Formation, Characterization and Stability of Natural Antimicrobial-Cyclodextrin Complexes

Cristián Samperio Torrebiarte

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Food Science & Technology

Dr. Joseph E. Marcy, Chairman

Dr. Sean F. O'Keefe

Dr. William N. Eigel

August 6th, 2009
Blacksburg, Virginia

Keywords: essential oil, cyclodextrin, preservative, inclusion complex, solubility, antimicrobial

Formation, Characterization and Stability of Natural Antimicrobial-Cyclodextrin Complexes

Cristián Samperio Torrebiarte

Abstract

As a response of the need for a natural antimicrobial to replace sodium benzoate's use as a preservative in beverages, twenty eight compounds known to have antimicrobial activity were evaluated to quantify their solubility. Twenty three of the compounds evaluated are components of plant essential oils and the remaining five compounds are alkyl esters of *para*-hydroxybenzoic acid. The test compounds were evaluated for aqueous solubility as well as their solubility in an acid-based beverage mixture. The compounds were found to be practically insoluble (< 100mg/L), very slightly soluble (100mg/L – 1,000mg/L) or slightly soluble (1,000mg/L to 10,000 mg/L).

o-Methoxycinnamaldehyde, *trans,trans*-2,4-decadienal, cinnamic acid, and citronellol were complexed with α- and β- cyclodextrin and evaluated through phase solubility analyses. The complexes formed showed improved aqueous solubility for all compounds. Complexation with α-CD resulted in an increase of aqueous solubility of *o*-methoxycinnamaldehyde by 10-fold, *trans,trans*-2,4-decadienal by 3.2-fold, cinnamic acid by 6.3-fold, and citronellol by 8-fold. In addition, complexation with β-CD resulted in an increase of aqueous solubility of *o*-methoxycinnamaldehyde by 1.6-fold, *trans,trans*-2,4-decadienal by 3.1-fold, cinnamic acid by 1.7-fold, and citronellol by 1.6- fold.

The storage stability of the α-CD complexes of *o*-methoxycinnamaldehyde, *trans,trans*-2,4-decadienal and citronellol were evaluated for 7 days in an acid-based beverage solution by SPME GC-MS. The complexes exhibited varying levels of degradation throughout the duration of the study all. The concentration of *o*-methoxycinnamaldehyde detected by SPME GC-MS decreased by 61.7%. Similarly, the concentration of *trans,trans*-2,4-decadienal and that of citronellol decreased by 62.7% and 43% respectively. Additionally, a comparison by UV/Vis of the storage stability of the complexes stored in glass and PET containers was performed. The storage stability comparison proved that absorption into the PET polymer membrane did not occur.

Acknowledgements

I would like to thank Dr. Joseph E. Marcy for his leadership throughout this project. I would also like to thank Dr. Sean F. O'Keefe for his help, guidance and availability throughout this process. Thanks to Dr. William N. Eigel for serving on my committee and also for his guidance throughout the entire project. It has been a pleasure to work with them.

Very importantly I would like to thank all the members of the research group working on this project. Thanks to all the staff for providing guidance and support specially Harriet Williams. Thanks to my office mates Kevin Holland, Paul Sarnoski and Laura Geise for their advice and their humor. A very important thank you to Dr. John Koontz who shared information and provided support and guidance in the early stages of this project.

I would like to acknowledge my family for their support. My parents Mario and Maria Jose who are simply the best parents I could ever ask for. In addition, I would like to thank my grandparents Carlos and Olga for always supporting my vision. Thanks to my brothers and sister Mario Estuardo, Julian and Maria Olga for always supporting me with their love. Moreover, a very special thank you to Adriana Serrano who is the person that motivates me to keep moving forward.

I would finally like to thank Pepsi Co. for funding and guidance throughout the entire process of my work.

Table of Contents

List of Tables and Figures

CHAPTER 1

Introduction and Justification

In November 2005, the Food and Drug Administration (FDA) received a small study conducted by private laboratory where the presence of low levels of benzene was reported in a small number of soft drinks that contained benzoate salts and ascorbic acid. As a follow-up, the FDA's Center for Food Safety and Applied Nutrition (CFSAN) conducted a study on beverages found in the market. Although the FDA has no standards for allowed levels of benzene, they have adopted the standards that the US Environmental Protection Agency (EPA) holds for drinking water, which dictates that the maximum contaminant level (MCL) should be below 5 μg/L. It was hypothesized that sodium benzoate, a common preservative, was reacting with ascorbic acid to produce free benzene (FDA, 2006).

