CHARACTERIZATION OF α-CYCLODEXTRIN INCLUSION COMPLEXES WITH *TRANS*-CINNAMIC ACID IN AN ACID-BASED BEVERAGE SYSTEM

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ABSTRACT

In response to a need for a natural antimicrobial to replace sodium benzoate, cinnamic acid was chosen. Due to cinnamic acid's solubility issues, α-cyclodextrin was used as a host molecule to form an inclusion complex with the cinnamic acid molecule. The cinnamic acid: α-cyclodextrin inclusion complex was then characterized using phase solubility analysis, proton nuclear magnetic resonance (H-NMR), and solid inclusion. Phase solubility analysis verified the maximum amount of cinnamic acid that α-cyclodextrin was able to host. H-NMR was used to determine the complex association constant, determine the chemical shifts of available protons, and yield a stoichiometry for the complex. The solid inclusion complex allowed for a physical formation of the complex, yielding further information in support of the complex stoichiometry. Microbiological tests were also performed to quantify the antimicrobial abilities of the complex, the guest, and the host against the yeast *Saccharomyces cerevisiae* and mold *Paecilomyces variotii*.

Results indicated that approximately 990.29 ppm in aqueous solution was the maximum amount of cinnamic acid in the complex. The 2:1 stoichiometry yields an association constant of 21.7 M^{-1}. Results also indicated that the cinnamic acid readily conformed to fit within the α-cyclodextrin host molecule, which remained a rigid structure. An 8.9% weight to weight of cinnamic acid was calculated for the solid inclusion again reinforcing a 2:1 stoichiometry. Microbiological studies showed little to no inhibition power by the complex at varying concentrations against *S. cerevisiae* and *P. variotii*. Free cinnamic acid showed greater antimicrobial activity compared with free α-cyclodextrin and the complex.

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CHAPTER 1: INTRODUCTION

The Problem

In November 2005, the Food and Drug Administration (FDA) conducted a study on beverages found in the market. Recent laboratory results showed low levels of benzene, a known carcinogen, in many soft drink and fruit beverages. Although there are no standards for allowed levels of benzene in beverages, other than water, which is allowed a maximum of 5 parts per billion (ppb), the FDA conducted further studies looking at benzene levels in common beverages on the market (CFSAN and Safety, 2006). It is believed that sodium benzoate, a common preservative used in beverages, was reacting with ascorbic acid in the beverages and was being broken down in to free benzene. It was further shown that these benzoic salts react with ascorbic acid and erythorbic acid as well, especially in the presence of elevated temperatures and light (CFSAN and Safety, 2006).

The study was conducted on 100 soft drinks and other beverages pulled from the shelves in Virginia, Michigan, and Maryland. Of these 100 tested, from multiple producers, only four soft drinks and one fruit drink were implicated as having above 5 ppb benzene levels in aqueous solution (CFSAN and Safety, 2006). These results were posted as of April 2006. The study then continued with the FDA adding 86 more samples from Maryland, Maine, and Massachusetts and results posted May of 2007. Five products were implicated in the second study which allowed for reformulations by the manufacturers resulting in four left on the shelves and one discontinued (CFSAN and Safety, 2006).

As the information began to reach the public bad publicity over the benzene scare started to affect the sales of soft drink and other fruit beverages. Though none of PepsiCo's products were implicated in the FDA study, they contracted the Food Science Department at Virginia Tech to work with a replacement antimicrobial that will give the consumer a natural substitute for sodium benzoate.

Cinnamic acid & cyclodextrins

With recent research and rising popularity of the compound cinnamic acid, a weak acid derived from the bark of cinnamon, PepsiCo contracted Virginia Tech to work with cinnamic acid as a natural replacement. Cinnamic acid is a known antimicrobial that is used in

pharmaceuticals and cosmetics in the market already (Hoskins, 1984). Although cinnamic acid has shown great stride as an antimicrobial its poor solubility and wettability have caused problems in its incorporation in to aqueous solutions. When placed in an aqueous solution the cinnamic acid tends to clump and stay clumped. With a solubility of only 0.5 grams per liter, cinnamic acid takes a good amount of time or good amount of solvent to solubilize the compound (Hoskins, 1984). To overcome the solubility problem, an inclusion complex is theorized using cyclodextrins as the host molecule.

Cyclodextrins are cyclic oligosaccharides made up of different numbers of glucose molecules. α-Cyclodextrin, which is to be used for this study, is made up of six glucose units to form a cylinder (Jimenez V, 2005). A highly polar molecule, due to the hydroxyl groups pointing towards the outside of the molecule, it has a high affinity for accepting guests in to its interior cavity (Jimenez V, 2005). Cyclodextrins have been used in industry for years in foodstuffs, pharmaceuticals, cosmetics, and packaging (Saenger, 1984).

Research Objectives

The cinnamic acid: α-cyclodextrin inclusion complex can then be tested using phase solubility analysis, Nuclear Magnetic Resonance (NMR) studies, and solid inclusion complexing to characterize the complex. The characterization will allow data acquisition to better determine the fit of the guest within the host, determine the stoichiometry of the complex, and provide a physical complex to incorporate in to a beverage, or aqueous, solution. Also, as a complex its antimicrobial properties must be tested to ensure no loss in its antimicrobial properties as compared to free cinnamic acid. The complex will also be tested against the two most prevalent spoilage microorganisms in soft drink and beverage facilities, *Saccharomyces cerevisiae* and *Paecilomyces variotii*, respectively.

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CHAPTER 2: REVIEW OF LITERATURE

trans-cinnamic acid

Trans-cinnamic acid is used due to its simplicity in nature, yielding an extended conjugated resonance and its inclusion effect as it is more commonly found in nature (Letizia CS, 2004; Uekama, 1975). Cinnamic acid is found to have a low solubility reported at 0.5 g l^{-1} in aqueous solution (Hoskins, 1984). For this reason it has not been used in extent in aqueous solution but in its original powder form. In solid form it has been used as an antimicrobial in fruits and as an additive to cosmetics (Roller and Seedhar, 2002; Hashimoto, 1996). Consequently, there has been little to no research performed on cinnamic acid concerning the actual mechanics causing its antimicrobial effect or how it prevents microbiological growth (Lambert and others 2001). The structure of cinnamic acid can be found in Figure 1.

Research states that the higher the pKa of a compound the higher the antimicrobial ability (Ramos-Nino and others 1996). Literature states that cinnamic acid has a pKa of 3.98-4.44. pKa attributes to 81% of cinnamic acid's inhibition effect (Kouassi Y, 1998). The low pKa corresponds to the high lipophilicity of cinnamic acid which in turn increases its antimicrobial effects. One theory projects cinnamic acid causes acidification of the bacterial cell as it alters the bacterial cell's membrane (Chambel, 1999). The alteration of the membrane allows the cinnamic acid to stimulate the influx of protons traveling across the cell membrane to increase its permeability. Once inside, the cinnamic acid, with its liposoluble acidic structure, can disrupt the bacterial cell's cytoplasm (Chambel, 1999). Cinnamic acid uses penetration of the cytoplasm in the bacterial cell to inhibit the amino and keto acid transport of the bacterial cell, rendering it useless (Sheu and others 1975).

It has also been reported that a long side chain indicates increased antimicrobial ability (Kouassi Y, 1998). Due to cinnamic acid's lipophilicity and long chain status higher antimicrobial ability occurs. The antimicrobial property corresponds to a higher proportion of the liposoluble undissociated weak acids present at the pH found in the solution (Chambel, 1999). In a comparison study done with benzoic acid there was a 30 mM difference in their ability to inhibit *Bacillus subtilis,* 30 mM for inhibition by cinnamic acid and 60 mM by benzoic acid. The study shows that although their pKa's are similar, 4.2 and 4.4, respectively, a longer lipophilic side chain allows more complete microbial inhibition (Kouassi Y, 1998). Long-chain fatty acids, such as cinnamic acid, are known to inhibit gram-positive bacteria, such as *Bacillus*

subtilis, but are not effective on gram-negative bacteria, for example *Escherichia coli* (Sheu and others, 1975). The Gram distinction is due to the fact that cinnamic acid cannot penetrate the cell's lipopolysaccharide membrane (Sheu and others, 1975). It is thought that there is no outer membrane disintegration or depletion of the ATP found within the cell.

As an antimicrobial, cinnamic acid works directly in the cytoplasmic membrane of the bacterial cell to inhibit its ability to perform (Chambel, 1999). Although cinnamic acid is usually paired with high acid foods it has also been shown to be effective at neutral pHs inhibiting the growth of microbial and yeast cells (Fujii T, 2001). In a study with *Rhodotorula minuta,* a fungus that prefers neutral pH, cinnamic acid was able to show complete inhibition of fungus, abruptly ceasing the growth (Fujii T, 2001). The study also gave rise to theory that cinnamic acid does not kill the microbial cell but instead keeps it from growing by reducing respiration of the cell mitochondria (Fujii T, 2001). Yeast inhibition occurs in amounts as little as 0.14 mM of cinnamic acid (Chambel, 1999). When treated at relatively low levels of 1 mM cinnamic acid on fruit slices, a prevention of visible spoilage and microbial flora was apparent for an extended time, showing that low level doses of cinnamic acid are effective as antimicrobials (Roller and Seedhar, 2002). There is also the added benefit of using cinnamic acid as an antimicrobial without changing the organoleptic properties associated with foods in which it is incorporated (Roller and Seedhar, 2002).

When studying the effect of cinnamic acid on inhibition of pathogens, such as *Listeria monocytogenes* and *Bacillus subtilis*, both were affected by low concentrations of cinnamic acid, 0.11% and 0.44% (30 mM, 4.44 g/L) respectively (Sheu and others, 1975). In a second study, working with *Bacillus subtilis*, statistics show that only 3 mM cinnamic acid is needed to inhibit 50% of the bacteria (Sheu and others, 1975). In this same study *Escherichia coli* was studied as well. Although many long chain fatty acids are not considered antimicrobials for gram-negative organisms such as these, results showed 9 mM cinnamic acid was needed to inhibit 50% of the bacteria (Sheu and others, 1975).

α-cyclodextrin

α-Cyclodextrin has been used for years as a host molecule in the pharmaceutical industry with some exposure in the food industry as well. Though there are multiple forms of cyclodextrin there have been many studies performed to compare them. α-Cyclodextrin, shown

in Figure 2 and Figure 3, is the smallest of the cyclodextrins, with only six glucose units and an average interior diameter of 5-Å forming a truncated cone (Jimenez V, 2005; Wood, 1976). α-Cyclodextrin has a higher dissolution rate than that of β-cyclodextrin, almost eight times as much, as well as a smaller cavity diameter, which allows a tighter fit within the cavity yielding enhanced solubility, chemical stability and absorption characteristics (Uekama, 1979). It is also shown that α-cyclodextrin has a more drastic conformational change to conform to a substrate than β-cyclodextrin (Saenger, 1984). The α-cyclodextrin's ability to conform is also shown by the number of protons available for bonding when complexed. β-Cyclodextrin is a very rigid structure due to the interior bonding of hydroxyl groups which creates a belt of hydrogen's for stability. α-Cyclodextrin does not have this hydroxyl group bonding due to one glucopyranose unit being distorted as the diameter interior is smaller. Therefore, only four hydrogen bonds are readily available for bonding which may explain why β-cyclodextrin has the lowest solubility index (Szejtli, 1998).

α-Cyclodextrin forms a structure similar in shape to a hexagon, as all six glucose units are identical in their C1 chair conformations (Wood, 1976; Saenger, 1984). It has also been shown that there is no other conformation that cyclodextrin takes on other than the C1 chair, whether the cavity is occupied or not (Saenger, 1984). The ring structure consists of six intramolecular, interglucosidic hydrogen bonds (Wood, 1976). It also mimics the shape of a cylinder with all of the hydroxyl groups found on the exterior of the molecule (Jimenez V, 2005). α-Cyclodextrin is unique in that its distribution of hydrophilic and hydrophobic groups allows the hydroxyl units to reside in both rims of the cone. The position of these hydroxyls allow α-cyclodextrin to become soluble when added to a solution (Saenger, 1984). The interior of the cavity also produces high electron density, yielding the ability to lend electrons to a substrate (Szejtli, 1998).

