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Selective Determination of Catechin among Phenolic Antioxidants with the Use of a Novel Optical Fiber Reflectance Sensor Based on Indophenol Dye Formation on Nano-sized TiO_2

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ABSTRACT: The optical sensor for "tea catechins" was built by immobilizing 2,2′-(1,4-phenylenedivinylene)bis-8-hydroxyquinoline (PBHQ) on TiO_2 nanoparticles (NPs). The sensor worked by "indophenol blue" dye formation on PBHQ-immobilized TiO_2 NPs as a result of *p*-aminophenol (PAP) autoxidation with dissolved O_2 at pH 10. Among quercetin, rutin, naringenin, naringin, gallic acid, caffeic acid, ferulic acid, *p*-coumaric acid, catechin, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, and trolox, only catechin group antioxidants delayed the color formation on NPs, as measured by the reflectance signal at 710 nm. For quantitative analysis, reflectance signal versus time was recorded, and the difference between the areas under curve (ΔAUC) in the presence and absence of catechin was correlated ($r = 0.98$) to catechin concentration. The selectivity of the sensor for catechins was shown in tea infusions compared to other plant extracts and was ascribed to the nonplanar structure of catechin interfering with the formation of perfectly conjugated indophenol blue on TiO_2 surface.

KEYWORDS: *tea catechins, nanoparticles, indophenol blue, reflectance sensor, antioxidant analysis*

INTRODUCTION

From its legendary discovery in the year 2737 BCE by the emperor Sheng Nung, when leaves fell into a pot of boiling water, to the universally enjoyed beverage it is today, tea has had a significant role in human history. The medicinal uses of tea were first reported by Chinese scholars in a text written by Pen T'sao ca. 24 to 221 CE, and the physiological effects of tea and tea flavonoids continue to challenge scientists. Tea was first used in China for its medicinal properties 5000 years ago.[1] Tea beverage is an infusion of the leaves of *Camellia sinensis*.[2] Many kinds of tea are produced, although these can be classified principally into three types: green (unfermented), oolong (semifermented), and black (fully fermented).[3] In addition to the direct consumption of tea either by brewing loose leaves or tea bags or in a ready-to-drink form, there have recently been more applications for tea extracts, especially in the nutraceutical and food areas.[4] Flavanols and flavonols, the main classes found in tea, are 30% of the dry weight of fresh leaf. Catechins (flavan-3-ols), the predominant flavonoids, are characterized by di- or trihydroxyl group substitution of the B-ring and the meta-5,7-dihydroxy substitution of the A-ring. Fresh tea leaf contains four major catechins: (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG), which are colorless, water-soluble compounds responsible for the bitterness and astringency of green tea.[5] Green tea is made by inactivating the enzymes in the fresh leaves, either by firing or by steaming, to prevent the enzymatic oxidation of catechins. Black tea is made by a polyphenol oxidase catalyzed oxidation of fresh leaf catechins, termed fermentation. This fermentation process results in the oxidation of simple polyphenols, that is, tea catechins, to more complex condensed molecules, which give black tea its typical color and strong, astringent flavor. Oolong tea, also called "semifermented tea", is prepared by shortly firing and then drying the leaves and, thus, its characteristics are between those of black and green teas.[6] During fermentation of black tea, polyphenol oxidase in the tea leaves catalyzes the oxidation of the majority of catechins into theaflavin, hence reducing its catechin content.[7] A growing body of evidence from both human and animal studies suggests that regular consumption of green tea can reduce the incidence of a variety of cancers, including those of the colon, pancreas, and stomach, as well as other diseases.[4,8−10] The traditional health claims for tea such as vasodialation, coronary heart disease prevention, and diuretic, antioxidant, anticholesterol, anti-inflammatory, antibacterial, antiviral, anticancer, and antineurodegenerative effects are mostly due to tea catechins.[5,11]

Liquid chromatography (LC) and capillary electrophoresis (CE) are the most cited techniques for catechin separation, identification, and quantitation. LC with UV detection is frequently used; however, mass spectrometry and electrochemical, fluorescence, and chemiluminescence detection are also utilized in cases requiring higher sensitivity or selectivity. Two modes of CE, capillary zone electrophoresis and micellar electrokinetic capillary chromatography, have been employed for the determination of catechins. Additional analytical techniques, such as gas chromatography, thin-layer chromatog-

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raphy, paper chromatography, spectrophotometry, biosensing, chemiluminescence, and nuclear magnetic resonance spectroscopy have also been utilized for the determination of catechins. All of these techniques requiring expensive, bulky, and sophisticated instruments have been extensively reviewed.[12]

Aside from the requirement for developing sensitive and selective methods of analysis of tea catechins in regard to recovery of natural chemotherapeutic agents to support preclinical efficacy and toxicity testing studies, catechin group compounds need to be analyzed in blood plasma for bioavailability, pharmacokinetics, and disease prevention studies.[12] The limited number of enzyme-based biosensors for catechin detection[13] has been criticized for suffering from low stability, sensitivity, and specificity.[14] Although some electrochemical sensors were previously developed for catechin with limited selectivity, such as the laccase-immobilized Au nanoparticle encapsulated−dendrimer bonded polymeric sensor,[14] there is no optical sensor in the literature for selective determination of catechin group antioxidants. The aim of this paper is to fill this literature gap and to develop an optical fiber reflectance sensor for the determination of "tea catechins" among other important phenolic antioxidants such as quercetin (QR), rutin (RT), naringenin (NGN), naringin (NG), gallic acid (GA), caffeic acid (CFA), ferulic acid (FRA), *p*-coumaric acid (PCA), and trolox (TR). For this purpose 2,2′-(1,4-phenylenedivinylene)bis-8-hydroxyquinoline (PBHQ), previously used for *p*-aminophenol detection,[15,16] was immobilized on TiO_2 nanoparticles (NPs) (Degussa P25). The proposed reflectometric sensor is expected to be superior to simple solution-phase spectrophotometry and may enable high-throughput real-time screening of catechin detection via an optical scanner. Automation and commercialization (such as reflectometric/colorimetric kits) of the proposed sensor may minimize costs. Advantages of absorptimetric or reflectometric sensors include ease of use, visual evidence of the detection event, and resistance to interferences.[17]