The study's results were posted in April 2006 reporting that out of the 100 beverages tested, four soft drinks and one fruit drink contained benzene in levels above five ppb in aqueous solution. The study had a second phase in which 86 more samples were tested and results were posted on May of 2007. The second phase of the study found five products implicated (FDA, 2006).

Sodium benzoate is the sodium salt of benzoic acid which is present as benzoic acid in aqueous environments. A wide variety of foods and beverages use sodium benzoate as an antimicrobial additive. It is most suitable in naturally acidic or acidified foods and beverages in the pH range from 4.0 to 4.5. Due to its broad availability and low cost, it is used in many different types of foods, including carbonated and still beverages. The usage level ranges from 0.05% to 0.10% (Chichester and Tanner, 1968).

The mechanism by which benzene may be produced is described by Gardner and Lawrence, 1993. The study suggests that transition metals can catalyze a one-electron reduction of oxygen by ascorbic acid to produce the superoxide anion radical, "which undergoes spontaneous disproportionation to produce hydrogen peroxide" (Gardner and Lawrence, 1993). The transition metals needed to catalyze the reaction would be present in the water used for the preparation of the beverages. The study goes on to show that subsequent metal-catalyzed reduction of hydrogen peroxide by ascorbic acid can generate a hydroxyl radical. The study concludes by showing that the hydroxyl radical generated by the metal-catalyzed reduction of oxygen and hydrogen peroxide by ascorbic acid "can attack benzoic acid to produce benzene under conditions prevalent in many foods and beverages" (Gardner and Lawrence, 1993). The reaction is highly dependent on the concentration of ascorbic acid, optimum level of transition metal catalyst, and pH. Benzene production increases with increasing ascorbic acid concentration until the ascorbic acid concentration becomes too high, and then it, competes with benzoic acid for the hydroxyl radical (Gardner and Lawrence, 1993). Benzene production is optimum at 1.0 mM concentration of copper sulfate (Gardner and Lawrence, 1993). The production of benzene decreases as pH increases. The maximum amount of benzene is produced at pH 2, and constantly decreases as pH reaches 7. Benzene is not detectable in mixtures with pH above 7 (Gardner and Lawrence, 1993). Benzene production also increases with exposure to elevated temperatures (Kyoung and others, 2008). Consequently, soft drinks and beverages with ascorbic acid and added sodium benzoate that are subjected to intense heat can be susceptible to the production of benzene.

Benzene is a known human carcinogen and neurotoxin (Fleming-Jones and Smith, 2003). Consequently, efforts have to be made in order to eradicate its presence in beverages.

Research Objectives

In the task to find a replacement for sodium benzoate 28, chemical compounds isolated from natural sources known to have antimicrobial activity were selected for evaluation (Table 1.1). All 28 compounds are currently ineffective as antimicrobials in beverages due to their low aqueous solubility. The first research objective is to accurately quantify the aqueous solubility of these compounds. The Minimum Inhibitory Concentration (MIC) of the compounds against *Zygosaccharomyces bisporus, Saccharomyces cerevisiae,* and *Zygosaccharomyces bailli* will be determined. Based on the solubility and MIC data, compounds with the best antimicrobial activity, but limited aqueous solubility, will be chosen to form molecular inclusion complexes with α-cyclodextrin (α-CD) and β-cyclodextrin (β-CD). Phase solubility analyses will be performed to determine if the compounds' complexation with α-CD and β-CD improves their aqueous solubility. Subsequently, solid inclusion complexes will be prepared for further characterization. The stability of the inclusion complexes will be studied in an acid-based beverage matrix. Finally, the stability of the complexes stored in PET containers will be compared to the stability of the same complexes stored in glass containers.

References

Chichester, D.F.; Tanner, F.W.,Jr.1968. Antimicrobial Food Additives. *In* Handbook of Food Additives; Furia, T.E., Ed,; The Chemical Rubber Co.: Cleveland, OH.