It has also been shown that α-cyclodextrin increases in its potential energy when complexed with another molecule (Wood, 1976). It has a hydrophobic interior which readily accepts lipophilic molecules to release the water that is usually stored in the cavity when in solution (Jimenez V, 2005). Its exterior is hydrophilic which enhances its solubility (Jimenez V, 2005).

α-Cyclodextrin has a maximum solubility of 149 mM (22.1 g/L) in distilled water yielding good overall solubility and solution stability (Saenger, 1984). The inclusion properties

are based on many weak forces, such as van der Waals forces, working together to form a preferable, affinity-based complex (Jimenez V, 2005). The complexation of α-cyclodextrin also incorporates the transfer of hydrophobicity and charge of the anions to differentiate the complexation of α-cyclodextrin with another molecule (Jimenez V, 2005). There are two different conformational states that α-cyclodextrin remain in depending on solution. If the α-cyclodextrin is empty, meaning there is no other molecule with which to complex, water takes its place inside the α-cyclodextrin cavity. The water collapses the torus and the ring of hydrogen bonds is altered thus producing steric strain on the α-cyclodextrin (Saenger, 1984). If the α-cyclodextrin cavity contains a substrate, then it takes on a round, or hexagonal, shape which takes minimal potential energy. The minimal energy allows for a relaxed structure state (Saenger, 1984). There are also three observed mechanisms for the transfer of substrate into the water-occupied α-cyclodextrin cavity (Saenger, 1984). The first involves a direct replacement of water for the substrate. The second involves the formation of the relaxed state followed by expulsion of the water by the substrate. Third, the substrate binds to the exterior of the cavity and then enters (Saenger, 1984). The association-dissociation process is very rapid and reversible between the α-cyclodextrin and substrate (Saenger, 1984).

Cyclodextrins are considered favorable solutes due to the cavity's highly ordered solvent molecules (Uekama, 1975). When complexed, the α-cyclodextrin can form channels with one another, creating larger structures. With a molecule like cinnamic acid, which contains a carboxyl group, the α-cyclodextrin will form cages that are structurally sound. The cage formation is due to the hydrogen bonds forming sheets of O(2)H/ O(3)H on one side of the sheet and O(6)H on the opposite side. The sheets then orient themselves to face one another to close the sides of the α-cyclodextrin with adjacent molecules (Saenger, 1984).

Cyclodextrins have also shown browning inhibition properties when paired with fruit beverages that undergo heat treatments (Irwin PL, 1993).

trans-cinnamic acid: α-cyclodextrin complex

Cinnamic acid and α-cyclodextrin complement one another in size and shape yielding a complex that is effective and yields higher magnitudes of extrinsic optical activity (Uekama, 1975). Within the inclusion complex, the diameter of the α-cyclodextrin molecule is smaller and will better host the cinnamic acid molecule, due to the benzene ring, as compared to the other

cyclodextrin varieties (Uekama, 1975). Benzene rings will fit in both α and β versions of cyclodextrin as their interiors range from 6 to 8 Å. A benzene structure measures approximately 7 x 7 x 3.4 Å and therefore is able to fit in to the interior of the cyclodextrin cavity (Lach and Cohen, 1963). It is shown that molecules made up of benzene rings have the propensity to bond with the α-cyclodextrin cavity and will lead to an elliptical distortion of the benzene ring with the O(4) hexagon elongated by approximately 0.8 Å̊ (Saenger, 1984). Although it is theorized that the benzene ring is the portion of the molecule to bind within the cyclodextrin cavity, it is also theorized that the side chains may fit in to the cavity and complexation occur which allow for the cinnamic acid molecule to be included in solution (Lach and Cohen, 1963). Studies theorize that hydrogen bonding, due to the many hydroxyl and carboxyl groups of the cyclodextrin is the primary mechanism in the inclusion complex formation. If the guest is too small, forces holding it in place within the cyclodextrin cavity are too weak to form a stable inclusion complex (Lach and Cohen, 1963). With the benzene ring diameter being roughly the same size as the cyclodextrin cavity a strong stability is expected, though strain is necessary due to the tight fit (Lach and Cohen, 1963).

If the complex is formed as a solid state complex, rehydrating the complex for the ability to increase solubility or increase bioavailability of the substrate is shown to be non-problematic as opposed to using the complex for protection or stabilization of the substrate (Saenger, 1984). Solubility studies propose that α-cyclodextrin is capable of interacting with the substituent groups as well as benzene rings, yielding a 2:1 molar ratio (Uekama, 1979). The interation with other groups may be due to the ability of α-cyclodextrin to interact with many molecules, including those that are derivatives of cinnamic acid (Uekama, 1979). Studies have shown that due to a 2:1 molar ratio, when complexed, there is a change in pKa from 4.35 when uncomplexed to 6.27 (Uekama, 1975).

Characterization Methodology Rationale

Phase-solubility analysis. Phase-solubility analysis is a measurement of the equilibrium solubility of a chemical substance in a given solvent, at a given temperature and pressure which is used to identify the purity of the substance (Higuchi, 1971). The interactions between the two molecules used in the inclusion complex are held together with weak induction forces, such as van der Waals forces and charge transfer (Higuchi, 1971). The electron transfer between the two

molecules creates electrostatic forces of attraction (Higuchi, 1971). These charge transfers have been linked to overall solubility effects with the inclusion complexes, especially with aromatic molecules (Higuchi, 1971). The ability for a compound to become soluble is closely related to the molecular interactions of the compound. For example, if a compound has a lower solubility it is expected to have a higher solute to solute interaction (Higuchi, 1971). Placed in solution it is much more likely for two molecules in an aqueous solution to interact with one another as opposed to their environment. Though little analytical application is available with these methods it has been used in research for gaining more information about the substrate, including association and stability constants (Higuchi, 1971). It has also been applied as a quantitative measure for purity determination of the substrate in question, which in turn can prepare the sample for further characterization.

In running analysis, successive amounts of a sample are added to a solvent which remains at a constant volume in which it is slightly soluble. The solution is then brought to equilibrium through agitation at a constant temperature and the solution phases are then analyzed for total solute content (Higuchi, 1971). A phase diagram can then be constructed by plotting the weight of the solute found per unit of solution against the weight of the sample added per unit of solvent. There are many different solubility paths the cinnamic acid: α-cyclodextrin complex could follow. The diagram is then read and interpreted (Higuchi, 1971). A phase solubility experiment then yields the maximum solubility of the substrate in question.

Nuclear magnetic resonance (NMR). NMR is a spectroscopic technique that is element-selective and noninvasive. It is capable of characterizing the structural differences between many molecules that are present in a sample (Bertmer and others 2006). Proton-NMR (H-NMR) is a procedure that allows for identification of single protons in a sample. With the ability to identify a compound's protons, a shift in those protons can be interpreted as a change in conformation of the molecule at different concentrations. NMR is based on the theory that protons act as magnets, giving off a magnetic pulse in response to a pulse. This pulse given off by the protons is then recorded and shown as a spectrograph. By viewing the spectra, a literal shift in spectral peaks, representing the compound's protons, is seen. Shifts are also an advantage as they provide insight into the conformation of the complex. Chemical shifting and conformational change are difficult processes to detect with any other analytical procedure

(Schneider and others 1998). For these reasons, NMR shift titrations are the most often used method for determining association constants with cyclodextrins (Schneider and others, 1998).

Pharmaceutical needs have caused an increase in NMR cyclodextrin research. NMR is now required in the pharmaceutical industry as the structural characteristics of compounds in use are legally mandatory (Schneider and others, 1998). NMR can structurally characterize compounds and therefore has become a necessary tool in the industry. Although it is too indirect to acquire a three-dimensional structural representation, models can be derived from the data provided from NMR spectroscopy (Schneider and others, 1998).

Literature holds many NMR spectra of α-cyclodextrin though few of cinnamic acid. Studies have shown that cyclodextrin has a shielding effect caused by the interior cavity which limit the parts per million (ppm) when a guest is included in the complex (Schneider and others, 1998). There are few proton shielding differences between the types of cyclodextrins available for research. For example, H-1 for α-cyclodextrin is the base line for movement and should remain constant. Due to this shielding effect that is produced, there is a possibility of a shift registered by NMR of up to 0.1 ppm (Schneider and others, 1998). Also, when reviewed in more detail, a shift is shown to occur for α-cyclodextrin H-6a and H-6b in the solvent D_2O of 0.02 to 0.06 ppm. D_2O is the solvent of choice for the cinnamic acid: α-cyclodextrin complex as it is most closely resembles the environment of a high-acid beverage. The solvents used have an effect on complexation of the compounds and can lead to fast exchange of protons, such as in water (Schneider and others, 1998). This fast proton exchange is why D_2O is such a desirable solution to work with. Other studies have shown that the binding constant increases when using D2O as opposed to H2O as the solvent for NMR, especially as compounds are complexed with cyclodextrins (Wang and Matsui, 1994). Although D2O is as close to a pure water system as can be achieved for NMR and that their properties are very similar there is much more structural order in D2O which can cause and increase in a compounds' binding affinity (Wang and Matsui, 1994). Tetramethylammonium salts (TMS) are used as an internal reference for the NMR to compare the complex to and determine if a shift occurs (Schneider and others, 1998).

Solid inclusion complex. A solid inclusion complex gives the researcher the complex as a physical solid to perform further structural characterization studies. With a solid inclusion complex, the actual structure and fit of the two molecules can be further determined with

differential scanning calorimetry (DSC), X-ray diffractometry, morphological analysis using a scanning electron microscope, UV-Vis Spectrometry and ^{1}H-NMR (De Azevedo, 2002). An energy change is then formulated which is associated with the formation of the complex (De Azevedo, 2002). Preparation of samples used for a solid inclusion complex study include freeze-drying of the complex to draw off all moisture and form a solid powder (Echezarreta-Lopez and others 2000). The main purpose of forming the solid inclusion complex is to create a pure sample of the complex which can be used for further testing and analysis, such as for determination of percent weight of certain compounds once complexed. It can also be used to determine stoichiometry of the complex.

Microbiological Methodology Rationale

Saccharomyces cerevisiae. *Saccharomyces cerevisiae* is a eukaryotic budding yeast which is found on humans and other mammals, birds, soil, in wine and in beer. It can survive and proliferate in environments of pH 2.0- 6.0 and a_w levels ranging from 0.69- 0.83 being optimum for growth (Imai and Ohno, 1995; Laroche and others 2004). *Saccharomyces cerevisiae* can also survive and proliferate in environments up to 50 °Brix due to their systems of enzymes which can produce solutes that allow them to grow at lower a_w (Battey and others 2002). Its vegetative cells are gram positive while the ascospores it produces are gram negative. Used for many purposes in the food and pharmaceutical industries, it is mostly widely known as baker's yeast as it is the key fermentative yeast for brewing beer and making wine.

In people, *Saccharomyces cerevisiae* can cause infections in those that are immunocompromised (Salonen and others 2000). An *S. cerevisiae* infection can cause pneumonia, liver abscess, sepsis, fungemia, and other diseases (Aucott and others 1990). It has also been shown to be isolated from periodontal lesions found in HIV-infected individuals (Jabra-Rizk and others 2001). A known spoilage microorganism in the food industry, especially specific to fruit juices, *Saccharomyces cerevisiae* is often the target microorganism for heat processing and other such treatments to rid a product of yeasts (Elez-Martinez and others 2004). With high water activities in beverages many hurdles are used, such as preservatives, high pH, and high sugar levels that *Saccharomyces cerevisiae* are able to overcome (Battey and others, 2002). For example, *Saccharomyces cerevisiae* has the ability to adapt its growth to the presence of acetic, propionic, and benzoic acid (Malfeito Ferreira and others 1997). With such high

fermentation rates it is possible that it is able to overcome obstacles these quickly, having been shown to repair itself during growth, showing adaption to its surroundings (Malfeito Ferreira and others, 1997). The adaption abilities may be one reason why there is such a spoilage factor with *S. cerevisiae* in the food industry (Malfeito Ferreira and others, 1997).

