Protective role of native bovine serum albumin and alpha-unsaturated fatty acids on catechin oxidation

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Catechin, a common ingredient of tea and coffee is a colourless water soluble compound which undergoes spontaneous oxidation by aerial O$_2$, heat and also by UV exposure or ammonium persulfate (APS) treatment resulting in a noticeable change to yellow colouration ($\lambda_{\max} = 440$ nm). Several $\alpha$-unsaturated fatty acids (ascorbic, maleic, sorbic but not acrylic), $\beta$-mercapto ethanol (BME) and native bovine serum albumin (BSA) block the colouring process whereas the alkylated BSA fails to do so, confirming the reducing role of thiol (-SH) group(s) within the protein molecule. But alkylation does not change either the affinity ($K_d$) or the capacity ($B_{\max}$) of catechin binding at pH 7.5 ($K_d \sim 0.8$ nM and $B_{\max} \sim 4.0$ mol/mol BSA). Interestingly, a large increment in $B_{\max}$ is observed at pH 4.5. Additionally, the native BSA acts as a non-competitive inhibitor during oxidation of catechin as analyzed by the Lineweaver-Burke plot. The oxidation by APS follows the Michaelis equation and the rate ($V_{\max}$) is favourable at pH 8.5 (order $\rightarrow$ pH: 8.5 $>$ 7.5 $>$ 10.5) whereas the activation energy ($\Delta E$) deduced from Arrhenius equation shows that the reaction is favourable at higher pH. These incidents insist to hypothesize that APS, UV illumination and aerial oxygenation produce the same yellow poly-phenolic species as proven by the identical spectral changes associated with any of these procedures. The reducing agents interfere by blocking the crucial oxidative pathway.

Keywords: Catechin, polyphenols, ascorbic acid, UV oxidation, singlet oxygen, free radicals, reactive oxygen species, BSA, kinetics, UV-Vis spectra, Michaelis constant, Lineweaver-Burke plot, Arrhenius equation

(+ ) Catechin, a phenolic compound abundant in tea leaves, chocolates and coca plant is an important member of the flavonoids family. Normally flavonoids consist of clusters of phenolic rings covalently joined by endogenous oxidative procedures that eventually evolve as low molecular weight polymer(s), occur naturally in fruits and vegetables exhibiting astringent tastes. In recent days these have gained much attention due to their beneficial role in health as anti-oxidant micronutrients enabling to prevent a number of serious ailments. In physiology, catechin and other flavonoids are proven to be anti-mutagenic, anti-carcinogenic, antiviral as well as anti-inflammatory due to their trapping ability of endogenously generated reactive oxygen species (ROS) that are deleterious to health. The trapping of generated ROS by the naturally occurring flavins offers protection from any of harmful influences. This phenomenon is the chief reason to include flavins enriched fruits or vegetables in the food menu. The ROS oxidizes phenolics to more complex flavonoids, leading to the formation of polymeric tannin(s) which adds further astringency to the taste.

List of Abbreviations:
Ammonium Persulfate (APS); Bovine Serum Albumin (BSA); Ethanol (EtOH); Methanol (MeOH); Dimethyl Sulfoxide (DMSO); Ultraviolet-Visible (UV-Vis); Reactive Oxygen Species (ROS); Dimethyl Formamide (DMF); N-Ethyl Maleimide (NEM); Room Temperature (RT).
are also known to play a defensive role by deactivating the generated ROS\textsuperscript{18-22}.

In nature, oxidation by metallo-enzyme(s) causes the formation of catechins, gallatechins and \( o \)-quinones, which react further among themselves resulting in the production of complex poly-phenols thereby adding more astringency to the taste\textsuperscript{1,3}.

In this way, the tannins or polyphenols produced during food processing or preparing red wines by \textit{in vivo} enzymatic oxidation cause varying extent of astringency\textsuperscript{3,23,24}. The event of chain elongation causes hydroxylation of mono-phenols to create \( o \)-di-phenols that oxidizes further to \( o \)-quinones. Being strong electrophiles, they are subjected to frequent attack by any nucleophile, creating varieties of quinoids\textsuperscript{25,26}.

The photo oxidation by UV or near UV absorption occurs mostly due to excitation energy transfer in normal oxygen\textsuperscript{27}. Phenolic oxidation follows similar pathways through the generation of singlet oxygen mostly, \( ^1\Sigma^+ \). The oxidation by peroxides generates the same reactive singlet oxygen (\( ^1\Sigma^+ \)). Whereas, in case of thermal radiation or heat energy, the process of oxidation may use the excited species of \( ^1\Delta_g \) state which is considered to be slightly more stable than the \( ^1\Sigma^+ \). It has been suggested that phenolics or aromatic dyes after absorbing the energy can convert molecular oxygen to reactive singlet states which may further react with the excited aromatic molecules causing oxidation and subsequently conjoining them creating large poly-phenols\textsuperscript{27-30}.

The present study involves the identification of the spectral pattern obtained during several oxidation schemes including UV irradiation (\( \lambda = 254 \) nm), aerial oxygenation, heating or APS treatment. The study is further directed to identify the inhibitory effects of several \( \alpha \)-unsaturated fatty acids, native BSA molecule or BME during oxidation of catechin. The stability provided by the above agents has immense physiological implications on the effects of naturally occurring phenolic compounds on human or animal health.