Fleming-Jones M, Smith R. 2003. Volatile Organic Compounds in Foods: A Five Year Study. *J. Agric. Food Chem.* 51: 8120-8127.

Food and Drug Administration (FDA). 2006. Data on Benzene in Soft Drinks and Other Beverages. Office of Food Additive Safety, CFSAN, Food and Drug Administration. Received October 2007 from http://www.cfsan.fda.gov/~dms/benzdata.html.

Gardner K. L.; Lawrence G. D. 1993. Benzene Production from Decarboxylation of Benzoic Acid in the Presence of Ascorbic Acid and a Transition-Metal Catalyst. *J. Agric. Food Chem.* 41(5): 693-695

Kyoung Hyun Ju, Jeong Hill Park, Sung Won Kwon. 2008. Evaluation of Headspace-Gas Chromatography/Mass Spectrometry for the Analysis of Benzene in Vitamin C drinks; Pitfalls of Headspace in Benzene Detection. *Biomedical Chromatography.* 22 (8): 900-905

Table 1.1 List of chemical compounds evaluated with CAS number and Sigma-Aldrich catalog number.

CAS #	Common Name	Sigma-Aldrich Catalog #	Molecular Weight
94-18-8	Benzyl-4-hydroxbenzoate (Benzyl paraben)	54670	228.24
104-55-2	Cinnamaldehyde	W228605	132.16
110861-66-0	Cyclohexanebutyric acid (Calcium Cyclohexanebutyrate)	228141	378.56
112-31-2	Decanal (Caprinaldehyde; Decyl aldehyde)	W236209	156.27
1504-74-1	o-Methoxycinnamylaldehyde	W318108	162.19
1731-84-6	Methyl nonanoate (Methyl pelargonate; Nonanoic acid methyl ester)	W272418	172.26
25152-84-5	Trans, trans, 2,4 decadienal	W313505	152.23
104-61-0	Nonanoic lactone (Gamma-nonalactone)	W278106	156.22
2315-68-6	Propyl benzoate	307009	164.20
104-67-6	Undecalactone (Undecanoic Gamma-Lactone)	U806	184.28
112-44-7	Undecanal	W309206	170.29
101-39-3	Methyl trans cinnamylaldehyde (alpha-methyl-trans-cinnamaldehyde)	112275	146.19
18031-40-8	Perillaldehyde	W355704	150.22
89-83-8	Thymol	W306606	150.22
103-41-3	Cinnamic acid benzyl ester (Benzyl Cinnamate)	W214205	238.28
140-10-3	Cinnamic acid (trans-3-Phenylacrylic acid)	W228818	148.16
110-44-1	Sorbic acid (2 4 hexadienoic acid)	W392103	112.13
99-76-3	Methyl paraben (methyl p-Hydroxybenzoate)	W271004	152.15
94-13-3	Propyl paraben (propyl 4-Hydroxybenzoate)	W295101	180.20
89-82-7	Pulegone (R)-(+)	W296309	152.23
106-22-9	Citronellol (3,7-Dimethyl-6-octen-1-ol)	W230901	156.27
5392-40-5	Citral (3,7-Dimethyl-2,6-octadienal)(Geranial and neral mixture)	W230308	152.23
97-53-0	Eugenol	W246700	164.20
94-26-8	Butyl paraben (butyl 4-Hydroxybenzoate)	W220302	194.23
120-47-8	Ethyl paraben (ethyl 4-Hydroxybenzoate)	54660	166.17
93-15-2	Eugenol methyl ester (4-Allyl-1,2-dimethoxybenzene)	46110	178.23
5989-27-5	(R) - Limonene	W263303	136.23
18172-67-3	β-pinene	402753	136.23

CHAPTER 2

Review of Literature

α-cyclodextrin and β-cyclodextrin

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of six, seven and eight α-D-glucopyranose units for α-CD, β-CD and γ-CD, respectively. The repeating glucopyranose units are linked by α-(1,4) linkages. α-CD, β-CD, and γ-CD are the three most common naturally occurring CDs often referred to as parent CDs. The chemical structures of parent CDs are shown in Figure 2.1. In addition, many CD derivatives have been produced by aminations, esterifications and etherifications. CDs containing less than six glucose units are too strained to exist and those containing more than eight units are hard to isolate and infrequently studied. Important characteristics of parent CDs have been summarized by Szejtli, (1998) and Del Valle, (2004).