There is a significant presence of *Saccharomyces cerevisiae* in the food production facilities as it is the main yeast spoilage concern in soft drink and juice plants (Battey and others, 2002). In one 2005 study within a processing facility, 99% of the samples showed contamination with *Saccharomyces cerevisiae* range of 10^6- 10^8 CFU per gram (Glover and others 2005). These values demonstrate its propensity to develop and populate in processing facilities. It has been shown to be ubiquitous in foods and raw materials. In juice studies it was shown that *Saccharomyces cerevisiae* showed more of a propensity for survival in heat processed juices than in liquid cultures of malt broth (Pieckova and Samson, 2000). Due to the fact that it is not a pathogenic organism there are no regulations in place for dealing with the yeast despite proper cleaning and sanitizing procedures.

Paecilomyces variotii. *Paecilomyces variotii* is a filamentous fungus with thick walled hyphae and ascopsore production. It is found in soil, decaying plant life, insects, and food products. *Paecilomyces variotii* has a pH range of 3.9 to 6.9 with an optimum growth at pH 5.9. Its water activity (a_w) maximum for growth is 0.793 as *Paecilomyces variotii* is a xerotolerant fungi (Wheeler and Hocking, 1988; Pieckova and Samson, 2000; Estevez and others 2005). It is also a heat tolerant fungi, therefore often found in heat treated products though it rarely forms ascospores as a result of treatment (Houbraken and others 2008). It is able to grow in temperatures up to 50 ºC, deeming it one of the most heat resistant fungi known due to its ability to produce ascospores. It has been shown to survive heat processes at 93 ºC for 5 minutes (Pieckova and Samson, 2000). The ability to survive is due to the thick-walled hyphae and chlamydospores which are produced in the budding phase of growth (Pieckova and Samson, 2000). The fungus is also able to grow at low oxygen levels and in the presence of preservatives, specifically sorbate, which proves a challenge in food manufacturing facilities (Houbraken and others, 2008; Pieckova and Samson, 2000).

Paecilomyces variotii is commonly associated with keratitis and soft tissue infections in immunocompromised patients, though no mycotoxins are known to be produced (Pieckova and

Samson, 2000). It is theorized to enter the body through the respiratory tract and can also be found in exposed wounds from catheters (Groll and Walsh, 2001). Once in the body, *Paecilomyces variotii* become distinctly similar to microconidia and can then travel freely within the patients bloodstream to cause infection (Groll and Walsh, 2001). It is most commonly associated with endocarditis which can lead to death (Houbraken and others, 2008). In food its route of contamination is unknown though it is found most frequently in pasteurized fruit beverages and is the most frequently encountered spoilage microorganism in multiple foods, especially oil based products. Examples include margarine, cheese, dried fruit, seeds, cereal, and peanuts (Pieckova and Samson, 2000). (Pieckova and Samson, 2000). Due to the fact that it is not a pathogenic organism there are no regulations in place for dealing with the fungus despite proper cleaning and sanitizing procedures in a processing facility.

Minimum inhibitory concentration (MIC) studies. Food spoilage is often contributed to the fermentation process microorganisms undergo producing CO_2 and ethanol which cause off tastes and textures in the food product (Malfeito Ferreira and others, 1997). MIC studies determine the minimum amount of antimicrobial concentration needed to inhibit visible growth of microorganisms over a set period of time. Therefore, the lower the number value derived by MIC studies the more effective the compound. There are tests for pathogens, yeasts, molds, etc. A study testing the antimicrobial ability of cinnamic acid against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium showed a relatively high MIC value, which is not desired, of 5.0 and 7.5 $mmol^{-1}$, respectively (Olasupo and others 2003). This study holds some of the first data for cinnamic acid against these compounds (Olasupo and others, 2003). As previously stated, cinnamic acid also shows inhibitory effects on kiwi and honeydew melon (Burt, 2004). A cinnamic acid derivative, cinnamaldehyde also holds a slight effectiveness in cooked shrimp, yogurt, and alfalfa seeds (Burt, 2004).

Another study working with the parent compound of cinnamic acid, as well as many of its derivatives, reported MIC study results of 2.34 log 1/MIC against *S. aureus*, 2.34 against *B.subtilis*, 2.39 against *E.coli*, 2.69 against *C. albicans*, and 2.69 against *A. niger* (Narasimhan and others 2004). The study also showed MIC values of 2.55-3.10 against certain fungi (Narasimhan and others, 2004). A similar study correlating pH with titratable acidity and antimicrobial presence found that *Saccharomyces cerevisiae* was still able to grow at levels as

low as pH 3.8 with a preservative concentration of 325 ppm (Battey and others, 2002). This study was able to show a correlation between pH and preservative concentration. When pH was not a factor and high levels of preservatives used they still showed growth and vice versa (Battey and others, 2002).

The MIC studies allow a researcher to define the range of effectiveness of a test compound or inclusion complex's concentration against specific microorganisms. The results then define which concentrations are the most effective, the lower the better, against the microorganisms in question and allow for a numerical value determination of effectiveness. These models can then be mimicked in the product to be produced in a processing facility. Although there are many different varieties of MIC studies, whether the researcher is using agar wells or aqueous solution, the results are able to predict contamination models in real time. Further studies can then be conducted using standard plate counts to determine the concentration of the microorganism and its growth rate at the different preservative levels tested.

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Tables & Figures

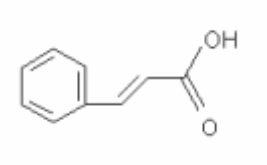

Figure 2.1 Chemical structure of *trans*-cinnamic acid.

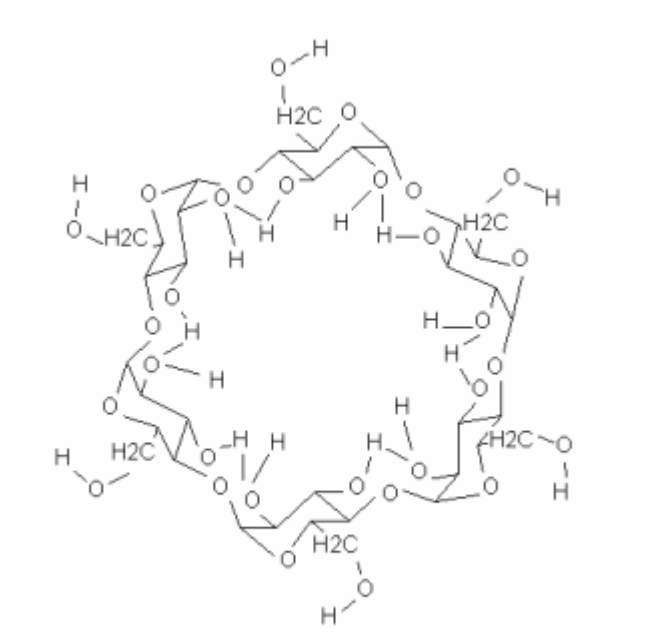

Figure 2.2 Chemical structure of α-cyclodextrin.

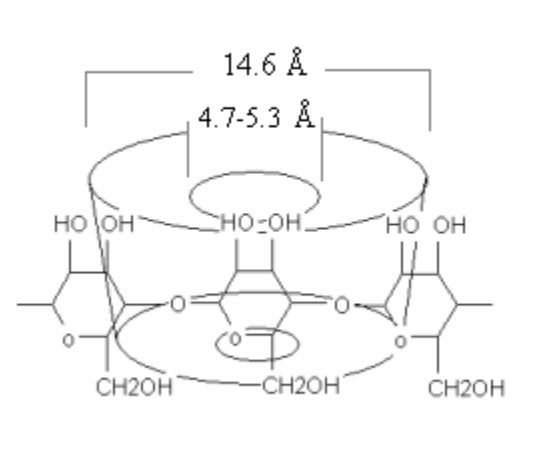

Figure 2.3 Molecular dimensions of α-cyclodextrin.

CHAPTER 3: THE FORMATION OF CINNAMIC ACID: A-CYCLODEXTRIN INCLUSION COMPLEXES AND THEIR CHARACTERIZATION

Abstract

Characterization of the cinnamic acid: α-cyclodextrin complex was important to investigate. Phase solubility tests were conducted to show the maximum amount of cinnamic acid that α-cyclodextrin was able to host and the maximum concentration of α-cyclodextrin that could host the cinnamic acid. Results indicated that 0.990 g/L of cinnamic acid in aqueous solution was able to be complexed. This concentration had two times the solubility of the initial 0.5 g/L of free cinnamic acid in aqueous solution. Proton nuclear magnetic resonance (H-NMR) was used to determine the conformation of the molecules, both guest and host, the affinity to bond, and the association constant of the reaction. Results indicated that the cinnamic acid guest molecule was shifting at the site of the benzene ring to conform to the rigid structure of the α-cyclodextrin host molecule. It was also shown that the α-cyclodextrin had an association constant of $K_{1:1}$= 817.3 M^{-1} and $K_{1:2}$= 21.7 M^{-1}. Further H-NMR studies were done to reflect the stoichiometry of the complex, which was a 1:2 ratio. Further studies to collect a solid inclusion complex allowed for information pertaining to the percent weight of cinnamic acid found within the complex. Results yielded an 8.9% weight to weight ratio of cinnamic acid in the complex. These results indicate that the cinnamic acid undergoes much more strain to bind with the interior cavity of the α-cyclodextrin. It also shows that the cinnamic acid undergoes fast action binding that leaves it free to incorporate in to multiple molecules of α-cyclodextrin.

Introduction

The Food and Drug Administration (FDA) released a study in November 2005 regarding benzene levels in soft drinks and fruit beverages on the market (CFSAN and Safety, 2006). Benzene, a known carcinogen, was being produced by a breakdown of the preservative sodium benzoate, which was reacting with ascorbic acid (FDA, 2006). The dissociation mechanism was accelerated by elevated temperatures and exposure to light (CFSAN and Safety, 2006). In want of a new, natural preservative cinnamic acid was chosen by industry. A poor solubility of 0.5 g/L aqueous solution and a poor wettability necessitated an inclusion complex with α-cyclodextrin. α-Cyclodextrin was chosen as the host molecule for cinnamic acid, as opposed to cyclodextrins many other forms, due to its smaller interior diameter, of approximately 5 Å and its solubility of 149 mg/L in a water-based solution (Hashimoto, 1996).

To characterize this inclusion complex of cinnamic acid and α-cyclodextrin in a water based solution, multiple methods of investigation were chosen. To determine the maximum amount of cinnamic acid that could be complexed with α-cyclodextrin, a phase solubility study was conducted. To quantify a conformational change on the molecular level between the guest and host molecules, proton nuclear magnetic resonance (H-NMR) was used. Two experimental methods were chosen for the H-NMR study to yield data of the complex stoichiometry as well as an association constant. Finally, a solid inclusion complex was formed to physically obtain the complex as well as to determine the ratio of cinnamic acid to α-cyclodextrin in the complex for future microbial studies.

Materials & Methods

Materials. *trans*-Cinnamic acid of 98 +% purity supplied by Arcos Organics (Geel, Belgium). α-Cyclodextrin, CAVAMAX® W6 pharma cyclohexaamylose supplied by Wacker Fine Chemicals (Munich, Germany). Ethyl alcohol, absolute, 99.5%, A.C.S. reagent supplied by Arcos Organics (Geel, Belgium). Puradisc 25PP disposable filter device, 0.45 µm polypropylene supplied by Whatman, Schleicher & Schuell (Florham Park, New Jersey, USA). Latex free 10 mL syringe supplied by Becton Dickinson & Co. (Franklin Lakes, New Jersey, USA). UV-Vis spectrophotometer, UV-2101PC supplied by Shimadzu (Kyoto, Japan). Deuterium oxide, 99.9 atom% D supplied by Aldrich (Steinheim, Germany). NMR Sample Tubes 507-HP supplied by

Chemglass (Landisville, New Jersey, USA). NMR Inova400 ROBOT supplied by Varian (Palo Alto, California, USA). Filtration Product, 75 mm Filter Unit- 500 mL supplied by Nalgene (Rochester, New York, USA). Sentry Freezemobile 12SL freeze dryer supplied by Virtis (Gardiner, New York, USA). NutsPro- NMR Utility Transform Software- Professional for Windows supplied by Acorn NMR, Inc. (Livermore, California, USA).