MATERIALS AND METHODS

Chemicals and Apparatus. TiO_2 Degussa P-25 (Aeroxide TiO_2 P25, hydrophilic fumed metal oxide, average particle size = 21 nm, composed of 75−80% anatase and 20−25% rutile, specific surface area (BET) = 50 ± 15 $m^2\ g^{-1}$) was supplied by courtesy of Evonik Degussa Ticaret Ltd. Sti., Turkey. The hydroxyquinoline derivative, 2,2′-(1,4-phenylenedivinylene)bis-8-hydroxyquinoline (PBHQ), was synthesized at the chemistry laboratory of the Department of Chemistry, Faculty of Science, Ankara University. The flavonoids quercetin, rutin, naringin, naringenin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate and morin were purchased from Sigma Chemical Co.; (−)-catechin and gallic acid from Fluka Chemicals; ferulic acid, *p*-coumaric acid, caffeic acid, ascorbic acid, and trolox from Aldrich Chemical Co.; and Na_2CO_3 and *p*-aminophenol from E. Merck.

Reflectance measurements were carried out using a commercially available miniature fiber optic based spectrometer (Ocean Optics Inc., HG4000CG-UV-NIR), which utilizes a small tungsten halogen lamp (Ocean Optics Inc.) as the light source and a CCD-based detector for reflectance measurements. Light reflected from the probe surface was transmitted by a bundle of optical fibers to a miniature fiber optic spectrophotometer (Ocean Optics HG4000CG-UV-NIR), which on the other hand was connected to a PC (Dell-compatible). For optical isolation, the probe and the detector were kept in a black box to minimize any interference from ambient light.

For HPLC analysis, a Waters Breeze 2 model HPLC system (Milford, MA, USA) equipped with a 1525 binary pump, a column thermostat, a 2998 photodiode array detector (Chelmsford, MA, USA), an ACE C18 column (250 mm × 4.6 mm × 5 μm) (Milford, MA, USA), and a Hamilton 25 μL syringe (Reno, NV, USA) was used. Data acquisition was accomplished using Empower PRO (Waters Associates, Milford, MA). The analytical wavelength of detection was λ = 280 nm.

Two different solutions of the mobile phase, that is, methanol (A) and 0.2% of *o*-H_3PO_4 in bidistilled water (B), were used in gradient elution.[18] The following working mode was adopted for gradient elution in the analysis of tea antioxidants[4] (V_{sample} = 20 μL; flow rate = 0.8 mL min^{-1}): 1 min, 0% A−100% B (slope 1.0); 20 min, 70% A−30% B (slope 1.0); 25 min, 0% A−100% B (slope 1.0).

Preparation of Solutions. PBHQ solution at 1.0 × 10^{-3} M concentration was prepared in tetrahydrafuran (THF) and *p*-aminophenol (PAP) solution at 1.0 × 10^{-2} M in ethanol (EtOH), the latter being diluted 10 times with EtOH just before analysis. Sodium carbonate at 1% (w/v) was prepared in distilled water. All polyphenolic compounds, except quercetin and morin, were freshly prepared in 96% EtOH at 10 mM concentration. All necessary dilutions, just before analysis, were made with distilled water with the exception of quercetin and morin, which were diluted with 1% Na_2CO_3 solution.

Infusion Preparation from Ready-To-Use Tea Bags. Tea bags supplied from local markets in Istanbul were weighed as such to yield masses ranging between 2.0575 and 2.5713 g. Tea bags were dipped into and pulled out of beakers containing 250 mL of freshly boiled water for the first 2 min and allowed to steep for the remaining 3 min in the covered beakers (total steeping time was 5 min). The bags were removed, and the partly turbid solutions were filtered through a black-band Whatman quantitative filter paper after cooling to room temperature.[19] One milliliter of sample was withdrawn from 250 mL tea infusions and diluted 10 times for green tea and 5 times for black tea (*Ca. sinensis*) samples. Herbal tea samples, namely, chamomile (*Matricaria chamomilla*), fennel (*Foeniculum vulgare*), mint (*Lamiaceae*), and sage (*Salvia*), known to be rich in phenolic antioxidants other than catechins, were used without any dilution. Only green and black tea samples caused a delay in the formation of the blue-colored product.

Preparation of Optical Fiber Reflectance Sensor. To develop the optical fiber reflectance sensor, PBHQ was immobilized on nano-sized TiO_2 (Degussa P25). Five milliliters of PBQH solution in THF was added to 1 g of stirred TiO_2 suspension, and the mixture was allowed to stand at ambient temperature to evaporate the solvent. PBQH-immobilized nanoparticles (NPs) were kept in the dark in a transparent film-covered beaker until use.

Reflectance Measurements and Quantification of Catechin Group Flavonoids. In the experiments, 0.2 g of PBHQ-immobilized TiO_2 NPs were weighed into a series of conical bottom centrifuge tubes, and then 2 mL of Na_2CO_3 and 0.5 mL of PAP solutions, followed by *x* mL of standard or sample were added and diluted to a final volume of 5 mL with distilled water. Each tube was continuously shaken up and down for different time periods, that is, 1, 3, 5, 7, 10, 15, 20, and 40 min. After shaking, the tubes were centrifuged at 4000 rpm (2500*g*) for 5 min. Upper liquid phases were decanted, and the reflectance of the "indophenol blue" formed on nano-sized TiO_2 was measured at 710 nm. All reflectances were measured against a blank nano-TiO_2 that does not contain either PAP or antioxidant sample. In blank preparation, exactly the same amounts of PBHQ-immobilized TiO_2 and Na_2CO_3 were used, and an optimal shaking time of 40 min was adopted. All solutions were kept saturated with dissolved oxygen during measurements, as O_2 was a critical parameter affecting the degree of oxidation of PAP and subsequently the reproducibility of sensing.