CDs are formed by the degradation of starch with the enzyme glucosyltransferase. The three parent CDs are crystalline, homogenous, nonhygrospcopic substances (Szejtli, 1998). Important physical properties of the parent CDs are summarized in Table 2.1. The glucopyranose units of CDs are in the $^4C_1$ (chair) conformation and, as a consequence, all secondary hydroxyl groups are located on one of the two edges of the ring and the primary ones are on the other edge. For that reason, CD rings are conical cylinders (Szejtli, 1988). A volumetric representation of the parent CDs is shown in Figure 2.2.

CDs can form intramolecular hydrogen bonds between their secondary hydroxyl groups. β-CD's water solubility is rather low compared to the rest of native cyclodextrins, most likely because it forms intramolecular hydrogen bonds. In the α-CD molecule, one glucose unit is in a distorted position which disrupts the hydrogen bond formation

making it more water soluble. The CD cavity has different diameters on each side. On the side containing the primary hydroxyl groups, the cavity is smaller due to free rotation of these groups. On the side where the secondary hydroxyl groups are located, the cavity is larger (Szejtli, 1988). The dimensions of the parent CDs are shown in Figure 2.2.

The exterior of the CD molecule has various hydroxyl groups, consequently it is quite polar. On the other hand, the interior of the CD cavity is non-polar compared to its exterior and common external environments such as water (Connors, 1997). These unique structural characteristics enable CDs to form inclusion complexes with a very wide range of solid, liquid and gaseous compounds (Del Valle, 2004). Geometrical and steric factors determine the fit of the guest molecule inside the CD cavity. For that reason, the proper fit, even if only partial, of a guest molecule in a CD cavity is the most important requirement for inclusion complex formation (Szejtli, 1998). There are no covalent bonds formed or broken by complexation. While hydrophobic interactions and Van der Walls are known to be the main interactions involved in complexation, steric forces and hydrogen bonding could also contribute (Szejtli, 1988). The main driving force of complex formation is the release of water molecules from inside the CD cavity and their replacement with more hydrophobic guest molecules. The replacement of enthalpy-rich water molecules by hydrophobic guest molecules decreases CD's ring strain. Hence, the complexation reaction takes place because it is energetically favorable and results in a more stable lower energy state species (Del Valle, 2004). Complexes formed achieve a dynamic equilibrium between guest and host molecules and the association is reversible (Saenger, 1984). The binding strength again depends on the fit

of the guest in the cavity and the specific interactions between surface atoms (Del Valle, 2004).

The formation of inclusion complexes has important effects on the physicochemical properties of host molecules. Some beneficial changes induced by complexation include: alteration of guest's solubility, stabilization against effects of light, heat, and oxidation, reduction of guest's physiological responses, and reduction of volatility (Hedges, 1998). The most common industrial use of inclusion complex formation with CDs is to increase solubility of functional ingredients. When a guest molecule forms a complex, it is essentially surrounded by the CD molecule. The hydrophobic groups of the guest molecule that would be in contact with the solvent interact with the hydrophobic groups inside of the CD cavity and let the hydrophilic groups on the outside of the CD cylinder interact with the solvent. This results in an increase in solubility of the complexed guest which can lead to an increase in bioavailability in an aqueous system. In many cases the increase in solubility proves to be insufficient to have an effect on bioavailability (Hedges, 1998). Generally, the lower the water solubility of a compound the greater the relative solubility increases gained by complexation (Kootnz, 2003). Inclusion complex formation also stabilizes compounds. Stabilization is achieved by the occupancy of the CD cavity by the guest molecule. Once the cavity is occupied by a molecule other reactive molecules are excluded from occupying the cavity at the same time preventing interaction and reaction. In addition, steric hindrance prevents the interactions with exposed portions of the guest molecule (Hedges, 1998). Furthermore, complexation can reduce the rate of photodegradation of some light sensitive compounds (Mielcarek, 1997).