**Materials and Methods**

**Materials**

All chemical ingredients including catechin, unsaturated fatty acids (ascorbic, sorbic, maleic and acrylic), buffering - salts (K-PO\textsubscript{4}, Na-BO\textsubscript{3}) were of ACS certified reagent grade quality (> 99.8% purity) and purchased from Sigma-Aldrich (St-Luis, MO). Solvents (methanol, ethanol, DMSO, BME) were also from Sigma and belong to either HPLC or certified analytical grade. BSA with high purity (> 99%) was purchased from ICN Biomedicals Inc. (Aurora, OH, Lot # 86828) and used after making it fatty acid free using Norit (Sigma-Aldrich) following the previous protocol\textsuperscript{31}. The absorbance measurements ranging from 200–765 nm were conducted manually on Hitachi U-2000 spectrophotometer using optically balanced 1.0 mL quartz cuvettes. Using a circulatory thermostat (Lauda – RP845, VWR International Boston, MA) the temperatures were controlled during all experiments, which is attached to the cell holder inside the spectrophotometer. All temperature fluctuations were kept within \( \pm 0.1^\circ \)C inside the quartz cuvettes during kinetic experiments. The dialysis tubing used for binding study to follow the equilibrium dialysis procedure was bought from Spectrapore (Spectrum Lab, CA) having cut-off molecular weight of 2.0 KD and 1.0 cm diameter. Before use the tubings were cut 3.0 inch long and afterward boiled in 1% bicarbonate in water for 10 min, washed thoroughly and equilibrated later in the desired buffer while storing in a refrigerator for use in future without adding any sodium azide as preservative\textsuperscript{31}. Temperature controlled (\( \pm 1^\circ \)C) Barnstead Labline platform shaker used during binding study was from Cambridge Instrument (Cambridge, MA). The hand held type
ultraviolet source UVGL 258 used for UV irradiation experiment is of dual wave-length 254 / 365 nm and supplied by VWR international (Boston, MA).

Methods

Purification of BSA: The protocol consisted of active charcoal treatment in mild acidic pH and published earlier. BSA solution (~ 40%) was prepared in 0.1 M K-PO4 buffers, pH 4.7. To it 0.1% Norit suspension in same buffer was slowly introduced and kept stirring at low speed with a magnetic bar for about 1 hr at RT. After filtering at first through Whatman filter paper # 1 and later with 0.2 µm Amicon filter under vacuum, the BSA solution was adjusted to pH 7.4 with 5.0 M KOH. It was then dialyzed against distilled water until electrolyte free, monitored by measuring the conductance of dialysate (~ 1.0 µMho). The dialyzed solution was lyophilized and stored in cold for future use. The purity was judged by SDS-PAGE as a single band.

The alkylation of BSA by NEM was carried in pH 7.4, 0.2 M K-PO4 buffers. About 20 mL of 10% of purified BSA solution was treated with 5.0 mL of NEM solution dissolved in DMSO (0.25 M NEM) adding drop-wise slowly and intermittently thus avoiding any precipitation at room temperature while mixing with a magnetic stirrer for ~ 4 hr period. The clear solution was then dialyzed against distilled water exhaustively to remove any NEM and lyophilized later to keep for further use in future.

UV irradiation and spectral analysis: In all cases, 3.0 mL samples were taken in the disposable plastic cups (10 mL) of 3.5 cm diameter, which were then kept inside the grooves of metal block attached to a circulator thermostat for maintaining the exact room temperature of 22°C. The UV illuminator (254 nm) was held 6 cm above the liquid surface level. The entire assembly was slowly shaken on a platform shaker. The time of exposure was varied according to the experiment but in most occasions it was ~ 2 hr unless otherwise mentioned. At the end UV/Vis spectra (200 to 600 nm) was recorded manually after balancing the quartz cuvettes using proper references.

Effect of APS: The treatment with APS was conducted for 5.0 min which was seen adequate for complete oxidization. The APS concentration was ~ 50 mg/mL and added to catechin solution ~ 2–5 mg/mL in 0.2 M KPO4 buffer, pH 8.5 at RT. In most cases the solution was often diluted to 1:10 or 1:20 fold before recording the spectra. The appropriate controls were maintained with buffer and no APS.

Effect of Protic Solvents: In all cases catechin solutions were prepared in 0.2 M KPO4, pH 8.5 and then diluted with either DMSO or EDE before keeping under UV light. The time of exposure was maintained as same ~2.0 hr. The final concentrations of organic solvents were about 3–5% during the reaction. The controls were either without the organic solvent with or no UV exposure or solvents unexposed to UV.

Kinetic Study: In case of UV oxidation a series of catechin solutions at desired pH and concentrations in buffer (3.0 mL) were kept under UV (λ = 254 nm) exposure as indicated earlier. Later each one of them were successively measured for a different time point and plotted as O.D (λ = 440 nm) against the time (t-min). During APS oxidation study, the reacting solutions were kept immersed at first separately inside the bath to reach preferred temperature. The quartz cuvettes were kept in the holder connected to the circulatory water bath kept at same temperature within ± 0.1°C fluctuations. Ten minutes after attaining the desired temperature, the APS solutions (50 µL final concentration – 3.0 mg/mL) were added in the both cuvettes having catechin or reference buffer solution continuously stirred with tiny bar magnets. The reaction was allowed to proceed inside the cuvette and the absorbance was recorded at various time intervals depending on the nature of experiment. The experiments were carried at several catechin concentrations and also at different pH.

