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Reaction of Caffeic Acid with Amino Acids

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REACTION OF CAFFEIC ACID WITH AMINO ACIDS

by

Yonggang Wang

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Arts
Department of Chemistry

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2000
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Yonggang Wang
REACTION OF CAFFEIC ACID WITH AMINO ACIDS

Yonggang Wang, M.A.
Western Michigan University, 2000

The reaction of caffeic acid with tryptophan was studied as a model for the reaction of caffeic acid with amino acids in the aquatic environment. Although the autoxidation reaction of caffeic acid in aqueous solution with subsequent dimerization had been previously studied, it was examined as well for comparison purposes. The present studies demonstrate that oxidized caffeic acid would react with tryptophan. The reaction, like the dimerization reaction, was catalyzed by the presence of iron(III) ions at low pH (<4) and by hydroxide ions at high pH (10). The reaction of caffeic acid with itself yielded six products, four of which were identified by mass spectroscopy as dimers, with the other two being trimers. The reaction of caffeic acid with tryptophan yielded three product peaks. Two of the product peaks had similar retention times to the dimers from the self-reaction of caffeic acid; the third was a new product resulting from the reaction of caffeic acid with the tryptophan.

A mechanism for the reaction of caffeic acid with tryptophan has been proposed. This mechanism was based on the analysis of the products and previous mechanisms presented by earlier workers for the caffeic acid self-reaction.
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CHAPTER I

INTRODUCTION

Caffeic Acid and Phenolic Compounds

The expression "phenolic compounds" embraces a wide range of substances which possess an aromatic ring with a hydroxyl substituent, including their functional derivatives. Among the natural phenolic compounds, of which several hundreds are known, the flavonoids and their relatives form the largest group. However phenolic quinones, lignins, xanthones, depsidones, and other groups also exist in considerable numbers as well as many simple monocyclic phenols. Two examples of phenolic compounds are caffeic acid (CA) and chlorogenic acid. In nature, caffeic acid and chlorogenic acid are degraded from plants and found naturally in various foodstuffs and beverages such as coffee beans and their soluble constituents, potatoes, fruits such as apples and their juices, tobacco leaves, olive oil, and wine (Van Buren et al., 1973; Challis and Bartlett, 1975; Woodring et al., 1990; Chi-Tang, 1992).

It is well known that some phenolic compounds, such as caffeic acid and chlorogenic acid contribute to both food browning (Hurrell and Finot, 1984) and the formation of humic substances in the natural environment (Jiang, 1996). There is a growing interest in the studies of the dietary phenolic compounds (Challis and Bartlett, 1975; Laranjinha et al., 1994). Generally, there are two types of phenolic
browning reactions. Enzymatic oxidation is the more important reaction in fresh fruits and juices and early in food processing, when poly-phenol oxidase is present (Coseteng and Lee, 1987; Matheis, 1987). In processed foods with the enzyme removed or in the natural environment, nonenzymatic autoxidation can take place. Autoxidation (the expression originally used by Cilliers in 1989 to refer to the reaction of caffeic acid with itself following its oxidation by oxygen. This terminology will be used throughout this thesis) can be catalyzed under alkaline condition or in the presence of copper(II) ions (Jiang, 1996).

Humic Substances

Humic substances (HS) are the most widespread and ubiquitous natural nonliving organic materials in soil and aquatic environments. They constitute the major fraction of soil organic material (up to 80%) and the largest fraction of natural organic matter in aquatic systems (up to 60% of dissolved organic carbon) (Thurman, 1986; Stevenson, 1982; Schnitzer, 1991; Stevenson, 1994).

Humic substances are a physically and chemically heterogeneous mixture of naturally occurring, biogenic, relative high molecular weight, mixed aliphatic and aromatic compounds. They are formed by humification during the decay process and transformation of biomolecules that originate from dead organism and microbial activity.

Based on solubility in acids and alkalis, HS can be divided into four fractions; (1) humic acid (HA), the portion that is soluble in dilute alkaline solution and is
precipitated upon acidification to pH 2; (2) fulvic acid (FA), the portion that is soluble at any pH value; (3) humin, the portion insoluble in both alkalis and acids; and (4) hymatomelanic acid, the alcohol-soluble portion of HA (Stevenson, 1965).

Since humic substances consist of a chemically heterogeneous mixture of compounds, it is difficult to describe uniquely the molecular formula of HA, FA and other HS species. However, it is possible to propose the general structure of a molecule of HA and FA on the basis of compositional, structural and functional group information. The functional groups in humic substances, especially those most reactive with protons and metals, have been well characterized. These functional groups include carboxyl, phenolic, and alcoholic hydroxide, quinone and ketonic carbonyls, amino, and sulfhydryl (SH) (Sposito, 1989). Table 1 lists these important functional groups in humus. A typical model structure for humic acid proposed by Stevenson (1982) is shown in Figure 1.

Several pathways have also been proposed for the formation of humic substances. The “lignin-protein theory” of Waksman (1932), considers lignin as the main source of HS, with the involvement of amino compounds produced by microbial synthesis. In one pathway, reducing sugars and amino acids formed as by-products of microbial metabolism are assumed to be the only precursors of HS. The current concepts of HS genesis favor the “polyphenol theory”, which involves polyphenols and quinones, either derived from lignin or synthesized by microorganisms. Figure 2 shows the detailed steps involved in polyphenol theory of soil humus formation (Cresser and Killham, 1993).
Figure 1. A Model Structure Proposed for Humic Acid.

Table 1

The Important Functional Groups in Humus

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Structural formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxyl</td>
<td>$-\text{COOH}$</td>
</tr>
<tr>
<td>Carbonyl</td>
<td>$-\text{C}=\text{O}$</td>
</tr>
<tr>
<td>Amino</td>
<td>$-\text{NH}_2$</td>
</tr>
<tr>
<td>Imidazole</td>
<td>Aromatic ring NH</td>
</tr>
<tr>
<td>Phenolic OH</td>
<td>Aromatic ring OH</td>
</tr>
<tr>
<td>Alcoholic OH</td>
<td>$-\text{OH}$</td>
</tr>
<tr>
<td>Sulfhydryl</td>
<td>$-\text{SH}$</td>
</tr>
</tbody>
</table>
Figure 2. Representation of the Polyphenol Theory of Humus Formation.