The reference (different from the blank, against which reflectance measurements were made) contained PAP but not antioxidants in addition to the blank constituents. To investigate the effects of standards or samples, selected compounds were tested in the presence of PAP, and the obtained reflectance values were compared to that of a reference containing only PAP at the same conditions. The reference showed the most negative (i.e., of greatest absolute value at 10 min, −13.31) reflectance, whereas the other phenolics, except catechin

group antioxidants, showed close values that were relatively independent of time. On the other hand, catechin group flavonoids gave a gradual increase (in absolute value) of reflectance with time (e.g., 10 min value, −9.83) and finally reached the reflectance of the reference. The catechin group showed the steepest reflectance differences during the initial phase of color development on TiO_2 NPs. For quantitation of the catechin group antioxidants, reflectance signal versus time curves were recorded for both catechin-containing samples and reference, and the difference between the areas under curve (ΔAUC) in the presence and absence of catechin was correlated to catechin concentration.

Determination of Optimal PAP Concentration for Indophenol Blue Formation. Different volumes of PAP solution between 0.1 and 1.0 mL were added to 0.2 g of NPs, followed by 2 mL of 1% Na_2CO_3. After dilution to 5 mL with H_2O, the tubes were shaken for 40 min, and the reflectance of the blue product formed on the solid surface was measured.

Reflectance Measurements of Mixtures. To investigate the effects of other phenolic antioxidants on catechin determination, different binary mixtures were prepared by adding 0.5 mL of selected phenolic compounds one by one (at 1.0×10^{-3} M concentration) to 0.5 mL of equimolar catechin; moreover, epicatechin + trolox and epicatechin + quercetin mixtures were examined under the same conditions. Reflectances of the above mixtures measured at the end of 5 min were compared to that of the reference not containing antioxidants but PAP. In a second group of experiments, three different concentrations of naringin, naringenin, quercetin, and ferulic acid were added to a constant amount of catechin solution, and reflectances were measured as described.

Catechin Sensing Applied to Green, Black, and Herbal Tea Samples Using the Method of Standard Additions. One milliliter each of 1.0×10^{-3} M catechin and trolox were added to two different tea samples, namely, 1 mL of 1:5 diluted green tea (GT) and 1 mL of nondiluted chamomile tea (CT) infusions. Reflectance measurements were performed for 1, 3, 5, 7, 10, 15, 20, and 40 min time periods for GT and CT tea samples with and without CAT and TR. Reflectance signal versus time was recorded, and the results were compared.

RESULTS AND DISCUSSION

Indophenol Blue Formation. The dye formation scheme is based on the reaction between *p*-quinone imine (i.e., that emerged from autoxidation of *p*-aminophenol with dissolved O_2 in alkaline solution) and PBHQ preimpregnated on TiO_2, as shown in Figure 1.

As reported before by Gil et al.,[20] air oxidation of PAP is fast at high pH values (9.0−10.0), so the reaction was performed at pH 10, achieved by using 1% Na_2CO_3 solution. Color stability of indophenol blue dyes usually lies within the pH range of 10−12,[21] but this is dependent on the nature and position of substituents of the phenolic substrate to which the oxidation product of PAP (quinone-imine) couples. Thus, the original assay depending on the reaction of *p*-aminophenol with PBHQ was developed in alkaline medium.[15] In this study PBHQ was immobilized on TiO_2 NPs, and the reaction occurred on the solid phase. PBHQ could be much better immobilized than 8-hydroxyquinoline (HQ) on an XAD-7 resin to build a solid reflectometric sensor.[16] HQ could easily desorb from the solid sensor surface under the conditions of sensing measurement (e.g., leach out as 8-hydroxyquinolinate anion at pH 10, considering that the consecutive acidity constants of free and silica gel-immobilized HQ were reported as $pK_{a1} = 4.0$, $pK_{a2} = 11.5$, and $pK_{a1} = 3.3$, $pK_{a2} = 7.0$, respectively,[22]) and a proper signal could not be obtained. Generally, TiO_2 surface is oleophilic and hydrophobic; that is, it exhibits "amphiphilicity".[23] Batch adsorption experiments revealed that under the predetermined conditions, the PBHQ adsorption capacity, at

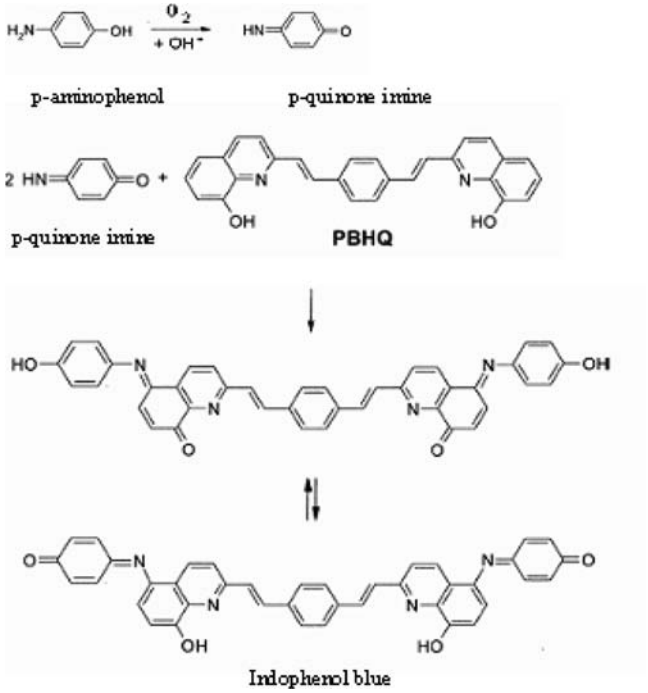

Figure 1. Autoxidation of *p*-aminophenol (PAP) to *p*-quinone imine with dissolved O_2 and formation of "indophenol blue".

saturation, of nano-TiO_2 was 9.4 μmol g^{-1} (calculated by UV spectrometry for the PBHQ remaining in solution). The actually used nanosensor in the experiments was undersaturated with respect to PBHQ, because 5.0 μmol g^{-1} PBHQ was retained by evaporation of solvent on TiO_2. This amount of PBHQ was shown not to leach out from the sorbent surface under the assay conditions. Maximum reflectance signal was recorded at 710 nm. The absolute value of negative reflectances increased with increasing intensity of the blue color on NPs.