The kinetic data were analyzed later by using Lineweaver – Burke plot (Eqn. – 1) to calculate Michaelis’ constant (Km) and the maximum reaction rate (Vmax).

\[
\frac{1}{V_i} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} 
\]

Thus plotting 1 / Vi (Vi – initial reaction rate) against 1 / [S] ([S] – substrate concentrations) will result in a straight line. Reciprocal of the intercept at Y-axis (1 / Vmax) provides Vmax (the maximum reaction rate). The intercept at X-axis (1 / Km) after reversing provides K_m (Michaelis’ constant or rate constant). The Km and Vmax were calculated at 37°C reaction. To observe the effect of BSA on APS reaction, 1.35% BSA (final) was kept in both cuvettes with or without catechin and the reaction was initiated by adding the similar level of oxidant.
The thermal oxidation rate fitted well as a second order kinetics by following the equation below (Eqn. – 2).

\[
\delta [S] / \delta t = - K [S]^2 \quad \text{... (2)}
\]

The integrated form is shown in equation below (eqn – 3) where [S] is the substrate or catechin concentration at any time t and \([S_o]\) is the substrate level at time when t = 0.

\[
1 / [S] = 1 / [S_o] + K t \quad \text{... (3)}
\]

Plotting \(1/[S]\) against t would yield a straight line and eventually the slope will provide the rate constant \(K\). Since concentration is directly proportional to the absorbance according to Beer’s law (Absorbance = \(\varepsilon \times \text{Concentration} \times \text{Path length}\); \(\varepsilon = \text{extinction coefficient of the molecule}\) so when \(1 / \text{[Absorbance]}\) was plotted against t it produced a straight line, and from the slope \(K\) was then calculated. All absorbance changes were monitored at constant wavelength of \(\lambda = 440\) nm.

The activation energy (\(\Delta E\)) of APS reaction was calculated by following the Arrhenius equation (Eqn. – 4).

\[
\ln K = - \Delta E / RT + \ln A \quad \text{... (4)}
\]

Where \(K\) is the rate constant for APS reaction, \(\Delta E\) denotes activation energy, \(T\) is the temperature in Kelvin scale and \(R\) is the universal gas constant (8.314 J/K/mol); \(A\); is a proportionality constant related to the collision frequency during reactions. Plotting \(\ln K\) against 1/T provided straight line and from the slope \((-\Delta E/R)\), \(\Delta E\) the activation energy was calculated.

**Binding Experiment: Catechin Binding:** The experiment was conducted following the past protocols.\(^{31}\) Exact 1.0 mL of BSA solution in 0.1M K-PO\(_4\) buffer of desired \(pH\) were taken in dialysis casings and ends were double knotted to prevent any leak. They were then immersed in catechin solutions (25 mL) with varying concentrations and kept on orbital shaker overnight in dark either at 22°C or any other settings. The binding equilibrium was reached within 12 hr. In order to construct a binding isotherm, a series of (50 mL) solutions were prepared with known ligand concentrations ranging from 0.1 \(\mu\)M to about 1.5 \(\mu\)M. The solutions were divided in half (25 mL) in two small bottles with screw cap. In one of the series were added BSA solution in dialysis casings and capped tightly for shaking. The other series were left in similar conditions. The catechin measurements were conducted next day using freshly prepared Folin-Ciultu’s reagent. The unknown concentrations from binding solutions were measured after constructing a linear standard curve from known catechin solutions against absorbance at 765 nm.

In a typical Folin assay, 200 \(\mu\)L of catechin solution of various concentrations were mixed with 3.0 mL water along with 400 \(\mu\)L of 10% Na\(_2\)CO\(_3\), then 50 \(\mu\)L of Folin reagent was added with vigorous shaking. The mixtures were left at 37°C for 30 min and absorbance values were recorded at 765 nm. The concentrations of the binding solutions in presence of BSA were assessed from the standard curve after knowing the O.D. All measurements were conducted at a time. The amount bound (\(\Gamma\)) was calculated from the following formula after knowing initial (\(C_i\)) and final equilibrium (\(C_f\)) concentration.

\[
\Gamma (\mu\text{g of catechin} / \text{mg of BSA}) = (V_i \cdot C_i - V_f \cdot C_f) / W
\]

Here, \(V_i\) = Initial or original volume (mL); \(V_f\) = original volume (mL) + BSA volume (mL) and \(W\) is the weight (mg) of BSA used in binding. The unit of \(\Gamma\) was later expressed as moles of catechin/mole of BSA in place of \(\mu\)g of catechin/mg of BSA, taking into account the molecular weight of BSA = 70 KD.

Each binding point was conducted in triplicate while each set of experiment was performed at least three times for proper evaluation of Scatchard’s analysis data providing standard deviation for both binding affinity (\(K_d\)) and capacity (n).

As per precaution, a blank experiment was conducted without using BSA in the dialysis bag to observe any catechin binding with the membrane component. No interaction was observed at any \(pH\) studied in this work.