Phase Solubility Analysis. Seven glass bottles with screwtop lids were each filled with 10 mL distilled H_2O and labeled as 0 mM α-CD, 25 mM α-CD, 50 mM α-CD, 75 mM α-CD, 100 mM α-CD, 125 mM α-CD, and 145 mM α-CD. The appropriate amount of α-cyclodextrin (α-CD) was then added to the distilled H_2O as the label describes. With the lids tight, the solutions were placed in the darkened mechanical shaker for 24 hours at 25 ºC at 250 rpm. After 24 hours the bottles were extracted from the shaker and an excess of cinnamic acid were added to each bottle with 0.05g/10 mL of the aqueous solution. Once the lids were replaced, the bottles were set back in the darkened shaker for 48 hours at 25 ºC at 250 rpm. The samples were then pulled and stored in a stationary, dark place to allow the excess cinnamic acid to settle out of solution for 24 hours. The solution was then extracted with a 10 mL syringe and filtered into a test tube using a 0.45 µm filter tip. For samples ranging from 0 mM- 75 mM, 1 mL of 50% ethanol was added to the aqueous solution. For samples ranging from 100 mM- 145 mM, 0.1 mL 50% ethanol was added. These solutions were placed into the shaker at 25 ºC at 250 rpm for 30 minutes. The samples were removed and 1 mL 100% ethanol was added to all samples. These samples were then transferred to UV-Vis disposable cuvettes and run on the UV-Vis spectrophotometer. Settings for the UV-Vis spectrophotometer included a wavelength range of 230-330 nm with a specific target wavelength of 269.5 nm. This procedure was done in triplicate.

Phase solubility allowed the observation of the maximum amount of cinnamic acid able to be complexed with α-cyclodextrin. Once the multiple concentrations of α-cyclodextrin with excess cinnamic acid were run through the UV-Vis spectrophotometer, data was extracted from the concentration data given at a wavelength of 269.5 nm. Each of these individual numbers was then added as the y-value in the equation $y = 0.3984x - 0.0565$, which has been derived from a standard equation of the line for cinnamic acid in ethanol. The newly calculated values determine the amount of cinnamic acid in mg/L found in the aqueous solution. These values

plotted on the y-axis against the concentration of α-cyclodextrin on the x-axis yield a phase solubility diagram.

Nuclear Magnetic Resonance (NMR).

The Benesi-Hildebrand Method. Two stock solutions were prepared by labeling two glass screwtop bottles 2 mM cinnamic acid stock and 140 mM α-cyclodextrin stock, respectively. For the 2 mM cinnamic acid stock solution 14.82 mg of cinnamic acid was placed in 50 mL of deuterated oxide (D_2O). The stock solution was placed in the mechanical shaker for 24 hours at 25 ºC at 250 rpm. For the 140 mM α-cyclodextrin stock solution 1362.2 mg was placed in 10 mL of deuterated oxide (D_2O). The stock solution was also placed in the shaker for 24 hours at 25 ºC at 250 rpm. After these compounds had solubilized they were removed from the shaker and NMR sample tubes were labeled Run 1- Run 8. These Runs were distinguished by the amount of cinnamic acid stock and α-cyclodextrin stock included in each of the sample NMR tubes. Each included 0.35 mL of the cinnamic acid stock. Stock α-cyclodextrin was added in the following quantities: 0 mL, 0.05 mL, 0.1 mL, 0.15 mL, 0.2 mL, 0.25 mL, 0.3 mL, 0.35 mL. These samples were processed by the Inova400 ROBOT. The spectrographs retrieved were processed using the NUTS computer program.

Method of continuous variation (Job's plot). Nine glass bottles with screwtop lids were acquired and labeled Run 9- Run 17. These aqueous samples were arranged with cinnamic acid concentrations and the α-cyclodextrin concentrations and 0.2 mM of cinnamic acid and 1.8 mM of α-cyclodextrin; 0.4 mM cinnamic acid and 1.6 mM of α-cyclodextrin; 0.6 mM cinnamic acid and 1.4 mM of α-cyclodextrin; 0.8 mM cinnamic acid and 1.2 mM α-cyclodextrin; 1 mM cinnamic acid and 1 mM α-cyclodextrin; 1.2 mM cinnamic acid and 0.8 mM α-cyclodextrin; 1.4 mM cinnamic acid and 0.6 mM α-cyclodextrin; 1.6 mM cinnamic acid and 0.4 mM α-cyclodextrin; 1.8 mM cinnamic acid and 0.2 mM α-cyclodextrin. Once both of these compounds were added to the appropriate vial, 10 mL D_2O was added to put them together in solution. They were then placed in the Innova mechanical shaker for 48 hours at 25 ºC at 250 rpm. Once they were finished, 0.7 mL was drawn from each of the bottles and placed in an appropriately labeled NMR sample tube. These samples were then taken to be processed by the Inova400 ROBOT.

The spectrographs retrieved were processed using the NUTS computer program. These procedures were done in triplicate.

Solid Inclusion Complex. Twenty-six grams of α-cyclodextrin were mixed in 1 L of distilled H_2O. The α-cyclodextrin solution was then shaken for 24 hours at 25 ºC at 250 rpm. After 24 hours, 15 grams of cinnamic acid were added and the aqueous solution placed back in the shaker for 48 hours at 25 ºC at 250 rpm. The aqueous solution was poured in to the filter sterilizer using vacuum pressure. The filtered solution was then separated into 500 mL portions and placed in two wide, shallow dishes, covered with plastic wrap, frozen, and the samples were lyophilized over four days. The samples were then removed and stored in a concealed bottle in a desiccator, which was kept in a darkened environment. This procedure was done in triplicate.

Results & Discussion

Phase Solubility Analysis

The curve shown in Figure 1 shows the maximum amount of cinnamic acid which can be complexed by α-cyclodextrin. The phase diagram curve shows the peak at approximately 990.29 ppm aqueous solution, which denotes the maximum amount of cinnamic acid able to be held by α-cyclodextrin. Cinnamic acid's optimum uptake was seen at 0.04 M of α-cyclodextrin in aqueous solution. Literature has shown results with estimations at 170 M aqueous soltuion, equal to 2,518 ppm, for a cinnamate ion: α-cyclodextrin inclusion complex though tris(hydroxymethyl)aminomethane base and hydrochloride were used as solvents to aid in complete solubility of the complex (Connors and Rosanske, 1980). This value is double the solubility that was found in the present study. One reason for this may be that the distilled water used to solubilize the complex was not acidified to mimic a soft drink or fruit beverage and no solvents were used making it much more difficult to incorporate the complex. The non-acidification of the solutions test was initially done to achieve a baseline test for solubility.

H-NMR

Benesi-Hildebrand Method

The Benesi-Hildebrand method conducted using H-NMR allowed further data processing to determine the chemical shift of specific protons within both guest and host molecules as well

as the calculation of the complex's association constant. Figure 2 shows the chemical shift noted between the smallest amount of α-cyclodextrin present and the highest amount. Table 1 shows the amount of conformation, or shift, performed by the cyclodextrin molecule. As can be seen, there is very little conformational shift present. The largest shift at 0.089 ppm, occurred with hydrogen 6a which is found on the exterior of the molecule. Hydrogen 6a is part of the chain which hangs off of the cyclic molecule and is oriented as a tail off of the molecule, as seen in Figure 3. The hydrogen is theorized to shift to accommodate a host (Irwin PL, 1993). Irwin (1993) has stated that little conformation is expected from the α-cyclodextrin as a very rigid structure will keep the molecule mostly stationary. Support for this argument can also be seen with the disruption of the aromatic protons which where more disturbed by the binding than the protons which had the largest shifts. When viewing the chemical shift, based on the Benesi-Hildebrand method, it is seen that the majority of the conformation is coming from the cinnamic acid molecule itself. The cinnamic acid shifts can be seen in Figure 4 and Table 2. One study found similar results, seeing the majority of movement in the shift of the host molecule than with a cyclodextrin molecule (Denadai and others 2007). For cinnamic acid, the major shifts of 0.178 ppm and 0.189 ppm occurred with hydrogen groups 4, 5, and 6 as well as hydrogen groups 3 and 7, respectively. As these hydrogens are found on the benzene ring, this then points to its conformation and ability to fit within the host of the α-cyclodextrin. Irwin (1993) has stated similar conformations, where the largest shifts were associated with the hydrogens on the benzene ring allowing the researcher to believe that this moiety inserts and attaches within the cyclodextrin cavity.

An association constant can be calculated from the information given in the Benesi-Hildebrand method. The association constant is a quantitative description of the affinity for the α-cyclodextrin and the cinnamic acid that binds to it. Stability constants are defined by the equations where *K* represents the association constant, [S] represents the substrate, [L] represents the ligand, and [SL] represents the substrate-ligand complex (Rosanske and Connors, 1980):

$$K_{11} = \frac{[SL]}{[S][L]}$$

$$K_{12} = \frac{[SL_2]}{[SL][L]}$$

Although the association constant can be determined through phase solubility it is a more defined number if calculated using the Benesi-Hildebrand Method. The association constant of the cinnamic acid: α-cyclodextrin inclusion complex came to $K_{1:1}$= 817.3 M^{-1} and $K_{1:2}$= 21.7 M^{-1}. These results were calculated using the equation of the line acquired from the double reciprocal plot plotting 1/Δδ (M) by 1/α-CD (M). The equation was derived from the Benesi-Hildebrand Equation, shown below, where ΔA represents the change in absorbance, G represents the guest, H the host, and K_a the association constant, and b based on the Beer-Lambert Law:

$$\frac{1}{\Delta A} = \frac{1}{b\Delta[G]_o K_a} \times \frac{1}{[H]_o} + \frac{1}{b\Delta[G]_o}$$

By solving for the x-intercept the association constant, K_a, setting $\frac{1}{\Delta A} = 0$, is found to be $K_a = -\frac{1}{[H]_o}$. The results then show the calculation of an association constant of 817.3 M^{-1} for a 1:1 linear association (Connors and Rosanske, 1980). These ratio's association constants are much smaller than those values that have been calculated by other studies which have found a 2:1 reaction to yield an association constant of 60 M^{-1} for cinnamic acid and 15 M^{-1} for the cinnamate ion (Connors and Rosanske, 1980; Rosanske and Connors, 1980). Though showing a very weak interaction the reason for the difference is that the association constant is very dependent on the environment of the test including sensitivity to pH, ionic strength, temperature, etc. (Connors and Rosanske, 1980).

Method of continuous variation

The method of continuous variation yields information regarding the stoichiometry of the complex (Connors and Rosanske, 1980). Once the spectra were processed and the values of the peaks were identified, plots of the chemical shifts above 0.5 ppm aqueous soltuion for both cinnamic acid and α-cyclodextrin were made. The change in shift observed multiplied by the concentration of cinnamic acid or α-cyclodextrin, depending on the plot, was then plotted against *x*, a value denoting an increase in concentration. The plot of the shift observed in α-cyclodextrin can be seen in Figure 5. As can be seen, there was very little shift of the protons in the α-cyclodextrin when complexed. As explained above, the shift value was expected due to the rigidity of the molecule. The molecule of major shift was the guest, cinnamic acid. This

cinnamic acid shift can be seen in Figure 6. Cinnamic acid has its major shift with the protons associated with the benzene molecule. The Job's plot design was developed to plot the data as a parabola. Based on the structure of the method as ratio based concentrations of cinnamic acid and α-cyclodextrin a parabola was expected. The plotted data in Figure 6 show a loosely based parabola with defined peaks. Each peak, for both proton shifts represented, represent an individual parabola. Therefore, each proton shift contains the peaks of two overlapping parabolas. The peaks of shift H4,5,6 are oriented over 0.3 and 0.5. The peaks of shift H3,7 are positioned over 0.3 and 0.7 on the x-axis. These values are highly significant as the two peaks suggest a 2:1 stoichiometry. A 1:1 stoichiometry ratio would not be off center from 0.5 as is shown in Figure 6 (Dodziuk, 2006). A 2:1 stoichiometry is expected of a cinnamic acid: α-cyclodextrin complex (Dodziuk and others 1999; Rosanske and Connors, 1980). The 2:1 stoichiometry also shows that the host is able to enter at either end of the α-cyclodextrin, and which end is preferred is unknown (Rosanske and Connors, 1980).