To obtain maximum reflectance on the solid phase, the optimal PAP concentration was investigated by recording reflectance values against final PAP concentration in a total volume of 5 mL (Figure 2). The reflectances were nearly

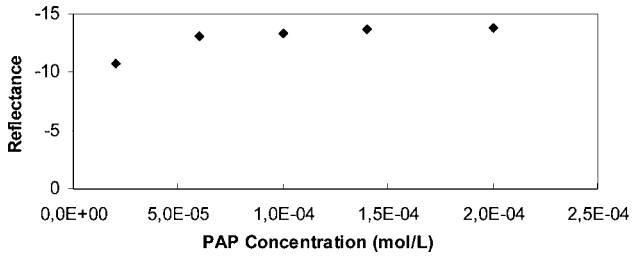

Figure 2. Reflectance versus final concentration of PAP.

constant for PAP concentrations between 1.0×10^{-4} and 2.0×10^{-4} so that 1.0×10^{-4} M PAP final concentration was selected for further experiments (0.5 mL of 1.0×10^{-3} M PAP was added, the total volume being 5 mL).

Effects of Phenolic Antioxidants on Reflectance of Indophenol Blue on NPs. To examine the effects of various antioxidant phenolic compounds on indophenol blue formation on NPs, selected antioxidants covering various classes of polyphenolics and flavonoids were investigated: quercetin

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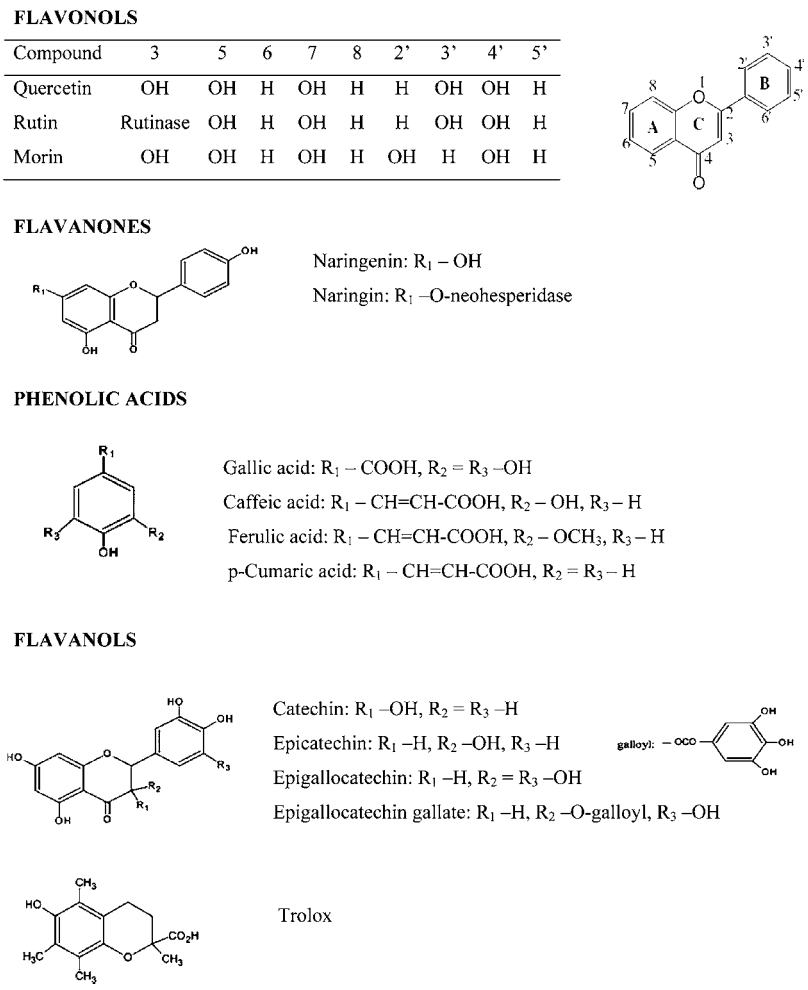

Figure 3. Structural formulas of the tested phenolic compounds.

(QR), rutin (RT), and morin (MR) were flavonols; naringenin (NGN) and naringin (NG) were flavanones; gallic acid (GA) was a phenolic acid; caffeic acid (CFA), ferulic acid (FRA), and *p*-coumaric acid (PCA) belonged to the hydroxycinnamic acids subclass of phenolic acids; catechin (CAT), epicatechin (EC), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) were flavanols. Trolox (TR) was also tested as being a reference standard. Structural formulas of the tested antioxidants are shown in Figure 3. Reflectance values were compared at the end of the 10 min reaction time in the presence and absence of 1 mL 1.0×10^{-3} M TR, QR, CAT, GA, CFA, RT, NG, NGN, EC, ECG, MR, FRA, EGCG, and EGC separately. Among all examined phenolic compounds, only catechin group antioxidants (CAT, EC, ECG, EGC, and EGCG) caused a drastic decrease in the color intensity on NPs, and the single interferent was a nonphenolic thiol compound, GSH (Table 1).

Additionally, time-dependent effects of the tested antioxidant compounds on indophenol blue formation on NPs were examined. For this purpose, four different concentrations of TR, QR, CAT, NGN, EC, and FRA (final concentrations were 0.5×10^{-4}, 1.0×10^{-4}, 1.5×10^{-4}, and 2.0×10^{-4} M in 5 mL solution) were tested. The reflectance signal was recorded in the presence and absence of selected compounds individually for 1, 3, 5, 7, 10, 15, 20, and 40 min. None of the mentioned compounds except CAT and EC caused a major change in reflectance signal at any concentration. As an example, reflectance signal versus time curves in the presence and absence of 1.0×10^{-4} M antioxidants are shown in Figure 4. In the absence of antioxidants, the reference (containing only PAP) curve showed almost a constant reflectance independent of time.