**Oxidized Catechin Binding:** At first native catechins were oxidized in aqueous solution by applying heat and APS. Exact amount of catechin after weighing was dissolved in methanol (21.1 mg/mL) by vortexing. The 10 mL of methanolic solution was then quickly added to 50 mL of boiling buffer, \(pH\) 7.4, 0.2M K-PO\(_4\) and the boiling continued for at least 10 min. After cessation of boiling 0.1 g of APS was added at warm condition and the solution was left for 3–4 hr at RT with occasional shaking. A change of colour from yellow to deep orange was
observed. At the end more buffer was added to make up to 100 mL. The attainment of absorbance to a steady level indicated complete oxidation of catechin, which was monitored spectrophotometrically at \( \lambda = 440 \) nm after diluting (1:1000) the sample in buffer (pH 7.5). The oxidized stock was successively diluted in buffer and expressed in terms of molarity of single catechin unit ranging from 2–30 mM. The binding experiment using BSA were performed in similar way by equilibrium dialysis procedure. The standard curve was prepared at different dilution by measuring absorbance at 440 nm and the unknown concentrations were calculated from the experimental samples after recording their absorbance. The amount bound per mole of BSA was calculated as mentioned above. The binding results were expressed in moles of catechin unit bound per mole of BSA.

**Data analysis:** All data were analyzed using the software, Prizm – 5 from GRAPHPAD (San Diego, CA). The linearity was judged by the least square with regression analysis and if necessary in case of binding, the nonlinear curve fitting was used. For other experimental data often ANOVA was applied. The spectral results were represented in a table format (Table I) for the sake of simplicity and better understanding.

**Results**

**Effect of UV irradiation, pH change, treating with APS, heating, BME and the medium:** Table I summarizes the spectral changes occurring under different treatment conditions including the effect of several reagents. Normally, the freshly prepared catechin is colourless with absorption maxima at the UV range, \( \lambda_{\text{max}} = 260 \) nm. The yellow colouration occurs if exposed to UV radiation or treated with any oxidizing reagent but no matter, in all situations the peak at 260 nm is always present. The generation of yellow colour with simultaneous appearance of a new peak at \( \lambda_{\text{max}} = 440 \) during any of the procedures signifies a radical change of the catechin molecule. The effect was noticed to be highly pH dependent. As shown in Table I, a, at acidic pH (4.7, 0.2 M K-PO\(_4\)) no colouration occurs when irradiated with the UV. But under identical exposure conditions, changing the media pH either to normal (pH 7.5) or higher (pH 8.5 or 10.5) caused colouring, generating a peak maximum (\( \lambda_{\text{max}}\)) at 440 nm. The presence of APS, a strong oxidant, produced the same effect without the UV exposure (Table I, b). Heating the catechin solution at normal pH also brought the yellow colour with similar spectral pattern and having \( \lambda_{\text{max}} = 440 \) nm. But adding the BME or switching to acidic pH completely blocked the colour formation without showing the peak maxima at 440 nm (Table I, c). Besides heating, BME also prevented colour generation by UV at normal pH (Table I, d). The low level of solvents like DMSO or EDA exerted no influence on the spectral changes compared to that at ordinary pH 7.5 showing peak maxima at 440 nm when exposed to the UV light (Table I, e). Further, it was noticed that prolong storage of catechin solution in semi-transparent poly-carbonate capped tube either at normal or higher pH also resulted in the same colouration (\( \lambda_{\text{max}} = 440 \) nm) at RT under ordinary light and the intensity was enhanced with time. The event could be prevented either by lowering the pH to 4.7 or keeping in the dark or cold at pH 7.5 – 8.5. The presence of BSA blocked the colour generation in all situations (Table I, f and g, Figure 1). But if treated with NEM, BSA failed to do so (Table I, g).

**Effect of unsaturated fatty acids:** Figure 2 shows the influence of unsaturated fatty acids like ascorbic, sorbic and maleic (0.05 mol L\(^{-1}\)) in blocking the UV induced colour change of catechin molecule at pH 8.5. But in the case of acrylic (0.05 mol L\(^{-1}\)) no blocking was ever observed.

**Differences in absorbance ratio (A\(_{260}/A_{440}\)):** In aqueous buffered media catechin solutions always followed the Beer’s law even when turned yellow by UV, APS treatment or infusion of thermal energy. For the respective wavelengths, \( \lambda = 260 \) nm or \( \lambda = 440 \) nm a strict linearity (\( r^2 \approx 0.99 \)) was observed in the absorbance vs concentration (mol L\(^{-1}\)) plot passing through the origin. The absorbance ratio, \( A\(_{260}/A_{440}\) or extinction ratio \( e\(_{260}/e_{440}\) in case of UV irradiated and APS treated samples were observed to be within the range of 2.2 - 2.6 (N= 5 observations) whereas the value was significantly elevated in case of thermally activated samples, \( A\(_{260}/A_{440}\) = 3.1 – 3.4 (N = 6 observations). This noteworthy rise under different oxidative conditions is evidently an interesting demarcation (p \approx 0.004, N = 5 observation) although the UV-Vis spectral pattern remains the same under any of the oxidative conditions.

**Kinetics of oxidation reaction:** The oxidation kinetics by UV is more favourable at pH 8.5. The pH order for reaction kinetics is, pH 8.5 > pH 10.5 > pH 7.5 >>>>>> pH 4.7 and the reaction rate increases in a linear fashion. Quite evidently no reaction was taking place under acidic condition (pH 4.7).
Table I — Summary for the generation of peak ($\lambda_{\text{max}} = 440$ nm) in the visible region of catechin solution undergoing oxidation under various treatments and conditions. The irradiation time for UV oxidation was 2.0 hr at RT.

