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An Investigation on Nonenzymatic Autoxidation of Phenolic Compounds in Natural Waters

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AN INVESTIGATION ON NONENZYMATIC AUTOXIDATION OF PHENOLIC COMPOUNDS IN NATURAL WATERS

by

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To my husband, my father and my sister, I extend my wholehearted appreciation. They are my source of inspiration and my continuous drive to finish in excellence.

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Lei Jiang

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The nonenzymatic autoxidation of the phenolic compounds chlorogenic acid in aqueous solution was studied. The study demonstrated that the oxidation rate of chlorogenic acid was strongly dependent on pH. The higher the pH, the faster the rate of oxidation. The involvement of phenolate ions and radicals in oxidation reactions are two possible reasons for the pH dependency. The presence of a trace amounts of reducible transition metal ions, such as copper(II), dramatically increases the oxidation rate of chlorogenic acid. Light also increases the oxidation rate of chlorogenic acid. However, the photochemical reaction was a much slower process.

The TLC and HPLC results show that the oxidation of chlorogenic acid produces a mixture. The percentage of the various components in the mixture was affected by oxidation time.

The NMR results indicate that under strong alkaline conditions, some chlorogenic acids were initially hydrolyzed into caffeic acid and quinic acid. The study also showed that the oxidation of caffeic acid was pH dependent. The oxidation rate of caffeic acid was much faster than that of chlorogenic acid under acidic and neutral conditions.

Both NMR and UV/VIS results demonstrated that the conjugated double bond on the side chain of chlorogenic acid was involved in the oxidation reaction.

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CHAPTER I

INTRODUCTION

Motivation

There are a variety of organic compounds and inorganic compounds in natural waters. Their existence has a tremendous influence on living creatures in natural waters. The functions of these various compounds and their interactions in natural waters has become an exciting area of research. In recent years this area of research has become more and more important because of the need to protect natural waters from pollution. Phenolic compounds, humic substances and transition metals are three large groups of compounds that merit further study, especially their interactions. Many researchers (Hatcher, 1988; Flaig, 1988; Hedges, 1988) have suggested that phenolic compounds play a key role in the formation of humic substances in the environment. Humic substances are polymeric compounds that can have an effect on the fate of the other constituents of natural waters, particularly metals. The relationships of phenolic compounds, humic substances and transition metals, though very complicated, has attracted more and more interest in the area of environmental research. This thesis was an experimental effort towards developing a better understanding of the relationship of these three compounds in natural waters.

Phenolic Compounds

In natural waters, most phenolic compounds come from natural sources such as the degradation of natural plants, vegetables and fruits. Some of them may also come from man-made sources such as discharges from industry.

Phenolic compounds, especially *ortho*- and *para*-dihydroxyphenols, are susceptible to oxidation (Cha et al., 1986; Cilliers, 1989). Many natural phenolic compounds, such as caffeic acid, chlorogenic acid and caftaric acid, have the aforementioned structures. Studies on the oxidation of natural phenolic compounds are important to both the environmental and food scientists. In the environment, scientists are interested in the pathway of formation from natural phenolic compounds to humic substances. In the food industry, scientists are interested in understanding the oxidative browning of polyphenols in fruits and vegetables. Such undesirable browning is a main cause of quality and nutritional loss which costs the food industries millions of dollars each year.

Although numerous studies have focused on the oxidation of phenolic compounds, the mechanism is still not fully understood. It is generally believed that two kinds of reactions are involved in this process, enzymatic oxidation and nonenzymatic oxidation. Enzymatic oxidation is the more important reaction in fresh fruits, juices and early in food processing when polyphenol oxidase is present (Matheis, 1987). In processed foods and natural waters where the enzyme is not present or inactivated, nonenzymatic autoxidation plays a key role (Cilliers, 1991). It has been found that nonenzymatic autoxidation could also be catalyzed by certain metal ions naturally present or picked up from processing equipment and can even mimic enzyme action (Pandell, 1983).

Humic Substances

Humic substances are believed to be the most widely distributed organic materials on earth (Stevenson, 1982). They are found not only in natural waters but also in soil, sewage, marine and lake sediments (Stevenson, 1982).

Although they are not believed to be physiologically harmful, they are

unacceptable in potable water. Humic-like materials also create important problems in water treatment plants. They react with chlorine (during chlorination) to produce the carcinogen chloroform and other halogenated organic compounds, some of which are also known to be toxic. Humic-like materials may also concentrate compounds such as pesticides and heavy metals. They can interfere with removal of odor-producing compounds by competing for adsorption sites during activated carbon treatment (Stevenson, 1982).

Based on solubility characteristics, humic substances are fractionated into four groups, humin, fulvic acid, humic acid and hymatomelanic acid (Stevenson, 1965) as shown in Figure 1.

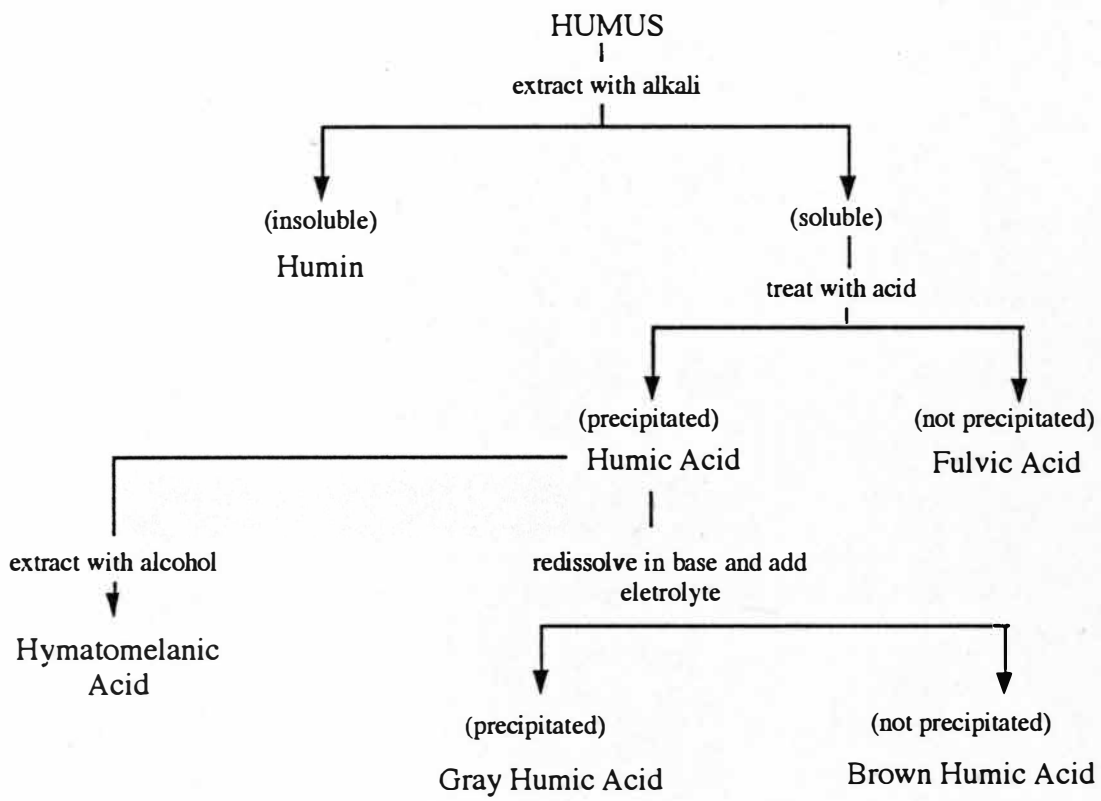


Figure 1. Scheme for the Fractionation of Humic Substances.

Because of their complex, multicomponent nature, humic substances are difficult to be described in specific molecular structure. Fuchs' scheme (Fuchs, 1931) for the structure of humic acid given in Figure 2 has been widely quoted in the literature. The structure of humic acid shows the polyphenol frame of humic substances.

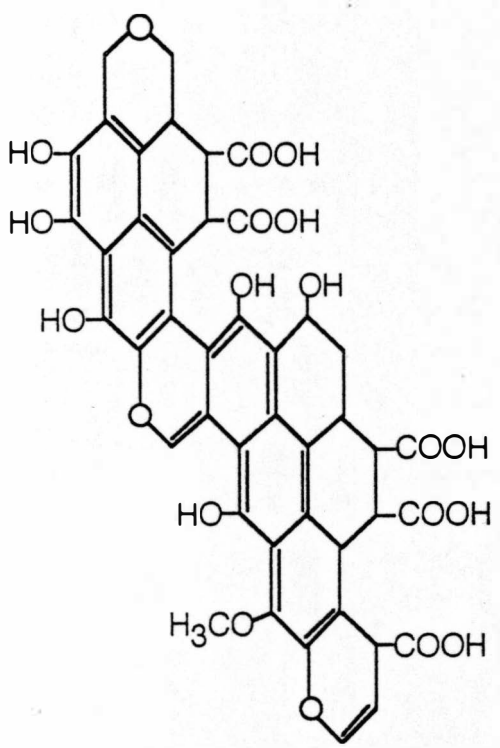


Figure 2. Structure of Humic Acid According to Fuchs.

The biochemistry of the formation of humic substances is one of the least understood aspects of humus chemistry. Continued research can be justified on theoretical and practical grounds. An understanding of the pathways of humus synthesis would result in greater comprehension of the Carbon cycle and of the changes that occur when plant residues and organic wastes undergo decay by microorganisms. It is generally agreed that humic substances originate from

decomposition of vegetation, but there is little consensus on the mechanisms by which humification occurs.

The classical theory is Waksman's "lignin-protein" scheme (Waksman, 1938). It is a degradative pathway. The lignin is attacked by microorganisms and its residue is most likely the precursor substance for humus. Proteinaceous materials, primarily from microorganisms are linked into this lignin-derived humus 'core' as the lignin is modified by the action of microorganisms. The humus thus formed has a continually changing structure depending on the degree of decomposition. The higher degree of oxidative degradation produces the most modified lignin products.

The majority of present day investigators favor a mechanism involving quinones (Hedges, 1988). The polyphenol model, which was popularized by Kononava, Flaig and Matin and Haider (Flaig, 1988), is the basic frame of this theory. In this model, the plant biopolymers first degrade to small molecules such as phenolic compounds. These small molecules then re-polymerize to form humic substances according to the synthetic pathway given in Figure 3 (Stevenson, 1982).

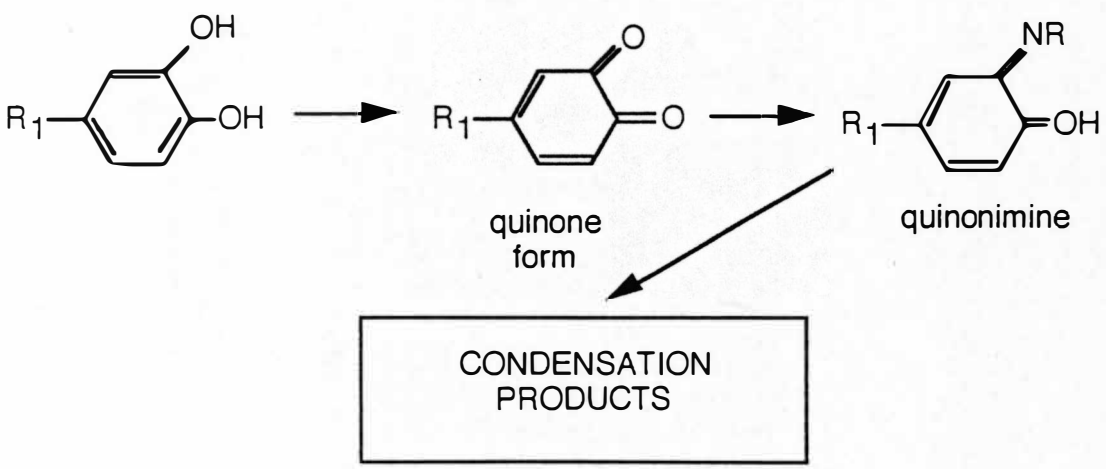


Figure 3. The Polyphenol Model.

In Figure 3, the phenolic compound changes to its quinone form first. Second, the quinone reacts with amine or amino compounds to form quinonimine and then condenses to humic products. One of the major strengths of the polyphenol model is that the polymerization of quinones is an extremely facile reaction under environmental conditions, especially at slightly basic pH or in the presence of nitrogenous substances.

In natural environments, the oxidation of polyphenols to quinones can either occur spontaneously in the presence of molecular oxygen or be enzymatically mediated by a wide variety of microorganisms. Such aerobic oxidation reactions are reportedly catalyzed by clay minerals, insoluble transition metal oxides and dissolved cation such as Mn^{2+} and Fe^{3+} (Lanson, 1980).

Transition Metals in Natural Waters

The availability of metal ions, either as micronutrients or as toxins, is determined by the physico-chemical form of the metal ion, its speciation. The speciation of a particular metal ion is strongly influenced by any coordinating ligands which may be present. It has been postulated that the release of caffeic acid from plant roots results in the reduction of iron(III) to iron(II), which is the necessary oxidation state of iron ions for absorption by plant root (Marschner, 1981).

The complexation between caffeic acid and copper(II), zinc(II), iron(II), Manganese(II), cobalt(II), Nickel(II) and cadmium(II) ions have been investigated by Linder et al. (1987, 1992). They reported that the metal ion is bound at the more strongly coordinating catecholate site on the caffeate ligand and not at the weaker carboxylate site at pH 6-9. Therefore, only mononuclear complex is formed at pH 6-9. At higher pH values, a minor binuclear complex, in which two metal ions are bound at both sites, is formed together with the mononuclear complex.

Copper complexes are important catalysts for the oxidation and oxygenation of

organic compounds in chemical and biological systems (Karlin, 1983; Nigh, 1973). The catalyst system, copper(I) chloride in pyridine, has been investigated as an oxidation and oxygenation catalyst by Rogic (1983). This catalyst is suitable for the oxidative coupling of phenols, the oxidative ring cleavage of catechols and/or *o*-quinone (Davies, 1979). A proposed mechanism (Speier, 1986) for the reaction of copper complexes with 3,5-di-t-butylcatechol can be shown in Figure 4.

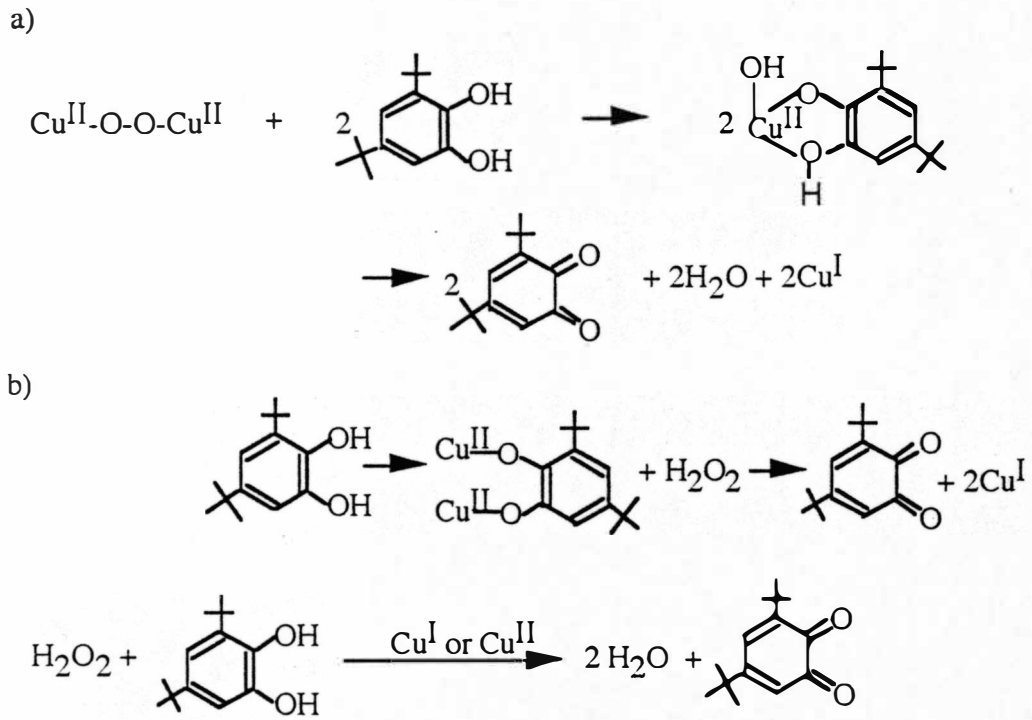


Figure 4. Proposed Mechanism for the Reaction of Copper Complexes With Catechol.

In natural waters, copper commonly occurs as a pollutant in surface waters and can be very toxic to fish. Experimental evidence suggested that only the soluble forms of copper are available to fish. The most likely soluble forms of copper are its complexes with carbonate, chloride, amino acids and polypeptides and humic substances as well as the free cupric ion (Stiff, 1971). Williams (1969) found that 5-28

per cent of the total soluble copper was associated with organic material. The decomposition of vegetation gives rise to both humic substances and peptide material. Humic substances and polypeptides can complex copper strongly enough to render them important at the copper concentration possible in polluted water.