Solid Inclusion Complex

A solid inclusion complex allows for a physical characterization of the complex. With the powder form of the complex mathematical equations were used to quantify the amount of cinnamic acid found in the powdered complex. The resulting complex was a lightweight, fluffy, white powder which was pulverized in a stomacher to condense it and make it a more manageable substance. The UV-Vis spectrophotometer was used to run the diluted cinnamic acid: α-cyclodextrin complex at wavelength 269.5 nm. The recorded cinnamic acid concentration at this wavelength was 890.37 mg/L in aqueous solution. This value was then used to determine the percent weight of cinnamic in the complex using the equation below:

$$\left(\frac{890.37\,mg}{1\,L}\right)\times\left(\frac{1\,g}{1000\,mg}\right)=0.89037\,g/L$$

$$\left(\frac{0.89037\,g}{1\,L}\right)\times\left(\frac{1\,L}{1000\,mL}\right)=\left(\frac{10\,mL}{1}\right)=\frac{0.0089037\,g}{10\,mL}$$

$$\left(\frac{0.0089037\,g}{10\,mL}\right)\div\left(\frac{0.1\,g}{10\,mL\ complex}\right)=0.089037$$

$$0.089037\times100\%=8.9\%\ \textit{weight to weight cinnamic acid}$$

Therefore, it was calculated that out of the complex in hand, due to lyophilization, 8.9% weight to weight in the complex can be attributed to the cinnamic acid. This is expected as the ratio of a smaller percentage of guest and a larger percent of host is needed to drive the reaction. This information also contributes to a 2:1 stoichiometry as much more of the α-cyclodextrin must be present compared to the cinnamic acid to fully coerce the inclusion complex formation. With this value it is shown that a 2:1 stoichiometry is present and at what weight value the cinnamic acid is present in comparison to α-cyclodextrin.

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Tables & Figures

	Proton Shift (ppm)						
α-Cyclodextrin (mM)	H1	H2	H3	H4	H5	H6a	H6b
10	0.000	0.000	0.000	0.000	0.000	0.000	0.000
20	0.002	0.021	0.016	0.004	0.003	0.080	0.002
30	0.002	0.015	0.024	0.013	0.009	0.083	0.006
40	0.003	0.017	0.028	0.015	0.011	0.084	0.008
50	0.003	0.020	0.028	0.017	0.015	0.086	0.010
60	0.003	0.020	0.031	0.018	0.017	0.087	0.012
70	0.004	0.021	0.031	0.020	0.019	0.089	0.013

Table 3.1 Individual proton shift calculations (ppm) of α-cyclodextrin (mM) in the inclusion complex at a set concentration of 1 mM cinnamic acid.

	Proton Shift (ppm)			
α-Cyclodextrin (mM)	H1	H 4,5,6	H 3,7	H2
0	0.000	0.000	0.000	0.000
10	0.050	0.089	0.098	0.058
20	0.053	0.105	0.153	0.089
30	0.032	0.183	0.186	0.087
40	0.031	0.197	0.204	0.088
50	0.025	0.197	0.206	0.086
60	0.020	0.179	0.190	0.075
70	0.020	0.178	0.189	0.075

Table 3.2 Individual proton shift calculations (ppm) of cinnamic acid set at 1 mM over increasing α-cyclodextrin concentrations (mM) within the inclusion complex.

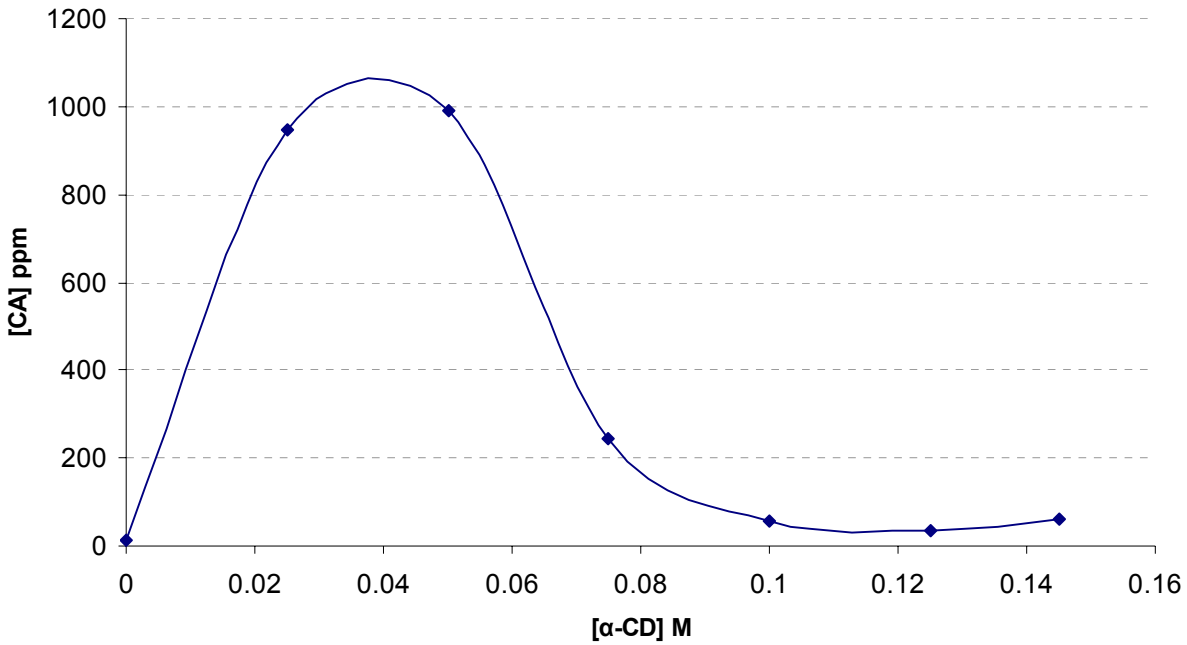

Figure 3.1 Phase solubility diagram of α-cyclodextrin: cinnamic acid complex plotting α-cyclodextrin concentration (M) against cinnamic acid concentration (ppm)

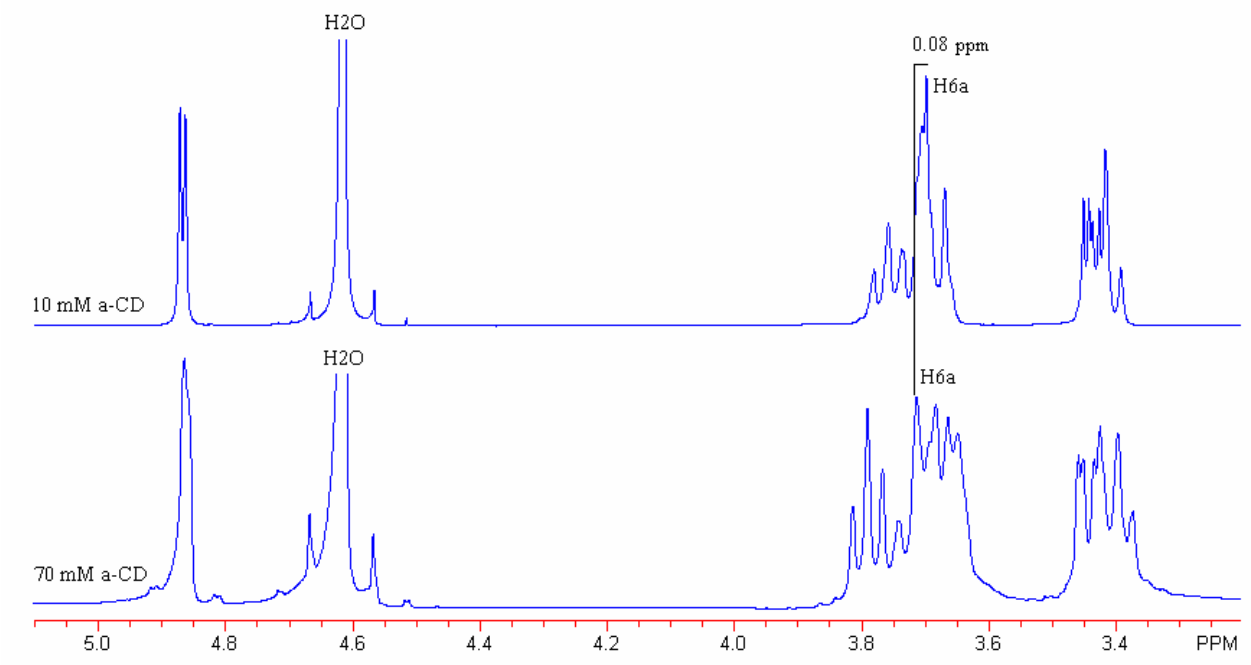

Figure 3.2 Benesi-Hildebrand Method chemical shift comparisons between 10 mM and 70 mM of α-cyclodextrin.

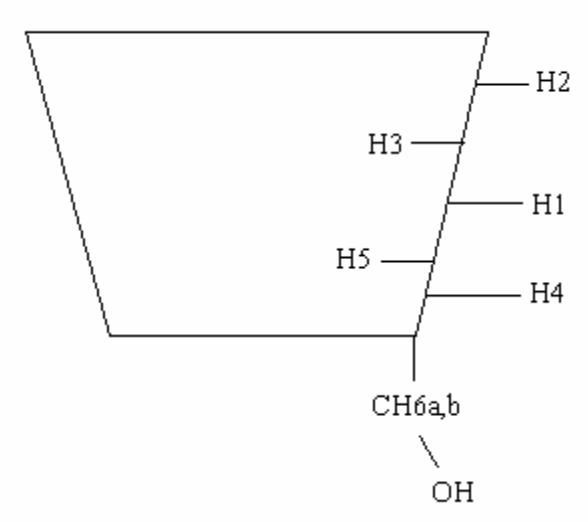

Figure 3.3 Two dimensional view of a cyclodextrin molecule and the orientation of protons found in the interior and exterior of the cyclic molecule.

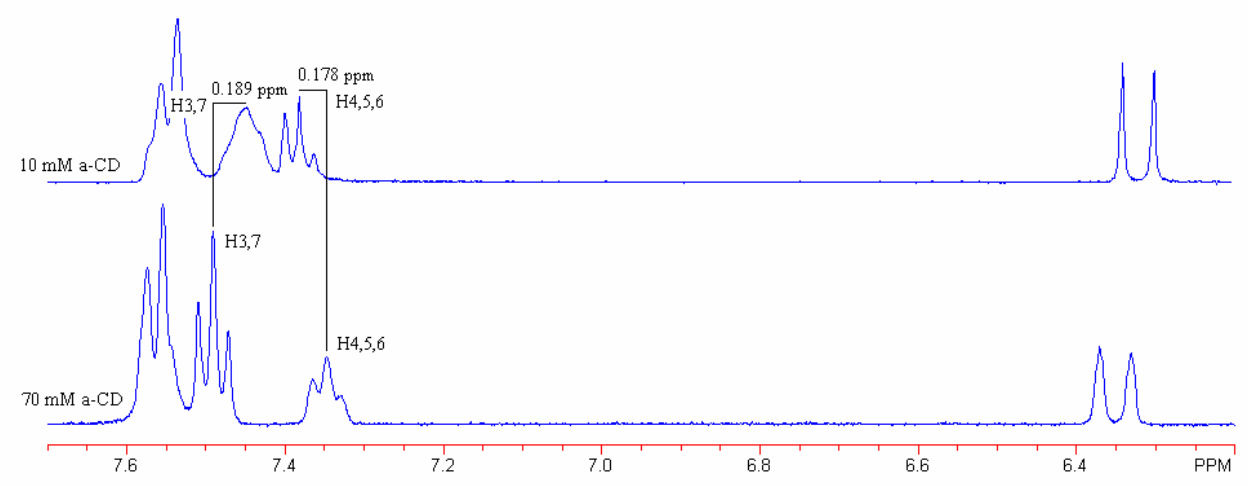

Figure 3.4 Benesi-Hildebrand Method chemical shift comparisons of cinnamic acid between 10 mM and 70 mM α-cyclodextrin.