Table 1. Reflectance of Indophenol Blue Formed on NPs at the End of the 10 min Reaction Time in the Presence and Absence of Selected Antioxidants at 2.0×10^{-4} M Final Concentration

antioxidant	reflectance	antioxidant	reflectance
ref (only PAP)	−13.603	FRA	−13.200
TR	−13.211	**CAT**	**-9.828**
QR	−12.753	**EC**	**-9.731**
GA	−12.214	**EGCG**	**-9.756**
CFA	−13.444	**EGC**	**-10.769**
RT	−13.409	**ECG**	**-8.907**
NGN	−12.478	PCA	−12.366
NG	−12.930	**GSH**	**-9.770**
MR	−12.955		

Reflectance Measurements of Mixtures. To investigate the effects of other phenolic antioxidants on cathechin determination, 0.5 mL volumes of selected compounds at 1.0×10^{-3} M concentration were added to 0.5 mL of 1.0×10^{-3} M CAT, and the reflectance values were recorded (as described before) at the end of 5 min of reaction time (Table 2).

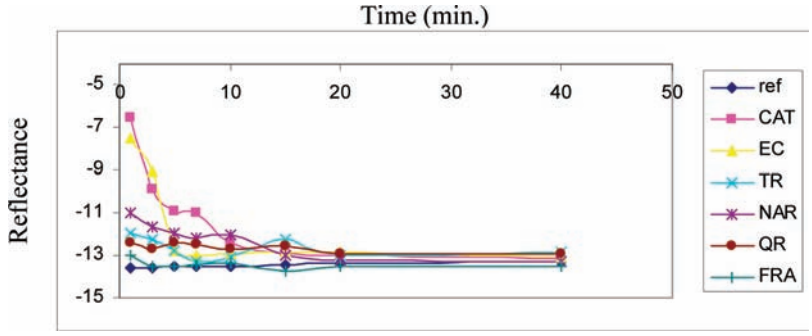

Figure 4. Reflectance signal versus time in the presence and absence of 1.0×10^{-4} M antioxidants.

Table 2. Reflectance Measurements of Different Binary Mixtures Containing 1.0×10^{-4} M CAT and Selected Antioxidants at 1.0×10^{-4} M Final Concentration (at the End of 5 min of Reaction Time)

compound	reflectance	compound	reflectance
ref (only PAP)	−13.150	CAT + CFA	−10.117
only CAT	−9.083[a]	CAT + GA	−10.178
CAT + NGN	−9.537	CAT + QR	−10.112
CAT + NG	−9.097	CAT + EC	−9.055
CAT + FRA	−10.163	CAT + TR	−9.239

[a]RSD = 4.90% for $N = 7$.

In the second experiment, 0.25, 0.5, and 1.0 mL volumes of 1.0×10^{-3} M NG, NGN, QR and FRA were added to 1.0 mL of 1.0×10^{-3} M CAT, and reflectances were measured at the end of the fifth minute (Table 3). As can be seen from Tables 2 and 3, selected phenolic compounds caused relatively minor changes on the CAT reflectance at the tested concentrations.

Plausible Reaction Mechanism. The reaction of *p*-benzoquinone monoimines with monohydric phenols gives indophenol dyes by a process involving a rate-controlling electrophilic coupling of the monoimine to the para-position of the phenol, followed by rapid oxidation of the resulting leuco-dye by a second molecule of the monoimine.[24] At pH >10, the neutral monoimine and the phenolate ion are the major reactive species, whereas below pH 10, the reaction between the conjugate acid of the monoimine and the phenolate ion becomes significant. Because the pK_a for catechin was reported as 7.8 by Salimi et al.,[25] catechin phenolate anion at the working pH (pH 10, achieved in Na_2CO_3 solution) of the proposed method would compete with PBHQ for monoimine, thereby distorting the coplanar structure of the PAP−PBHQ dye formed on TiO_2 surface (i.e., it should be remembered that catechins have a noncoplanar, in fact, perpendicular, geometry of the flavanol A- and C-rings to the B-ring[26] due to the absence of 2,3-double bond, hindering conjugation of the whole molecule). This competition was apparent in preliminary experiments where catechin at high concentrations was noted to yield a purple color with PAP in the absence of PBHQ (e.g., possibly due to PAP−catechin coupling under oxygenated alkaline conditions). However, the utilized reaction cannot be carried out in solution under the predetermined conditions, because when PBHQ dissolved in THF, PAP dissolved in alcohol, Na_2CO_3, and CAT dissolved in water are brought together, a green-colored precipitate is formed, the turbidity of which hinders spectrophotometric measurements. Thus, sensing on a solid sorbent surface was preferable.

Quercetin, rutin, and morin include the C_2-C_3 double bond in conjugation with the 4-oxo function in the C-ring, responsible for the electronic delocalization involving the A- and B-rings through C and for spreading conjugation over the entire molecule; therefore, their structural properties do not hinder the formation of a coplanar dye on derivatized rutile surface. Structural aspects of quercetin investigated at a theoretical level[27,28] have shown a completely planar structure, characterized by an extended conjugation over the whole molecule. Even though naringin and naringenin lack this C_2-C_3 double bond (like catechin group flavonoids), they have very few phenolic −OH groups to effectively interact with *p*-aminophenol. Additionally, they lack the −OH or −O-galloyl substituents (present in catechin derivatives) attached to the C_3-position of the C-ring that are also expected to interfere with coplanar dye formation. Thus, the proposed sensor selectively detects the catechin group flavonoids, because only this group of antioxidants delayed the color formation on nano-sized TiO_2 by interfering with the formation of the indophenol dye.