Purpose of Study

Previous research has shown that phenolic compounds, humic substances and transition metals are associated and can have a great influence on one another. Humic substances and phenolic compounds are good ligands for many transition metals and they could affect the fate of transition metals in natural waters. On the other hand, some transition metals in natural waters can have a catalytic effect on the oxidation of phenolic compounds. To fully understand the significance of their effect in natural waters, more systematic studies are needed to fully resolve some important issues. For example:

1. What kind of condition (physical factors) favor the phenolic compound in its oxidation to humic substance?

2. What kind of compounds are formed during the early oxidation?

3. How do metal ions, associated with phenolic compounds, catalyze their oxidation?

These are the kinds of problems that triggered this project. The objective of this research was to investigate autoxidation of phenolic compounds in natural waters. Chlorogenic acid and caffeic acid were chosen for this study since they are found in relatively high concentrations as normal metabolites in many plants, fruits and plant-derived foods.

The effects of three physical factors: metal ion, pH and light, on the autoxidation of chlorogenic acid are the main subject of my research. These factors are

very important to the degree of oxidation in natural water systems.

Copper(II) was chosen as the metal ion in this study for comparison with previous results in our group which had shown that copper ions in natural waters have a catalytic effect on the oxidation of caffeic acid to form a caffeic acid dimer as a product (Xu, 1994).

Several experimental techniques were used in this study to pave a way for eventual separation and identification of final compounds.

This study tries to demonstrate the importance of nonenzymatic oxidation and subsequent polymerization of phenolic compounds in the environment. The results may provide a clue on the mechanism of humic acid formation. It can also facilitate further study of participation of phenolic compounds in non-enzymatic browning in processed foods.

CHAPTER II

LITERATURE REVIEW

Chlorogenic Acid

The term chlorogenic acid has over the years been used to describe a range of depside-linked esters formed between quinic acid and variously substituted cinnamic acid derivatives. However, originally the term applied specifically to a single compound identified as 3-caffeoylquinic acid given in Figure 5. But under current IUPAC recommendations more correctly designated as 5-caffeoylquinic acid (Griffiths, 1992).

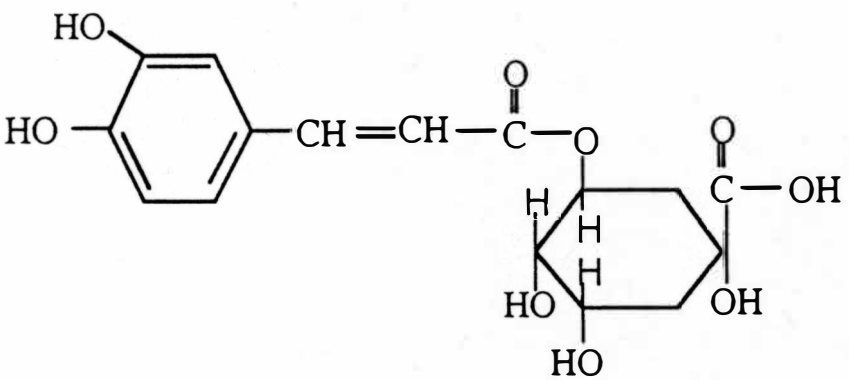


Figure 5. The Structure of 3-Caffeoylquinic Acid (IUPAC: 5-Caffeoylquinic Acid).

Chlorogenic acid was first isolated from coffee beans but is very widely distributed. Chlorogenic acid and its derivatives may constitute up to 0.1% of many plant tissues (Griffiths, 1992).

Marishita et al. (1986) separated chlorogenate extract from crude green coffee beans by HPLC and found at least 11 components. They found the UV spectra of these components were almost identical to chlorogenic acid (5-caffeoylquinic acid) and

showed λ_{max} at 325 nm. Nine of the components were identified as chlorogenic acid derivatives by ^{1}H NMR and fast atom bombardment mass spectroscopy. They include 5-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, 4-*O*-feruloylquinic acid, 3-*O*-feruloylquinic acid, 4,5-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid and 3-*O*-feruloyl-4-*O*-caffeoyl-quinic acid. Similar results were reported by Clifford (1986).

Chlorogenic acid is a secondary product of plant metabolism. It has been found that the enzyme, hydroxycinnamoyl D-glucose:quinate hydroxycinnamoyl transferase, produces chlorogenic acid by transesterification between caffeoyl D-glucose and D-quinic acid in sweet potato roots (Bradfield, 1956).

Biologically, chlorogenic acid has a variety of functions and its chemistry is very complicated. It reversibly associates with a wide range of substrates including proteins and polysaccharides during metabolism. Studies of these phenomena are of increasing interest because of their possible involvement in a functional role and practical significance (Martin, 1987).

Chlorogenic acid has been implicated in both enzymatic and non-enzymatic browning of fruits and vegetables (Mathew and Parpia, 1971). It was found that ascorbic acid can prevent or delay these oxidative reactions by reduction of the quinones. Other compounds, e.g. sulfhydryl compounds, may also reduce the *o*-quinone and/or inhibit the enzyme directly by blocking the active site containing copper (Mathew, 1971). It has also been found that the water activity and temperature have an effect on the nonenzymatic browning in a model food system (Monsalve, 1990). The higher moisture and temperature the system, the higher the browning or discoloration of food. The activation energy for nonenzymatic browning in dried foods increased from 20 kcal/mol up to 45 kcal/mol with decreasing moisture. It has been suggested that water is an important reactant in the nonenzymatic conversion of the

o-quinone, formed by enzymatic oxidation of chlorogenic acid, to hydroxyquinones. Polymerization of the hydroxyquinones forms the brown pigment (Pierpoint, 1966).

In his review article, Matheis (1987) suggested that the rate of enzymatic browning depends on the following factors: (a) Concentration and type of phenolic compounds, (b) Concentration and substrate specificity of PPO, (c) Concentration of naturally occurring inhibitors of browning, (d) Concentration of oxygen and (e) pH, storage time and temperature. He also suggested that the storage time seems to have a greater influence on changes of the rate of browning than storage temperature.

Susceptibility to oxidation is not always undesirable. Recent studies have shown that caffeoylquinic acids can be used as antioxidants to inhibit lipid peroxidation. Maruta et al.(1995) has demonstrated the following behavior with respect to caffeoylquinic acids. First, caffeic acid derivatives are more effective than α-tocopherol as an antioxidant. Second, activity of caffeoylquinic acids on a molar basis increases with an increase in the number of caffeoyl residues. Third, esterification of caffeic acid with quinic acid lowers its activity in this system. They conclude that the antioxidant efficiency increases in the order α-tocopherol < chlorogenic acid < caffeic acid < isochlorogenic acid. Since caffeoylquinic acids are widely distributed in various foods, detailed investigations on the biological effects of the compounds would be quite valuable.

Enzymatic Oxidation of Phenolic Compounds

Enzymatic oxidation of phenolic compounds results from the catalytic action of an enzyme called Polyphenol Oxidase (PPO). PPO can be found in most plant tissues.

In intact cells, PPO is spatially separated from phenolic compounds. If the cell is injured or damaged, the PPO can come into contact with phenolic compounds. When oxygen is present, PPO will then catalyze the oxidation of the phenolic compounds to

form quinones. After the conversion to a quinone, the phenolic compound can then recombine with another phenolic compound or with other organic molecules around it, such as amino acids, peptides and proteins, to yield a nitrogenous polymer.

It has been reported that quinone formation is reversible in the presence of reducing agents; however, the polymerization step is not (Shepherd, 1995). The molecular structure and composition of the polymers has not been defined but the polymers have similar properties to those of natural humic acids.

Villegas et al. (1987) studied the enzymatic reaction of chlorogenic acid catalyzed by chlorogenate caffeoyl transferase. The enzyme was extracted from sweet potato roots and purified. It was found to convert chlorogenic acid into isochlorogenic acid in a one step reaction in vivo as shown in Figure 6.

Nonenzymatic Oxidation of Phenolic Compounds

Nonenzymatic oxidation is a spontaneous process in the presence of molecular oxygen. Unlike enzymatic oxidation, nonenzymatic oxidation have been much less studied.

Cilliers, et al. (1989, 1992) investigated nonenzymic autoxidation in a caffeic acid model system. They found that the oxidation products have larger retention times than caffeic acid on reversed-phase columns. This indicates that the oxidation products are less polar than caffeic acid and are probably not ring-opened products which would increase polarity. The identification results from NMR and mass spectroscopy indicated that the major oxidation products are different dimers as given in Figure 7 and trimers. They proposed the following mechanism given in Figure 8.

They also found that the proportion of different oxidation products formed from caffeic acid was affected by pH, concentration and temperature. But the major products are formed in each case and give a constant chromatographic profile under a given set

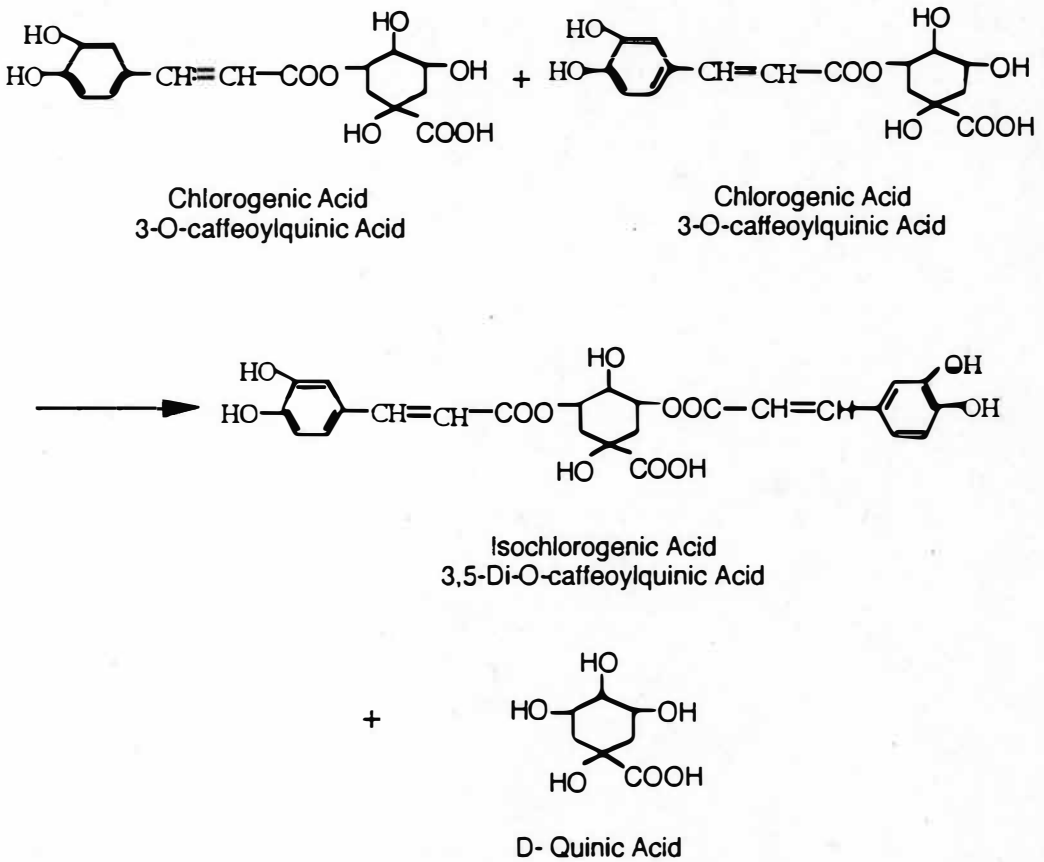


Figure 6. Enzymatic Reaction Catalyzed by Chlorogenate Caffeoyl Transferase.

of conditions. The rate of reaction is increased by increasing pH and temperature. Some of the oxidation products were formed at equal final concentrations independent of the pH but at rates that were highest at high pH. Others were dependent on pH, and the highest concentrations and rates were found at high pH. The controlling factor in the rate of autoxidation was indicated to be the phenolate anion concentrations.

Oxidation of phenols under alkaline conditions readily leads to the formation of the radical anions of the corresponding quinones. Atherton and Willder (1993) reported the characterization using EPR and ENDOR of free radicals formed during the aerobic

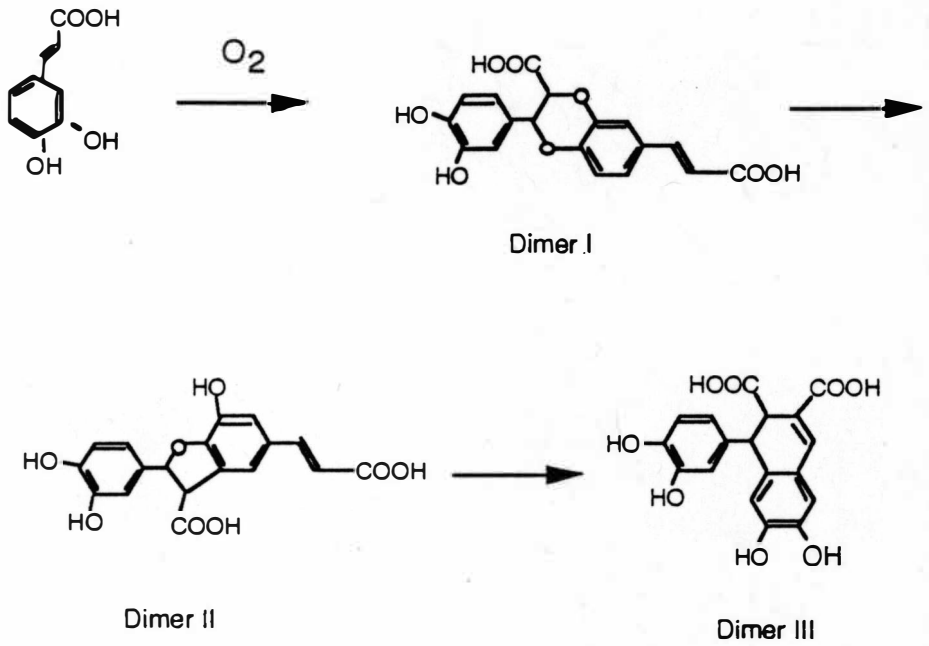


Figure 7. Structure of Dimer Isomers From the Oxidation of Caffeic Acid.

oxidation of strongly alkaline aqueous solutions of chlorogenic acid and caffeic acid. It was shown that radical I, II and III were formed in the oxidation of both caffeic acid and chlorogenic acid in strong alkali. This indicated that a major early step in the chemistry of chlorogenic acid under strong alkaline conditions was the loss of the quinic acid by hydrolysis. The formation of radical I, II or III was dependent on the degree of oxidation. Radical I is formed first and has five coupled protons which might result from hydroxy substitution in the side chain, presumably at the β-position. Radical II was formed from the decay of radical I. Radical III was formed from the decay of radical II. Since both radical II and III have four couplings protons, they might be the trianions derived from trihydroxy cinnamic acids, formed by hydroxy substitution into the ring in caffeic acid, 3,4,5-trihydroxy and 3,4,6-trihydroxy compounds.

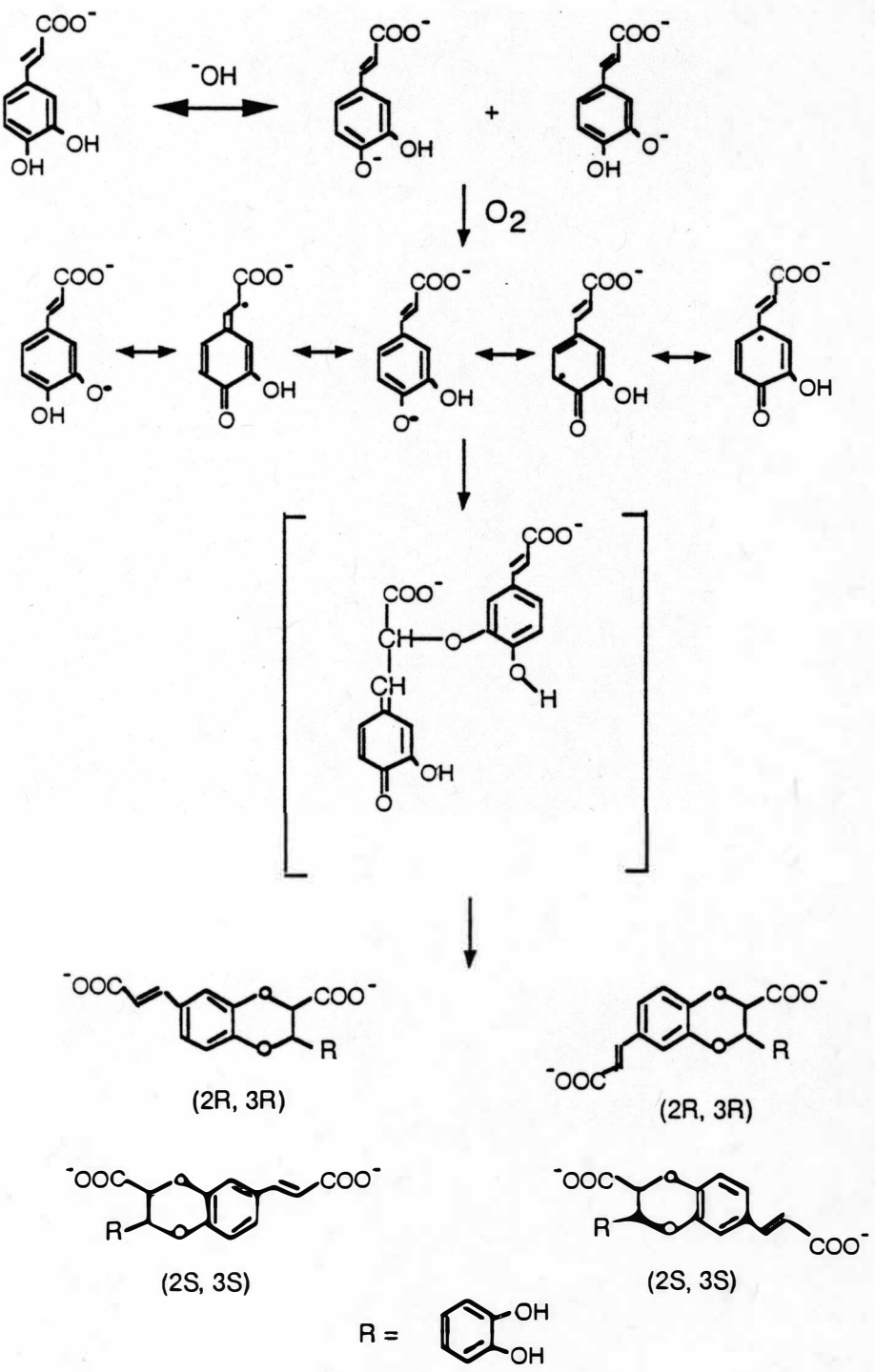


Figure 8. Proposed Mechanism of Dimer Formation.