An important observation for the use of CD complexes as antimicrobials is that these cyclic oligosaccharides are not mediums for microorganisms, especially yeasts and fungi. However, if CD prevents the guest molecule from getting into direct contact with cell membrane the complexed molecule, will lose its antimicrobial properties (Szejtli, 1988).

Wacker Biochem submitted to the U.S. Food and Drug Administration (FDA) an independent Generally Recognized As Safe (GRAS) determination for β-CD as a flavor carrier or protectant. The FDA did not question the self-affirmed GRAS status and assigned it the GRAS Notice No. GRN 000074 in October 2001 (FDA, 2001). Wacker Biochem also submitted to the FDA an independent GRAS determination for α-CD as a as a carrier or stabilizer for flavors (flavor adjuvant), colors, vitamins and fatty acids, and to improve mouth-feel in beverages. The FDA did not question the self-affirmed GRAS status and assigned it the GRAS Notice No. GRN 000155 in June 2004 (FDA, 2004).

Natural Antimicrobials

The field of antimicrobials derived from nature has been extensively studied throughout history. Since the fifth century BC, Hippocrates mentions 400 medicinal plants, many of them now known to synthesize biologically active chemical compounds (Shultes, 1978). The category of antimicrobials derived from nature includes compounds derived from plants, animals and microorganisms (Roller, 2003). The scope of this research will focus on antimicrobial compounds derived from plants.

Essential oils (EOs) are aromatic oily liquids obtained from plants and plant products such as fruits, seeds, and leaves. Essential oils, volatile oils and ethereal oils are terms used in scientific literature referring to one and the same. Steam distillation is the

most common extraction method of EOs from plant material (van de Braak and Leijten, 1999). The antimicrobial properties of some EOs has been recognized and studied for years (Boyle, 1955). EOs have exhibited antiviral, antibacterial, antimycotic, antitoxigenic, antiparasitic, and insecticidal properties (Burt, 2004; Corbo and others 2009).

Generally EOs are composed of sixty or more individual compounds. Typically a major component constitutes 85% or more of the EO while the remaining 15% is composed of minor components. Although most of the antimicrobial properties of an individual EO are attributed to its major compound, minor components are also known to contribute (Burt, 2004). The compositional nature of several EOs has been studied by gas chromatography and mass spectrometry (GC/MS) (Salzer, 1977; Wilkins and Madsen, 1991; Daferera and others, 2000; Juliano and others 2000; Bauer and others 2001; Delaquis and others, 2002; Adams, 2007). EOs are composed of complex mixtures of terpenoids (mono-, sesqui- and di-terpenes), alcohols, ketones and aldehydes of terpenoids. Aromatic compounds arising from the phenyl-propanoid pathway are very common. Many individual compounds of plant EOs are common to many species (Adams, 2007). Adams' (2007) work has a very comprehensive analysis by GC/MS of most compounds found in plant EOs. Compounds derived from EOs that were included in this study are listed in table 2.2 along with a plant species source. In addition table 2.2 shows the compounds' GC/MS retention time and Kovats retention index.

The antimicrobial activity of EOs is mostly attributed to phenolic compounds and their derivatives (Cowan, 1999; Corbo and others, 2009). Phenolics exercise their antimicrobial activity by injuring lipid membranes resulting in leakage of cellular

contents (Vigil and others, 2005). There is a great deal of published literature the effects of different EOs on different bacteria. Deans and Ritchie (1987) studied the effect of 50 plant EOs on 25 genera of bacteria concluding that both Gram-positive and Gram-negative bacteria are susceptible. The level of susceptibility was highly variable depending on the bacteria and EO in question. A review by Nychas (1995) summarizes the effect of the EOs from linden flower, orange, lemon, grapefruit, mandarin, sage, rosemary, oregano, thyme, cinnamon, cumin, caraway, clove, allspice, mastic gum and onion against the foodborne pathogens *Staphylococcus aureus, Listeria monocytogenes, Aremonas hydrophilia, Salmonella typhimurium and Clostridium botulinum.* The study concludes that to some degree all are sensitive to the EOs, again with major variability in the levels of inhibition. Many researchers concluded that the effectiveness of EOs decreased when experiments were conducted in vivo due to the complex components of food matrices (Smid and Gorris, 1999).