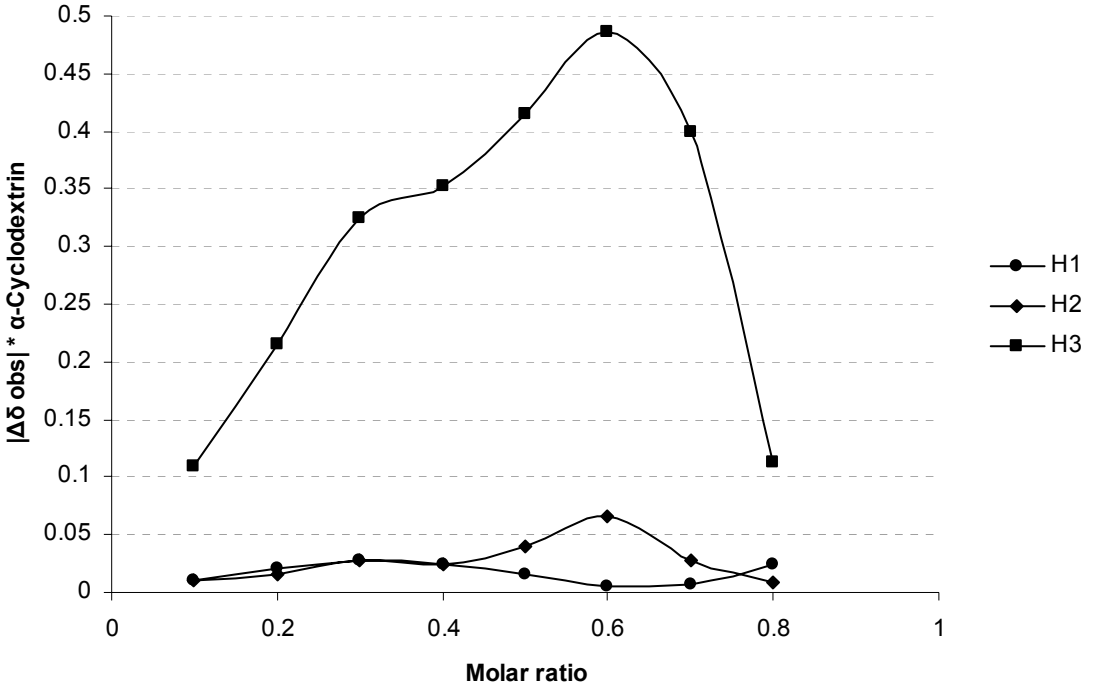

Figure 3.5 Continuous variation plot (Job's plot) for protons of α-cyclodextrin complexed with cinnamic acid.

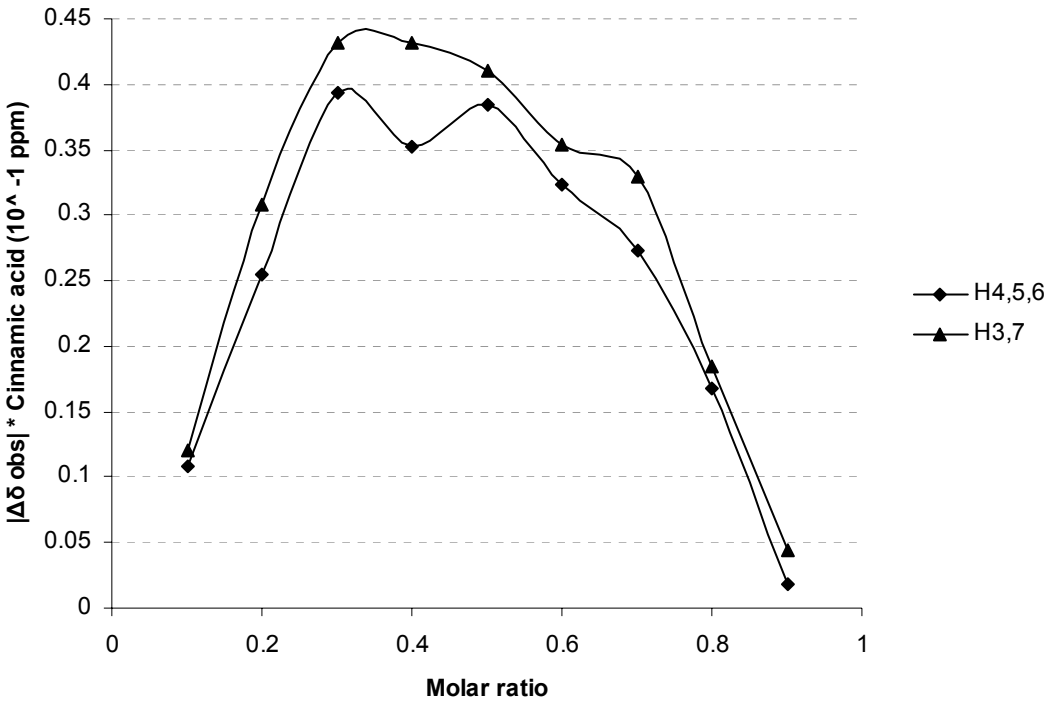

Figure 3.6 Continuous variation plot (Job's plot) for protons of cinnamic acid complexed with α-cyclodextrin.

CHAPTER 4: MICROBIOLICAL STUDIES OF CINNAMIC ACID: α-CYCLODEXTRIN INCLUSION COMPLEXES

Abstract

Minimum inhibitory concentration studies were performed using cinnamic acid: α-cyclodextrin inclusion complex and microrganisms *Saccharomyces cerevisiae* and *Paecilomyces variotii*. Three methods were designed to determine the effectiveness of the complex at different concentrations. The first method tested different inoculation levels against increasing concentrations of the inclusion complex. Results were highly variable therefore poor indicators of this method as small aqueous amounts of 4 μL were pulled to be incubated and surveyed for visible turbidity denoting growth. A second method was designed to mimic this study through the use of larger sample sizes up to 3 mL for determination of growth. It also restricted the total colony count amount to 3.3 CFU/mL aqueous solution. This study showed that at the 3.3 CFU/mL inoculation level little to no inhibition ability was seen by the complex at varying concentrations for both organisms. To test the complex against its guest and host compounds a third method was designed. This method tested free cinnamic acid against free α-cyclodextrin against the inclusion complex. Results showed high inhibition of the microorganisms by free cinnamic acid, no inhibition by free α-cyclodextrin and little to no inhibition by the complex, as initially seen in Method 2. The limited complex inhibition effects may have been due to the inability of the complex to deliver the guest effectively to exhibit its antimicrobial effects. Another theory is due to the presence of α-cyclodextrin in the complex; it may act more as a food source for the microorganisms, as opposed to an inhibitor.

Introduction

With the release of a Food and Drug Administration (FDA) November 2005 study regarding benzene levels in soft drinks and fruit beverages, a want for a natural antimicrobial became top priority (CFSAN and Safety, 2006). Benzene, a known carcinogen, was being produced in the beverages by a breakdown of the preservative sodium benzoate. The dissociation mechanism of sodium benzoate reacting with ascorbic acid was accelerated by elevated temperatures and exposure to light resulting in free benzene (CFSAN and Safety, 2006). Cinnamic acid was the choice of the natural preservative to use but, with a poor solubility of 0.5 g/L in aqueous solution and a poor wettability it necessitated an inclusion complex with α-cyclodextrin. α-Cyclodextrin was chosen as the host molecule as opposed to cyclodextrins many other forms, due to its smaller interior diameter, of approximately 5 Å and its solubility of 149 mg/L in a water-based solution (Hashimoto, 1996).

Once complexed with α-cyclodextrin, it is theorized that the cinnamic acid will still be able to hold its antimicrobial properties. A strong antimicrobial in its powder form, it is now being used in aqueous form against the two most difficult microorganisms for soft drink and fruit beverage processing facilities: *Saccharomyces cerevisiae*, a yeast, and *Paecilomyces variotii*, a mold (Deak, 1996; Chambel, 1999; Battey and others, 2002). Three separate methods have been developed for the experimentation of the effectiveness of the complex. The first method will determine the ability of the α-cyclodextrin: cinnamic acid complex, at increasing concentrations, to stunt the growth of both *S. cerevisiae* and *P.variotii*. The second method will further study this same retardation of the microorganisms at a specific level of inoculation as determined by the processing facility. The third method will compare the effectiveness of the complex against free cinnamic acid as well as free α-cyclodextrin.

Materials & Methods

Materials. *trans*-Cinnamic acid of 98 +% purity supplied by Arcos Organics (Geel, Belgium). α-Cyclodextrin, CAVAMAX® W6 pharma cyclohexaamylose supplied by Wacker Fine Chemicals (Munich, Germany). Solid inclusion complex supplied by Dina L. Romano's research (Blacksburg, Virginia, USA). *Saccharomyces cerevisiae* yeast culture supplied by Pepsi Co. (Valhalla, New York, USA). *Paecilomyces variotii* fungus culture supplied by Pepsi Co. (Valhalla, New York, USA). Microcentrifuge tubes 0.5 mL with secure

closure, supplied by Fisher Scientific (Pittsburg, Pennsylvania, USA). Innova 4230 Refrigerated incubator shaker (Edison, New Jersey, USA). Premium 100% Pure Apple Juice, 100% Vitamin C calcium enriched, Pressed from fresh apples, not from concentrate, 64 Fl.oz (1/2 gallon) 1.89 L, Pasteurized supplied by White House (Winchester, Pennsylvania, USA). Alpha-D(+)-glucose, anhydrous 99+% supplied by Acros Organics (Geel, Belgium). D-Fructose, reagent grade (crystal) supplied by Fisher Scientific (Fair Lawn, New Jersey, USA). Sucrose (α-D-Glucopyranosyl; β-D-fructofuranoside; Saccharose; Can Sugar) supplied by Sigma Chemical Company (St. Louis, Missouri, USA). Malic acid, FG, powder supplied by Presque Isle Wine Cellars (North East, Pennsylvania, USA). Nylon, 0.45 µm filter tips supplied by Fisher Scientific (Pittsburg, Pennsylvania, USA). Amsco Eagle 3011-C Gravity Steam Sterilizer supplied by Steris (Mentor, Ohio, USA). Maxi Mix II Type 37600 Mixer Vortex, 120 Volts, 40 Watts supplied by Barnstead/Thermolyne (Dubuque, Iowa, USA). Research pipettes, 10-100 µL, 100-1000 µL, supplied by Eppendorf (Hamburg, Germany). Redi-Tip pipette tips, 200 µL, 50- 1000 µL supplied by Fisher Scientific (Pittsburg, Pennsylvania, USA).

Minimum Inhibitory Concentration Studies.

Method 1. This method, dilution, and inoculation scheme was supplied by PepsiCo. Preparation of the media included 900 mL of distilled H_2O, 100 mL of preservative-free apple juice, 46.8 grams D-glucose, 59.4 grams fructose, 1.8 grams D-sucrose. This media was similar to the beverages a complex would be added to in respects to the pH, °Brix, and titratable acidity. The media was then stirred with no heat to incorporate all of the ingredients. Because the media contains such a high level of sugar it could not be autoclaved and was filter sterilized instead. The pH was taken once all was dissolved and adjusted to a pH of 3.4 using 1 M Malic acid in aqueous solution. Once the media was prepared it was separated into 200 mL amounts in four separate bottles with screwtop lids. Each bottle was labeled 1 mM [CA:αCD], 2 mM [CA:αCD], 3 mM [CA:αCD], and 4 mM [CA:αCD]. The appropriate amount of solid inclusion complex was added to each of these bottles and incubated at 25 ºC at 250 rpm for 24 hours. Once this was done the test tubes were organized with six test tubes per concentration per microorganism. Controls were formulated as well with a positive and negative control for both microorganisms. A standard plate count showed the amount of CFU per 2.7 mL in the aqueous stock solutions of *Saccharomyces cerevisiae* and *Paecilomyces variotii*. This was then used to diagram the dilution

scheme found in Figure 1. After the dilutions were made and inoculated, 4 µL of each aqueous dilution was pipetted into a microcentrifuge tube which was incubated at 25 ºC for five days. The microcentrifuge tubes were vortexed and visually checked for turbidity daily. A positive symbol (+) denoted visible turbidity therefore colony growth. A negative symbol (-) denoted no visible turbidity therefore no colony growth. This procedure was done in duplicate.