Under acidic conditions the nonenzymatic reaction was very slow, taking weeks to months (Oszmianski et al., 1985). The reaction takes place at an appreciable rate under acid conditions if some metal catalysts are present (Speier, 1986; Pandell, 1983). Xu (1994) reported that copper(II) had a catalytic effect on the oxidation of the caffeic acid to form a caffeic acid dimer as a product.

CHAPTER III

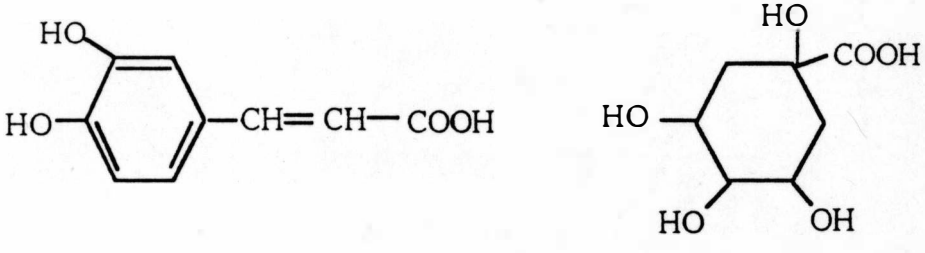
EXPERIMENTAL METHODS

Materials

Chlorogenic acid, caffeic acid and quinic acid were purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. Copper(II) chloride was purchased from Fisher Scientific, Fair Lawn, NJ. Milli-Q water was used for all the aqueous samples.

Properties of Materials

The structure of chlorogenic acid has been shown in Figure 5. Figure 9 shows structures of caffeic acid and quinic acid. Comparing with Figure 5, it is easy to see that chlorogenic acid is the ester of caffeic acid and quinic acid. Chlorogenic acid, caffeic acid and quinic acid are polar compounds and have relative high melting points.



Caffeic Acid Quinic Acid

Figure 9. Structures of Caffeic Acid and Quinic Acid.

Chlorogenic acid is partially soluble in cold water and soluble in warm water. It is a gray-white powder with a molecule weight of 354.31 g/mol. The solubility of

Chlorogenic Acid was tested in different solvents as shown in Table 1. Caffeic Acid is a pale gray powder with a molecule weight of 180 g/mol. It is partially soluble in cold water. Quinic acid is a white powder and has a molecule weight of 192.17 g/mol. It is soluble in water.

Table 1

Solubility of Chlorogenic Acid in Different Solvents

Solvent Name	Solubility	Appearance
DMSO	soluble	light pale yellow solution
Acetone	insoluble	
Dioxane	insoluble	
Methyl-Alcohol	soluble	colorless solution
Pyridine	soluble	pale yellow solution
Acetonitrile	insoluble	
Benzene	insoluble	
Water	mostly soluble in cold H_2O	colorless solution
Chloroform	insoluble	

Purity Checking

Since most natural compounds are not pure, the purity of materials were tested by Differential Scanning Calorimetry (DSC), Thin-layer Chromatography (TLC) and High-performance Liquid Chromatography (HPLC). Figure 10 shows the DSC thermogram of chlorogenic acid. The single sharp peak at the temperature of 210 ^{o}C indicates the sample was pure. In addition, chlorogenic acid produced only a single

spot in TLC and a single peak in HPLC; no further tests were performed.

Both caffeic acid and quinic acid also produced only a single spot in TLC and a single peak in HPLC indicating that they were pure. Therefore the above materials were used without further purification.

Sample Preparation

Aqueous Sample

In natural water, the typical concentration range of organic species and transition metals is from 0.1 ppm to 10 ppm. Low concentration samples were used to match the natural system in this study. Considering the instrument sensitivity, 0.1 mM and 0.05 mM chlorogenic acid solution, 0.05 mM caffeic acid and 0.05 mM 1:1 ratio of caffeic acid & quinic acid were prepared. A low concentration solution of chlorogenic acid (0.005 mM) was also used for some studies.

The sample pH values were fixed by addition of 50% (by weight) NaOH to obtain the desired pH. The pHs were determined using a Corning Model 240 pH meter calibrated with standard pH buffers (pH = 4, 7 and 10.1). The pH range of samples was from 3 to 12.

Aqueous solutions of chlorogenic acid and caffeic acid were colorless at low pH. As pH was increased, the solution color changed to yellow and after several days to dark brown. The higher the pH, the darker the solution. After several months, a very small amount of brown or black precipitate was formed in the solution. This precipitate was thought to be the polymerization product of chlorogenic acid or caffeic acid.

Copper(II) chloride was added to the chlorogenic acid solution in order to check the effect of transition metals on oxidation. Three different molar ratios of chlorogenic acid and copper ion were prepared. They were 1:0.5, 1:1 and 1:2. Because copper ion

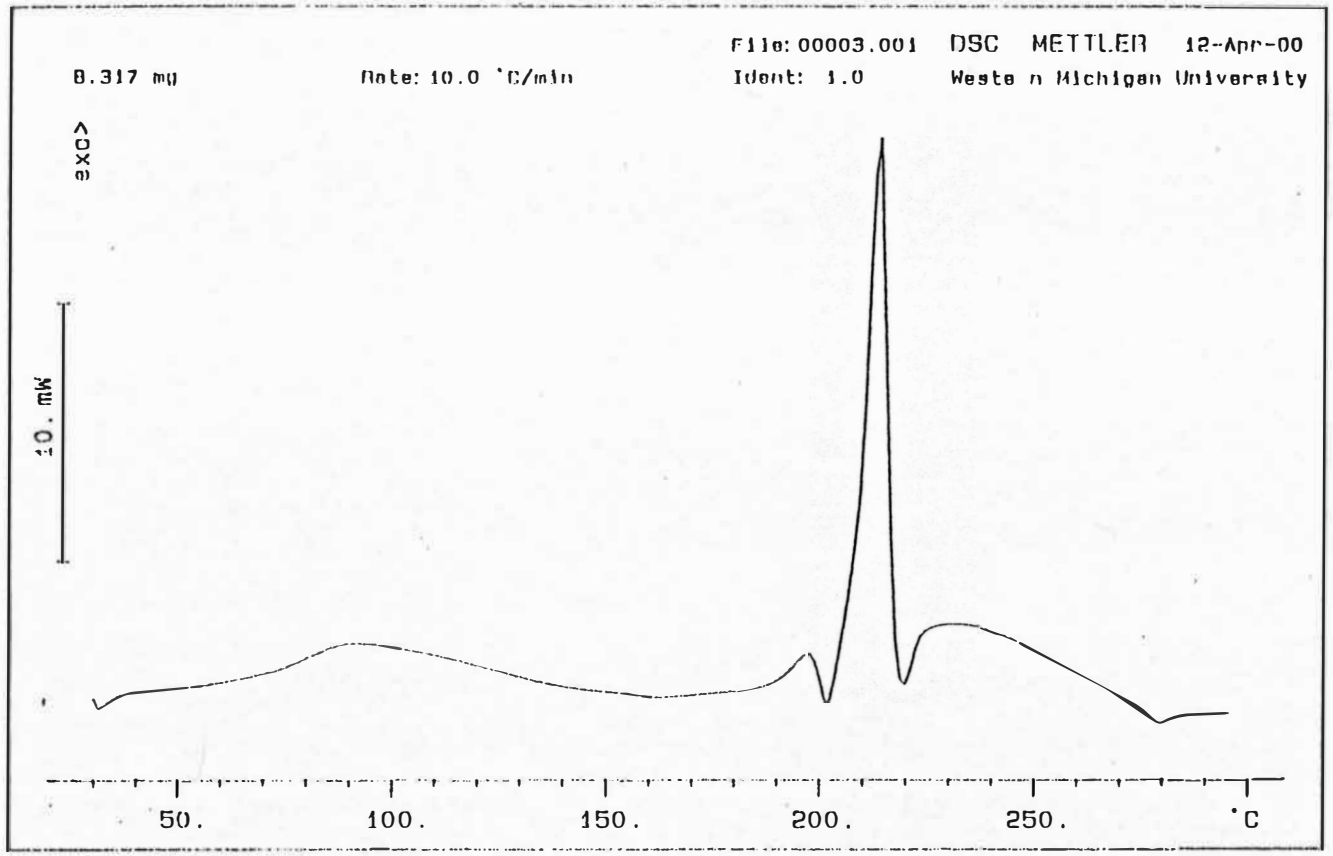


Figure 10. Differential Scanning Calorimetry Thermogram of Chlorogenic Acid.

reacts with hydroxyl groups and affects the sample pH, a buffer was added to the chlorogenic acid solutions to control pH.

Recovery of Oxidation Products

In order to obtain a large amount of oxidation products, an aqueous solution of chlorogenic acid with a higher concentration was prepared. This solution was prepared by dissolving 0.5 g chlorogenic acid in 100 mL Milli-Q water. The pH of the solution was adjusted to 10.5 to obtain products quickly. The solution was then stored at room temperature for more than one week for complete reaction. Before recovering the sample from solution, the pH was adjusted to 7. The recovery of the sample was done by freeze drying techniques to avoid the heat sensitivity of natural compounds.

Instrumental Technique

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry has found widespread use in the pharmaceutical industry for testing the purity of drug samples(Skoog, 1992). A METTLER TA4000 system Differential Scanning Calorimetry(DSC) was used for purity checking. The biggest advantage of this method is that no standard (100% pure product) is needed for the DSC purity test. The DSC determination of purity is principally in the range of 98 to 99.99 mol% purity (METTLER Instrument Corp., 1987). There is less than 2% error in this method.

Beside the purity checking, the DSC graph can also give the information about the melting point, decomposition and dehydration of compounds. If the isomer percentages are higher than 2%, the curve will show the different peaks of the isomers.

Chlorogenic acid (8.317 mg) was weighed and sealed in an aluminum pan. The

pan was heated in the DSC furnace from 30 °C to 300 °C at a rate of 10.0 °C/min. To avoid oxidation, nitrogen was used as a purge gas at 50 ml/min.

Freeze Dry

Freeze drying is a process whereby water is removed from frozen materials by subliming water directly into its vapor without the intermediate formation of liquid water. Of the various methods of dehydration, freeze drying is especially suited for substances that are heat sensitive. Freeze drying has been used extensively in the area of pharmaceuticals and biology and food processing.

A VirTis Bench Top Freeze Dry system was used in this study. The system vacuum pressure was controlled at 30 to 60 millitorr. The system temperature was maintained at -30 °C. The aqueous solutions were first frozen using dry ice and then connected to a valve on the drying chamber until the sample was completely dry.

Ultraviolet-Visible Spectrophotometry (UV/VIS)

Dao and Friedman (1992) found that the use of ultraviolet spectrophotometry to estimate chlorogenic acid concentration appears more reproducible than HPLC since chlorogenic acid undergoes a time and light dependent change in concentration during sample preparation. The instrument used was a Cary-14 UV-Visible Spectrophotometer equipped with OLIS on-line software in this study. The UV spectrum, from 200 nm to 500 nm, was used to monitor the aqueous samples. Milli-Q water was used as the blank. The samples were measured every week to monitor for the degree of reaction.

Thin-layer Chromatography (TLC)

Thin layer chromatography is one of the most popular and widely used separation techniques. It is easy to use and has found wide application to a great

number of different samples at relatively low cost. Compared to GC, TLC and HPLC are not limited by the lack of volatility or thermal stability of the sample. Compared to HPLC, TLC uses less solvent, has a shorter development time and is simpler to change the mobile phase. Therefore, TLC is often used to develop a solvent system for HPLC (Touchstone, 1992). In this study, TLC was used to check the purity of the compounds under study and to separate and identify the components of oxidation products.

Eastman chromatogram 13181 silica gel with fluorescent indicator sheet (size 20 cm x 20 cm) was used as the thin-layer chromatography plate in the study. Silica gel is the most widely used TLC sorbent. It is a polar sorbent and slightly acidic in nature.

About 1 mg of the dry sample from freeze drying was dissolved in 10 mL methanol. 15 μL of solution was then applied as a single spot to the silica gel plate along with chlorogenic acid, caffeic acid and quinic acid standards. The standard solutions were prepared in the same way as the sample.

The plate was developed with ethyl acetate, formic acid, glacial acetic acid and water in volume ratio 100:11:11:27 (Wagner et al., 1984) until the solvent front was 13 cm from the starting point. It took about two hours for elution. The plate was then dried. The visualization was carried out under UV light at 366 nm. The volume ratio of ethyl acetate and water was varied to get an optimum condition for separation.

High-performance Liquid Chromatography (HPLC)

A Varian Model 5020 High Performance Liquid Chromatography with a Hewlett Packard integrator and a UV- Visible variable -wavelength detector was used in this research. Two columns were used in this study. One was a 30 cm x 4 mm Micropak MCH-10, a reversed-phase column utilizing a C-18 hydrocarbon monolayer covalently bonded to 10 μm microparticulate silica as the stationary phase. Another was a 25 cm x 4 mm NH-10, a normal phase column.

About 10 mg of the dry sample from freeze drying was dissolved in 25 mL methanol and then diluted to 100 mL with acetonitrile. The standard solutions of chlorogenic acid and caffeic acid were prepared in the same way. The samples were filtered with a 0.5 μm MF-MILLIPORE filter before it was injected into column. The sample was injected via a 10 μL loop.

Two solvent systems were used as the mobile phase which consisted of Milli-Q water/acetonitrile, 1% Acetic acid buffer (pH=2.6)/acetonitrile and 1% Phosphoric Acid buffer (pH=0.7)/acetonitrile. The acetonitrile was HPLC grade. The buffers were filtered through a 0.6 μm MF-MILLIPORE filter under vacuum before introducing them into the reservoir. The purpose of this treatment was to remove gases as well as suspended matter. The flow rate was 1 mL/min. The UV/VIS detector absorbance scale was set at 0.2 AUFS at a wavelength of 320 nm.

Nuclear Magnetic Resonance

A Bruker AC 200 nuclear magnetic resonance spectrometer (NMR) was used with ^{1}H frequency of 200.132. The spectrum of pure chlorogenic acid, caffeic acid and quinic acid were obtained as the standards. DMSO-d_6 was used as solvent and TMS was used as an internal reference.

CHAPTER IV

RESULTS AND DISCUSSIONS

Factors Related to Nonenzymatic Oxidation

Effect of pH

In order to study the effect of pH on the nonenzymatic oxidation of phenolic compounds, a series of chlorogenic acid and caffeic acid solutions were prepared. The pH of the solution was adjusted to a desired value. Experimental results are discussed in the following paragraphs.

Oxidation of Chlorogenic Acid

Figure 11 shows an UV/VIS spectrum of a freshly prepared chlorogenic acid solution (0.1 millimolar, pH 3). Several absorption peaks can be seen in the spectrum. The absorption maximum, which is due to π to π^* transition of electron of conjugated double bond in chlorogenic acid, is at 320-325 nm. This peak, which is also observed in the caffeic acid spectrum, is a fingerprint of cinnamic compounds. The change of absorbance around this wavelength range usually indicates reaction of the conjugated double bonds of the side-chain in cinnamic compounds.

