Stability Study of Green Tea Natural Extract in Aqueous Solutions and its Chemical Kinetics

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ABSTRACT

The current study aims at stabilizing the green tea natural extract in aqueous solutions using a validated method of High Performance Liquid Chromatography (HPLC). Catechins, which is a group of phenolic constituents of green tea, was used as markers to monitor the changes in the assay of green tea with time.

The effects of different factors on the tea catechins stability were examined in this work including atmospheric air, temperature, pH value, buffer species and the effect of adding different pharmaceutical expedients to the tea aqueous solutions. The results show significant effect of atmospheric air and temperature on the stability of catechins. The later was directly proportional to the amount of air and temperature due to the oxidation reaction of catechins. On the other hand, it was found that catechins were stable at low pH (pH less than 3) but unstable at pH values above 6. Tea catechins solutions were more stable in citrate buffer than in phosphate buffer. Enhancement of the tea catechins stability was achieved by adding ethanol, propylene glycol, sorbitol and refined sugar at 0.2 M ionic strength.

Different kinetic’s parameters have been determined in this work including reaction order, rate constants and activation energies of the oxidation reaction of green tea catechins confirming pseudo first order reaction.

Keywords: Green Tea, Catechins, Stability, HPLC, Oxidation Reaction, Co-Solvents, Chemical Kinetics.

1. INTRODUCTION

Tea (Camillia Sinensis) is one of the most widely consumed beverages in the World after water, coffee and carbonated soft drinks. Tea can be classified into three categories; green (unfermented) tea, oolong (partially unfermented) tea and black (fermented) tea depending on the level of fermentation (Cheng, 2005).

Green tea has been found to be superior to black tea in terms of antioxidant activity owing to the higher content of catechins. The quality and health properties of the green tea are associated with the chemical components, particularly the poly phenols and caffeine extracted from the leaf. The primary components under study in this respect are the flavonoid polyphenols, which have been found to have strong antioxidant effect in vitro studies (Henning et al., 2003). Several investigators at various laboratories studied the bioactivity of tea compounds; as the relationship between tea and metabolism (Tsuneki et al., 2004), the effect of tea on blood glucose and cholesterol levels (Yang and Koo, 2000), the impact on the ability of the bodys cells to handle oxidative stress (Sabu et al., 2002), and the effect of green tea on slowing development of abnormal blood vessels in certain diseases, including cancer (Moyers and Kumar, 2004), (Hakim et al., 2003). The results of these investigations showed that tea catechins can act as antioxidants due to its ability to act as hydrogen atom donor, and free radicals acceptor and therefore interrupting oxidation chain reactions or by chelating metals. This is attributed to annexation of hydroxide groups to catechins molecules as the main factor causing their strong antioxidant proprieties (Gramza and Korczak, 2005).

Other remedial effects of green tea have been studied such as anti-endothelial dysfunction effect (Chen et al., 2004), (Kim et al., 2004), and anti-inflammatory effect (Sueoka et al., 200) (Aneja et al., 2004), thus, reducing the risk of developing Hypertension (Yang, 2004) and acting as antidiabetic agent (Waltner et al., 2002), (Anderson and Polansky, 2002).

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kinds of phenolic constituents such as flavanols, flavadiols, flavonoids, condensed tannins flavonoids and their glycosides. These phenolic compounds constitute approximately 30-40% of the dry weight of green tea leaves and contribute to their beneficial health effects. Flavonoids include many groups like chalcone, flavanone, dihydroflavonol, flavan-3-ol, flavones, flavon-3-ol and tea catechins that are part of flavan-3-ol family as shown in Figure (1). The major tea catechins are (-)-epigallocatechin 3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin (EC), (-)-gallocatechin (GC), and (+)-catechin (C) as shown in Figure (1).

Our goal is to study the effect of the atmospheric air, temperature, pH value, buffer species and adding co-solvents on stability of catechins in green tea extract, in order to find the ideal factors that can increase the shelf life of green tea extract active components. In addition the chemical kinetics were studied on the whole catechines groups in the tea extra matrix.

2. EXPERIMENTAL METHODS

2.1 Chemicals

Acetonitrile, ethyl acetate (HPLC grade), citric acid, ethanol and orthophosphoric acid were obtained from MERCK, propylene glycol and sorbitol were from KIMIA, while sulphuric acid was from ACROS and refined sugar was from ANDELEX and used without further purification. Water used was deionised.