(a) Catechin spectra at different pH after UV ($\lambda = 254$ nm) irradiation: Catechin: 2.5 mg/mL, Buffer: 0.2 M K$_4$PO$_4$

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH</th>
<th>UV peak $\lambda_{\text{max}}$ = 260 nm</th>
<th>Visible peak, $\lambda_{\text{max}}$ = 440 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4.7</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Normal</td>
<td>7.5</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Normal</td>
<td>8.6</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Normal</td>
<td>10.6</td>
<td>Present</td>
<td>Present</td>
</tr>
</tbody>
</table>

(b) Treatment with APS: Catechin: 2.6 mg/mL, APS: 52 mg/mL (final), reaction time: 5.0 min, $\text{pH}$ 0.2 M K$_4$PO$_4$ buffer

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH</th>
<th>UV peak $\lambda_{\text{max}}$ = 260 nm</th>
<th>Visible peak, $\lambda_{\text{max}}$ = 440 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>With APS</td>
<td>8.5</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Without APS</td>
<td>8.5</td>
<td>Present</td>
<td>Absent</td>
</tr>
</tbody>
</table>

(c) Effect of heating in absence and presence of BME: Catechin: 2.77 mg/mL, BME: 0.1% (final), $\text{pH}$ 7.5 0.2 M K$_4$PO$_4$, heating time: 20 min (in boiling water bath, inside temperature ~ 80°C)

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH</th>
<th>UV peak $\lambda_{\text{max}}$ = 260 nm</th>
<th>Visible peak, $\lambda_{\text{max}}$ = 440 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>No heat</td>
<td>7.54</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Heating</td>
<td>7.54</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>No heat + BME</td>
<td>7.54</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Heat + BME</td>
<td>7.54</td>
<td>Present</td>
<td>Absent</td>
</tr>
</tbody>
</table>

(d) Effect of BME on UV exposure: Catechin: 2.7 mg/ mL, $\text{pH}$ 7.5, 0.2 M K$_4$PO$_4$

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH</th>
<th>UV peak $\lambda_{\text{max}}$ = 260 nm</th>
<th>Visible peak, $\lambda_{\text{max}}$ = 440 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>No UV</td>
<td>7.56</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>With UV</td>
<td>7.56</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>UV + BME</td>
<td>7.56</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>No UV + BME</td>
<td>7.56</td>
<td>Present</td>
<td>Absent</td>
</tr>
</tbody>
</table>

(e) Effect of protic solvents like, DMSO or EDA on UV irradiation: catechin: 2.59 mg/mL, Buffer: 0.2 M K$_4$PO$_4$, pH 7.5, DMSO or EDA: 3 - 5% (final)

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH</th>
<th>UV peak $\lambda_{\text{max}}$ = 260 nm</th>
<th>Visible peak, $\lambda_{\text{max}}$ = 440 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>No UV</td>
<td>7.5</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>No UV + DMSO</td>
<td>7.5</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>UV + DMSO</td>
<td>7.5</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>UV – DMSO</td>
<td>7.5</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>No UV + EDA</td>
<td>7.5</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>UV + EDA</td>
<td>7.5</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Only UV</td>
<td>7.5</td>
<td>Present</td>
<td>Present</td>
</tr>
</tbody>
</table>

(f) Effect of BSA during UV exposure of catechin solution 2.5% in 0.2 M K$_4$PO$_4$ buffer, pH 4.7, time of UVexposure was 2 hr

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH</th>
<th>UV peak $\lambda_{\text{max}}$ = 260 nm</th>
<th>Visible peak, $\lambda_{\text{max}}$ = 440 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>No UV</td>
<td>4.7</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>No UV + BSA</td>
<td>4.7</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>UV + BSA</td>
<td>4.7</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>UV + BSA</td>
<td>4.7</td>
<td>Present</td>
<td>Absent</td>
</tr>
</tbody>
</table>

(g) Effect of native and alkylated BSA on the heating behavior of catechin solution: 2.35%, 0.2 M K$_4$PO$_4$, pH 7.5, temperature ~ 45°C, time: 20 min.

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH</th>
<th>UV peak $\lambda_{\text{max}}$ = 260 nm</th>
<th>Visible peak, $\lambda_{\text{max}}$ = 440 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>No heat</td>
<td>7.54</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Heating</td>
<td>7.54</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>No heat + BSA</td>
<td>7.54</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Heat + BSA</td>
<td>7.54</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Heat + NEM-BSA</td>
<td>7.54</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>No heat + NEM-BSA 7.54</td>
<td>Present</td>
<td>Absent</td>
<td></td>
</tr>
</tbody>
</table>


Figure 1 — UV-Vis spectra showing the effect of BSA during UV exposure of catechin solution at RT. Catechin 2.66 mg/mL, BSA 1.0%, pH 8.5, 0.2 M K-PO$_4$ buffer. Time of UV exposure: 2 hr, $\lambda=254$ nm.

Figure 2 — UV-Vis spectra indicating the effect of various $\alpha$-unsaturated fatty acids on UV oxidation of catechin solution at RT. Catechin 1.22 mg/mL, fatty acids 0.05 mol L$^{-1}$, pH 8.55, 0.2 M K-PO$_4$ buffer. Time of UV exposure: 2.0 hr, $\lambda=254$ nm.
The oxidation rate by APS at 37°C was also seen to be linear as well as pH dependent like in the above case of UV kinetics. Therefore expectedly, it did not proceed at pH 4.7. The pH dependency of reaction velocity ($V_{\text{max}}$) as calculated by the Linewever-Burke Plot followed almost the similar order, pH 8.5 (0.38) > pH 10.5 (0.30) > pH 7.5 (0.26). The addition of BSA (1.35%) hindered the oxidation process by acting as a non-competitive inhibitor while lowering the oxidation rate almost four fold ($V_{\text{Max}} = 0.28$ - BSA compared to 0.071 + BSA) and its role was already viewed during UV induced spectral change (Figure 3).