Figure 12 shows UV/VIS spectra of chlorogenic acid solution (0.1 millimolar, pH 10.5). An important feature of this figure is that the spectra are changing with time. The graph shows that the absorption of chlorogenic acid at the 325 nm peak rapidly decreases as time increases. This decrease in absorption indicates the involvement of the chlorogenic acid side chain in the reaction. In Figure 12, it can be

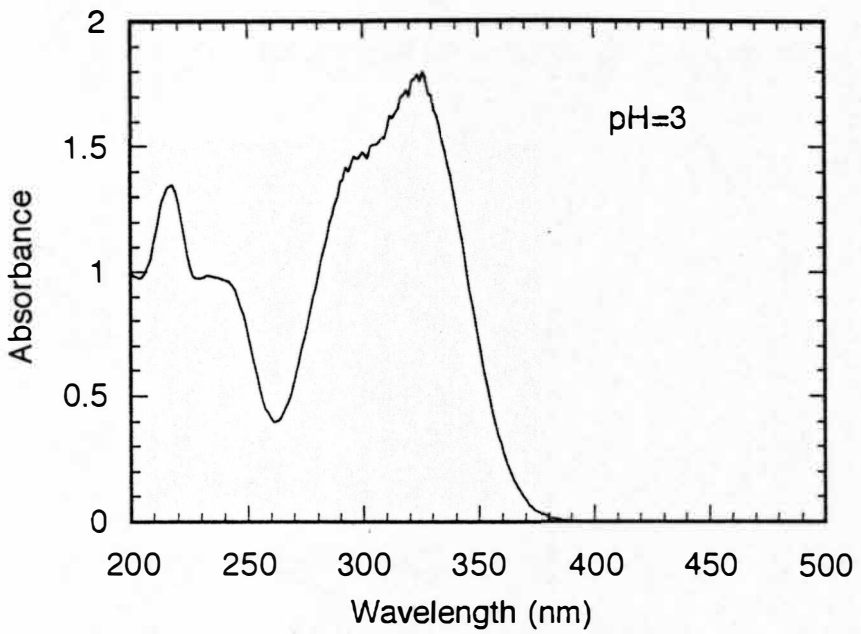


Figure 11. UV/VIS Spectrum of Chlorogenic Acid (0.1 mM) at pH = 3.

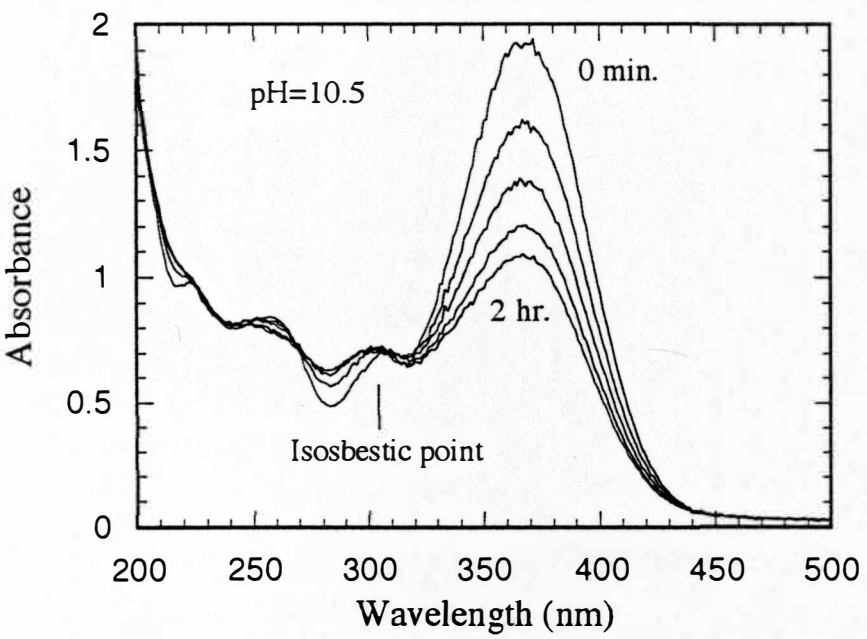


Figure 12. UV/VIS Spectra of Chlorogenic Acid (0.1 mM) at pH = 10.5.

seen that the absorbance of chlorogenic acid dropped to 50% of the original value after 2 hours. Because the absorbance is proportional to the concentration (Beer-Lambert Law), the concentration of chlorogenic acid has also decreased by half within 2 hours. This means that the reaction was very fast at pH 10.5.

The absorption maximum has been shifted in Figure 12 to a higher wavelength. This is a bathochromic shift due to high pH. An absorption tail at 450 nm can also be seen in Figure 12. This tail originated from the absorption of reaction products with visible brown colors. It was observed during this study that the color of the solution was changing from yellow to dark brown as time increased. After an extended period of time (2 months), a small amount of brown precipitate was observed in the solution.

Pierpoint (1966) reported that polymerization of the hydroxyquinones, which formed from enzymatic oxidation of chlorogenic acid, produced brown compounds. In this study, the brown precipitates was thought to be the polymeric compounds which were formed from nonenzymatic oxidation of chlorogenic acid or its fragments.

One interesting feature of Figure 12 are the isosbestic points. An isosbestic point is located on the spectra where the absorbance at the corresponding wavelength is not changed with time. The existence of isosbestic points indicates that there is an equilibrium reaction taking place. Figure 12 shows that absorption peaks between two isosbestic points either decreases or increases as time increases. For example, the absorbance at 280 nm increases while the absorbance at 370 nm and 260 nm decrease with time. This illustrates that concentrations of intermediate species are changed during the reaction.

In Figure 12, spectrum curves are not smooth at the absorption maximum. This suggests that radicals be formed during the oxidation reaction at alkaline conditions. The formation of radicals can increase the rate of polymerization of hydroxyquinones which are formed from oxidation of chlorogenic acid. The polymerization of

hydroxyquinones produced brown compounds which first turn the color of solution to yellow then formed precipitates.

The UV/VIS spectra of chlorogenic acid, 0.1 millimolar, at pH 12 are shown in Figure 13. Comparing Figure 13 with Figure 12, it can be seen that the absorption maximum of chlorogenic acid solution at pH 12 decreases at a greater rate than that at pH 10.5. At pH 12, it takes only 30 minutes to drop the absorbance to 50% of the original absorbance while it takes 2 hours at pH 10.5. It was also observed during this study that the color of the solution at pH 12 was much darker than that at pH 10.5. These results indicate that the rate of oxidation of chlorogenic acid was pH dependent. The higher the pH, the faster the oxidation rate.

In addition, the bathochromic shift of the maximum peak was larger at pH 12 than that at pH 10.5. The higher bathochromic shift at pH 12 was due to the higher concentration of phenolate ions in the solution.

The UV/VIS spectra of chlorogenic acid, 0.1 millimolar, at pH 7 are shown in Figure 14. The absorption maximum decreases slowly as time increases. It took almost six weeks for the absorbance of chlorogenic acid to drop to 50% of the original value. In addition, the bathochromic shift of maximum peak was almost unobservable in Figure 14. The brown precipitates were observed in this solution after several months. But the amount of precipitate in this solution was less than those in the solutions with higher pH.

For a more extensive study of the pH effect, a series of 0.05 millimolar chlorogenic acid solutions at different pH were prepared and monitored with UV/VIS spectrophotometry for several months. The absorbance of these solutions at 320 nm, as a function of time, are shown in Figure 15. The plot clearly illustrates the effect of pH on the rate of oxidation.

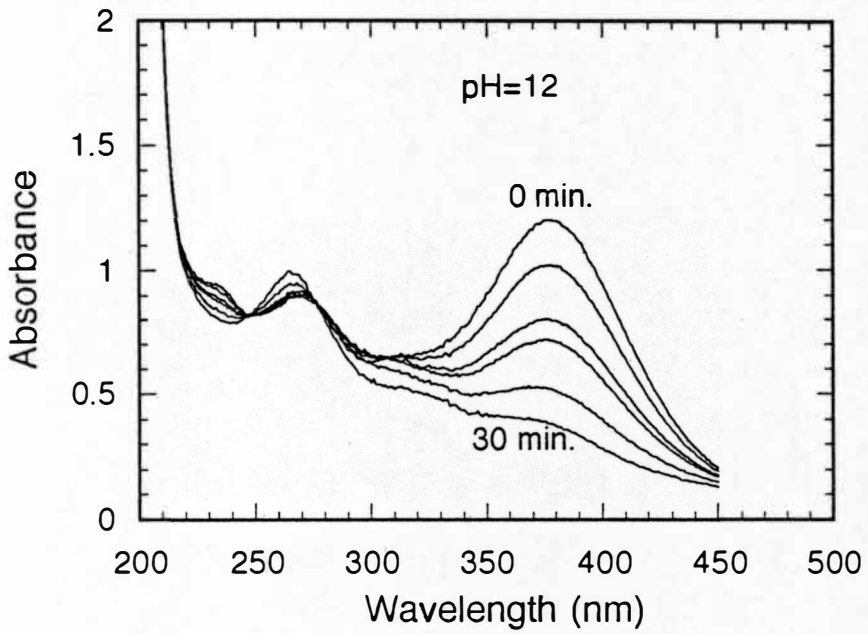


Figure 13. UV/VIS Spectra of Chlorogenic Acid (0.1 mM) at pH = 12.

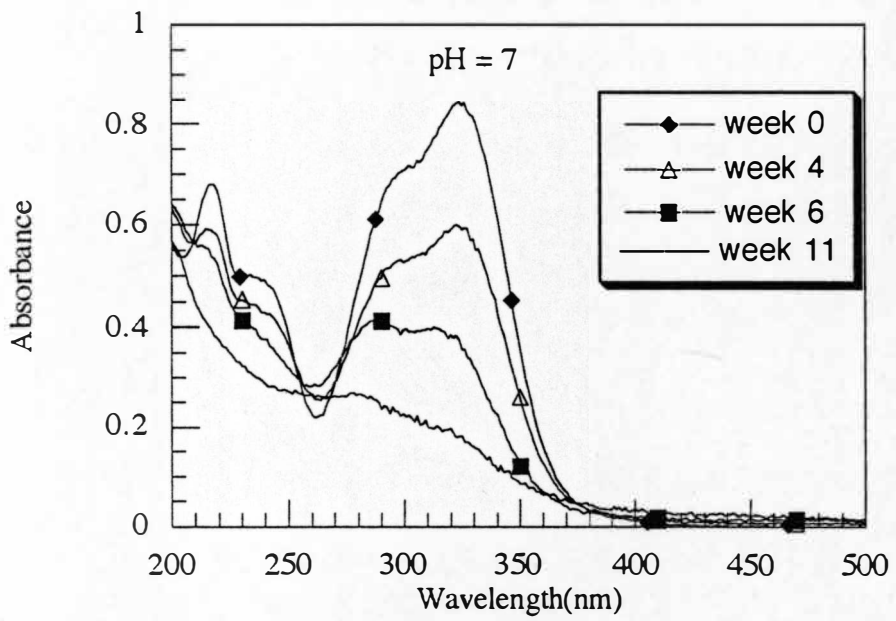


Figure 14. UV/VIS Spectra of Chlorogenic Acid (0.05 mM) at pH = 7.

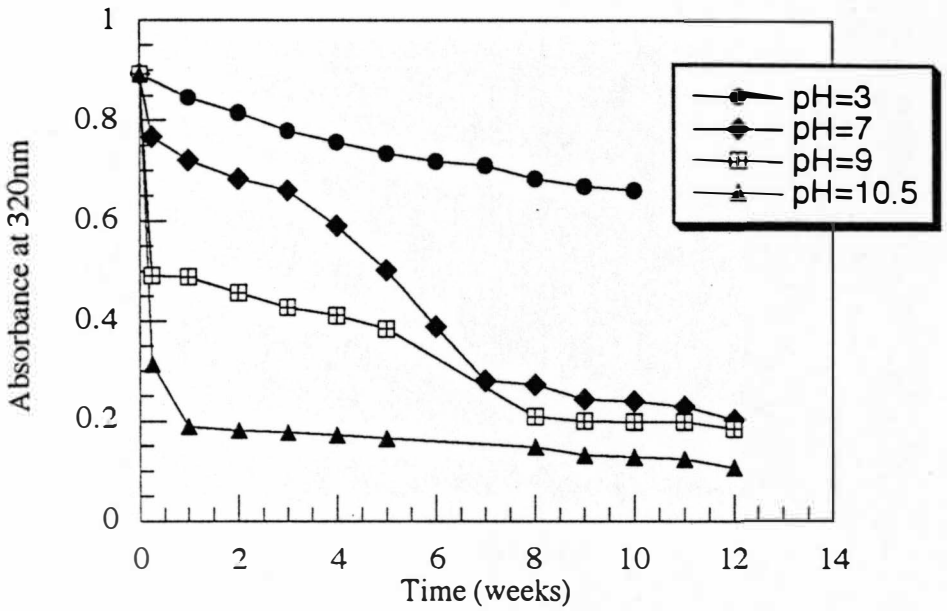


Figure 15. Effect of pH on Oxidation of Chlorogenic Acid (0.05 mM).

As shown in Figure 15, the reaction was very fast at high pH (pH = 10.5 curve). After a week, the absorbance was almost stable and the reaction was completed. Since not all absorption at 320 nm is lost during the oxidation, this indicates that some oxidation products of chlorogenic acid might contain some side chain conjugated ethylenic groups or not all chlorogenic acid is converted.

This result was also observed by Cilliers et al. (1991) on the oxidation of another phenolic compound: caffeic acid. They reported that roughly 30% side chain ethylenic conjugation existed in the oxidized compound of caffeic acid.

At lower pH (pH = 3 curve), the reaction was very slow. After four months, the absorbance was still high which means most of the compound was not altered. There was no significant amount of precipitate observed. However, there is a clear trend of oxidation. The color of the solution at pH 3 changed from colorless to yellow

after four months. It can be seen that time was an important factor for oxidation in acidic conditions. This result shows chlorogenic acid can be nonenzymatically oxidized after a long time period even at low pH.

At neutral condition (pH = 7 curve), oxidation rate was much quicker than that at acidic condition. After 7 weeks, the absorbance became stable and the reaction was completed. Although the time was long compared to that of an alkaline solution, seven-weeks is actually a short time in natural systems. The above results point out the significance of nonenzymatic oxidation in natural water systems.

Our results demonstrated that the rate of oxidation of chlorogenic acid was pH dependent. The involvement of phenolate ion during the oxidation reaction can theoretically explain why the oxidation reaction was pH dependent (Cilliers, 1991). It was found that the phenolate ion can react directly by charge transfer with oxygen to form a semiquinone and dramatically cut down the activation energy of reaction (Cilliers,1991). In contrast, the phenolic compound has a higher activation energy for reaction with oxygen to form a semiquinone (Cilliers, 1991). As the solution pH increases, more chlorogenic acid was converted to its phenolate form and the oxidation rate increased.

Oxidation of Caffeic Acid

Plots of the results of the reaction of caffeic acid (0.05 mM) experiments at pH values of 3, 7 and 10.5 is given in Figure 16. At higher pH, the reaction rate was fast. At lower pH, the reaction rate was slow.

Cilliers et al. (1989) have also reported the oxidation of caffeic acid. The result of our study was in reasonable agreement with their report. Thus, the reliability of our results are confirmed. Some minor differences can be attributed to variations in the conditions of the two studies. First, the concentration of their study is 120 times higher

than this study. Second, in their study, the solution was oxidized under a 100% oxygen atmosphere while this study was performed under an air atmosphere. Both differences will have an effect on the reaction rate. Therefore, the oxidation rate they reported around neutral conditions are higher than in this study.

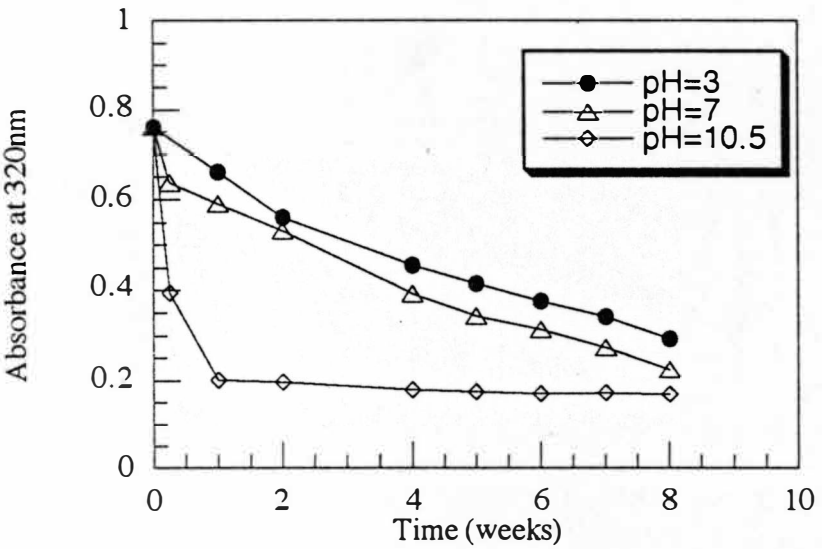


Figure 16. Effect of pH on Oxidation of Caffeic Acid (0.05 mM).