2.2 Green Tea Material

A commercial Chinese green tea powdered extract was provided from Ningbo/ China. It was extracted commercially using chloroform as the organic layer and ethyl acetate as the aqueous layer. The extract has been standardized with respect to phenolic compounds (catechins). The used green tea extract contains 95% (w/w) polyphenols.

2.3 Instrumentations

An HPLC instrument equipped with Alliance 2690 pump and UV-PDA detector model 996 from WATERS/ USA, pH meter from Mettler Toledo/ Switzerland, incubator from HOTPACK/ USA, climatic chamber VC0150 from VOTSCH and analytical balance Genius ME2155 from SARTOREUS/ Germany.

2.4 Methods

2.4.1 HPLC Analysis

Monitoring the assay of polyphenols was done by using a reversed phase Reprosil (C18, 250 x 4.6 mm, 5µm) column. The HPLC instrument equipped with UV-PDA detector at 280 nm setting the injection volume for samples to 10 µl. The temperature of the samples was set at 6.0 ºC to avoid the polyphenols degradation during analysis. The flow rate was set at 1.0 ml/ min. using a mixture of 0.5 % (v/v) H2SO4 in water, acetonitrile and ethyl acetate as a mobile phase with a ratio of (86 : 12 : 2) (v/v), respectively. The chromatographic data was processed using Mellinium Software designed for WATERS corporation (USA).

2.4.2 Stability Markers

Figure (2) shows a chromatogram of, four sharp peaks at different retention times (tR) representing four different green tea catechins as a result of HPLC analysis. These peaks are assigned to (-)-epicatechin (EC) at tR = 5.62 min., (-)-epigallocatechin 3-gallate (EGCG) at tR = 7.25 min., non identified catechin (NC) at tR = 8.29 min. and (-) -epigallocatechin at (EGC) at tR = 13.25 min. as compared to the chromatogram of the green tea standard materials. Although, four catechins have been eluted from the sample, three catechins have only used as markers. The low natural abundance of EC eluted at 5.6 min in tea extract prevented its accurate quantitation, therefore, the quantitation was done only for EGCG and EGC peaks at tR of 7.3 and 13.5 min., respectively. Since the unknown peak eluted at tR = 8.3 min. has similar properties to EGCG and EGC, therefore, it was assumed to be one of the catechins extracts named as non identified catechin (NC).

2.5 Investigating the Effects of Different Factors on the Stability of Green Tea Catechins in Aqueous Solutions

In order to fulfil the requirements of method suitability to the examined catechin, method validation has been performed considering the following parameters; linearity, precision, reproducibility, selectivity in addition to determination of detection limit and quantitation limit. Method validation indicates the robustness and accuracy of the used HPLC method.

2.5.1 Atmospheric Air Effect

A stock solution of 0.05% (w/v) green tea extract was prepared by dissolving 125.0 mg of the extract in 250.0 ml water. The solution was distributed into different
sealed glass vials with rubber septums and aluminium caps. The amounts of atmospheric air in the vials were adjusted by varying the volumes of the solutions in the vials. Some of the vials were 25% filled with the solutions, others were 50% and 75% filled and the rest were completely filled with the solution. All vials were stored at 40.0 ºC and suppressed to duplicate analysis after five days of storage using HPLC.

2.5.2 Temperature Effect
The same solution in section 2.5.1 was distributed into different sealed glass vials with rubber septums and aluminium caps and 95% filled with the solution. The vials were stored in dark at different temperatures 5.0 ºC, 25.0 ºC and 40.0 ºC. Samples were analyzed in duplicates in five days time interval for two weeks.

2.5.3 pH Effect
Samples of 0.05% (w/v) green tea extract were prepared by dissolving 25.0 mg extract in 50.0 ml of 0.05 M phosphate buffer solution of 0.2 M ionic strength and different pH values range from 2 to 9. The ionic strength value was adjusted by adding suitable quantities of NaCl and the desired pH value was adjusted by adding drops of 2.0 M NaOH. The green tea extract samples were 95% filled vials and stored at 40.0 ºC for two weeks. The analysis was done in five days time interval for two weeks.