Method 2. This method was a modified version of Method 1 as supplied by PepsiCo. Preparation of the media included 900 mL of distilled H_2O, 100 mL of preservative-free apple juice, 46.8 grams D-glucose, 59.4 grams fructose, 1.8 grams D-sucrose. This media was similar to the beverages a complex would be added to in respects to the pH, °Brix, and titratable acidity. The media was then stirred with no heat to incorporate all of the ingredients. The pH was adjusted to a pH of 3.4 using 1 M malic acid. Once the media was prepared it was separated in to seven bottles of 200 mL with screwtop lids. Each of these bottles were labeled 1 mM [CA:αCD], 2 mM [CA:αCD], 3 mM [CA:αCD], and 4 mM [CA:αCD], control with cinnamic acid and no α-cyclodextrin, control with α-cyclodextrin and no cinnamic acid, and control with no cinnamic acid and no α-cyclodextrin. The solutions were mixed as defined by the labels. These aqueous solutions were then placed in the shaker for 24 hours at 25 ºC and 250 rpm. Once the media was prepared, it was filter sterilized into the test tubes with eight test tubes for each concentration and two for control media. The dilution schemes can be found in Figure 2. The final three test tubes, of the same dilution, each contained 3.3 CFU/mL of aqueous *Saccharomyces cerevisiae* and *Paecilomyces variotii*, respectively. These last tubes per concentration per microorganism inoculated were incubated at 25 ºC for five days. The test tubes were vortexed and visually checked for turbidity daily. A positive symbol (+) denoted visible turbidity growth. A negative symbol (-) denoted no visible turbidity therefore no growth. This procedure was done in triplicate.

Method 3. Preparation of the media included 900 mL of distilled H_2O, 100 mL of preservative-free apple juice, 46.8 grams D-glucose, 59.4 grams fructose, 1.8 grams D-sucrose. This media was similar to the beverages a complex would be added to in respects to the pH, °Brix, and titratable acidity. The media was then stirred with no heat to incorporate all of the ingredients. The pH was taken once all was dissolved and adjusted to a pH of 3.4 using 1 M

Malic acid. Once the media was prepared it was divided among 15 glass bottles. One set of five was labeled free cinnamic acid with each of those bottles divided by concentration of cinnamic acid alone; 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm. The second set of five bottles was labeled free α-cyclodextrin with concentrations of 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm. The last set of five was labeled [CA:αCD] with concentrations of 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm. Each bottle was then filled with the appropriate amount of free cinnamic acid, free α-cyclodextrin, and [CA:αCD] complex, all aqueous solutions. Controls were formulated as well with a positive and negative control for both microorganisms per method. These bottles were placed in the mechanical shaker for 24 hours at 25 ºC at 250 rpm. Once these were all prepared the dilutions were made for each compound, free cinnamic acid versus free α-cyclodextrin versus the complex, for all concentrations. Each of these were inoculated with 20 µL aqueous solutions of both *Saccharomyces cerevisiae* and *Paecilomyces variotii* as shown by Figure 3. These last tubes per concentration per microorganism inoculated were incubated at 25 ºC for five days. The test tubes were vortexed and visually checked for turbidity daily. A positive symbol (+) denoted visible turbidity therefore colony growth. A negative symbol (-) denoted no visible turbidity therefore no colony growth. This procedure was done in triplicate.

Results & Discussion

Method 1

After the full incubation period of five days was finished, the overall picture of results was not as expected. Found in Table 1, results, with a (+) or (-) indication for visible turbidity therefore growth, seemed to vary drastically and not follow a typical growth pattern as is expected with microorganisms. The stock solutions were given a plate count for *S. cerevisiae* of $1.13x10^7$ CFU per five mL stock and for *P. variotii* of $1.4x10^6$ CFU per five mL stock as enumerated by PepsiCo. As these values were not verified once received the dilution scheme became more of an estimate than an exact cell count. It is most commonly expected to see growth where a majority of cells are found first, followed by a breakthrough of the antimicrobial compound by the smaller total cell count. For this method, the microcentrifuge tubes inoculated with the highest number of yeast or fungi cells seemed to show turbidity after those tubes with fewer known cells, which indicated a possibility of sampling error.

Due to such a small amount, 4 μL of aqueous solution, drawn from a sample of up to 3.0 mL aqueous solution, which contained the initial inoculum, there is reason to doubt the proper sampling of the yeast and mold colonies. The fraction of a sample drawn from the dilution tubes may not have included the same amount of cells that would better represent the overall environment that was initially intended. While one sample, which was intended to have to more cells, may have only picked up the diluent and few yeast or mold cells, another sample may have done just the opposite. Where there are more cells there is more of a chance for those cells to proliferate despite inhibition ability by the complex. This pattern, however, was not shown as is apparent by the results of the experiment. Therefore, the second MIC method was produced to better represent the contamination of a water-based, acid-adjusted sample.

Method 2

This second method was developed to duplicate the industry acceptable level for yeasts and molds in beverage processing. Tolerance levels are acceptable for yeasts and molds in products that have undergone heat treatments as no food product is completely sterile. These tolerance levels, as stated by PepsiCo., were incorporated in to the dilution/inoculation scheme matching the upper range of contamination accepted, at 3.3 CFU/mL aqueous solution. A total plate count was made of the stock solutions prior to use. The plate count for *S. cerevisiae* was 3.7×10^6 CFU per mL stock and for *P. variotii* of 1.13×10^7 CFU per mL stock. Once incubated for the full five days the overall results showed little microbial inhibition, shown in Table 2. Although the higher concentrations of the complex yielded better inhibition power against both *S. cerevisiae* and *P.variotii* it could not maintain its inhibition longer than three to four days. In the food industry, this is a short shelf-life, especially for beverage life. One study shows that complexing with cyclodextrin led to a decrease in antimicrobial ability with every microorganism used in the investigation (Kral'ova and Bujdakova, 1996). It has also stated that an excess of cyclodextrin in the solution leads to complete elimination of antimicrobial ability (Kral'ova and Bujdakova, 1996). One study states the reasoning behind this low to no inhibition ability is due to the inclusion complexes being unable to penetrate through the cell membranes leaving the effector incapable of reaching the necessary site of inhibition (Kral'ova and Bujdakova, 1996). This may also have been the case in the low inhibition power of the cinnamic

acid: α-cyclodextrin inclusion complex tested as the conformation of the cinnamic acid within the α-cyclodextrin molecule cannot be completely defined.

The complex itself, at varying concentrations, showed a positive trend toward a higher level of inhibition ability with a higher concentration of complex. This positive trend was as expected but the fact that it did not inhibit the yeast or mold more than five days was disappointing. It has been shown in past studies that levels of free cinnamic acid, when added to an aqueous solution, exhibit antimicrobial properties though only to inhibit the maximum growth of *Saccharomyces cerevisiae* (Chambel, 1999). Using small concentration levels of lower than 1 mM it only showed enough inhibition of *S. cerevisiae* to slow its growth, not to completely inhibit it (Chambel, 1999). Other studies have shown weak inhibition of up to 50% of the bacteria in question, namely *E. coli* and *B. subtilis* (Sheu and others, 1975). Studies have shown that inhibition ability has come from cinnamic acid in its powder form, not in aqueous form, in the presence of spoilage microorganisms on fruit slices (Roller and Seedhar, 2002). Free cinnamic acid showed potential in the study, though concentrations could be much higher as it was not dissolved in water or a solvent to aid in solubility (Roller and Seedhar, 2002). Although these studies show potential for cinnamic acid alone they do not show cinnamic acid working in tandem with α-cyclodextrin. With cinnamic acid complexed to increase solubility this led to the development of the third method for the MIC studies.

Method 3

Method 3 was designed to compare the antimicrobial abilities of the complex against free cinnamic acid as well as free α-cyclodextrin. The importance of this study was to take all of the literature, which stated the benefits of free cinnamic acid as an antimicrobial and compare this data against Method 2 of this study, which showed little inhibition power by the complex. The method also allowed for a maximum of 500 ppm of free cinnamic acid in solution. The results, summarized in Table 3, showed the difference between their abilities. A total plate count was made of the stock solutions prior to use. The plate count for *S. cerevisiae* was 3.7×10^6 CFU per mL stock and for *P. variotii* of 1.13×10^7 CFU per mL stock.

As can be seen in Table 3, free cinnamic acid showed antimicrobial abilities against both *S. cerevisiae* and *P. variotii*. Although smaller parts per million (ppm) measurements of the compounds were used due to solubility restraints of the free cinnamic acid, a comparison can be

made. Over the five day period no growth was seen from the test tubes inoculated with either yeast or mold in the free cinnamic acid tubes. The ability of the free cinnamic acid to inhibit both *S. cerevisiae* and *P. variotii* was just as purposed by literature (Burt, 2004; Castelli and others 1999; Guerzoni and others 1990). All of these studies showed that free cinnamic acid could be used as an effective antimicrobial. Much of that data was derived from the powder form of the compound as opposed to aqueous solution, which is an interesting point and could be studied further.

Table 3 also shows the results of the test conducted with free α-cyclodextrin. These results were not a surprise as α-cyclodextrin has no antimicrobial properties whatsoever. These samples showed turbidity denoting growth as early as day two of the experiment giving the yeast and mold more of a food source than a preservative effect. Although α-cyclodextrin has many important properties dealing with solubility, protection from oxidation, stability, etc., the results were expected as no antimicrobial properties have been attributed to α-cyclodextrin (Connors, 1997; Hashimoto, 1996).

For the cinnamic acid: α-cyclodextrin complex results, they exhibited similar behavior to that of the previous method as far as the time the tubes lasted before visible turbidity was demonstrated. Although the two method's results are similar, it does show that the free cinnamic acid is much more effective as an antimicrobial than the complex itself. Though further studies could be designed to look at this more closely, it is possible that the α-cyclodextrin itself is affecting the antimicrobial properties of the complex. Although it aids drastically in the solubility of the cinnamic acid it could be acting as a starchy food source for the yeast and molds it is trying to inhibit. One study showed that β-cyclodextrin, when complexed with hydroxypropyl, also showed a reduction in the antimicrobial properties of the complex (Lehner and others 1994). Low concentrations of the complex showed no inhibition of the microorganisms and at higher concentrations minimal inhibition of the microorganisms was shown with no damage to the bacteria itself observed (Lehner and others, 1994). This study also used relatively low inoculation levels, similar to the present methodology, and still low inhibition ability was seen when the antimicrobial compound was complexed with cyclodextrin (Lehner and others, 1994). The study theorized that the reason for poor inhibition power when complexed was due to the poor bond strength of the molecules. On the other hand, this study also showed that a Gram-positive bacteria, *S. aureus*, showed complex inhibition ability due to

its gram orientation (Lehner and others, 1994). *S. cerevisiae* is a Gram-positive yeast with Gram-negative spores. If the Gram-positive trend held true it would seem there would be an increase in the complex inhibition power, due to the complex interaction with the cell wall. The inhibition, however, was not seen.

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Tables & Figures

Saccharomyces cerevisiae[1]						pH 3.4
[CA:αCD]						
1 mM	D2	n.g.	n.g.	n.g.	n.g.	n.g.
2 mM	D4	n.g.	n.g.	n.g.	n.g.	n.g.
3 mM	D4	D4	n.g.	n.g.	n.g.	n.g.
4 mM	D4	D4	D5	D5	n.g.	D4
	6E+02	6E+01	6E+00	6E-01	6E-02	6E-03
Total CFU calculated per test tube						
Paecilomyces variotti[1]						pH 3.4
[CA:αCD]						
1 mM	D3	D3	D4	D4	n.g.	n.g.
2 mM	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.
3 mM	D4	D3	D3	D3	D4	D5
4 mM	D2	D4	D4	D4	D5	D5
	8.4E+01	8.4E+00	8.4E-01	8.4E-02	8.4E-03	8.4E-04
Total CFU calculated per test tube						

[1]n.g. indicates no growth; D1 indicates growth day 1; D2 indicates growth day 2; D3 indicates growth day 3; D4 indicates growth day 4; D5 indicates growth day 5

Table 4.1 Data table recording day turbidity (D1-D5) was seen to indicate growth over a five day incubation period at 25 °C with *Saccharomyces cerevisiae* and *Paecilomyces variotii*. Each trial tested in duplicate with calculated inoculation levels from stock solution plate count conducted for both organisms against the strength of different concentrations of [CA:αCD].