Oxidation of Caffeic Acid in the Presence of Quinic Acid

Chlorogenic acid is the ester of caffeic acid and quinic acid. This structural connection triggered the investigation of the role of quinic acid in the oxidation of chlorogenic acid. Quinic acid was added to a caffeic acid solution (0.05 mM) to obtain a 1:1 molar ratio of caffeic acid to quinic acid. The solutions' pH was then adjusted to 3, 7 and 10.5 respectively. Plots of the absorbance versus time for these solutions are given in Figure 17. The graph shows that the higher the pH, the faster the rate of the oxidation.

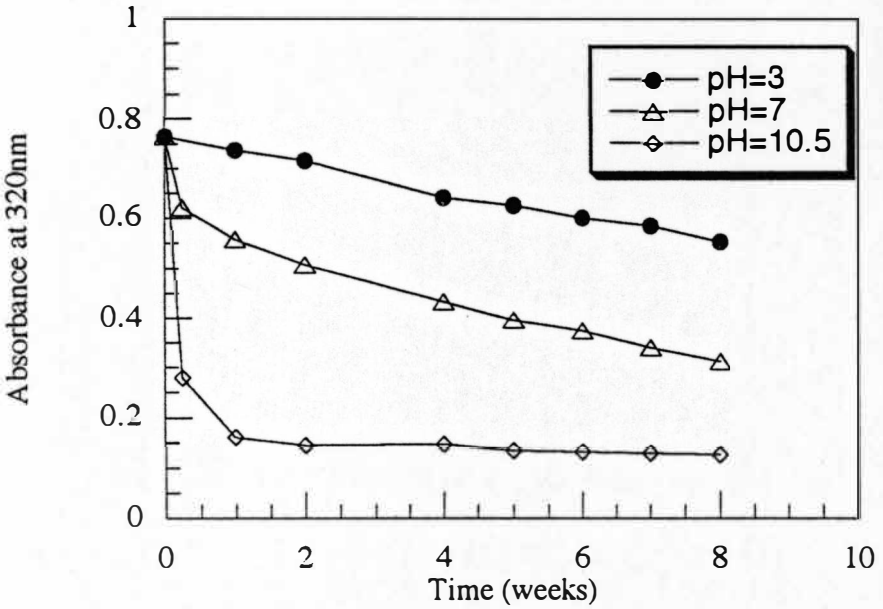


Figure 17. Effect of pH on Oxidation of Caffeic Acid (0.05 mM) in the Presence of Quinic Acid.

Comparison

The general features of Figure 15, 16 and 17 are very similar although the figures represent three different systems, chlorogenic acid, caffeic acid and caffeic acid with quinic acid respectively. In order to compare the pH effect on different systems, Figure 15, 16 and 17 are reorganized as shown in Figure 18.

Figure 18 illustrates several important features. First, at acidic conditions (pH 3), caffeic acid is oxidized more quickly than the compounds in the other two systems. The presence of quinic acid has a strong inhibition effect on caffeic acid oxidation. Second, at neutral conditions (pH 7), the oxidation rates of the two systems: caffeic acid and caffeic acid with quinic acid, are almost the same. The presence of quinic acid

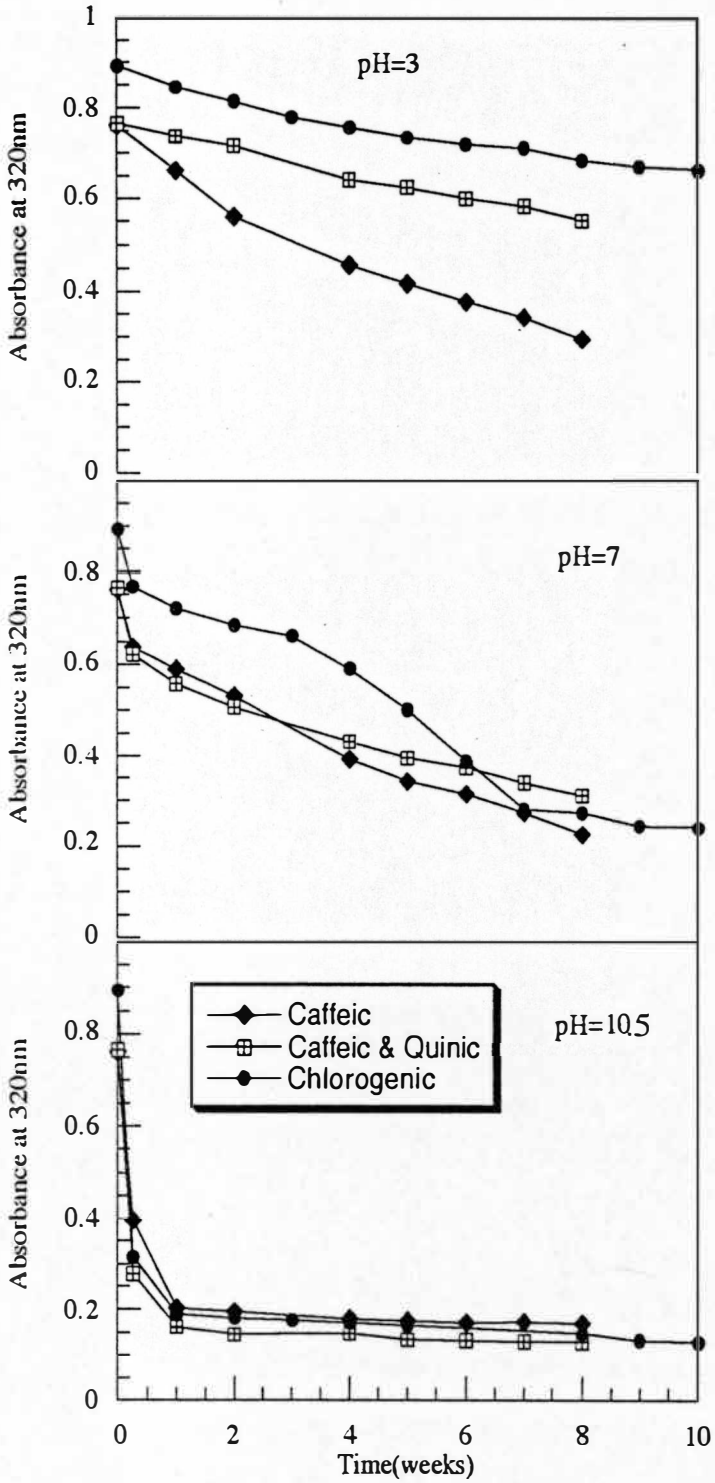


Figure 18. Comparison of the Effect of pH on Different Systems.

does not affect the oxidation of caffeic acid. However, the oxidation rate of caffeic acid in the presence of quinic acid is still faster than chlorogenic acid. Third, at alkaline conditions (pH 10.5), the oxidation rates are the same for all three systems. This behavior can be explained as follows:

In the basic conditions, hydrolysis is the first step of chlorogenic acid oxidation. The hydrolysis is very quick at highly alkaline conditions. After hydrolysis, the oxidation rate of chlorogenic acid depends on the caffeic acid oxidation rate. Therefore all the curves are overlapped to each other.

In the neutral condition, a slow hydrolysis of chlorogenic acid took place and deferred chlorogenic acid oxidation. Therefore the rate oxidation of chlorogenic was slower than the other two system at the beginning. Cilliers (1990) has reported that a slow hydrolysis of chlorogenic acid in apple juice would occur with time. Since there is no hydrolysis for the system - caffeic acid with quinic acid, the rate of this system is the same as the rate of caffeic acid system.

In the acidic conditions, hydrolysis is less possible for chlorogenic acid and its oxidation would be largely affected. Also the mechanisms of chlorogenic acid and caffeic acid might be not the same. Thus, the system of chlorogenic acid and caffeic acid have very different oxidation rates. When quinic acid is added into caffeic acid, the estification of caffeic acid with quinic acid lowers the activity of caffeic acid (Maruta, 1995). Because quinic acid inhibited caffeic acid forming quinone, the oxidation rate of caffeic acid system is larger than the system-caffeic acid and quinic acid.

Effect of Transition Metal Copper(II)

In order to study the effect of copper(II) on the reaction of chlorogenic acid, two groups of samples were prepared. Both groups had the same concentration and pH. The difference was that in one group copper(II) chloride was added while in the

other one it was not. The molar ratio of chlorogenic acid to copper (II) was 1:1. The results of reaction are given in Figure 19. When copper ion was present the reaction rate is increased dramatically. It was observed that the color of the solutions were much darker when the copper(II) was present. Also, it took less time to see precipitates formed in the solutions when copper(II) was present. These results clearly illustrate the strong catalytic effect of copper (II) on the oxidation reaction of chlorogenic acid.

The UV/VIS spectra of 0.1 millimolar chlorogenic acid solution with a 1:1 molar ratio of copper(II) as a function of time are shown in Figure 20. When Figure 20 is compared with Figure 12, a lot of similarities are found between them, such as the peak absorbance decreases as time increases etc. However, the absorbance drops to about 50% of the original value after 2 days in Figure 20 while it takes only two hours in Figure 12. This indicates that the reaction rate was slower in the presence of copper than that in a highly alkaline condition. This indicates that the effect of the hydroxyl ion on the reaction was much stronger than that of the copper ion.

In Figure 20, it is clearly seen that the absorbance at 450 nm increases with time. This absorbance could originate from the absorption of oxidation products. As discussed earlier in Figure 13, the absorption of oxidation products was also at wavelength 450 nm. This suggested that the structures of the oxidation products might be similar in spite of the two different conditions. In the previous studies in our group, it was found that the presence of copper(II) in oxidation products seriously interfered with the NMR spectra and attempts to identify the structure of the oxidation products of caffeic acid using NMR was unsuccessful (Xu, 1994). The above result provides an alternate way to identify the oxidation products using NMR.

Also, in Figure 20, there are more isosbestic points which indicates that the number of the intermediate species was increasing. One explanation of this might be the complexation between copper ion and chlorogenic acid since chlorogenic acid has two

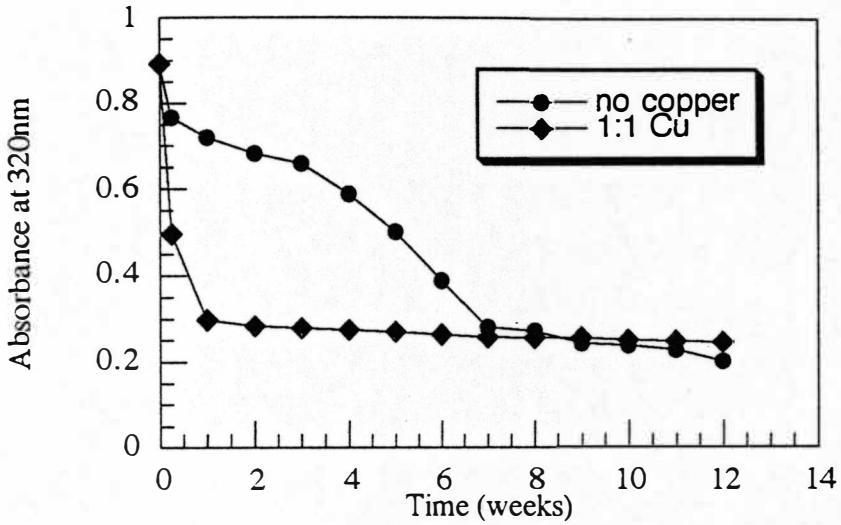


Figure 19. Catalytic Effect of Copper(II) on Oxidation of Chlorogenic Acid (0.05mM).

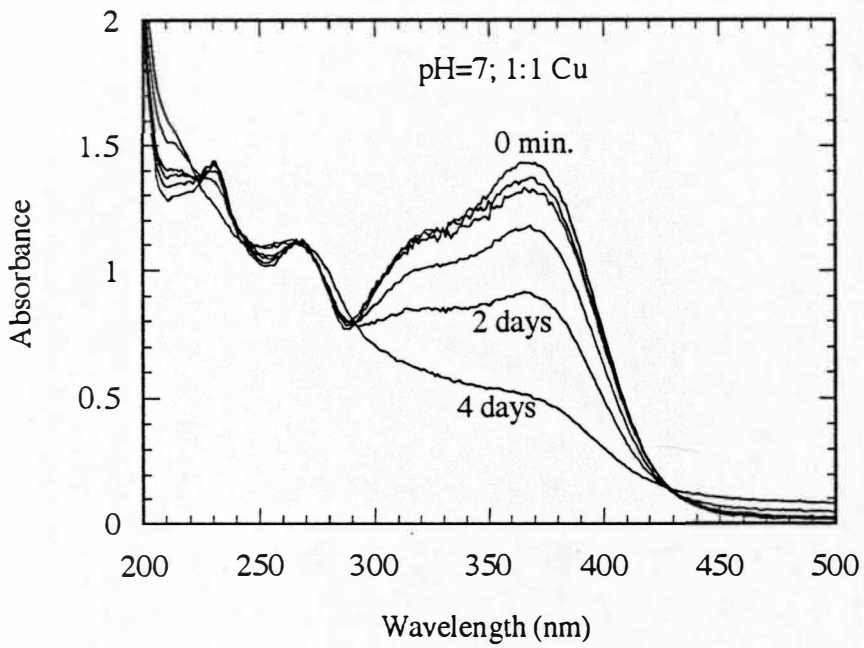


Figure 20. UV/VIS Spectra of Chlorogenic Acid (0.1 mM) at pH = 7 in the Presence of Copper(II).

adjacent hydroxyl groups, which are potential coordinating sites for the copper(II). The formation of complexation products during the oxidation would increase the number of the intermediate species.

The concentration of copper ion also has some effect on the beginning rate of oxidation. The effect of copper concentration on the oxidation results of chlorogenic acid is shown in Figure 21.

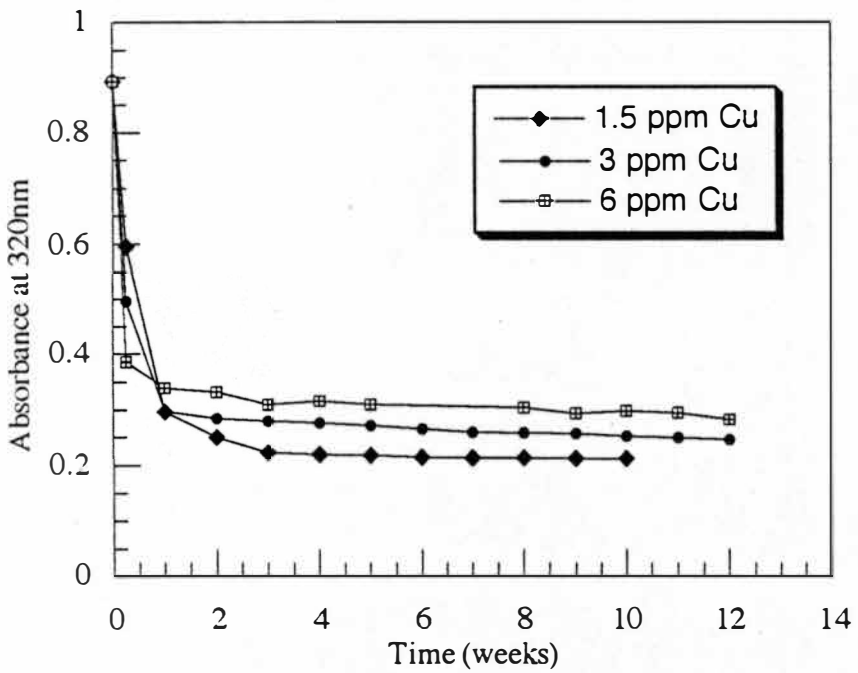
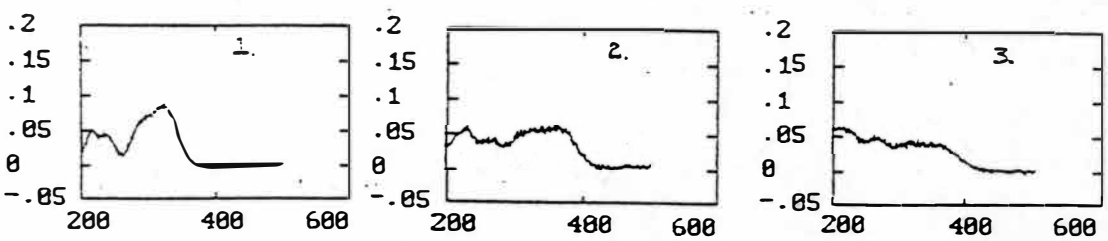


Figure 21. Oxidation of Chlorogenic Acid (0.05 mM) at Different Copper(II) Concentrations.

Initially, the higher the concentration of copper(II), the faster the reaction. Also, the higher the copper(II) concentration, the higher the final absorbance which means the oxidation products contain more ethylenic conjugated side chain units.

Even though the concentration of copper (II) in chlorogenic acid solution is very low, the catalytic effect of copper ion on the oxidation can still be seen. Figure 22

demonstrates this. In this plot, the UV/VIS spectrum shows that with the presence of 0.3 ppm copper(II) in solution, the chlorogenic acid was quickly oxidized in several days.



No.1: Initial spectrum; No.2: One day later spectrum No.3: Four days later spectrum

Figure 22. Chlorogenic Acid Oxidation With a Trace of Cu(II) (0.3 ppm).