The study of humic substances is a worldwide activity with many implications for our survival, environment and human health. Much progress has been made in humic substance research recently, particularly with regard to trying to deduce HS structures with the help of sophisticated analysis and molecular modeling (Cresser and Killham, 1993). Studies of highly purified humic substances have indicated that they have reproducible and accountable properties and appear to have common metal binding sites (Sposito, 1989). Humic substances are nature’s fertilizers. Interest in humic substances will continue to grow in the future.
Iron in Natural Waters

Iron is a common constituent of rocks. Fossil fuels, ores, municipal sewage and industrial effluent also contain iron and other trace elements. Iron is introduced to natural waters through the weathering of rocks and by human activities. In many rivers and lakes, the human input of iron is many times greater than the natural input (Stumm and Baccini, 1978). The water in equilibrium with the iron mineral ferrihydrite \([\text{Fe(OH)}_3]\), produces a certain dissolved iron concentration, which is present primarily as the Fe(III) species. Under certain specific conditions, Fe(III) can also exist as ferric-organic (humic-fulvic) complexes and as colloidal ferric oxyhydroxides. Compared to Fe(III), there is a much smaller amount of uncomplexed Fe(II) existing in natural waters (Stumm and Baccini, 1978).

Objective of Study

Caffeic acid is a natural occurring phenolic compound. Amino acids and transition metals can also be found in natural environment. Humic substances are the major fraction of natural organic matter in both soil and aquatic systems. Environmental scientists have done numerous studies of the formation of humic substances. It is suggested that phenolic compounds play an important role in one proposed pathway (polyphenol model) of the formation of humic substances (Flaig, 1988). Previous studies have also demonstrated that there is an interaction between caffeic acid and transition metals like copper(II) and iron(III) (Deiana et al., 1992). However, the reaction products have not been well characterized. What are the
relationships of natural existing phenolic compounds with amino acids, transition metals and humic substances? How are the humic substances formed in natural waters? What role do amino acids play in the formation of humic substances? This study attempts to answer some of these questions by studying the reaction of caffeic acid with tryptophan; investigating the effect of hydroxide and iron(III) ion on the reaction of caffeic acid with amino acids; characterization of the products of the reaction of caffeic acid with tryptophan. A possible reaction mechanism of caffeic acid with tryptophan has also been proposed.
CHAPTER II

LITERATURE REVIEW

Caffeic Acid and Iron (III) Complexation

Phenolic compounds with low molecular weight have considerable biological importance because they are involved in many processes that take place in the soil-plant systems. They increase the mobilization of important micro elements, such as Fe and Mn, primarily by complexation or reduction reactions converting insoluble compounds that are unavailable to plants to those that are available to plants (Lehmann et al., 1987). Among these phenolic compounds, caffeic acid is of special interest because it takes part in the transport of various metal ions from the soil to the plant roots (Linder and Voye, 1987). Olsen et al. (1982) demonstrated that caffeic acid underwent an oxidation reaction in aqueous solution whereby Fe(III) was reduced to Fe(II). In order to obtain a better understanding of the mechanisms involved in complexation of caffeic acid and iron(III), Deiana et al. (1992) studied the redox properties of caffeic acid and other phenolic compounds. They determined the stoichiometry and proposed a probable mechanism for the oxidation-reduction reaction (Figure 3). The interactions in aqueous solution of caffeic acid with copper(II), zinc(II), iron(II) and iron(III) were investigated by Linder and Voye (1987). They found that Fe(III) was reduced to Fe(II) in caffeic acid solution even at
very acidic conditions. Complexation of the metal ions predominantly involved chelation by the catecholic site of caffeic acid.

\[
\text{HO--O-CH==CH-COOH + 2Fe}^{3+} \rightarrow \left[\text{O}\right] + \text{2Fe}^{2+} + 7\text{Fe}^{3+}
\]

Deamination Products + 7Fe\(^{2+}\)

Figure 3. Suggested Interaction Mechanism between Caffeic Acid and Fe(III) by Linder and Voye (1987).

Non-enzymatic Oxidation of Phenolic Compounds

The oxidative browning of polyphenols in plant derived foods and beverages generally results in a loss of nutritional value, bitter taste and the appearance of undesirable brown colors (Hurrell and Finot, 1984). The first step in browning is the oxidation of o-diphenols to the corresponding highly reactive o-quinones (Pierpoint, 1966). Enzymatic oxidation is the most important reaction in fresh fruits and juices when polyphenoloxidase is present (Coseteng and Lee, 1987). However, non-enzymatic autoxidation can also take place in the presence of oxygen, especially when the medium is alkaline (Cillers and Singleton, 1989; Jiang, 1996). The different steps leading to the formation and further reaction of quinones to produce condensation products that are not fully understood and only a few hypotheses have
been proposed about their mechanism (Cilliers and Singleton, 1991; Fulcrand et al., 1994; Singleton, 1987).

In 1964, Cohen found that the photodimerization of ring-substituted cinnamic acids led to trans- and cis- dimers by breaking the double bond of the ethylene group on the side chain of caffeic acid. Figure 4 shows the structure of these two dimers (cyclobutane linkage dimers) (Cohen et al., 1964).

![Figure 4. Two Cyclobutane Linkage Dimers Proposed by Cohan (1964).](image)

Cilliers et al. (1991) reported the possible nonenzymic autoxidation products of caffeic acid in 1991. They found that the rate of reaction was increased by raising pH and/or temperature. The phenolate anion was believed to be a key factor in the formation of autoxidation products and the rate of reaction. The products they proposed were dimers linked via the double bond on the side chain of one molecule with the oxygens on the aromatic ring of another (ether linkage dimers). The mechanism proposed by Cilliers et al. (1991) is shown in Figure 5.
Figure 5. Mechanism of Dimer Formation Proposed by Cilliers (1991).
Xu (1994) studied the caffeic acid oxidation reaction in the presence of Copper(II). The reaction rate was increased when copper(II) was added to the reaction solution. Xu (1994) proposed a mechanism of caffeic acid autoxidation that involved both ether linkage and/or cyclobutane linkage dimerization in the presence of copper(II). Jiang (1996) found that hydroxide ion also had a catalytic effect on the chlorogenic autoxidation reaction.

Sodium Periodate oxidation of caffeic acid was investigated in acidic conditions by Fulcrand et al. (1994). It was confirmed that the rate and the yield of the reaction increased with increasing pH. In particular, the two products obtained at pH values lower than 4.6 were analyzed and isolated by reverse phase HPLC. Using $^1$H and $^{13}$C NMR and mass spectrometry, the products were believed to be two stereoisomers of 2,5-(3', 4'-dihydroxyphenyl) tetrahydrofuran 3,4-dicarboxylic acid (Figure 6). They also found that at pH values above 4.6, the products were same as shown in Cilliers's study (1991).

![Figure 6. Two Possible Structures of Products Proposed by Fulcrand (1994).](image-url)
Reaction of Phenolic Compounds With Amino Acids

Unlike the caffeic acid oxidation reaction, the reaction of caffeic acid with amino acids has been less studied. Flaig et al. (1975) reviewed the literature pertaining to the reaction of phenolic compounds with amino acids to form humic acids.