Although many EOs have been tested for their efficacy as antimicrobials, only major EO components like eugenol, cinnamaldehyde, thymol, citral, and perillaldehyde have been evaluated individually (Burt, 2004). Table 3 provides a list of these major EO compounds with the microorganisms that were tested against and the reported Minimum Inhibitory Concentration (MIC). In addition to antibacterial activity, some of the major EO components have been researched for antifungal activity. Iso-eugenol, cinnamaldehyde, carvacrol, eugenol and thymol revealed strong antifungal activity against common food contaminating fungi *Penicillum* sp., *Fusrium* sp., and *Aspergillus* sp. (Pauli and Knobloch, 1987). EO components have been tested in a number of food systems including: meat and meat products, fish, dairy products, vegetables, rice, and

fruits (Burt, 2004). Due to their low water solubility there are no studies done in aqueous systems.

In the United States, the legal aspects of the use of EO components in foods are very encouraging. All the compounds listed in table 2.2, except eugenol methyl ester, are either approved as food additives or listed or affirmed as GRAS by the FDA (EAFUS).

Parabens

The remaining antimicrobial compounds included in this study are a group of alkyl esters of *para*-hydroxybenzoic acid commonly referred to as parabens. Methyl paraben and propyl paraben are directly added to commercial food systems as antimicrobial agents (Davidson, 2005). The interest in these compounds in the development of a beverage preservative is because some are already in commercial use as food preservatives. However, their low aqueous solubility is a limiting factor.

The list of the compounds to be evaluated in this study with their reported water solubilities is in Table 2.4. Parabens are commercially available as odorless white powders. Parabens are stable to air and are resistant to cold and heat, including sterilization temperatures. The first reports on the antimicrobial activity of parabens were published in the 1920s by Sabalitschka and others (Davidson, 2005). The optimum pH for antimicrobial activity has been reported from three to eight (Chichester and Tanner, 1968). The antimicrobial and antifungal activity of parabens has been evaluated against a very wide range of bacteria and fungi (Table 2.5). Many inhibition studies use different experimental parameters so direct comparison is not representative of real results. However, relative comparison of reported MICs give a good overview of the effectiveness of these compounds. Generally, the longer the alkyl chain of the paraben

the greater its antimicrobial activity. Inversely, the longer its alkyl chain, the lower its water solubility.

The exact mechanism by which parabens function as antimicrobials is yet to be determined but many studies agree that most of their activity occurs at the cytoplasmic membrane (Furr and Russell, 1972; Freese and others, 1973). Furr and Russell determined that the presence of parabens causes intracellular RNA leakage in *Serratia marcescens* indicating disruption of cytoplasmic membrane. Other mechanism proposed by Freese and others concluded that parabens inhibit the uptake of serine and the oxidation of α-glycerol phosphate and NADH in *Bacillus subtilis.*

In the United States the regulatory status of methyl, propyl, and butyl paraben are GRAS at maximum concentration of 0.1%. (EAFUS). In the European Union methyl, propyl, and ethyl are approved for use in foods (Davidson, 2005).

Methodology Rationale

Solubility Studies

The aqueous solubility of a compound is defined as the extent to which a substance mixes with pure water to form a molecular homogeneous system at a given temperature. For pure compounds water, solubility is an equilibrium state (ASTM). Solubility studies will follow the ASTM (E1148) standard method for aqueous solubility measurement. An excess of compound is shaken or stirred in a flask of water to obtain equilibrium. Since equilibrium times depend on the compound's physical properties, the solution should be stirred for at least 24 hours. After equilibrium is reached, any suspended solute should be removed either by centrifugation or filtration. A standard curve can be prepared by producing a suspension of varying concentrations of test

compound by mixing a known concentration in a water miscible solvent and diluting the suspension in water. A plot of absorbance versus concentration should yield a straight line. Standard techniques of linear regression are then used to estimate concentration of test compound in water, that value represents water solubility (ASTM).