Effect of Light

In order to study the effect of light, two groups of samples were prepared at the same time. One group was kept in the dark in amber bottles and another group was kept under light in clear bottles. The two samples were measured at the same time by UV/VIS spectrophotometry for several weeks.

The effect of light on the reaction of chlorogenic acid in the absence of copper(II) is shown in Figure 23. In this graph, open symbols represent the samples stored under light and solid symbols represent the sample stored in the dark.

In Figure 23, all the curves show the absorbance decreases with time. At low pH, the change of absorbance are not affected by light. Similarly at high pH, there is no light effect. Under neutral conditions (pH 7), light does have an effect on the change of absorbance. As time increases, the effect of light was significant on oxidation. This result illustrates the importance of the photochemical effect on the nonenzymatic oxidation.

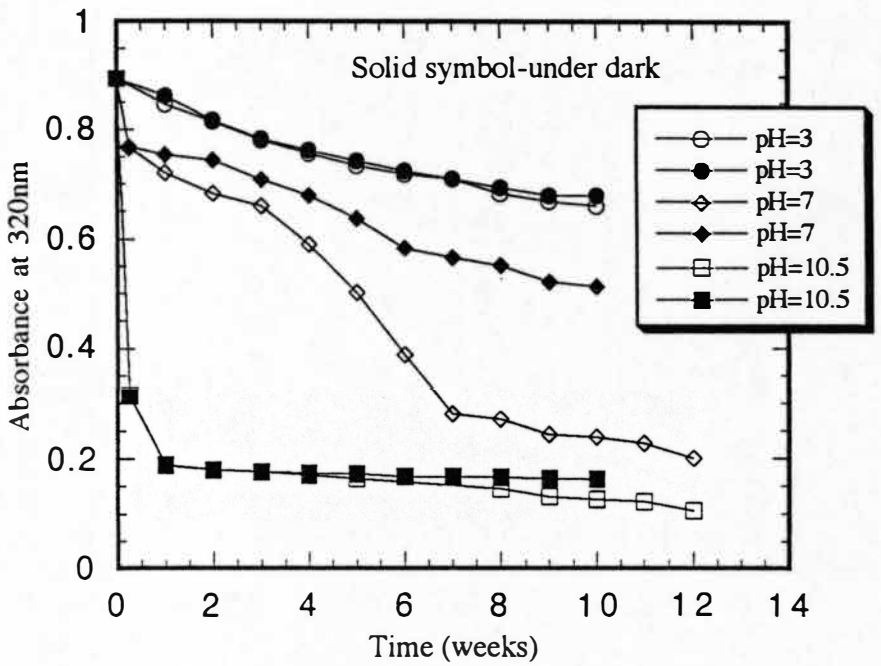


Figure 23. Effect of Light on Oxidation of Chlorogenic Acid (0.05 mM) Without Copper (II).

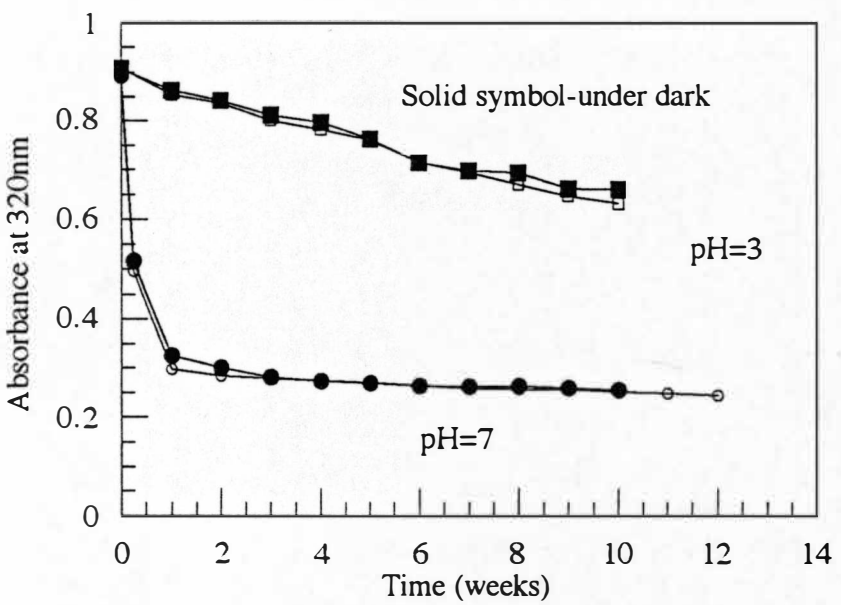


Figure 24. Effect of Light on Oxidation of Chlorogenic Acid (0.05 mM) With 3 ppm Copper(II).

The effect of light on the oxidation of chlorogenic acid in the presence of copper(II) (3 ppm) is shown in Figure 24. The light does not appear to have any effect on the reaction either in neutral or acidic conditions.

Separation of Reaction Mixtures

TLC

The results of the TLC separations of a reaction mixture and standards are listed in Table 2. The reaction mixture was recovered from chlorogenic acid solution after one month reaction under strong alkaline condition.

Table 2

TLC Results* for a Reaction Mixture of Chlorogenic Acid

	R_f Value			
	Oxidation product	Caffeic acid	Quinic acid	Chlorogenic acid
Spot 1	0.958	0.957		
Spot 2	0.890			
Spot 3	0.791			
Spot 4	0.532			
Spot 5	0.479			0.489
Spot 6	0.266		0.102	

* The elution solvents: 100:11:11:27 (by volume) ethyl acetate-formic acid-glacial acetic acid-water

TLC was used for separation of reaction mixtures. The separation occurs when one substance in a mixture is more strongly adsorbed by the stationary phase than the other components in the mixture. If a component has a polarity equal to that of the mobile phase, it will move with the solvent front. Conversely, if the polarity matches that of the sorbent, it will not move. For non-polar components, reversed-phase separation is required since nonpolar components will not interact with the silanol groups of the silica gel.

Since the R_f values for the standards decrease in the order caffeic acid > chlorogenic acid > quinic acid, the polarity of standards increases in the order caffeic acid < chlorogenic acid < quinic acid.

Table 3

TLC Results* for Different Reaction Mixtures

	Product 1 (one month Oxidation)		Product 2 (one week Oxidation)	
Spot	R_f	Intensity**	R_f	Intensity**
1	0.958	1st	0.967	1st
2	0.890	5th	0.800	6th
3	0.791	6th	0.680	4th
4	0.532	2nd	0.584	5th
5	0.479	4th	0.501	2rd
6	0.266	3rd	0.200	3rd

* The elution solvents: 100:11:11:27 (by volume) ethyl acetate-formic acid-glacial acetic acid-water

** The smaller the number, the higher the intensity.

Table 4

The Effect of Elution Solvents on Separation of a Reaction Mixture

Ratio*	100:11:11:27	90:11:11:37	80:11:11:47	70:11:11:57
Spot		R_f Value		
1	0.958	0.945	0.927	0.911
2	0.890	0.859	0.840	0.859
3	0.791	0.742	0.688	0.756
4	0.532	0.656	0.594	0.681
5	0.479	0.453	0.435	0.503
6	0.266	0.148	0.159	0.155

* Volume ratio of ethyl acetate-formic acid-glacial acetic acid-water

There were six spots totally separated on the TLC plate for the reaction mixture. This indicates that there were at least six components in the mixture. Spot 1 has a similar R_f value as caffeic acid. Spot 5 has a similar R_f value as chlorogenic acid. Because the resolution of the TLC method is limited, these components can not be considered as a single compound. It is quite possible that they contain several components. Therefore, the TLC method can only serve as a guide to develop optimal conditions for performing separations by column liquid chromatography.

Table 3 lists TLC results of the separation of reaction mixtures. Both reaction mixtures were recovered from chlorogenic acid solution, but the reaction periods of the two mixtures are different . Product 1 was recovered after one month oxidation under strong alkaline conditions while product 2 was recovered after one week oxidation under strong alkaline condition. The results of the two samples are similar. However,

the intensities and R_f values of some components do vary with oxidation time. This result illustrates that during the oxidation, the major products do not vary with time although their proportion does.

The results of varying the solvent polarity on the separation of a reaction mixture is given in Table 4. The mixture was recovered from chlorogenic acid solutions after one month of oxidation under strongly alkaline conditions. The results indicate little variation of the R_f value when the elution solvent increases its polarity. Also, it was observed during our study that the elution time increased as the water percentage in the solvent increased.

HPLC

HPLC experiments were performed in an attempt to separate the oxidation products of chlorogenic acid in a reaction mixture. The reaction mixture was recovered from chlorogenic acid after one month of oxidation under strong alkaline conditions. Table 5 summarizes the HPLC results. The retention times of all the components in the reaction mixture were small when a using reversed-phase C-18 column. The resulting resolution was insufficient for the separation. In addition, peak broadening was observed during the separation. The broadening was due to the interaction of components with unreacted SiOH groups in the C-18 column. An end-capped C-18 column can reduce this effect.

The result also indicates that some components in the reaction mixture have strong interactions with the column when using a polar stationary phase NH-10 column. This causes a long retention time for the components and decreases the efficiency of the column. However, when a strong acidic buffer was applied into the mobile phase, the interaction of components and stationary phase decreased and the retention time was reduced.

Four totally separated peaks were obtained when the NH-10 column was used with a phosphoric acid buffer as one of the mobile phases. Some peaks have shoulders which means the separation was not complete when using NH-10 column. From the above HPLC results, it can be concluded that a column whose polarity is between NH-10 and C-18 might be a good choice for the separation.

Table 5

HPLC Results of the Separation of a Reaction Mixture of Chlorogenic Acid

	Retention time (min.)		
Conditions	Oxidation Products	Chlorogenic Acid	Caffeic Acid
C-18 50% H_2O 50% CH_3CN	2.706 (l)* 3.171 (l) *Acidify sample pH=3	2.105	1.965
NH-10 Buffer (pH = 2.6)	11.706 (l) 34.00 (l)	36.36	10.00
NH-10 50% Buffer (pH = 0.7) 50% CH_3CN	2.502 (s)* 2.801 (s) 5.067 (l) 10.704 (l)	11.18	4.98
NH-10 70% Buffer (pH = 0.7) 30% CH_3CN	2.520 (s) 5.744 (l) 10.904 (l)	11.34	5.02
NH-10 100% Buffer (pH = 0.7)	2.400 (s) 5.604 (l) 10.761 (l)	11.78	5.12

* l = large peak area
* s = small peak area

Although the components of the reaction mixture were not totally separated by using HPLC experiments due to several limitations, these studies can be used to suggest optimum conditions for separations in future investigations.

Identification of Reaction Mixtures

Table 6 lists ^1H NMR data (in ppm) of standards and reaction mixtures. The original spectra of this study are shown in Appendix A. The peak assignments according to SADTLER (1978) for chlorogenic acid are shown in Figure 25.

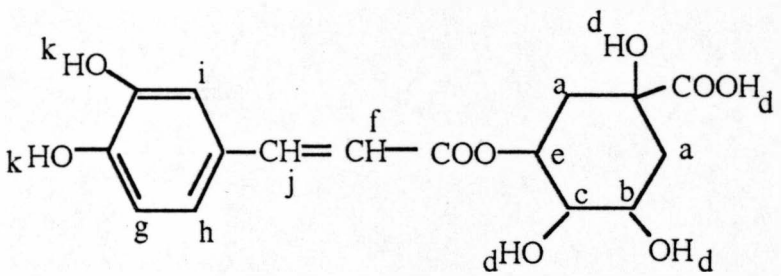


Figure 25. Peak Assignments for Chlorogenic Acid According to SADTLER.

Four reaction mixtures were tested and listed in Table 6. Product No.1 was the reaction mixture from a chlorogenic acid solution and was recovered from a neutral solution (pH 7) after one week. Product No.2 was the reaction mixture from chlorogenic acid solution and was recovered from an acidic solution (pH 3) after one week. Product No.3 was the reaction mixture from chlorogenic acid solution and was recovered from a neutral solution (pH 7) after two weeks. Product No.4 was the reaction mixture from chlorogenic acid solution and was recovered from a neutral solution (pH 7) after one month.

Comparing the chemical shift values of reaction mixtures with standards, it can be seen that product No.2 contains caffeic acid. This confirms that chlorogenic acid hydrolysis was the first step in the oxidation reaction under alkaline conditions.

Table 6

Chemical Shifts (δ, ppm) of ^1H NMR for Standards and Reaction Mixtures

						δ, ppm
Quinic Acid	Caffeic Acid	Chlorogenic Acid	Product No.1	Product No.2	Product No.3	Product No.4
~ 1.74		~ 1.70 a*	~ 1.75	~ 1.60	~ 1.70	~ 1.73
3.20				3.36	3.12	3.16
3.71		3.69 b	3.64		3.55	3.60
3.82		4.07 c	3.80		4.04	3.93
4.54		4.75 d	4.77			
5.34		5.02 e	5.26	5.20		
	6.17	6.17 f	6.17	6.19	6.10	6.15
	6.73	6.75 g	6.73	6.78	6.71	6.75
	6.91	6.93 h	6.92		6.96	6.90
	6.98	7.00 i	7.02	7.04	7.04	7.09
	7.42	7.42 j	7.48	7.45		
		> 7.60 k				
	9.15			9.35		
	9.65			9.70		

* Peak assignment is shown in Figure 25 according to SADTLER (1978).
~ Peak is around the listed chemical shift.

Cilliers (1991, 1989) had reported that oxygen was consumed during caffeic acid autoxidation. So, we believed that the newly formed caffeic acids, which were

hydrolyzed from some chlorogenic acids under strongly alkaline conditions, were quickly oxidized by the oxygen in the solution. The TLC separation results of this study showed that some components had similar or less polarity than caffeic acid in the oxidation products. This confirmed that nonenzymatic oxidation occurred in the chlorogenic acid reaction under strongly alkaline conditions.

The results in Table 6 also indicates that after long periods of reaction, the peak at 7.42 ppm for the product No.3 and No.4 was lost. As shown in Figure 25, this peak was due to the proton of double bond which is located on the side chain of chlorogenic acid. The missing peak suggests that the side chain of chlorogenic acid is involved in the oxidation reaction.

Table 7

Relative Peak Area at Different Chemical Shifts in ^1H NMR Spectra

δ, ppm	7.43	6.50 - 7.20	6.20	1.5 - 2.0
	Relative Peak Area			
Product No.2	1.24	3.50	1.26	5.38
Chlorogenic Acid	1.39	3.42	1.32	4.24

Table 7 lists relative peak areas of chlorogenic acid and its reaction mixture from our NMR spectra. Product No.2 was the reaction mixture from chlorogenic acid solution and was recovered from an acidic solution (pH 3) after one week. The result indicates that the peak intensity of double bond CH=CH (δ = 7.43 ppm & δ = 6.20 ppm) decreased after one week reaction while the peak intensity of aliphatic β–substituent's methine proton CH-C-C(=O)R (δ = 2.0 ppm) increased. This result

further demonstrates the involvement of the double bond during the oxidation.

CHAPTER V

CONCLUSIONS

This thesis has concentrated on investigating the nonenzymatic oxidation of phenolic compounds in natural water system. Specifically, chlorogenic acid and caffeic acid were studied. An understanding of these processes is of importance to both the environmental protection and food industry.

The results domonstrate that pH, reducible metal ion such as copper(II) and light are three major physical factors affecting the nonenzymatic oxidation.

The oxidation of chlorogenic acid and caffeic acid was dependent on pH. The higher the pH, the faster the oxidation. The involvement of phenolate ion in the formation of semiquinone, which then undergoes a further polymerization reaction, was the main reason for pH dependency. The UV/VIS spectra indicated that at strong alkaline conditions, radicals were formed in the chlorogenic acid solution. These radicals would increase the rate of polymerization of semiquinone which is another reason for the oxidation dependence on pH. The results also demonstrate that the oxidation rate of caffeic acid was much faster than that of chlorogenic acid under acidic and neutral conditions

Copper(II) had a strong catalytic effect on the oxidation of chlorogenic acid even at a very low concentration. This result was significant since it points out that the existence of trace metals such as copper(II) in natural water systems can affect the decomposition path of organic species.

Light had a strong effect on the oxidation of chlorogenic acid at neutral conditions. However, the photochemical effect on the oxidation is much slower than

the catalytic effect of metal or hydroxyl ion on the oxidation.

A tremendous effort has been devoted for the separation and identification of the oxidation products of chlorogenic acid. Both TLC and HPLC results show that the oxidation product is not a single compound but a mixture. It contains at least six components with quite different polarities. Oxidation time had an effect on the relative percentage of these components.

The NMR results indicate that under strongly alkaline conditions, some chlorogenic acid was initially hydrolyzed into caffeic acid and quinic acid and was then oxidized.

The NMR results also show that after a long time of oxidation, the peak, which is due to the proton of the conjugated double bond on the side chain of chlorogenic acid, was absent. The result agrees with the UV/VIS studies. The UV/VIS spectra show that the absorption maximum, which is due to a π to π^* transition of an electron of the conjugated double bond of chlorogenic acid, was decreased during the oxidation. Therefore, both results indicate that during the oxidation of chlorogenic acid, the conjugated double bond was involved.