**Kinetics of heat activation of catechin and energy of activation:** The rate of colour formation was monitored at various temperatures and pH. The reaction rate was significantly enhanced with the rise of temperature and also occasionally seen to be nonlinear. The thermal activation kinetics data fitted best with the second order rate equation $1/[S] = 1/[S_0] + Kt$, (Eqn. – 3). The rate constant $K$ was calculated from the slope by plotting $1/[S]$ against $t$ which was seen linear in all cases. The values of $K$ thus obtained was later plotted according to the Arrhenius Equation, $\ln K = -\Delta E/RT + \ln A$ (Eqn – 4) (Figure 4) by using $\ln K$ against $1/T$. The plot also shows that the lowering of temperature slows down the reaction rate. The activation energy $\Delta E$ thus obtained showed linear pH dependency ($R^2 \sim 0.94$) (inset Figure 4).

**Catechin binding to BSA:** The analysis of catechin binding to BSA at pH 7.5 and 4.7 showed almost identical affinity ($K_d \sim 8.0 \times 10^{-8}$ mol L$^{-1}$) but with varying capacity ($B_{\text{max}}$) ranging from ~ 3.4 to 10.2 mol/mol of BSA (Table II). Evidently, the binding affinity, remained the same at all conditions ($K_d \sim 8.0 \times 10^{-8}$ mol L$^{-1}$) whereas changing of pH significantly altered the binding capacity ($B_{\text{max}}$) but the values are same either for native or NEM treated BSA (Table II). In both cases, higher binding values were observed at the acidic range, pH 4.7.

An interesting complexity arose when oxidized poly-catechin molecule binds to the BSA. The ratio $\Gamma$ (mol of catechin bound / mol BSA) / $C_\Gamma$ (catechin units mol L$^{-1}$ = $r \sim 0.22$) remained constant and stays linear with increasing concentration of oxidized poly-catechin molecules almost upto its solubility limit. The binding to alkylated BSA showed slight increment in the ratio, $r \sim 0.26$ but the values was not significant ($p \leq 0.056$). In either case, the binding level reached to almost 7 mol of catechin monomer units bound per mole of BSA.

**Discussion**

The transition from colourless solution to yellow-orange ($\lambda_{\text{max}} = 440$ nm) colouration of catechin is an indication of oxidation along with simultaneous conjugation of the monomeric units producing more
complex poly-phenols. The oxidation studied at present by observing the spectral change occurred either by UV irradiation, APS treatment, heating or prolonged storing at normal or higher pH under ordinary light when capped inside a glass or semi-transparent plastic bottle. The colour formation under ordinary light at RT (pH 7.4) is very slow compared to the UV irradiation. The incident of colour generation by UV is inhibited by low pH (4.7) but enhanced considerably at higher pH (Table I, a).

Considering the resonance configurations of phenols, it seems logical to predict that the creation of phenoxide radical leading to quinone formation at neutral or high pH could be the initial factor in the coupling procedures. Like the oxidative enzyme polyphenol polymerase, UV irradiation, thermal activation and APS can also polymerize the phenolics in the same manner following Michael type nucleophilic addition as proposed by McDonald and Hamilton (Scheme II).

The pKₐ of most phenolics are ~ 9.5, therefore at a pH near or higher than pKₐ, the compound dissociates fully or partially enhancing the possibility of phenoxide or semiquinone radical generation that results in different sets of oxidation products. But low pH disfavour the quinone formation therefore producing no spectral change at pH 4.7. Interestingly, the colour change by UV illumination also occurs

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**Table II** — Catechin binding to native and NEM treated BSA at acidic (pH 4.7) and normal pH (pH 7.5) in 0.2 M K-PO₄ buffer^a^

<table>
<thead>
<tr>
<th>Nature</th>
<th>pH</th>
<th>K_d (mol L⁻¹)</th>
<th>B_max (mol/mol BSA) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native BSA</td>
<td>4.7</td>
<td>8.2 ± 0.7 × 10⁻⁸</td>
<td>10.24 ± 0.3</td>
</tr>
<tr>
<td>Native BSA</td>
<td>7.5</td>
<td>8.9 ± 0.4 × 10⁻⁸</td>
<td>3.46 ± 0.07</td>
</tr>
<tr>
<td>NEM treated BSA</td>
<td>4.7</td>
<td>8.0 ± 0.2 × 10⁻⁸</td>
<td>9.98 ± 0.4</td>
</tr>
<tr>
<td>NEM treated BSA</td>
<td>7.5</td>
<td>8.4 ± 1.0 × 10⁻⁸</td>
<td>4.1 ± 0.08</td>
</tr>
</tbody>
</table>

^a^ The binding data were analyzed using GRAPHPAD, PRIZM – 5. Each point was in triplicate and repeated three times (N = 3, p =

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**Figure 4** — The Arrhenius plot (lnK = −ΔE/RT + lnA --- eqn - 4) at different pH during thermal oxidation of catechin solution. The reaction temperatures T: 37°C, 52°C, 61°C and 71°C. The pH of the reaction medium are, 7.5, 8.5 and 7.5, 0.2 M K-PO₄ buffer. The reaction was monitored for a period of 60 min in most of the cases. Inset plot shows the linear nature of activation energy (ΔE) during thermal change of the catechin molecule with increasing alkalinity.
without the prevention in presence of protic solvents, (3 ~ 5%) DMSO or EDA (Table I, e). In either case, the DMSO or EDA is unable to capture the UV generated ROS. But it remains unverified whether much higher solvent concentrations would play any significance in the colouring process.