2.5.4 Buffer Species Effect
Samples of 0.05% (w/v) green tea extract were prepared in phosphate and in citrate buffers of different pH values and 0.2 M ionic strength. The analysis of the samples was done every five days for two weeks.

2.5.5 Co-solvents Effect
The effect of adding ethanol, propylene glycol, sorbitol and refined sugar as expedients on the stability of green tea extracts in aqueous solutions was studied. A 0.05M of citrate buffer of pH 3.0 and 0.2 M ionic strength was mixed with different co-solvents at 10%, 20%, 40%, 60% and 80% (w/w). Green tea extract was dissolved in each solution obtaining 0.625% (w/v) solution. The samples were 95% filled sealed vials stored at 40.0 ºC for two weeks and analyzed every five days.

2.6 Kinetics for Catechins Degradation in Green Tea Extract's Aqueous Solution
Stock solution of 0.05% (w/v) of green tea extract aqueous solution, 95% filled in sealed vials, were stored at different temperatures of 25.0, 30.0, 35.0 and 40.0 ºC. In addition, different concentrations of green tea extract 0.05, 0.07 and 0.1 % w/v were prepared in aqueous solutions and stored at 35.0 ºC.

3. RESULTS AND DISCUSSION

3.1 Atmospheric Air Effect
Figure (3) shows a significant decrease in the stability of catechins as the amount of air increased at 40 ºC. This decrease has exceeded 50% loss of stability for EGCG in the case of 50% filled samples, but did not exceed 20% loss of stability in the case of 100% filled. The loss in the stability for the three catechins was in the order EGCG > NC > EGC indicating that EGC is the most stable one of the tea catechins. These results indicate that oxygen in atmospheric air causes degradation of catechins extracts. Therefore, in studying other factors (temperature and pH), all samples were 95% filled to enhance the stability of catechins in solution.

3.2 Temperature Effect
Figure (4) shows significant instability for the samples stored at 40.0 ºC, and significant stability for samples stored at 5.0 ºC for two weeks. It is worth mentioning that the catechins have followed the same order of stability noticed in the atmospheric air effect. The previous observation states that it is most probable that the oxidation reaction of catechins takes place steadily at high temperatures, indicating that 5.0 ºC is the optimum temperature that ensures the maximum stability for catechins.

3.3 pH Effect
Stability of green tea catechins was studied in 0.05 M phosphate and citrate buffers at 0.2 M ionic strength. It is obvious from Figure 5 and 6 that green tea catechins are more stable at low pH values and unstable at pH values higher than 6. The high stability of catechins at low pH can be attributed to the high concentration of H+ which causes the oxidation reaction [1] to shift to the left, thus inhibiting the oxidation of catechins (Mochizuki et al., 2002).
Although oxygen radical (O$_2^-$) is formed as a product of oxidation reaction, it does not enhance the oxidation due to its reaction with H$^+$ ions available at low pH values according to reaction [2].

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad [2]$$

Another reason for the higher stability of green tea extracts at low pH is the inhibited dissociation of the phenolic groups of the reactant, as the dissociated phenolic groups has a stronger electron donating properties than the undissociated one. Since O$_2^-$ propagates the catechines oxidation reaction and has higher oxidative activity than oxygen, the scavenging of O$_2^-$ by hydrogen ion to inhibit the reaction at low pH .On the other hand, the noticeable degradation of catechins at high pH values can be attributed to formation of semiquinones as degradation products according to the following reactions [1], [3] and [4] (Mochizuki et al., 2002):

$$\text{This was also confirmed with the electron spin resonance (ESR) signals (Mochizuki et al., 2002), which indicated that the thermodynamic stabilization of the semiquinones generated from the previous reactions [1] and [2], increases at high pH values; This is consisted with the hypothesis that suggest the decrease in forming the degradation products at low pH values (Mochizuki et al., 2002).}$$

3.4 Buffer Species Effect

Figure (5) and Figure (6) show the stability of
catechines in citrate and phosphate buffers. The greater stability in citrate as compared to phosphate buffer is due to antioxidant property of citrate in aqueous solution.

The difference in stability between EGC and EGCG is attributed to the electron donating property of the additional phenolic group at ring B as shown in Figure (1). (Mochizuki et al, 2002) Ring B in EGCG has three hydroxyl groups while EGC has two hydroxyl groups which makes EGCG more acidic than EGC. The higher acidity of ring B in EGCG makes it more ionized at higher pH than EGC, hence EGCG will be more active to react with the free radical than EGC.