Saccharomyces cerevisiae					pH 3.4
Incubation Period	Day 1	Day 2	Day 3	Day 4	Day 5
[CA:αCD]					
1 mM	-/-/-	-/-/-	+/+/+	+/+/+	+/+/+
2 mM	-/-/-	-/-/-	+/-/-	+/+/+	+/+/+
3 mM	-/-/-	-/-/-	-/-/-	+/+/+	+/+/+
4 mM	-/-/-	-/-/-	-/-/-	-/-/-	+/+/+

Paecilomyces variotii					pH 3.4
Incubation Period	Day 1	Day 2	Day 3	Day 4	Day 5
[CA:αCD]					
1 mM	-/-/-	-/-/-	+/+/+	+/+/+	+/+/+
2 mM	-/-/-	-/-/-	+/+/+	+/+/+	+/+/+
3 mM	-/-/-	-/-/-	-/-/-	+/+/+	+/+/+
4 mM	-/-/-	-/-/-	-/-/-	-/-/-	+/+/+

Table 4.2 Data tables recording turbidity to indicate growth over a five day incubation period at 25 °C with *Saccharomyces cerevisiae* and *Paecilomyces variotii*. Each trial in triplicate tested with known inoculation levels of 3.3 CFU/mL of both organisms against the strength of different concentrations of [CA:αCD].

***Saccharomyces cerevisiae*- Free cinnamic acid**					pH 3.4
Incubation Period	Day 1	Day 2	Day 3	Day 4	Day 5
Free-[CA]					
100 ppm	-/-	-/-	-/-	-/-	-/-
200 ppm	-/-	-/-	-/-	-/-	-/-
300 ppm	-/-	-/-	-/-	-/-	-/-
400 ppm	-/-	-/-	-/-	-/-	-/-
500 ppm	-/-	-/-	-/-	-/-	-/-

***Saccharomyces cerevisiae*- Free α-cyclodextrin**					pH 3.4
Incubation Period	Day 1	Day 2	Day 3	Day 4	Day 5
Free-[α-CD]					
100 ppm	-/-	+/+	+/+	+/+	+/+
200 ppm	-/-	-/+	-/+	+/+	+/+
300 ppm	-/-	+/-	+/+	+/+	+/+
400 ppm	-/-	+/+	+/+	+/+	+/+
500 ppm	-/-	+/+	+/+	+/+	+/+

***Saccharomyces cerevisiae*- cinnamic acid: α-cyclodextrin complex**					pH 3.4
Incubation Period	Day 1	Day 2	Day 3	Day 4	Day 5
[CA:αCD]					
100 ppm	-/-	-/-	+/-	+/-	+/-
200 ppm	-/-	-/-	-/-	-/+	-/+
300 ppm	-/-	-/-	-/-	+/-	+/-
400 ppm	-/-	-/-	-/-	+/+	+/+
500 ppm	-/-	-/-	-/-	+/-	+/-

***Paecilomyces variotii*- Free cinnamic acid**					pH 3.4
Incubation Period	Day 1	Day 2	Day 3	Day 4	Day 5
Free-[CA]					
100 ppm	-/-	-/-	-/-	-/-	-/-
200 ppm	-/-	-/-	-/-	-/-	-/-
300 ppm	-/-	-/-	-/-	-/-	-/-
400 ppm	-/-	-/-	-/-	-/-	-/-
500 ppm	-/-	-/-	-/-	-/-	-/-

***Paecilomyces variotii*- Free α-cyclodextrin**					pH 3.4
Incubation Period	Day 1	Day 2	Day 3	Day 4	Day 5
Free-[α-CD]					
100 ppm	-/-	+/+	+/+	+/+	+/+
200 ppm	-/-	+/+	+/+	+/+	+/+
300 ppm	-/-	+/+	+/+	+/+	+/+
400 ppm	-/-	+/+	+/+	+/+	+/+
500 ppm	-/-	+/+	+/+	+/+	+/+

***Paecilomyces variotii*- cinnamic acid: α-cyclodextrin complex**					pH 3.4
Incubation Period	Day 1	Day 2	Day 3	Day 4	Day 5
[CA:αCD]					
100 ppm	-/-	+/+	+/+	+/+	+/+
200 ppm	-/-	-/-	+/+	+/+	+/+
300 ppm	-/-	-/-	+/+	+/+	+/+
400 ppm	-/-	-/-	+/+	+/+	+/+
500 ppm	-/-	-/-	-/-	+/+	+/+

Table 4.3 Data tables recording turbidity to indicate growth over a five day incubation period at 25 °C with *Saccharomyces cerevisiae* and *Paecilomyces variotii* in three media types: free cinnamic acid, free α-cyclodextrin, and the complex. Each trial in duplicate tested with known inoculation levels of both organisms against the strength of different concentrations of [CA:αCD].

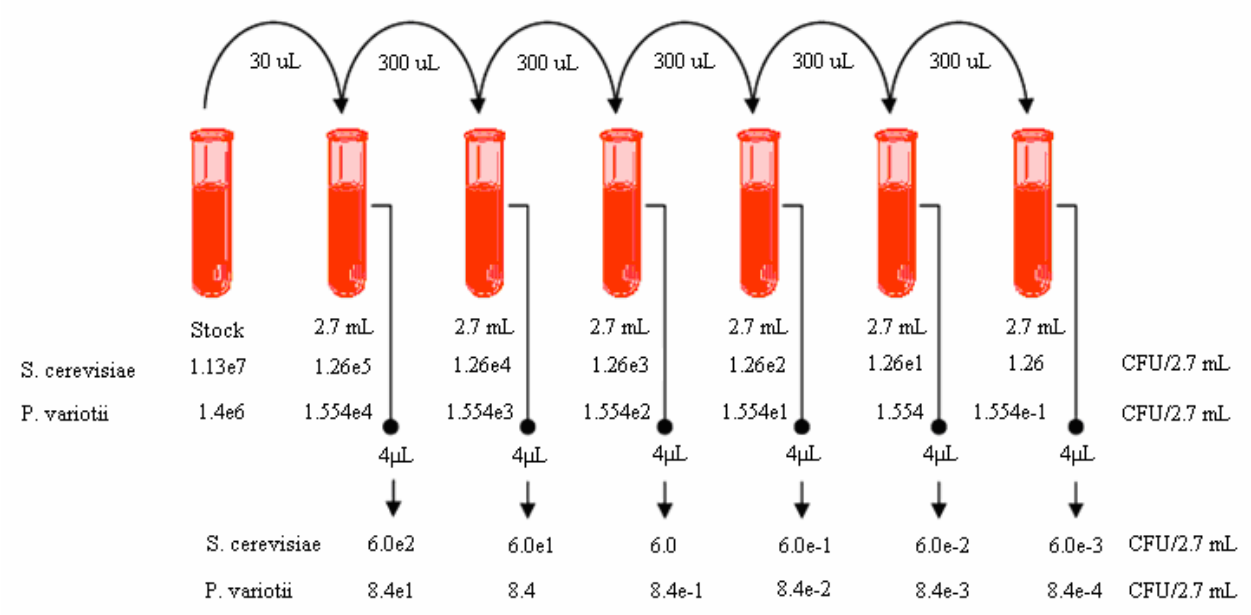

Figure 4.1 MIC Method 1 dilution and inoculation scheme supplied by PepsiCo. for *S. cerevisiae* and *P. variotii* including total CFU/2.7 mL per dilution test tube and incubation microcentrifuge tube.

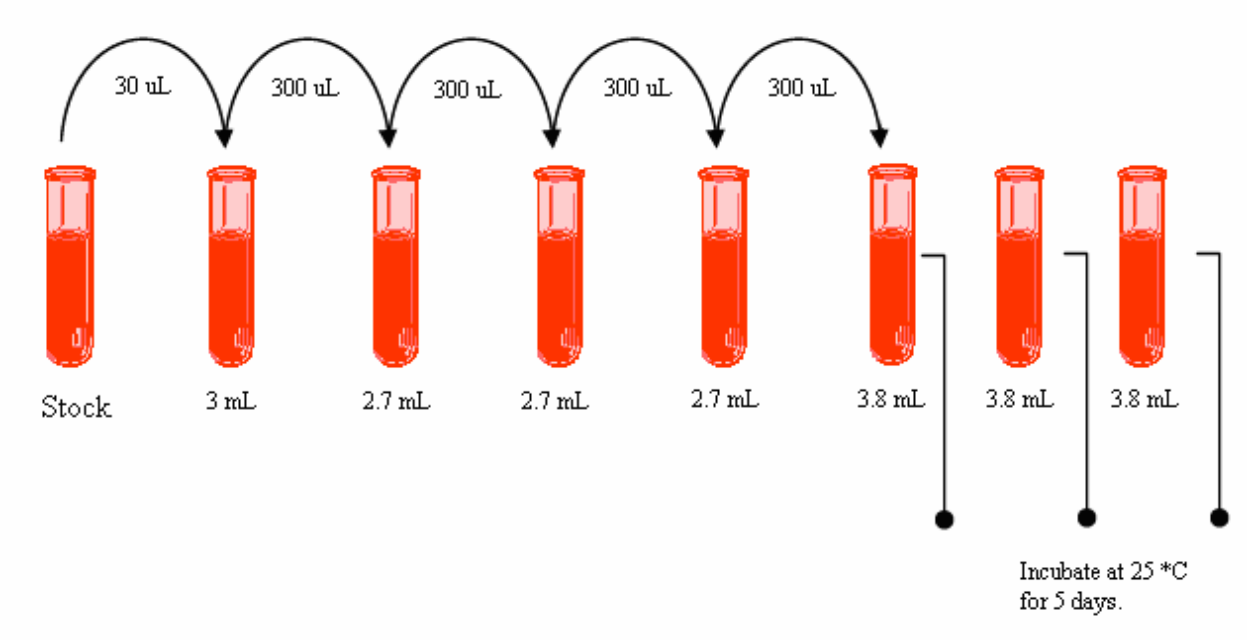

Figure 4.2 MIC Method 2 dilution and inoculation scheme for *S. cerevisiae* and *P. variotii*

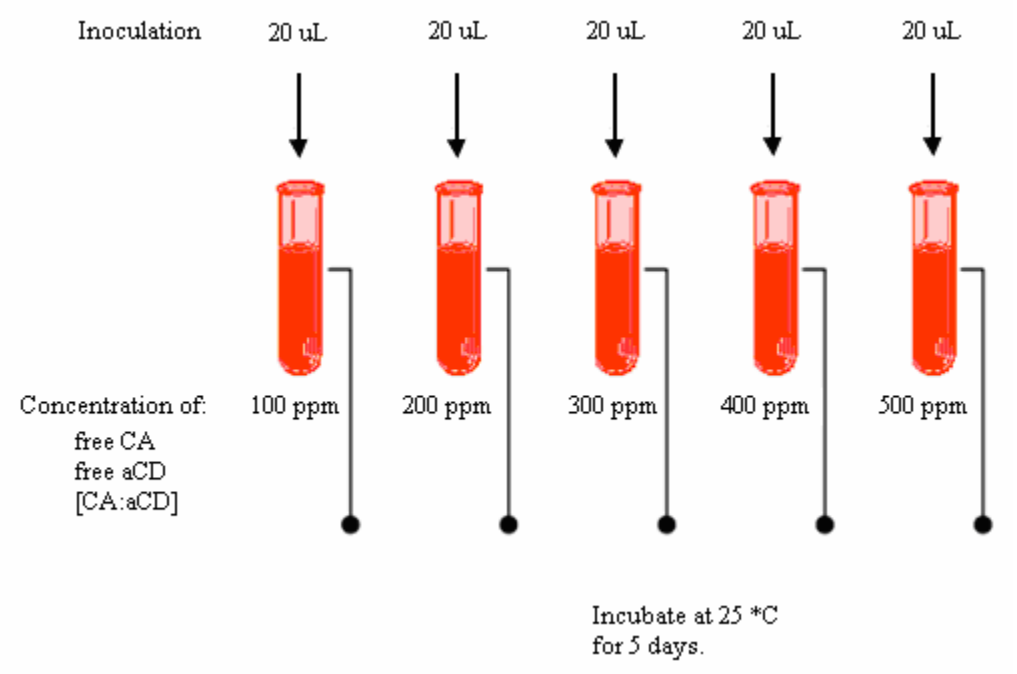

Figure 4.3 MIC Method 3 dilution and inoculation scheme for *S. cerevisiae* and *P. variotii*