Appendix A

Proton NMR Spectra of Caffeic Acid, Quinic Acid, Chlorogenic Acid and Its Oxidation Products

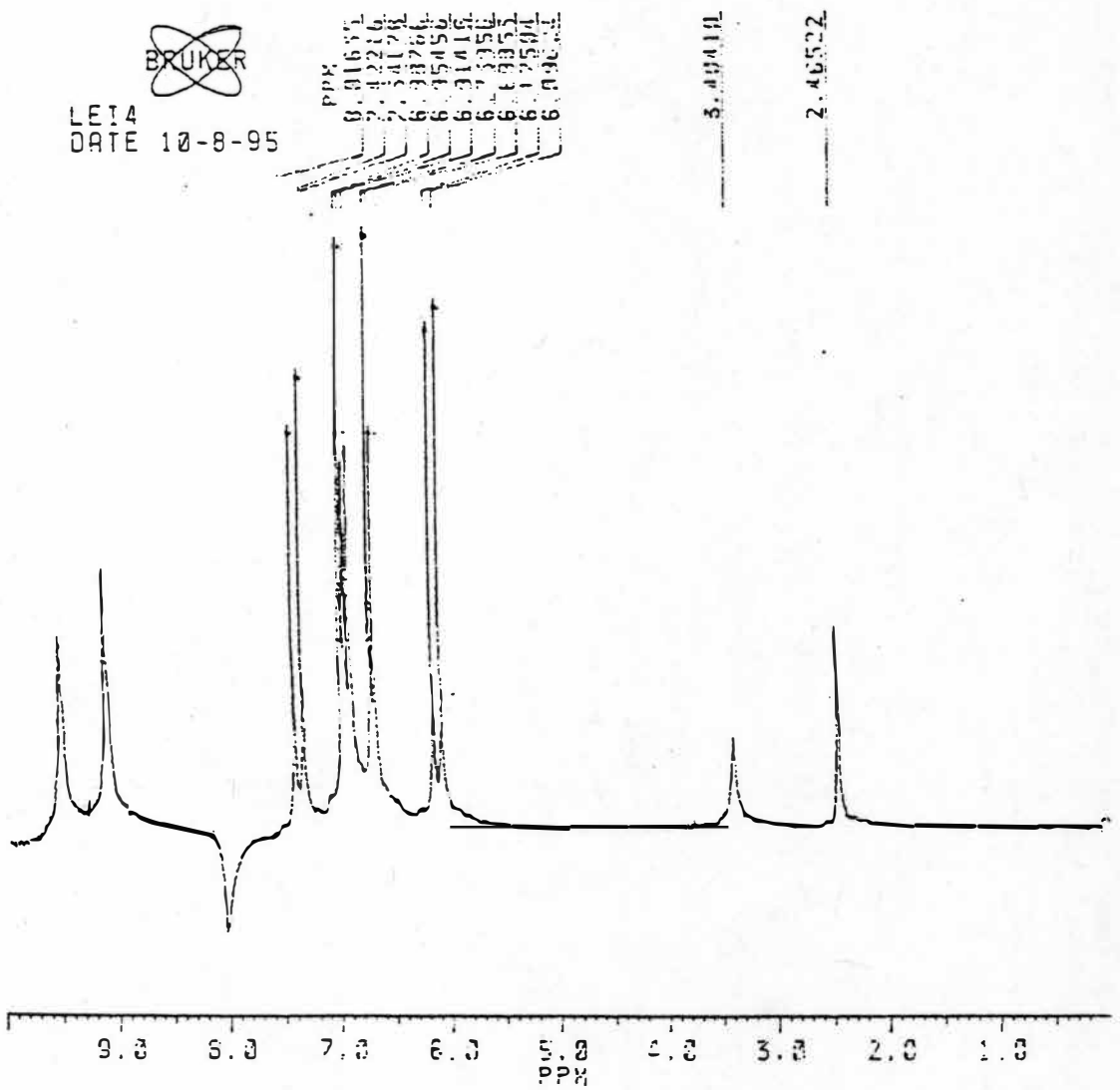


Figure A-1. ^{1}H NMR Spectrum of Caffeic Acid.

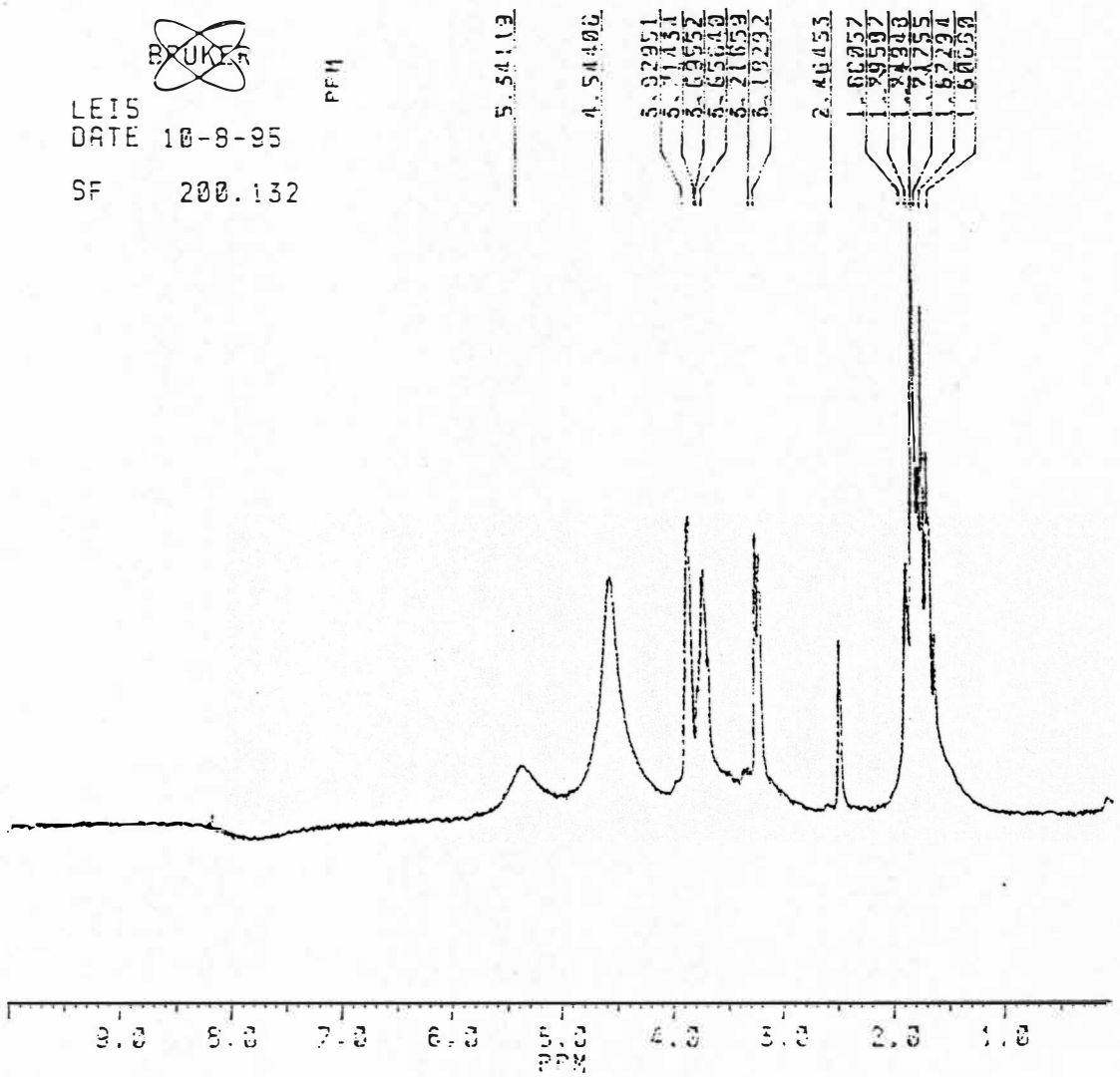


Figure A-2. ^{1}H NMR Spectrum of Quinic Acid.

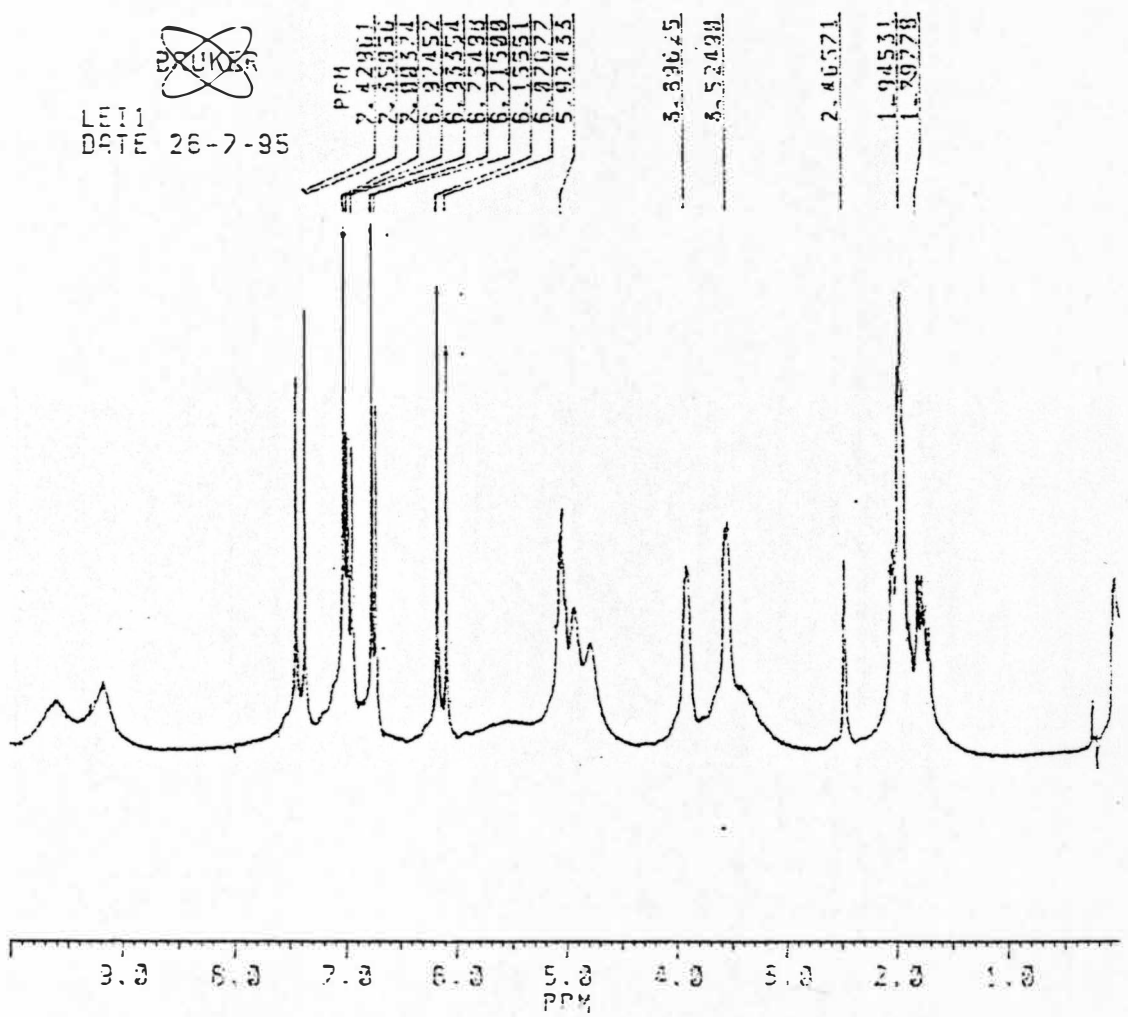


Figure A-3. ^{1}H NMR Spectrum of Chlorogenic Acid.

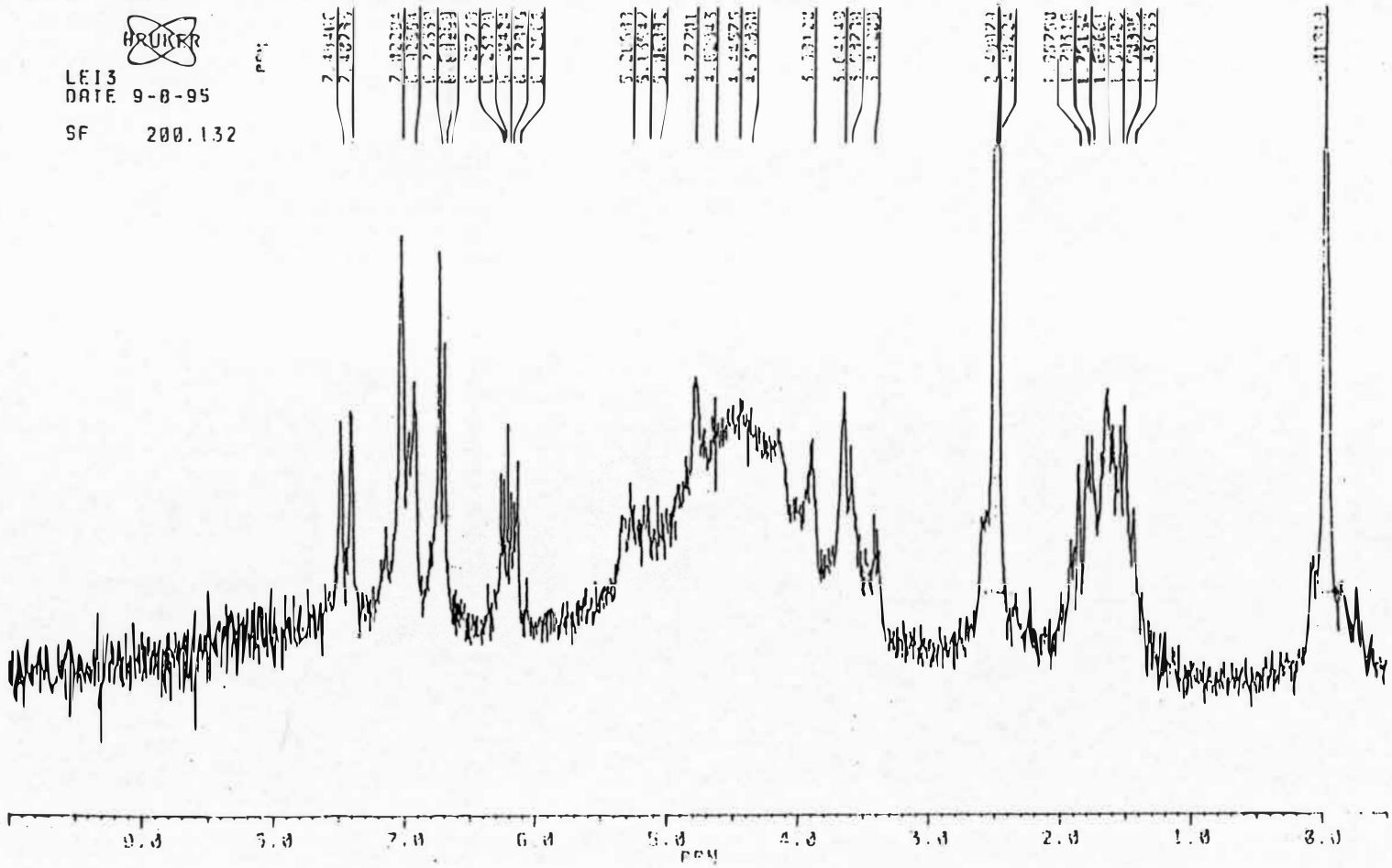


Figure A-4. ^{1}H NMR Spectrum of Oxidation Product of Chlorogenic Acid(Product No.1).