3.5 Co-solvents Effect

The effect of adding different co-solvents of different (w/w) concentrations to catechins is shown in Figure (7). The results indicate that the addition of co-solvents to the catechins buffer solutions increases their stability. The enhancement achieved was in the following order; ethanol > propylene glycol > sorbitol > refined sugar. The trend of co-solvents on the stability of catechins could be related to the dielectric constant (\(\varepsilon\)) of the solutions listed in Table (1). (Akhadov, 1981) Table (1) and Figure (7) show that the stability of the catechins increases as the dielectric constant of the solution decreases, it is also can be referred to the Hydrophilic-Lipophilic Balance (HLB) values of the solutions used (9.850, 12.225, 21.250 and 31.800) for ethanol, propylene glycol, sorbitol and refined sugar respectively (Attwood and Florence, 1983). The HLB values for the used co-solvents were calculated from the following equation (Attwood and Florence, 1983).

\[
\text{HLB} = \Sigma \text{(hydrophilic group numbers)} - \Sigma \text{(lipophilic group numbers)} + 7
\]

The co-solvents are ordered in terms of increasing the hydrophilicity, thus ethanol has the lowest HLB value and thus more lipophilic, while sugar has the highest HLB value and thus the more hydrophilic. It was found that as the hydrophilicity of the medium decreases, the stability of the tea catechins increases. The HLB values and the \(\varepsilon\) values of the solutions used to stabilize the natural products are found to have strong relationship with their stability as mentioned in earlier books and studies of (Boylan, 1979) and (Neradovic et al., 2003).

3.6. Kinetics of the Oxidation Reaction for Green Tea Catechins

Catechin Reaction Order

Figure (8) shows straight lines to be obtained when \(\ln \left[\frac{a_0}{a_{0-x}}\right]\) is plotted versus time for the three catechines, where \(a_0\) and \(a_{0-x}\) are the initial and final concentration of the catechin respectively. The slopes were obtained at various temperatures of 25.0 °C, 30.0 °C, 35.0 °C and 40.0 °C. The results indicate a first order reaction for the three examined catechins.

The dependence of the oxidation reaction rate on the catechin concentration is listed in Table (2). The plot of \(\ln \text{[rate]}\) versus \(\ln \text{[catechin concentration]}\) is shown in Figure (9). These results confirm a pseudo-first order reaction, for which it is approximation to take the concentration of \(O_2\) as constant through the reaction because its present in a large excess. The oxidation reaction has the form of first order rate law.

Table (3) shows the rate constants and their correlation coefficients at different temperatures. Plotting of \(\ln k\) against the reciprocal of temperature is shown in Figure (10). The activation energies were calculated from the slopes, \((-\frac{E_a}{R})\) to be 55.84, 57.67 and 59.5 kJ mol\(^{-1}\) for EGCG, NC and EGC respectively. The highest activation energy for EGC confirm higher stability for which more energy is needed as compared to NC or EGCG that reflects larger energy barrier of its oxidation reaction.

4. CONCLUSIONS

It was found that the low stability of the tea catechins aqueous solutions stored at 25.0 °C for two weeks is caused by the oxidation of the tea catechins. Increasing the temperature of storage has resulted in decreasing the stability of tea catechins in aqueous solutions; The oxidation reaction of tea catechins is found to be first order reaction and obeys Arrhenius equation.

Tea catechins have higher stability in low pH while they are extremely unstable at pH higher than five. It was also found that the addition of ethanol, propylene glycol, sorbitol and refined sugar to tea catechins aqueous solutions enhances their stability. The stability enhancement accompanying the addition of co-solvents was found to be inversely related to the dielectric constant of the different concentrations of the added
materials mixtures. Moreover, the stability was found to be inversely related with the Hydrophilic-Lipophilic balance (HLB) values of the added materials.

The results indicate that there is a need to focus on the stability on tea catechins in commercial tea drinks and to be considered in shelf life assignment. The traditional tea making method gives good amount of antioxidants, since tea immediately served, and also proves that adding lemon to tea drink is a good habit since it gives an extra stability for tea catechins.

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### Table 1.