Generally, the reaction can take place in one of two ways. One is by the addition of amino acids to the oxidized phenol:

\[
\begin{align*}
\text{O}_2 & \quad \text{Phenols} \\
& \text{Phenolase} \\
& \text{Quinones} \\
\end{align*}
\]

\[
\text{Quinone + Amino Acid} \rightarrow \text{Humic Substance}
\]

The other is via a deamination and decarboxylation of one amino acid; while the second is added to the phenol:

\[
\begin{align*}
\text{OH} & \quad \text{OH} \\
\text{I} & \quad \text{I} \\
\text{+} & \quad \text{+} \\
\text{\text{-Xe}} & \quad \text{-xH} \\
\text{-xH} & \quad \text{R} \\
\text{R} & \quad \text{N-6-cooH} \\
\text{I} & \quad \text{I} \\
\text{H} & \quad \text{H} \\
\text{H} & \quad \text{H} \\
\text{+NH}_3 & \quad \text{CHO} \quad \text{+CO}_2
\end{align*}
\]

Figure 7. Deamination and Decarboxylation of Amino Acids.

Which of the above two reactions is predominant depends upon many factors such as the specific amino acid, the phenolic compounds used, the pH, and the ratio of
the two reactants (Linder and Voye, 1987). Most of the amino acids studied by previous researchers were neutral and aliphatic. In this study, tryptophan was selected as a model for amino acids as tryptophan possesses an α-amino group and an indol group. Since both groups are very active, it was anticipated that it would react with the caffeic acid and give a high yield of products.
CHAPTER III

EXPERIMENTAL SECTION

Materials and Physical Properties

Caffeic acid (99%, Aldrich Chemical Co., Inc., Milwaukee, WI), L-(-)-tryptophan (99%, Aldrich Chemical Co., Inc., Milwaukee, WI), and ferric chloride (99.5%, General Chemical Division, New York, NY) were used in these studies. All aqueous samples were prepared using 18 MΩ Milli-Q water. Acetonitrile (HPLC Grade), 0.1% formic acid (made from 88% formic acid, Certified ACS) and Milli-Q water were used as solvents in HPLC separation and isolation.

Caffeic acid is a pale gray powder with a molecular weight of 180 g/mol. It is slightly soluble in cold water and soluble in boiling water. Tryptophan is a white powder with a molecular weight of 204 g/mol. It is also partially soluble in cold water and soluble in boiling water.

Sample Preparation

Caffeic Acid Solution

0.1 mM and 14 mM of caffeic acid solutions were prepared by adding 0.0018 g and 0.251 g of caffeic acid to 100 mL of water respectively. The solutions were heated on a hot plate until they were completely dissolved. The solution pHs were
raised by addition of 50% (w/w) NaOH to obtain the desired pH. The pH values were measured using a Corning Model 240 pH meter pre-calibrated with standard pH buffers (4.00 and 7.00). The pHs of all samples were adjusted to about 7.00 by addition of 6 M HCl before running the HPLC.

**Caffeic Acid and Tryptophan Mixture**

Two different concentrations of caffeic acid and tryptophan mixture were made: (1) 100 mL of 0.1 mM caffeic acid and 1 mM tryptophan; (2) 100 mL of 1 mM caffeic acid and 10 mM tryptophan. The solution pH values were raised to the specified pH, then adjusted back to neutral prior to introducing to the HPLC system.

**Caffeic Acid and Iron(III) Ion Mixture**

Iron(III) chloride was added to 0.1 mM caffeic acid solution so that the molar ratio of the iron(III) ion and caffeic acid was 1:2. A 0.1 mM of caffeic acid as a blank was also prepared at the same time. Then both were capped and stored in the dark place for UV-Visible absorption tracking and product comparison in two months.

**Caffeic Acid, Tryptophan and Iron(III) Ion Mixture**

The following two solutions were prepared for comparison study. First, a 100 mL of a solution containing 0.1 mM caffeic acid and 1 mM tryptophan and second 100 mL of a mixed solution containing 0.1 mM caffeic acid, 1 mM tryptophan and 0.05 mM iron(III). Both were capped and stored in the dark place for UV-Visible
tracking and product comparison in the following two months.

Physical Appearance of the Samples

The color changes of both 14 mM acid solution and 1 mM caffeic acid with 10 mM tryptophan solution was observed and recorded. When raising the solution pH value, the color changed from clear to greenish yellow, then brown and finally to dark brown. The higher the solution pH, the faster the color changed. Without adjustment of the solution pH or addition of iron(III), the solution slowly turned into brown within two months.

Instrumental Methods

UV-Visible Spectrophotometry (UV/Vis)

A HP 8451A diode array spectrophotometer equipped with a HP 7470A plotter and a HP ThinkJet printer was used to monitor the catalytic effects of iron(III) and hydroxide ions on the caffeic acid autoxidation reaction and the reaction of caffeic acid with tryptophan. It was also used for absorption measurements of isolated products of the above two reactions. The absorption spectrum of 0.1 mM caffeic acid is shown in Figure 8.

The first absorption measurements were taken right after the sample solutions were made. The absorption values at 320 nm, which is the maximum absorption wavelength of caffeic acid, were recorded twice a week for two months. The complete spectra were also obtained as the reaction of caffeic acid with tryptophan proceeded.
Figure 8. UV/Visible Spectrum of 0.1 mM Caffeic Acid Solution.

Analytical High Performance Liquid Chromatography (Analytical HPLC)

Caffeic Acid Autoxidation Reaction

A Varian 5060 High Performance Liquid Chromatography system (Varian, Walnut Creek, CA) with Milton Roy UV-Visible Detector and Hewlett-Packard Intergrator was used in this project. An Adsorbosil C18 (Alltech Associates, Inc., Deerfield, IL) 25 cm × 4.6 mm i.d. column with 5 µm packing materials was used in this study. The injection volume was 10 µL with a flow rate of 1.0 mL/min. A 0.1% formic acid buffer solution was used as one mobile phase (A) and acetonitrile (HPLC Grade) as the second mobile phase (B). The pH of 0.1% formic acid solution was
2.71, which was much lower than the pKₐ of caffeic acid and its derivatives. The function of 0.1% formic acid buffer was to suppress the ionization of caffeic acid and its derivatives. The buffer and all samples were vacuum filtered through a 0.45 Millipore filter before introducing them to HPLC system.

A gradient elution method was used in this study. The pump was programmed to start with a 90% mobile phase A and 10% mobile phase B, then ramped up to a 50/50 mixture in 30 minutes. After the column was washed with 100% mobile phase B for 10 minutes and equilibrated with 90% mobile phase A and 10% mobile phase B for additional 10 minutes, the next 10 µL sample was injected. The UV-Visible detector was set at a wavelength of 280 nm. The standard caffeic acid solution was chromatographed to determine its retention time.