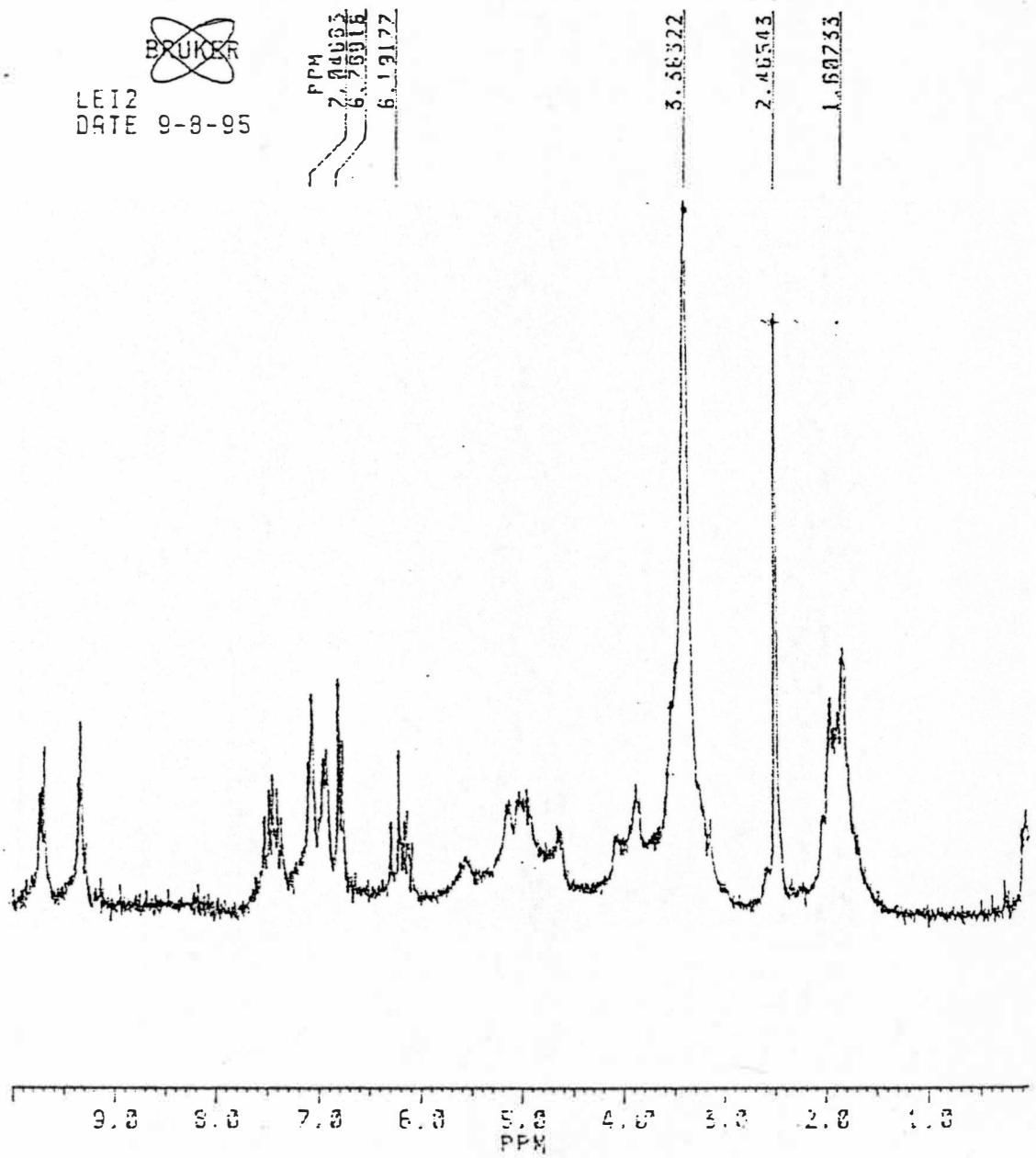


Figure A-5. ^{1}H NMR Spectrum of Oxidation Product of Chlorogenic Acid (Product No.2).

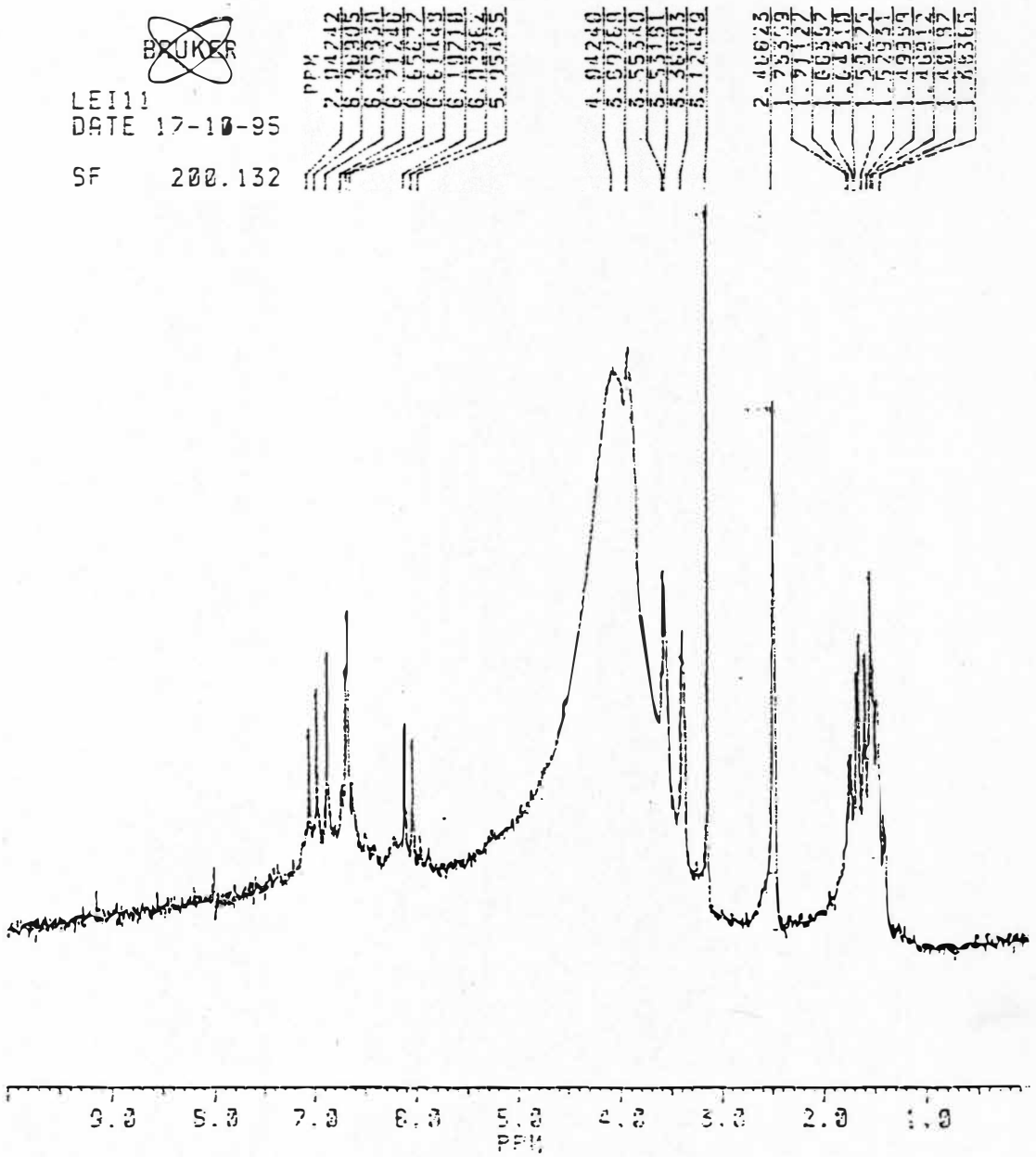


Figure A-6 ^{1}H NMR Spectrum of Oxidation Product of Chlorogenic Acid (Product No.3).

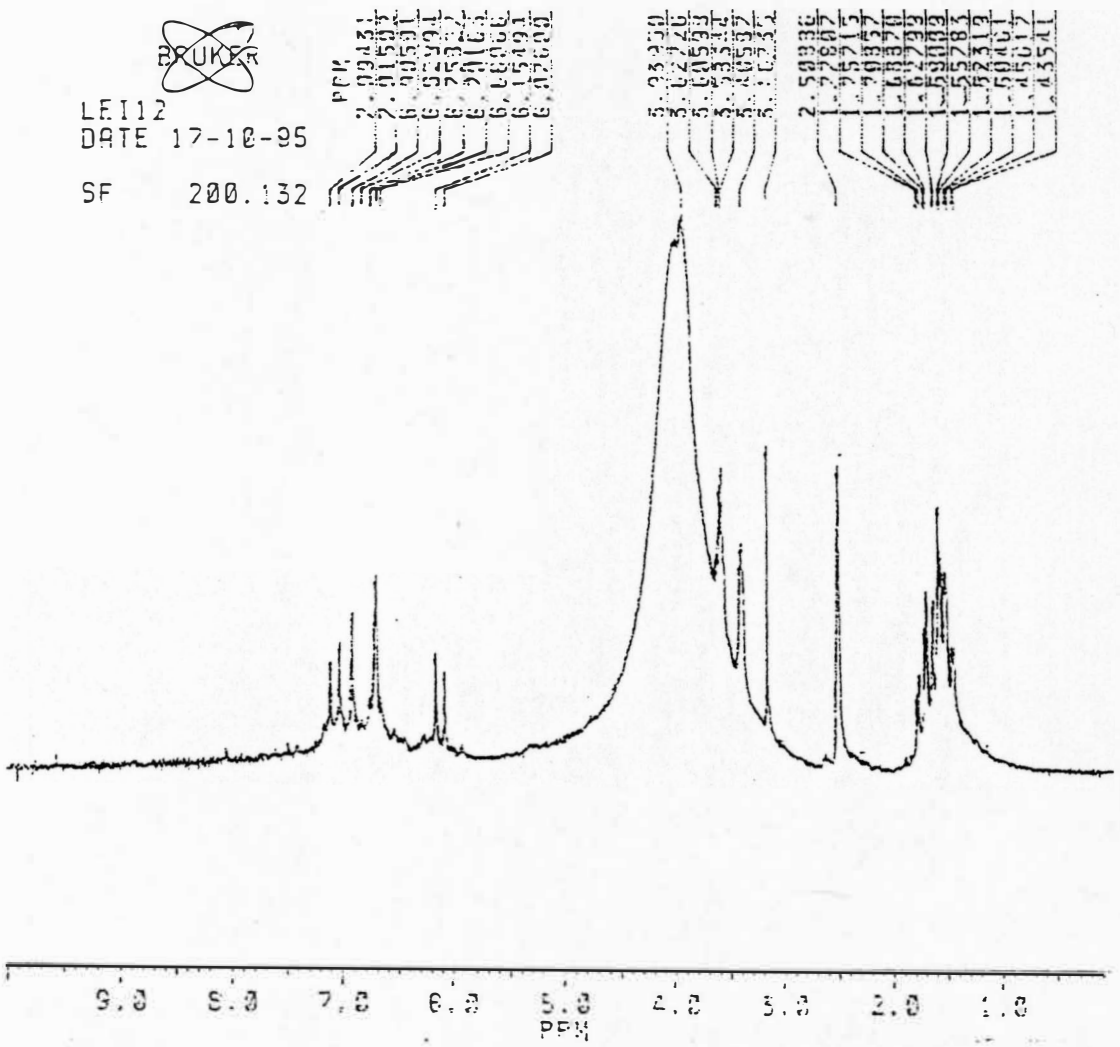


Figure A-7. ^{1}H NMR Spectrum of Oxidation Product of Chlorogenic Acid (Product No.4).

BIBLIOGRAPHY

Bradield, A.F. et al. (1952). Nature, Vol. 170, pp. 168.

Cha, J.A. et al. (1986). Aerobic Coupling of Aqueous Phenols Catalyzed by Binuclear Copper: Ring Substituent Effect and the Kinetics of the Coupling of o-Methylphenol. AICHEJ. 32, 477-485.

Cilliers, J.J.L. (1989). Nonenzymic Autoxidatiive Phenolic Browning Reactions in a Caffeic Acid Model System. J. Agric. Food Chem., 37, 890-896.

Cilliers, J.J.L. (1990). Total Polyphenols in Apples and Ciders: Correlation with Chlorogenic Acid. J. Food Sci., Vol. 55, No. 5, pp. 1458-1459.

Cilliers, J.J.L. (1991). Characteization of the Products of Nonenzymic Autoxidative Phenolic Reactions in a Caffeic Acid Model System. J. Agric. Food Chem., 39, 1298-1303

Clifford, M.N. (1986). Coffee Bean Dicaffeoylquinic Acids. Phytochemistry, Vol. 25, no. 7, pp. 1767-1769.

Dao; Friedman, (1992). Chlorogenic Acid Content of Fresh and Processed Potatoes Determined by Ultraviolet Spectrophometry. J. Agric. Food Chem., 40, 2152-2156.

Davies, G. (1979). Adv. Chem. Ser., 173, pp. 178.

Flaig, W. (1988). Generation of Model Chemical Precursors. Humic Substances and Their Role in the Environment, John Wiley & Sons, pp.75-92.

Fuchs, W. (1931). Die Chemie der Kohle, Springer, Berlin.

Griffiths, D.W. (1992). Development of Rapid Colorimetric Method for the Determination of Chlorogenic Acid in Freeze-Dried Potato Tubers. J. Sci. Food Agric., 58, 41-48.

Hatcher, P.G. (1988). Selective Degradation of Plant Biomolecules. Humic Substances and Their Role in the Environment, John Wiley & Sons, pp.59-74.

Hedges, J.I. (1988). Polymerization of Humic Substantces in Natural Environments. Humic Substances and Their Role in the Environment, John Wiley & Sons, pp.45-58.

Karlin, K.D. (1983). Copper Coordination Chemistry: Biochemical and Inorganic Perspectives, Adenine Press, Guilderland, NY.

Lanson, R.A. (1980). Oxidative Polymerization of Dissolved Phenols by Soluble and Insoluble Inorganic Species. Limnol. Oceanogr., 25, 505-512.

Linder, P.W. (1987). Polyhedron, 6, 53.

Linder, P.W. (1992). Potentiometric Investigations of Equilibria Between Caffeic Acid and Manganese(II), Cobalt(II), Nickel(II) and Cadmium(II) Ions in Aqueous Solution. J. Coord. Chem. Vol. 25, pp. 211-220.

Marschner, H. et al. (1981) J. Plant Nutr., 3, 551

Maruta, Y. et al. (1995). Antioxidative Caffeoylquinic Acid Derivatives in the Roots of Burdock. J. Agric. Food Chem. Vol. 43, no.10, pp. 2595.

Matheis, G. (1987). Polyphenol Oxidase and Enzymic Browning of Potatoes (Solanum Tuberosum).II. Enzymic Browning and Potato Constituents. Chem. Mikrobiol. Technol. Lebensm., 11, 33-41.

Mathew, A.G.and Parpia, H.A. (1971). Food Browning as a Polyphenol Reaction. Adv. Food Res. 19, pp. 75.

Matin, R. et al. (1987). The Caffeine-potassium Chlorognate Molecular Complex. Phytochemistry, Vol. 26, no. 1, pp. 273-279.

METTLER Instrument Corporation (1987). TA4000 System Operating Instructions, pp. 104.

Monsalve, A. (1990). Browning of Dehydroascorbic Acid and Chlorogenic Acid as a Function of Water Activity. J. Food. Sci. Vol. 55, no. 5, pp. 1425.

Nigh, W.G. (1973). Oxidation in Organic Chemistry, Academic Press, New York.

Oszmianski, J. et al. (1985). Changes in Grape Seed Phenols as Affected by Enzymatic and Chemical Oxidation in Vitro. J. Food Sci. Vol. 50, pp. 1505-1506.

Pandell, A.J. (1983). Mechanism of the Fe(III)-catalyzedPeracetic Acid Oxidation of Catechol. A Biomimetic Reaction for Pyrocatechase. J. Org. Chem., 48, 3908-3912

Pierpoint,W.S. (1966). The Enzymic Oxidation of Chlorogenic Acid and Some Reactions of the Quinone Produced. Biochem. J. 98, pp. 567.

Rogic, M.M. (1983). Copper Coordination Chemistry: Biochemical and Inorganic Perspectives, Adenine Press, Guilderland, NY., pp. 209.

SADTLER Research Laboratories INC. 1978.

Shepherd, K.M. (1995). Novel Capillary Electrophoresis Technique for the Study of Plant Phenolic Enzymic Oxidation Mechanisms. J. Agric. Food. Chem. Vol. 43, pp. 657-661.

Skoog, D. A. (1992). Principles of Instrumental Analysis 4th ed. Saunders College Publishing.

Speier, G. (1986). Copper-catalysed Oxidation of Catechols by Dioxygen. Journal of Molecular Catalysis, 37, pp. 259-267.

Stevenson, F.J. (1965). Gross Chemical Fractionation of Organic Matter. Methods of Soil Analysis, American Society of Agronomy, Madison. Wisc., pp. 1409-1421.

Stevenson, F.J. (1982). Humus Chemistry (Genesis, Composition, Reactions). New York: John Wiley-Interscience.

Stiff, M.J. (1971). The Chemical States of Copper in Polluted Fresh Water and a Scheme of Analysis to Differentiate Them. Water Research Pergamon Press, Vol. 5, pp. 585-599.

Touchstone, J.C.(1992). Practice of Thin Layer Chromatography. 3rd ed. John Wiley & Sons, Inc.

Villegas, R.J. A. et al. (1987). Purification and Characterization of Chlorogenic Acid: Chlorogenate Caffeoyl Transferase in Sweet Potato Roots. Phytochemistry, Vol. 26, no. 6, pp. 1577-1581.

Wagner, H. et al. (1984). A Thin-Layer Chromatography Atlas. Plant Drug Analysis. Springer Verlag: Berlin, pp. 163-194.

Waksman, S.A. (1938). Humus, Origin, Chemical Compositions and Importance in Nature. Baltimore: Williams & Wilkins Co.

Williams P.M. (1969). Association of Copper With Dissolved Organic Matter in Seawater. Limnol. Oceanogr. 14, pp.156-158.

Xu, L. X. (1994). The Reaction of Caffeic Acid and Copper(II) in Natural Water. M.A. thesis, Western Michigan University, Kalamazoo, MI.