The prior knowledge indicates that most phenolic oxidation perhaps follow the same route via photo-oxidation, phenolic molecule at first possibly attains a sensitized state under the photo-exposure by absorbing energy ($h\nu$) of the irradiated photons. The excited phenolic later interacts with molecular O$_2$ at lowest energy ($\sum_g^-$) state and transforms itself to the more reactive singlet ($\sum_g^+$) condition. The reactive singlet species thus produced reacts afterwards either with the sensitized phenolic compounds or else its normal counterparts imposing the colour changes while simultaneously undergoing conjugation increasing the sizes of poly-phenolic complexes. Concerning the peroxide (-O – O-) oxidations, reports showed that the reactions occur via the formation of similar singlet oxygen at different energy levels $\sum_g^+$ and $\Delta_g$. It was already documented that $\sum_g^+$ state is relatively short lived in comparison to the other singlet specimen $\Delta_g$, but can perform the similar act. The application of thermal energy mostly creates $\Delta_g$ thus causing identical colour change in the heating process. It can be safely speculated that the addition of thermal energy like its counterpart, UV irradiation raises the catechin molecule also to the high energy level which in presence of aerial or dissolved oxygen releases an active oxygen molecule either in $\sum_g^+$ or $\Delta_g$ state that eventually imposes further actions during the oxidation pathway. Normally, without the application of heat the oxidation at RT and at ordinary pH (7.5) is an extremely slow process which is considerably energized by the addition of thermal energy. The slow oxidation rate can be explained due to the possible lowering of photon absorption and concomitant ROS formation. So logically, if kept in dark or inside a refrigerator, the catechin solution rarely undergoes any photochemical changes therefore lasts longer. In a way, both could be equally effective in arresting the colourization of catechin. Obviously, irrespective of the nature of oxidative pathways, the UV-Vis spectral patterns are primarily the same which insists to hypothesize that identical reactions possibly occur during yellow colouration. The FT-IR analysis of purified yellow compounds isolated from different oxidative pathways showed similar banding pattern supporting the above fact (Mitra S. P. Unpublished data). But, despite the similarities in FT-IR data, the differences in absorbance ratio ($A_{260}/A_{440}$) of different oxidative pathways (UV and APS oxidation $A_{260}/A_{440}$ ~ 2.3; thermal oxidation $A_{260}/A_{440}$ ~ 3.2) deserve a logical explanation. It is possible that although the overall oxidation mechanism may be the same but thermal oxidation may differ slightly. The destruction of a few phenolic moieties may be a reasonable factor causing...
the lowering of absorbance at $\lambda = 440$ nm which eventually raises the ratio of $A_{260}/A_{440}$ to a high level ($\sim 3.2$). The preliminary unpublished data of HPLC confirms the presence of more UV ($\lambda = 260$ nm) absorbing materials than the visible ($\lambda = 440$ nm) components (Mitra S. P. Unpublished data). It is thus somewhat confirmative that infusion of thermal energy degrades the product molecule(s) considerably which subsequently lowers the absorbance at $\lambda = 440$ nm while enhancing the ratio ($A_{260}/A_{440} \sim 3.2$) to the higher level.

The addition of BSA (Table I, f and g, Figure 1) or BME (Table I, c and d) arrested the incidence of colour formation, an event caused due to the
contribution of thiol (–SH) group(s) from cysteine(s) which can efficiently trap the generated oxygen radicals. It is therefore speculated that catechin oxidation may follow the below Scheme III analogous to as dictated by Foote.\textsuperscript{28} 

As seen (Figure 2) that α-unsaturated fatty acids (0.05 mol L\textsuperscript{-1}) like ascorbic, sorbic or maleic efficiently blocked the colour formation of catechin solution (3.58 mg/mL) under UV irradiation (pH 8.55). The trapping of electron rich species by the olefinic bonds is likely the reason played during the anti-oxidant role. In this act, the positioning of olefinic bond is seen to be crucial for quenching the singlet species produced by UV illumination. The inability of acrylic acid (H\textsubscript{2}C=CH-CO\textsubscript{2}H) in preventing UV induced changes under identical condition further confirms the fact (Figure 2). Surely, the olefinic bond at extreme end fails to undergo the nucleophilic attack thereby stays un-reactive toward the electron rich reactive oxygen.

Lowering the pH to acidic level (pH 4.7) is seen to prevent the colouring event (Table I, a). It is conjectured that either the destruction of ROS or else the inefficiency of protonated phenol moiety taking part in the reaction could be the underlying reason since at low pH there is least chance of phenoxide formation (see above). As per pH dependency the oxidation rate is higher at pH 8.5 but lower at 10.5. Argumentatively, the molecular damage is claimed to be the exact cause at high above the pK\textsubscript{a} limit owing to quinone generation.