**Reaction of Caffeic Acid With Tryptophan**

The same column was used in product separation for the reaction of caffeic acid with tryptophan. The injection volume was also 10 µL with a flow rate of 1.0 mL/min. In comparison with the separation of caffeic acid autoxidation products, an isocratic elution method was chosen in this separation instead of the gradient elution method. A composition of 70% mobile phase A (0.1% formic acid buffer solution) and 30% mobile phase B (Acetonitrile, HPLC Grade) was maintained throughout the separation. The detector was again set at 280 nm. Both standard caffeic acid and tryptophan samples were also injected under these conditions to determine their retention times.
Comparison Study of the Three Pairs of Reactions

Three pairs of reactions were established in order to compare the products in each pair. For each pair of reactions, the products of individual reactions were analyzed separately on analytical HPLC system under the same conditions.

1. 0.1 mM Caffeic acid autoxidation reactions with and without addition of 0.05 mM Fe$^{3+}$ were sampled after two months. Both reaction mixtures were injected at the optimum separation parameters used in the separation of caffeic acid autoxidation products.

2. Reactions of 0.1 mM caffeic acid and 1 mM tryptophan with and without addition of 0.05 mM Fe$^{3+}$ were sampled after two months. Both reaction mixtures were analyzed at the separation conditions for reaction of caffeic acid with tryptophan.

3. Caffeic Acid autoxidation reaction and the reaction of caffeic acid with tryptophan. Both reactions were set up at pH=10, terminated after 2 hours. The reaction products were analyzed by analytical HPLC method under the separation conditions used in 2.

Preparative High Performance Liquid Chromatography (Preparative HPLC)

A C18 preparative 25 cm × 10 mm i.d. column (Alltech Associates, Inc., Deerfield, IL) was used to collect the fractions of caffeic acid autoxidation reaction. The injection volume was 100 µL with a flow rate of 2.0 mL/min. The mobile phases and elution method were the same as in the analytical HPLC method. A 5 mM
solution diluted from a 14 mM caffeic acid solution was manually injected into HPLC system.

A 100 µL sample was injected on the C18 preparative column for fraction collection of reaction products of caffeic acid with tryptophan. A higher flow rate of 3.0 mL/min of 70% of 0.1% formic acid buffer solution and 30% of acetonitrile (HPLC Grade) was used as the mobile phase.

The fractions collected from more than twenty injections were combined for each fraction. All fraction solutions were kept in the dark and air-dried for further identification with $^1$H nuclear magnetic resonance. Each collected fraction was also analyzed using UV-Visible spectrophotometry.

**Liquid Chromatography/Mass Spectrometry (LC/MS)**

In order to obtain the mass spectra information for each product, a Waters 2690 liquid chromatography system (Waters, Milford, MA) and a Micromass Quattro II mass spectrometer (Micromass UK Limited, United Kingdom) with an electrospray ionization source was used. The conditions used in these LC-MS separations were identical to that in earlier HPLC separations. The reaction mixtures of both caffeic acid autoxidation and caffeic acid with tryptophan were run on this LC/MS system. Mass and fragment information of each fraction including starting materials of caffeic acid were obtained.
A JEOL ECLIPSE 400 MHz nuclear magnetic resonance spectrometer (JEOL, Peabody, MA) was utilized to identify the collected fractions. The proton spectra of pure caffeic acid and tryptophan were collected as standards. D₂O was used as solvent and pre-saturation method was chosen. Due to the limited amount of collected samples, 128 and 256 scans were performed for the different fractions.
CHAPTER IV

RESULTS AND DISCUSSIONS

Effect of pH

The effect of pH on both the caffeic acid autoxidation reaction and the reaction of caffeic acid with tryptophan were studied. A series of caffeic acid solutions and caffeic acid with tryptophan solutions at different pH values were prepared and monitored over a period of two months with a UV-Visible spectrophotometer.

A caffeic acid UV-Visible absorption spectrum was given earlier in Figure 8. There are two maximum absorption peaks, one at about 320 nm and the other at 280-290 nm. These absorption peaks are so-called fingerprint peaks of cinnamic compounds in UV-Visible spectra region. The effect of pH on reaction rates of caffeic acid autoxidation reaction and the reaction of caffeic acid with tryptophan was studied primarily by measurement of UV-Visible absorption values at 320 nm. The existence of the aromatic ring and conjugated double bond in cinnamic compounds allows these compounds to absorb UV-Visible electromagnetic radiation resulting in a $\pi$ to $\pi^*$ electron transition. This was also the reason a UV/Vis detector was chosen for the HPLC separations. The loss of absorbance at 320 nm may indicate loss of the conjugated double bond of the side-chain in cinnamic compounds (Jiang, 1996).
Caffeic Acid Autoxidation Reaction

The UV/Visible absorption values at 320 nm are plotted as a function of time for three different pH conditions for the caffeic acid autoxidation reaction (Figure 9).

![Graph showing the effect of pH on the autoxidation reaction](image)

**Figure 9.** Effect of pH on 0.1 mM Autoxidation Reaction.

For the reaction at pH 3.2, which was the original pH value of 0.1 mM caffeic acid solution, as the time increases, the absorption at 320 nm slowly decreases. This indicates that the reaction proceeded slowly even at this pH. The decrease in absorbance indicates that the conjugated double bond of the side-chain in caffeic acid is lost during the autoxidation reaction. As the pH increases, the absorption at 320 nm
decreases more rapidly. After half a week, at pH 10, more than half of the absorption of caffeic acid at 320 nm had already disappeared. At pH 3.2, there was only a small decrease in the absorption in the first half a week period. The fact that the caffeic acid autoxidation reaction rate increased with increasing pH indicates the involvement of the reactive phenolate ion in this reaction (Cilliers and Singleton, 1989). The phenolate ion is thought to react directly, by charge transfer with triplet oxygen, to form a semiquinone radical, which will then undergo further reaction (Cilliers and Singleton, 1991).

In the first half week, the reaction rate was very high at pH 10. Then, the absorbance dropped very slowly and remained nearly stable after a week indicating the reaction was close to completion. However, after two months, there was still absorption at 320 nm for all three reactions (pH=3.2, 7 and 10). This suggests that either, not all caffeic acid is depleted, or that some oxidation products absorb in this region, or both possibilities exist.

These results are consistent with that of Jiang’s study (1996). In Jiang’s experiment, pH 3, 7 and 10.5 were chosen as different pH conditions to study the pH effect of the caffeic acid oxidation reaction. The effect of pH on chlorogenic acid oxidation reaction was also reported. Jiang found both chlorogenic acid and caffeic acid oxidation reaction were pH dependent. The reaction rates increased as the pH value increased. In 1991, Cilliers et al. reported that approximately 30% side chain ethylenic conjugation existed in the oxidized products of caffeic acid.
Reaction of Caffeic Acid With Tryptophan

The effect of pH on reaction of caffeic acid with tryptophan is illustrated in Figure 10. The three pH values at which reactions were run are pH 3.75, 7 and 10. The pH 3.75 was the pH of the original mixture of caffeic acid with tryptophan. The absorbances at 320 nm were recorded for eight weeks for each of the three pH values. As observed in caffeic acid oxidation reaction, the higher the pH, the faster the reaction rate. Again at low pH, the reaction still proceeds, although slowly. This indicates that the reaction of caffeic acid with tryptophan is also a pH dependent reaction. The loss of absorption attributed to caffeic acid suggests that either caffeic acid underwent an autoxidation reaction, or reacted with tryptophan, or both.

