysis in comparison of other like method like HPLC. The method could be explored for a new definition of tea quality and gradation.

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