The ability of catechins to form multiple hydrogen bonds on the lipid bilayers is known from the literature.[11] Thus, in addition to the main mode of interaction (basically electrostatic), catechin having five −OH groups may also form H-bonds primarily with PAP in solution and secondarily with

Table 3. Reflectance Measurements of 2.0×10^{-4} M CAT in the Presence of 0.5×10^{-4}, 1.0×10^{-4}, and 2.0×10^{-4} M Final Concentrations of Selected Phenolic Antioxidants

compound	reflectance	compound	reflectance
ref (only PAP)	−13.282	CAT + 1.0 mL of NGN	−10.074
CAT (alone)	−9.707	CAT + 0.25 mL of QR	−10.596
CAT + 0.25 mL of NG	−9.86	CAT + 0.50 mL of QR	−9.914
CAT + 0.50 mL of NG	−9.706	CAT + 1.0 mL of QR	−9.892
CAT + 1.0 mL of NG	−9.666	CAT + 0.25 mL of FRA	−9.012
CAT + 0.25 mL of NGN	−9.974	CAT + 0.50 mL of FRA	−9.014
CAT + 0.50 mL of NGN	−9.418	CAT + 1.0 mL of FRA	−9.556

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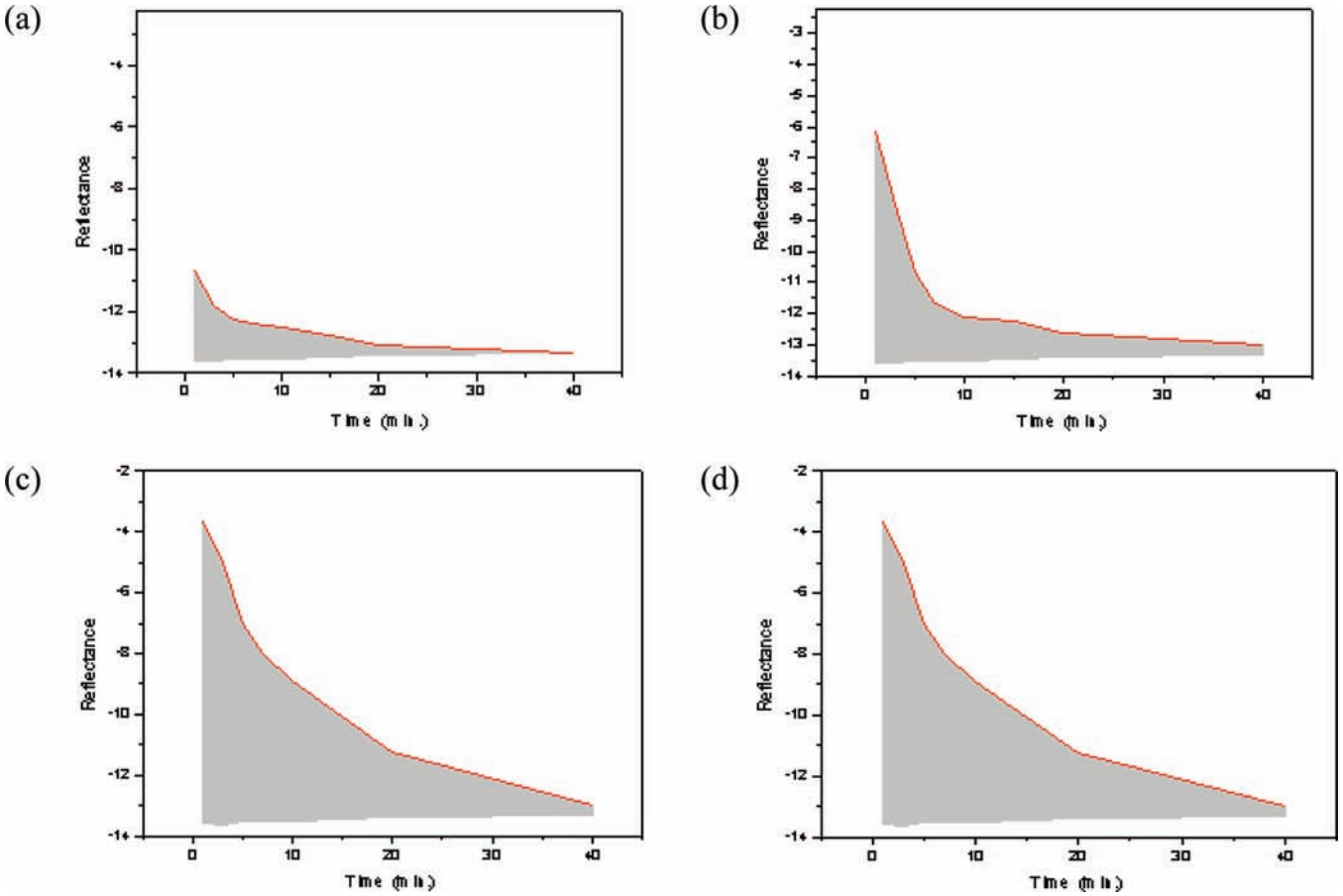

Figure 5. Reflectance versus time in the presence (higher curve) and absence (lower curve) of catechin. Shaded regions show the difference between the areas under curve (ΔAUC) in the presence and absence of catechin. Curves a, b, c, and d show reflectance signals versus time for CAT at 0.5×10^{-4}, 1.0×10^{-4}, 1.5×10^{-4}, and 2.0×10^{-4} M final concentrations, respectively, in 5 mL total aqueous phase.