Dielectric constants (ε) of different concentrations of aqueous solutions of co-solvents at 20°C. (Akhadov., 1981)

<table>
<thead>
<tr>
<th>Co-solvent</th>
<th>0%</th>
<th>10%</th>
<th>20%</th>
<th>40%</th>
<th>60%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>80.37</td>
<td>74.60</td>
<td>68.66</td>
<td>56.49</td>
<td>44.67</td>
<td>33.84</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>80.37</td>
<td>77.46</td>
<td>74.60</td>
<td>68.40</td>
<td>61.08</td>
<td>50.64</td>
</tr>
<tr>
<td>Refined sugar</td>
<td>80.37</td>
<td>78.00</td>
<td>75.40</td>
<td>69.80</td>
<td>61.20</td>
<td>-----</td>
</tr>
</tbody>
</table>

### Table 2.

Catechin oxidation reaction rates at different catechin concentrations

<table>
<thead>
<tr>
<th>Conc. M</th>
<th>Rate M.s⁻¹</th>
<th>Conc. M</th>
<th>Rate M.s⁻¹</th>
<th>Conc. M</th>
<th>Rate M.s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCG</td>
<td>0.1189</td>
<td>0.0236</td>
<td>0.0543</td>
<td>0.0085</td>
<td>0.0424</td>
</tr>
<tr>
<td>NC</td>
<td>0.1624</td>
<td>0.0331</td>
<td>0.0742</td>
<td>0.0115</td>
<td>0.0579</td>
</tr>
<tr>
<td>ECG</td>
<td>0.2293</td>
<td>0.0430</td>
<td>0.1047</td>
<td>0.0147</td>
<td>0.0817</td>
</tr>
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<td>0.1189</td>
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<td>0.0147</td>
<td>0.0817</td>
</tr>
</tbody>
</table>

### Table 3.

The rate constants of catechins oxidation reaction and their correlation coefficients at different temperatures

<table>
<thead>
<tr>
<th>Temp.</th>
<th>1/T</th>
<th>K (s⁻¹) x 10⁷</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EGCG</td>
<td>NC</td>
</tr>
<tr>
<td>298</td>
<td>0.00336</td>
<td>6.19</td>
<td>3.58</td>
</tr>
<tr>
<td>303</td>
<td>0.00330</td>
<td>8.97</td>
<td>4.36</td>
</tr>
<tr>
<td>308</td>
<td>0.00325</td>
<td>11.91</td>
<td>5.70</td>
</tr>
<tr>
<td>313</td>
<td>0.00319</td>
<td>18.75</td>
<td>11.39</td>
</tr>
</tbody>
</table>
Figure 1. The Major tea catechins

Figure 2. HPLC chromatogram of 0.05 % (w/v) green tea extract in water (C_{18} column, mobile phase of (86: 12: 2) 0.5%H_{2}SO_{4}; acetonitrile: ethyl acetate and flow rate of 1.000 ml/min.)
Figure 3. Variation of the stability of the three catechins in 0.05% (w/v) tea extract solution with the amount of atmospheric air at 40°C:
(a) EGCG, (b) NC, (c) EGC
Figure 4. Variation of the stability of the three catechins in 0.05% (w/v) tea extracts solution (95% filled) with temperature: (a) EGCG, (b) NC, (c) EGC
Figure 5. pH stability profile of 0.05% (w/v) green tea solutions in 0.05M phosphate buffer and 0.2 M ionic strength after 5 days at 40°C

Figure 6. pH stability profile of 0.05% (w/v) green tea solutions in 0.05M citrate buffer and 0.2 M ionic strength after 5 days at 40°C
Figure 7. Variation of the stability of the three catechins in 0.05% (w/v) tea extract solution in 0.05M citrate buffer at pH 3 and 0.2M ionic strength in the presence of different co-solvents of different (w/w) concentrations: (a) EGCG, (b) NC, (c) EGC.
Figure 8. The plot of $\ln \left( \frac{a_0}{a_0-x} \right)$ catechins versus time for 0.05% (w/v) tea extract in aqueous solution at (a) 25°C, (b) 30°C, (c) 35°C, (d) 40°C
Figure 9. The plot of ln rate of catechin oxidation reaction versus catechin concentration

Figure 10. The plot of ln k versus 1/T for tea catechins oxidation reaction

REFERENCES