Figures 11, 12 and 13 show the absorbance change of a mixture of tryptophan and caffeic acid as a function of time. The absorbance of tryptophan decreased a little as the absorption of caffeic acid decreased. Considering the excess amount of tryptophan used and the overlap of caffeic acid and tryptophan absorption peaks, it is difficult to attribute this to the loss of tryptophan. However, NMR experiments and comparison studies were performed to determine the involvement of tryptophan during the reaction.

The colors of both the caffeic acid oxidation mixture and the mixture of caffeic acid with tryptophan were observed during the reaction processes. It was found that the solution colors were darker under strong alkaline conditions in both reactions. The higher the pH of the reactions, the darker the reaction solutions.
Effect of Transition Metal Iron(III)

In order to investigate the effect of iron(III) on both caffeic acid autoxidation and reaction of caffeic acid with tryptophan, two pairs of reaction samples at pH 3.72 were prepared: caffeic acid solution with or without iron(III); Mixture of caffeic acid and tryptophan with or without iron(III). Effect of iron(III) on caffeic acid autoxidation reaction and effect of iron(III) on reaction of caffeic acid with tryptophan was both investigated by measurement of 320 nm absorbance once a week until the reactions started two months. The experiment results are illustrated in Figure 14 and Figure 15.

Figure 10. Effect of pH on Reaction of 0.1 mM CA With 1 mM Tryptophan.
Figure 11. UV/Visible Spectrum of Mixture of Caffeic Acid With Tryptophan at 0 min, pH10.

Figure 12. UV/Visible Spectrum of Mixture of Caffeic Acid With Tryptophan at 1 Hour, pH 10.
Figure 13. UV/Visible Spectrum of Mixture of Caffeic Acid With Tryptophan at 1 Week, pH 10.

Figure 14. Effect of 0.05 mM Fe\(^{3+}\) on 0.1 mM Caffeic Acid Autoxidation Reaction.
As shown in Figure 14 and Figure 15, both caffeic acid oxidation reaction and reaction of caffeic acid with tryptophan were catalyzed by addition of small amount of iron(III) ion. The colors of reaction mixtures were also observed to be darker with the presence of Fe\(^{3+}\). The effect of Fe\(^{3+}\) ions on the reactions was similar to the effect of hydroxide ions on these two reactions. The absorbance at 320 nm decreased with time. With Fe\(^{3+}\) present, the reaction rate increases dramatically, especially in the first two weeks. However, in comparison with pH effects on these two reactions, the Fe\(^{3+}\) effects are somehow weaker, which indicates a stronger catalytic effect of hydroxyl group than that of iron(III) ion.

![Graph](image)

Figure 15. Effect of 0.05 mM Fe\(^{3+}\) on the Reaction of 0.1 mM Caffeic Acid With 1 mM Tryptophan.
Separation and Isolation of Reaction Products

Caffeic Acid Autoxidation Reaction

Analytical HPLC

In order to obtain a good separation of the oxidation products of caffeic acid, much work was done on the development of an analytical HPLC method, which included selection of column, mobile phases, elution method, detector, etc. The reaction mixture was recovered after sixteen hours at pH 10. A sample chromatogram of caffeic acid and its oxidation products is shown in Figure 16. Peak 1 is the solvent peak. Peak 3 is non-consumed caffeic acid, which was confirmed by comparison with the retention time of pure caffeic acid at the same separation conditions. Peak 5 to peak 10 are final oxidation products of caffeic acid. Peak 2 and peak 4 are immediate oxidation products that disappeared after two months. However, since peak 2 elutes earlier than caffeic acid, its polarity must be greater than caffeic acid. While peak 4 to peak 10 have longer elution time than caffeic acid, indicating these products are less polar than caffeic acid, especially peak 9 and peak 10. Since the ring-opened products of caffeic acid would increase polarity and therefore have shorter retention time on reversed phase HPLC system, these products are likely not ring-opened products. The reproducibility of the separation was determined by comparison of chromatographic profiles of repeated injections.
Preparative HPLC

The preparative HPLC separation was developed based on analytical HPLC chromatography. The preparative separation chromatogram was similar to that of analytical HPLC except for the appearance of a few additional peaks that were not seen in analytical chromatography. This was due to the higher concentration of the sample in the preparative separation. However, the major peak profiles in both analytical and preparative HPLC separation were the same. A pure caffeic acid sample was again run on preparative HPLC system to confirm the caffeic acid peak. The collected fractions (5-10) were air-dried before further identification with UV-Visible spectrophotometry and proton nuclear magnetic resonance methods.

Figure 16. Separation of Caffeic Acid and Its Oxidation Products at pH 10 After 16 h. (Analysis of a 10-µL Sample by C18 Reversed-Phase HPLC System. Mobile Phases: A=0.1% Formic Acid, B=Acetonitrile. Gradient: 90% to 50% Mobile Phase A in 30 min. Detection at 280 nm. Peak 1 is the Solvent Peak. Peak 3 is Caffeic Acid. The Others are Oxidation Products.)
Reaction of Caffeic Acid With Tryptophan

Analytical HPLC

The isocratic elution method was chosen to separate the reaction products of caffeic acid with tryptophan. The chromatogram is given in Figure 17. Tryptophan was used in excess to insure that caffeic acid would react with it after it was oxidized. Peak 1 is a solvent peak. Peak 5 is tryptophan. The peak between peak 1 and peak 2 is caffeic acid. Peaks 2, 3 and 4 are reaction products.

As shown in Figure 17, tryptophan has a very long retention time and a broad peak. This is caused by two factors: one was the tryptophan concentration overload effect. The other was the strong interaction between the amino group in tryptophan and the silanol group in packing materials of the C18 column. The reaction mixture of caffeic acid autoxidation was also run under the same condition in order to compare the results with those of this reaction. A detailed discussion is given in comparison study section.

Preparative HPLC

Peaks 2, 3 and 4 (from now on the peak is referred as fraction) were collected using the preparative HPLC system. Approximately twenty injections were collected and the collected fractions were air-dried prior to identification with UV-Visible spectrophotometry and proton nuclear magnetic resonance methods.
Figure 17. Reaction of Caffeic Acid With Tryptophan at pH 10 After 10 h. (Analysis of 10-µL Sample by C18 Reversed Phase HPLC. Mobile Phases: 70%A, 0.1% Formic Acid; 30% B, Acetonitrile. Detection at 280 nm.)