The colour change by UV irradiation or APS follows first order kinetics and is seen to be favourable at pH 8.5 but unfavourable under acidic condition (pH 4.7). The Lineweaver- Burke Plot has been applied to understand the role of H\textsuperscript{+} ions and native BSA during catechin oxidation. Presumably at different pH the catechin molecules may undergo structural or conformational changes that eventually reflect in changing values of V\textsubscript{max} (pH 8.5 \rightarrow V\textsubscript{max} = 0.384; pH 10.5 \rightarrow V\textsubscript{max} = 0.30 and pH 7.5 \rightarrow V\textsubscript{max} = 0.26). The values for K\textsubscript{M} also change which can be correlated to the dissociation rate of the transitory complex formed between generated ROS and the catechin molecule. The rate is somewhat higher at pH 7.5 (K\textsubscript{M} = 2.94 \times 10\textsuperscript{-3}) compared to pH 8.5 (K\textsubscript{M} = 2.1 \times 10\textsuperscript{-3}) or pH 10.5 (K\textsubscript{M} = 1.6 \times 10\textsuperscript{-3}). Apparently, the low dissociation phenomena favours the oxidation rate possibly due to longer stabilizing effect of the complex for enhanced interacting affinity between catechin and ROS at high pH. Although an optimum condition in the reaction rate eventually arrives at pH 8.5 which favours the most. Therefore the low value of V\textsubscript{max} at pH 10.5 could be due to possible degradation of catechin. No reaction was ever noticed at pH 4.5. Further, the Lineweaver-Burke plot also helps understand the inhibitory role of native BSA showing its noncompetitive nature. In presence of BSA, the reaction rate, V\textsubscript{max} is significantly reduced (V\textsubscript{max} = 0.28 – BSA and V\textsubscript{max} = 0.071 + BSA) (Figure 3). Thus the native protein acts as a non-competitive inhibitor only due to the existence of reducing thiol (–SH) groups. Interestingly, the molecular binding phenomenon between BSA and catechin offers no effect in that regard.

The thermal effect of catechin oxidation follows the increment of colourization rate with rising of temperature as studied by observing the increase in O.D. at λ\textsubscript{max} = 440 nm. The process of colourization follows mostly the second order rate equation (eqn – 4). The activation energy (\Delta E) calculated by using the Arrhenius plot (logK vs 1/T) (Figure 4) shows a pH dependency displaying a negative slope (Figure 4), indicating the reaction favourability at higher pH. The event provides additional support in favour of phenoxide generation initiating the colouring event.

It is already mentioned that native BSA molecule can prevent catechin oxidation in solution at normal or high pH. It is concluded now that the reducing action of thiol (–SH) within the protein molecule plays the most crucial role because neutralizing that –SH by alkylating with NEM abolished the arresting effect. In fact, catechin binds with native BSA molecule almost the same way like its alkylated counterpart (Table II). Obviously, the molecular binding phenomena have the least interference in the blocking event in course of UV induced spectral shift. Contextually, the event has a significant impact in physiology because large abundance of albumin in circulation might help protect the catechin or other natural phenolics from any endogenous or exogenous sources of oxidation since they impart beneficial effects to the health. Interestingly, the binding affinity (K\textsubscript{d} ~ 0.8 nM) remains the same in either cases of both native and alkylated BSA even at the acidic (pH 4.7) or normal pH (7.5) which points to the fact that the nature of interacting sites are the same and far apart from the cysteine rich domain of the protein molecule. The variation in binding capacity (B\textsubscript{max}) is pH dependent. This may further suggest that there could be a structural alteration within BSA molecule at acidic pH enhancing the hydrophobic interactions which may
result in the increase of $B_{\text{max}}$. The alkylation by NEM does not cause any severe change in the binding parameters (Table II) possibly because of the limited number of free –SH within BSA. Therefore alkylation did not enhance much hydrophobicity. On the other hand, binding of oxidized catechin or poly-catechin offers an interesting phenomenon. The binding shows no saturation and increases almost linearly up to the solubility range of poly-catechin. Interestingly, the ratio ($r$), ($\Gamma$ mol of poly-catechin bound / mol BSA) / $C_F$ (Free poly-catechin units, mol L$^{-1}$) remain constant throughout the process ($r = 0.22$). No significant difference is observed in case of NEM treated BSA ($r = 0.25$). It is possible that extreme cooperativity might arise during the binding process of poly-catechin that can impose structural orientations of the protein molecule thus simultaneously increasing the extent of oxidized poly-catechin binding. It is also noteworthy that the aqueous solubility of poly-catechin is significantly enhanced in presence of BSA which lent stronger support regarding the cooperativity during binding (Mitra S. P. Unpublished data).

Conclusion
The entire study proves conclusively that under ordinary conditions catechin molecule in aqueous solution can undergo oxidative colour change absorbing at $\lambda_{\text{max}} = 440$ nm. The change is rapid under UV exposure, APS treatment or by the application of heat. In all situations the absorption maxima always remains the same, $\lambda_{\text{max}} = 440$ nm. The spectral change can be inhibited either by native BSA or $\alpha$-unsaturated fatty acids like, sorbic, maleic, ascorbic (but not acrylic) or other reducing agents like BME. Besides reducing agents, the acidic pH (4.7) can also block the spectral changes. The binding interaction with BSA molecule did not play any role because although alkylated BSA binds to the catechin but was unable to prevent any UV induced colour formation. The high pH favours the oxidation process by lowering the energy of activation ($\Delta E$).

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