PBHQ preimpregnated on the TiO_2 surface and subsequently hinder the formation of the coplanar indophenol dye for a limited time, after which the main redox reaction of indophenol dye formation on TiO_2 sensor prevails. Redox reactions generally have much greater equilibrium constants than those of other intermolecular interactions; for example, for the redox equilibrium constant K of an antioxidant compound having ΔE^o reduction potential difference with the antioxidant capacity assay reagent (e.g., cupric neocuproine reagent of the CUPRAC assay[19]) the Nernst equation states that $\Delta E^o = (RT/nF)\ \mathrm{Ln}\ K = (0.05916/n)\ \mathrm{Log}\ K$. Taking the standard reduction potential of Cu(II,I)-neocuproine as 0.6 V and that of dehydroascorbic acid/ascorbic acid (AA) as 0.28 V, the 2 e^- oxidation equilibrium constant of AA with the CUPRAC reagent may be expected to be $K \approx 6.58 \times 10^{10}$, which is much higher than the corresponding stability constants of intermolecular complexes. The presence of the *p*-hydroxyl group modifies the oxidation pathway of PAP in comparison to other aromatic amines such that the para-substituent adds to the ease of oxidation of PAP to a *p*-quinone imine product in the presence of air,[29] and once the quinone-imine forms, it rapidly couples with PBHQ to form indophenol dye (Figure 1); therefore, it is possible to retard this predominant reaction for only a limited time. The hypothesized catechin−PAP interactions in Na_2CO_3 solution may be responsible for the selectivity of the proposed sensor for catechin and other similar tea catechins over other antioxidants, because the other tested flavonoids and phenolics would not be expected to distort coplanarity of indophenol dye formation on the TiO_2 surface. However, thiol-type antioxidants, which are not present in tea, are expected to cause interference, because during the reaction of the quinone-imine of *p*-aminophenol with reduced glutathione (GSH), sequential oxidation/addition reactions were reported to occur, as revealed by a variety of polysubstituted thio-adducts.[30] Because the reflectance signal of the dye on the TiO_2 surface measured at 710 nm mainly arises from $\pi \rightarrow \pi^*$ transitions corresponding to charge transfer (resonance) between the quinoid and phenolic rings,[29] this conjugation/coplanarity to yield maximal reflectance within a prescribed period of time and the possible distortion of this conjugation with catechins to retard this reflectance signal are of vital importance to the working principle of the sensor. The relatively higher aqueous solubility of catechins,[31] compared to that of other tested phenolics, in the course of catechin−PAP interactions may also have retarded the formation and/or adsorption of the PAP−PBHQ indophenol dye on TiO_2.

Quantitative Determination of Catechins. Catechin quantitation was made by recording reflectance signal versus time and correlating the difference between the areas under curve (ΔAUC) in the presence and absence of catechin to catechin concentration. When a time-dependent signal (i.e., fluorescence or absorbance increase or decay) is a measure of antioxidant activity, the AUC of signal/time or the integrated signal over a fixed time interval is correlated to antioxidant concentration rather than the direct signal itself, for example, as in the ORAC assay of antioxidant activity.[32] For CAT at 0.5 ×

10^{-4}, 1.0×10^{-4}, 1.5×10^{-4}, and 2.0×10^{-4} M final concentrations (in 5 mL total volume), reflectance signal versus time curves are shown in Figure 5, panels a, b, c, and d, respectively.

The ΔAUC was calculated by means of Origin 6.0 graphic program, and the values were linearly correlated ($r = 0.98$) to catechin concentration (Figure 6). The linearity of this correlation is satisfactory in view of the quadratic−polynomial character of ORAC test responses (as ΔAUC vs antioxidant concentration).[33]

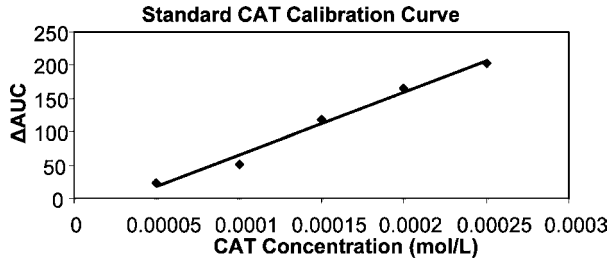

Figure 6. ΔAUC versus CAT concentration (mol/L) (ΔAUC = 9.47×10^5C − 29.67; $r = 0.98$).

Validation of the Sensing Method through Linearity, Precision, And Accuracy. Calibration curves (as ΔAUC vs concentration of catechin derivative) of CAT, EC, EGCG, EGC, and ECG were constructed with relatively high linear correlation coefficients (r = 0.97−0.98) and are compiled in Table 4, in which limit of detection (LOD) and limit of quantification (LOQ) values for each catechin derivative are also given. In a real sample (e.g., tea infusion), ΔAUC linearly correlated with the number of milliliters of extract. The ΔAUC of tea extract was sensitive to the added catechin concentration, as ΔAUC showed a regular increase with standard catechin addition; however, the results were not strictly additive (i.e., catechin-added tea extract did not show as much ΔAUC increase as a pure catechin solution per unit concentration of CAT added), probably due to the quenching effects of tea tannins.

CAT contents of standard solutions and synthetic mixtures of catechin derivatives were found with the aid of the proposed sensing method and statistically compared with the results of a reference HPLC method[18] for validation. The HPLC retention times for CAT and derivatives were as follows: EGC, 14.18 min; CAT, 14.55 min; EGCG, 15.58 min; EC, 16.52 min; ECG, 17.61 min. Two synthetic mixture solutions were prepared for statistical comparison, and $N = 5$ replicate measurements were made using the proposed and reference methods. (CAT + NG + TR) ternary mixture solution (containing 1.0×10^{-4} M of each constituent) and (CAT + EC + EGCG) mixture solution (containing 5.0×10^{-5} M of each constituent) were analyzed by the two methods, yielding $(9.25 \pm 1.14) \times 10^{-5}$ and $(1.45 \pm 0.094) \times 10^{-4}$ M concentrations of catechin derivatives (using the proposed method) in accordance with the expected values. The t and F tests yielded experimental values less than the tabulated values at 95% confidence level (e.g., the experimental F values for the first and second synthetic mixtures were 4.14 and 4.2, respectively, less than the tabulated value of F (6.39) for $(N - 1) = 4$ degrees of freedom, and also t values were 1.9 and 1.1, respectively, less than the tabulated value of t (2.78) for $(N - 1) = 4$ degrees of freedom), confirming that the proposed sensing method was as accurate and precise as the reference HPLC method. The intraday variability of the sensing method for 2.0×10^{-4} M pure CAT solutions was 2.0% (as RSD). These results should be accepted with the reserve that strict additivity in sensor response to catechin derivative concentrations may not be obeyed in real solutions such as tea extracts, probably due to the interference effects of tea tannins.