Identification of Reaction Products

Caffeic Acid Autoxidation Reaction

**UV-Visible Spectrophotometry (UV/Vis)**

The UV/Visible spectra of fraction 6 to 10 are shown in Figures 18 to 23. It was found that these spectra were similar, suggesting fraction 6 to 10 might be structural homologues. Their comparison with the caffeic acid spectrum indicates the possibility of a modified caffeic acid or oligomers. In the spectra of fraction 6-10, there are two maximum absorption peaks in each spectrum: 280-290 nm and 320 nm as was seen in the caffeic acid spectrum. This was indicative of the presence of the conjugated side chain in the oxidation products. However, in comparison with the caffeic acid spectrum, the magnitude of 320 nm absorbance in these spectra was
smaller than that of caffeic acid at 280-290 nm, indicating the loss of side chain
conjugation during the oxidation. Fraction 5 shows there was a very weak absorption
at 320 nm, suggesting that it has a different structure from fraction 6-10. The side
chain conjugation might not contained in its structure.

Figure 18. UV/Visible Spectrum of Fraction 5 Collected in CA Autoxidation
Reaction.

Figure 19. UV/Visible Spectrum of Fraction 6 Collected in CA Autoxidation
Reaction.
Figure 20. UV/Visible Spectrum of Fraction 7 Collected in CA Autoxidation Reaction.

Figure 21. UV/Visible Spectrum of Peak 8 Collected in CA Autoxidation Reaction.
Figure 22. UV/Visible Spectrum of Fraction 9 Collected in CA Autoxidation Reaction.

Figure 23. UV/Visible Spectrum of Fraction 10 Collected in CA Autoxidation Reaction.
Liquid Chromatography/Mass Spectrometry (LC/MS)

The mass spectra of all fractions including caffeic acid are given in the Appendix A. The MS data for product fraction 5 to 8 are summarized in Table 2. As shown in the Table 2, fractions 5-8 have the same molecular weight. They all contain the fragments 179 and 135. The fragment of 179 is m/z of caffeic acid. This confirms that the products possess at least one unit of caffeic acid. The fragment of 135 results from \([179 - 44 (CO_2)]\). Since m/z was only set in the range of 75 to 500 in the experiment, the fragment of 44 (COOH group) could not appear in mass spectra of caffeic acid or fractions 5-8. Peaks 5-8 can be assumed to be dimers of caffeic acid with a molecular weight of 358: 313 +1 + 44 (CO_2) = 358. Fractions 5-8 contain a major peak at 313 which is the product peak of the dimer (357) with loss of a CO_2 (44). Although the 357 was present, it was a small peak as it can easily lose a CO_2 of one of the acid groups. In fraction 9 and 10, fragments of 489 and 179 are found. The molecular weight of trimer of caffeic acid is 534, which is not shown in MS spectra because the m/z range was only set up to 500. However, it could also be derived that fraction 9 and 10 are trimers of caffeic acid according to their UV/Visible spectra and LC/MS data information: 489 + 1 + 44 (CO_2) = 534.

\(^1\)H Nuclear Magnetic Resonance Spectrometry (\(^1\)H NMR)

The \(^1\)H NMR spectra of standard caffeic acid and all oxidation products are listed in Appendix B. Table 3 summarizes the \(^1\)H NMR data (in ppm) of standard and reaction products. The peak assignments for caffeic acid are shown in Figure 24.
Table 2

LC-MS Data for Caffeic Acid and Its Oxidation Products

<table>
<thead>
<tr>
<th>[M–H]$^-$</th>
<th>Caffeic Acid</th>
<th>Fraction-5</th>
<th>Fraction-6</th>
<th>Fraction-7</th>
<th>Fraction-8</th>
<th>Fraction-9</th>
<th>Fraction-10</th>
</tr>
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<td>489</td>
<td></td>
<td>100</td>
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</tr>
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<td>313</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tr>
<tr>
<td>179</td>
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<td>100</td>
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<td>34</td>
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<td>41</td>
<td>30</td>
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<td>11</td>
<td>31</td>
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<td>42</td>
<td>40</td>
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<tr>
<td>89</td>
<td></td>
<td>68</td>
<td>42</td>
<td>79</td>
<td>91</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The protons at $d$ positions in caffeic acid could not be seen in the proton NMR spectrum because they are free to exchange with the D$_2$O solvent:

$$-\text{COOH} + \text{D}_2\text{O} \rightleftharpoons -\text{COO}^- + \text{HD}_2\text{O}^+$$

$$\text{HO} \begin{array}{c} \text{HO} \\ \text{HO} \end{array} + \text{D}_2\text{O} \rightleftharpoons \text{DO} \begin{array}{c} \text{DO} \\ \text{DO} \end{array} + \text{H}_2\text{O}$$
Figure 24. Peak Assignments for Caffeic Acid.

Table 3

$^1$H NMR Spectra Data ($\delta$, ppm) in D$_2$O at 400 MHz

<table>
<thead>
<tr>
<th>Caffeic Acid</th>
<th>Fraction 5</th>
<th>Fraction 6</th>
<th>Fraction 7</th>
<th>Fraction 8</th>
<th>Fraction 9</th>
<th>Fraction 10</th>
</tr>
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<tbody>
<tr>
<td>5.95 $d$</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>6.30(a) $d^*$</td>
<td>6.38 $q^*$</td>
<td>6.38 $d$</td>
<td>6.37 $d$</td>
<td>6.38 $d$</td>
<td>6.30 $d$</td>
<td>6.31 $d$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.42 $d$</td>
<td>6.42 $d$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.59 $d$</td>
<td>6.59 $d$</td>
</tr>
<tr>
<td>6.90(c$_1$) $d$</td>
<td>6.86 $d$</td>
<td>6.60 $d$</td>
<td>6.53 $d$</td>
<td>6.54 $d$</td>
<td>6.87 $d$</td>
<td>6.88 $d$</td>
</tr>
<tr>
<td>7.06(c$_2$) $d$</td>
<td>6.94 $d$</td>
<td>6.71 $d$</td>
<td>6.62 $d$</td>
<td>6.62 $d$</td>
<td>6.94 $d$</td>
<td>6.95 $d$</td>
</tr>
<tr>
<td>7.15(c$_3$) $d$</td>
<td>6.97-7.21</td>
<td>6.95-7.22</td>
<td>7.03-7.19</td>
<td>6.93-7.20</td>
<td>7.03-7.13</td>
<td>7.05-7.14</td>
</tr>
<tr>
<td></td>
<td>7.27 $d$</td>
<td>7.30 $d$</td>
<td>7.30 $d$</td>
<td>7.32 $d$</td>
<td>7.20-7.33</td>
<td>7.24-7.35</td>
</tr>
<tr>
<td>7.41(b) $d$</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>8.43 $s^*$</td>
<td>8.42 $s$</td>
<td>8.42 $s$</td>
<td>8.42 $s$</td>
<td>8.42 $s$</td>
<td>8.42 $s$</td>
</tr>
</tbody>
</table>

# Letters in parenthesis refers to assignments on the structure.

$s =$ singlet; $d =$ doublet; $q =$ quartet.
In Table 3, compares the NMR data of product fraction 6 with those of caffeic acid. The alkene side chain is seen at 6.38 and 7.30 ppm as two doublet peaks. This indicates that the protons on alkene side chain are in trans form. The appearance of two doublets at 6.60 and 6.71 ppm indicates the loss of the second alkene side chain conjugation in fraction 6. This result demonstrates the involvement of the double bond on the side chain of caffeic acid during the oxidation. The couplings of side-chain protons could be confirmed with decoupling experiments. Fraction 6, 7, and 8 give very similar NMR data. Small differences are observed due to their steric effects. Fraction 9 and 10 also have similar NMR spectra but different from those of fraction 5 to 8. We have shown that fraction 5 to 8 were dimers of caffeic acid and fraction 9 and 10 were trimers of caffeic acid based on their LC-MS data. However, the spectrum of product fraction 5 is different from those of fractions 6, 7 and 8. Loss of two alkene side chain conjugations is indicated by the appearance of two strong doublet peaks at 6.86 and 6.94 ppm. Doublet peaks shifted from 6.30 ppm to 5.95 ppm suggest the change of protons from alkene to alkane structure. These results illustrate fraction 6 to 8 are structural analogues. Fraction 9 and 10 are also structural analogues. The NMR results are consistent with UV-Visible spectroscopic results. In Xu’s study, cyclobutane linkage dimers and ether linkage dimers were suggested as two different structures of dimers of caffeic acid oxidation products (Xu, 1994). This NMR experiment provides the further evidence to show that some of the products (fractions 6, 7 and 8) are ether linkage dimers (Figure 5) and one product (fraction 5) is a cyclobutane linkage dimer (Figure 4).
Reaction of Caffeic Acid With Tryptophan

UV/VIS

The UV-Visible spectra of standard tryptophan and products of fraction 2 to 4 are given in Figures 25, 26, 27 and 28. Fractions 2 and 3 have two absorption peaks at 280-290 nm and 320 nm, suggesting structural analogues with caffeic acid. Fraction 2 and 3 might be dimers or trimers or mixture of dimers and trimers, which were produced by caffeic acid oxidation reaction. Fraction 4, on the other hand has a UV/Visible spectrum different from those of fractions 2 and 3. The spectrum of fraction 4 consists of both caffeic acid peak and tryptophan absorption peak. It has a strong absorption peak at 305 nm, indicating that the side chain double bond is not present in its structure. The fraction 4 spectrum is very similar to the standard spectrum of indolepropionic acid acid. According to the UV/Visible data, fraction 4 could be an indolepropionic acid.

LC/MS

LC/MS data for reaction products of caffeic acid with tryptophan was very confusing. The reaction products were well separated on HPLC using UV-Visible detection. However, on the LC/MS system, no separated peaks were observed using the mass detector. This was caused by the strong background noise in LC/MS system that was not UV active. The complicated sample matrix was the primary factor that resulted in the strong background noise.
Figure 25. UV/Visible Spectrum of Standard Tryptophan.

Figure 26. UV/Visible Spectrum of Fraction 2 Collected in Reaction of CA With Tryptophan.
Figure 27. UV/Visible Spectrum of Fraction 3 Collected in Reaction of CA With Tryptophan.

Figure 28. UV/Visible Spectrum of Fraction 4 Collected in Reaction of CA With Tryptophan.
The $^1$H NMR spectra of standard caffeic acid and three products are listed in Appendix B. Figure 29 (a) shows the structure of tryptophan and NMR peak assignments for tryptophan in D$_2$O solvent. Table 4 summarizes the $^1$H NMR data (in ppm) of standard caffeic acid and tryptophan, as well as reaction products. Proton NMR data of fraction 4 shows both caffeic acid and tryptophan units. Peaks at $\delta=3.31$, 3.49 and 4.07 of fraction 4 correspond to peaks at $\delta=3.31$, 3.50 and 4.08 of tryptophan. Small differences for chemical shifts at these peaks are observed. NMR peaks at $\delta=6.87$ and 7.43 of fraction 4 correspond to NMR peaks at $\delta=6.90$ and 7.41 of caffeic acid. A large peak at $\delta=9.77$ is also found in $^1$H NMR spectrum of fraction 4. The large peak could be a shifted peak caused by the linkage of caffeic acid with tryptophan. Fraction 4 is an incorporated compound between caffeic acid and tryptophan. Fraction 4 is one of indolepropionic acids. Structure of fraction 4 and peak assignments for it are shown in Figure 29 (b). The fact that $^1$H NMR data of fraction 2 and 3 in Table 4 are almost identical with $^1$H NMR data of fractions 6, 7 and 8 of the caffeic acid autoxidation reaction in Table 3 suggests that of fraction 2 and 3 have the same structures with fraction 6 to 8 in caffeic acid autoxidation reaction, which were shown to be ether linkage dimers of caffeic acid.

Comparison Study

Comparison studies were performed on the analytical HPLC system. In the first pair of reactions, caffeic acid autoxidation reaction with and without addition of
iron(III) ion, the products of reactions were analyzed after two months. Two very similar chromatograms were obtained. The similarities of chromatograms and UV study suggest that the iron(III) ion was only involved in catalysis of the reaction.
<table>
<thead>
<tr>
<th>Caffeic Acid</th>
<th>Fraction 2</th>
<th>Fraction 3</th>
<th>Fraction 4</th>
<th>Tryptophan</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>3.31(a) q*</td>
<td>3.30(a) q</td>
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</tr>
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</tr>
<tr>
<td>4.07(c) d</td>
<td>4.08(c) d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.30(a) d*</td>
<td>6.39 d</td>
<td>6.38 d</td>
<td>6.42(d) d</td>
<td></td>
</tr>
<tr>
<td>6.61 d</td>
<td>6.62 d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.71 d</td>
<td>6.70 d</td>
<td>6.87(f_2) d</td>
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<td></td>
</tr>
<tr>
<td>6.90(c_1) d</td>
<td>6.98-7.24</td>
<td>6.97-7.23</td>
<td>7.20(f_1, g_1)</td>
<td>7.18(d_1) t</td>
</tr>
<tr>
<td>7.06(c_2) d</td>
<td>7.30 d</td>
<td>7.31 d</td>
<td>7.30(f_3, g_2)</td>
<td>7.32(d_2) q</td>
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<td>8.44 s</td>
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<td>9.77 s</td>
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</table>

# Letters in parenthesis correspond to the proton assignment in Figure 29.

* s = singlet; d = doublet; t = triplet; q = quartet.
This result confirms the catalytic function of iron(III) during the reaction. Similar results were obtained during the study of reaction of caffeic acid with tryptophan with and without addition of iron(III) (Figure 30 and 31). The products of two reactions were also found to be same, indicating the iron(III) ion played a catalytic role during the reaction of caffeic acid with tryptophan.