Application of the Method to Green, Black, and Herbal Tea Samples. Green tea samples were diluted 10 times and black tea sample 5 times before analysis; herbal teas were not diluted before analysis. Reflectance measurements against time were recorded as in catechin quantitation, and ΔAUC values were calculated for each sample. CAT (or CAT derivatives) contents of tea were calculated by means of the linear calibration equation shown in Figure 6 and are given in Table 5 in the units of mg CAT/g dry tea. The developed method was also applied to herbal tea bags, namely chamomile (*M. chamomilla*), fennel (*F. vulgare*), mint (*Lamiaceae*), and sage (*Salvia*). All samples were examined as five replicates, and there was no significant delay observed in blue color formation during the analysis period.

Table 5. CAT Contents of Tea (*Camellia sinensis*) Samples[a]

tea sample	CAT content (mg CAT/g tea)
Dogadan green tea	95.1 ± 0.3
Beta green tea	102.9 ± 0.5
Of Çay breakfast (black) tea	64.4 ± 0.3

[a] CAT = $\bar{x} \pm (t_{0.95}s/\sqrt{N})$; $N = 5$ ($\bar{x}$ = mean, s = standard deviation).

Standard Addition Method Applied to Tea Samples. Of the two different tea samples used in experiments, green tea but not chamomile (*M. chamomilla*) tea responded to the method. Standard additions of TR and CAT to green tea (GT) were recorded as reflectance versus time (Figure 7). GT caused a decrease in the absolute value of reflectance with respect to the reference (i.e., caused a delay in blue-colored dye formation on the TiO_2 sensor) because it contained CAT-type flavonoids; TR addition did not make any change on the reflectance versus time curve of GT, whereas CAT addition caused an additive decrease (during the first 10 min) in the absolute value of reflectance (Figure 7), suitable for quantitative analysis of catechins with the proposed sensing method. It may also be

Table 4. Calibration Equations ($N = 5$), Limits of Detection (LOD), and Limits of Quantification (LOQ) for Catechin Derivatives (Concentration in mol L^{-1})

catechin derivative	calibration equations for catechin derivatives	LOD	LOQ
CAT	ΔAUC = 9.47×10^5C − 29.67 ($r = 0.98$)	3.72×10^{-7}	1.24×10^{-6}
EC	ΔAUC = 8.65×10^5C + 84.53 ($r = 0.98$)	4.16×10^{-7}	1.38×10^{-6}
EGCG	ΔAUC = 8.68×10^5C + 38.24 ($r = 0.97$)	4.15×10^{-7}	1.38×10^{-6}
EGC	ΔAUC = 5.52×10^5C + 46.24 ($r = 0.99$)	6.52×10^{-7}	2.17×10^{-6}
ECG	ΔAUC = 8.31×10^5C − 92.19 ($r = 0.97$)	4.32×10^{-7}	1.44×10^{-6}

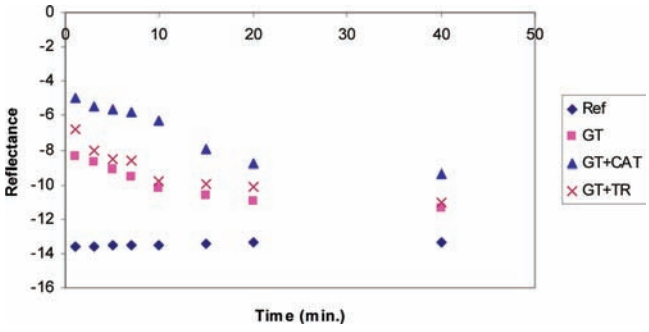

Figure 7. Reflectances of 1:5 diluted green tea sample (GT), GT + TR (at 2.0×10^{-4} M final concentration) and GT + CAT (at 2.0×10^{-4} M final concentration) versus time.

observed from Figure 7 that the optimal time period of determination should be strictly followed, as in the later phase, the reflectance of GT + CAT got closer to that of GT only, and signal additivity was not conserved.

Results for chamomile tea (CT) are shown in Figure 8. Either CT or CT + TR curves were very close to that of the

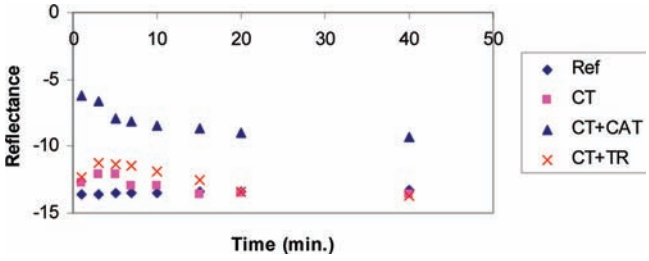

Figure 8. Reflectances of chamomile tea (CT) (nondiluted), CT + TR (at 2.0×10^{-4} M final concentration), and CT + CAT (at 2.0×10^{-4} M final concentration) versus time.

reference, meaning that both CT and TR were nonresponsive to the proposed sensing method. On the other hand, only CT + CAT produced a significant difference depending on the presence of CAT (Figure 8).

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ABBREVIATIONS USED

HQ, 8-hydroxyquinoline; PBHQ, 2,2′-(1,4-phenylenedivinylene)bis-8-hydroxyquinoline; NPs, TiO_2 nanoparticles; THF, tetrahyrofuran; EtOH, ethanol; PAP, *p*-aminophenol; ΔAUC, difference between areas under curve; LC, liquid chromatography; CE, capillary electrophoresis; QR, quercetin; RT, rutin; MR, morin; NGN, naringenin; NG, naringin; GA, gallic acid; CFA, caffeic acid; FRA, ferulic acid; PCA, *p*-coumaric acid; TR, trolox; CAT, catechin; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; GSH, reduced glutathione; AUC, area under curve; ORAC, oxygen radical absorbance capacity; LOD, limit of detection; LOQ, limit of quantification.

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