The last pair of reactions, caffeic acid autoxidation reaction and reaction of caffeic acid with tryptophan, were studied under the same separation conditions used in separation of reaction products of caffeic acid with tryptophan. By comparing these two chromatograms (Figure 32 and 33), fraction 4 in reaction of caffeic acid with tryptophan was identified as a previously unreported compound in the reaction. Fraction 2 and 3 in reaction of caffeic acid with tryptophan are ether linkage dimers of caffeic acid based on their proton NMR data.

Figure 30. Separation of Reaction Products of CA and Tryptophan Without Fe^{3+} (After 2 Months).
Figure 31. Separation of Reaction Products of CA and Tryptophan With Addition of Fe$^{2+}$ (After 2 Weeks).

Figure 32. Caffeic Acid Autoxidation Reaction at pH 10 After 16 h. (Analysis of 10-µL Sample by C18 Reversed Phase HPLC. Mobile Phases: 70% A, 0.1% Formic Acid; 30% B, Acetonitrile. Detection at 280 nm.)
Figure 33. Reaction of Caffeic Acid With Tryptophan at pH 10 After 10 h. (Analysis of 10-µL Sample by C18 Reversed Phase HPLC. Mobile Phases: 70%A, 0.1% Formic Acid; 30% B, Acetonitrile. Detection at 280 nm.)
CHAPTER V

PROPOSED MECHANISM

Based on UV-Visible spectra, proton NMR spectra and the comparison study, Peak 4 in reaction of caffeic acid with tryptophan was identified to be an incorporated compound between caffeic acid with tryptophan. Because of the pH dependence, the phenolate anion was believed to be involved in the reaction. Under alkaline condition, oxidation of caffeic acid easily led to the formation of radical anions of the corresponding semiquinones. Then, tryptophan was added to the semiquinones as a whole molecule to give an Indole-Propionic Acid (IPA). The reason we believe tryptophan attached to the side alkene chain of caffeic acid is that the side chain of caffeic acid is more reactive. The loss of the side alkene double bond was verified by UV/Visible data. The mechanism we proposed is different form that proposed by Flaig et al (1975) because caffeic acid contains an active side alkene chain. It should be noted that although our proposed final product is in agreement with our results, it may not be the only one. The mechanism and final product were proposed as a possibility and further analysis is needed to confirm them.

Under acidic conditions, the reaction is very slow, taking weeks and months. The reaction rate is accelerated under acidic condition with presence of iron (III). Xu (1994) reported another transition metal ion-copper (II) had a catalytic effect on caffeic acid autoxidation reaction. A catalytic reaction mechanism was also
Figure 34. Proposed Mechanism for Reaction of Caffeic Acid With Tryptophan.
postulated by Xu (1994) for caffeic acid autoxidation reaction with presence of copper (II).
CHAPTER V

CONCLUSIONS

The reactions of caffeic acid with tryptophan and caffeic acid autoxidation reaction were studied. Products of the two reactions were compared on HPLC under the same reaction and separation conditions.

The experimental data demonstrates that both hydroxide and transition metal iron(III) ion catalyzed both caffeic acid autoxidation reaction and reaction of caffeic acid with tryptophan. The probable first step in reaction of caffeic acid with amino acids must be the oxidation of caffeic acid. This suggests that the OH\textsuperscript{-} and Fe\textsuperscript{3+} must catalyze the oxidation of caffeic acid. This would also suggest that the oxidation of caffeic acid is the rate determining step in reaction of caffeic acid with amino acids.

In this study, caffeic acid autoxidation reaction gave six final products, three of which were found to be ether linkage dimers of caffeic acid and one was cyclobutane linkage dimer of caffeic acid. The other two were thought to be trimers of caffeic acid. Of the three products in reaction of caffeic acid with tryptophan, two are caffeic acid ether linkage dimers of caffeic acid. By identification with UV-Visible spectrophotometry and proton NMR, the new product was identified to be an incorporated compound between caffeic acid and tryptophan.

The fact that caffeic acid can react with tryptophan indicates that this mechanism could be used to incorporate nitrogen into humic substances in natural
waters. This also suggests the possibility of the reaction of caffeic acid with man-
made compounds such as pesticides in natural waters. This study also demonstrated
that although the oxidation reaction is slow at neutral pHs, it can be catalyzed by
transition metal ions like Fe$^{3+}$, which are widely present in the environment, at low
pHs.
Appendix A

LC-MS Spectra of Products of Caffeic Acid Autoxidation Reaction and Reaction of Caffeic Acid with Tryptophan
Total Ion Chromatogram of CA Autoxidation Reaction Mixture in LC-MS System
MS Spectrum of Caffeic Acid in Its Oxidation Reaction
MS Spectrum of Peak 5 Collected in Caffeic Acid Oxidation Reaction
MS Spectrum of Peak 6 Collected in Caffeic Acid Oxidation Reaction
MS Spectrum of Peak 7 Collected in Caffeic Acid Oxidation Reaction
MS Spectrum of Peak 8 Collected in Caffeic Acid Oxidation Reaction
MS Spectrum of Peak 9 Collected in Caffeic Acid Oxidation Reaction
MS Spectrum of Peak 10 in Caffeic Acid Oxidation Reaction
Appendix B

$^1$H NMR Spectra of Products of Caffeic Acid Autoxidation Reaction and Reaction of Caffeic Acid with Tryptophan
'H NMR Spectrum of Fraction 5 Collected in Caffeic Acid Autoxidation Reaction
$^1$H NMR Spectrum of Fraction 6 Collected in Caffeic Acid Autoxidation Reaction
$^1$H NMR Spectrum of Fraction 7 Collected in Caffeic Acid Autoxidation Reaction
$^1$H NMR Spectrum of Fraction 8 Collected in Caffeic Acid Autoxidation Reaction
$^1$H NMR Spectrum of Fraction 9 Collected in Caffeic Acid Autoxidation Reaction
"""H NMR Spectrum of Fraction 10 Collected in Caffeic Acid Autoxidation Reaction""
**H NMR Spectrum of Standard Tryptophan**
$^1$H NMR Spectrum of Fraction 2 Collected in Reaction of Caffeic Acid With Tryptophan
$^{1}$H NMR Spectrum of Fraction 3 Collected in Reaction of Caffeic Acid With Tryptophan
$^{1}H$ NMR Spectrum of Fraction 4 Collected in Reaction of Caffeic Acid With Tryptophan
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