Complex Formation

Inclusion complexes can be prepared in solution. The procedure is to stir or shake an aqueous solution of cyclodextrin with an excess of guest molecule. This may be performed with or without the use of a solvent. Equilibrium is reached after intense stirring. The mixture is stored immobile under constant temperature for 12 hours to allow any excess guest compound to settle out of solution (Szejtli, 1988). The quantitative determination of the guest's concentration can be made by spectophotometry at ultraviolet and visible ranges (UV/Vis), gas chromatography (GC) or high performance liquid chromatography (HPLC). However the complex has to be dissolved and dissociated. For UV/VIS, the complex must be dissolved in 50% ethanol. The determined amount of guest may be fully or partially complexed or fully uncomplexed. characterization methods can be applied to verify that a true inclusion complex is formed (Szeijtli, 1988).

Binding Constants

The binding constant of a non-covalently bound species of definite substrate-to-ligand stochiometry can be calculated when the complex formed is at equilibrium in solution (Connors, 1997). The substrate, S, is the guest and the ligand, L, is the host (CD) whose concentration is the independent variable. Stoichiometric ratios are given in the order S:L. CD complexes are commonly assigned a 1:1 stochiometric ratio, however

other ratios such as 1:2, 2:1, and 2:2 are known. Complexes with those stochiometries are formed according to the following equilibria:

$$S + L \leftrightarrow SL$$
$$SL + L \leftrightarrow SL_2$$
$$S + SL \leftrightarrow S_2L$$

The stepwise binding constants for these equilibria, denoted K_{11}, K_{12}, and K_{21} are defined by the following equations each constant has the unit M^{-1}:

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

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

$$K_{21} = \frac{[S_2L]}{[S][SL]}$$

The formation of higher complexes directly from the substrate and ligand is represented by the following:

$$mS + nL \leftrightarrow S_mL_n$$

with an overall binding constant β_{mn}:

$$\beta_{mn} = \frac{[S_mL_n]}{[S]^m\,[L]^n}$$

In CD complex studies binding constants are expressed in terms of stepwise binding constants (Connors, 1997).

Phase Solubility Analysis

The interactions between the two molecules in an inclusion complex are weak induction forces. The electron transfer between the two molecules creates electrostatic forces of attraction. These charge transfers have an overall solubility effect especially with aromatic molecules (Higuchi, 1971). The procedure of phase solubility analysis is

to add successive amounts of sample to a solvent which remains at a constant volume. In the case of this study, successive amounts of CD are added to a constant volume of water. The guest compound is added in excess to each aqueous-CD solution. The solution is then brought to equilibrium through agitation at a constant temperature and the solution phases are then analyzed for total guest content (Higuchi, 1971). A phase solubility diagram (solubility isotherm) can be prepared by plotting the weight of solute (aqueous-CD solution) by the weight of guest found per unit of solvent (Higuchi, 1971). A phase solubility analysis yields the maximum solubility of the complexed substrate.

Solid Inclusion Complex

A physical solid of the complex can be formed in order to further characterize its structure. Preparation of solid inclusion complexes involves freeze-drying the complex in solution 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 which can be used for further testing and analysis, such as for determination of percent weight of complexed guest. It can also be used to determine stoichiometry of the complex. Thermal analysis such as thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) can be used to verify complex formation with a solid inclusion complex.

Storage Stability Studies

The purpose of this study is to determine the percentage of guest compound in an acid-based beverage matrix remaining over a period of seven days. A known amount of cyclodextrin complex is added to a mixture of water, glucose, fructose, sucrose and ascorbic acid. The acid-based beverage matrix is adjusted to a pH of 3.4 and results

between 12^{o} and 13^{o} brix, simulating a commercial beverage system where the complexes could be used as antimicrobial agents. The concentration of guest compound is then evaluated in daily intervals by solid phase microextraction gas chromatography mass spectrometry (SPME GC/MS). The mixture is filtered before each evaluation to ensure that anything coming out of solution is removed. This method requires the complete dissolution and dissociation of the complex in a solvent preferably in the mobile phase (Szente, 1996). A time-course plot of remaining compound (%) is then produced (Ajisaka and others, 2000). A standard curve is prepared by dissolving increasing concentrations of the compounds in ethanol and diluting them in the acid-based beverage mixture to ensure that the standards have the same pH and soluble solids content as the samples.

An aqueous solution with a determined amount of complex is prepared to evaluate the storage stability of the complexes in different packages. The aqueous solution is acidified to a pH of 3.4 to represent the pH of a commercial acid based beverage. The complex-aqueous solutions are stored in polyethylene terephthalate (PET) and glass vials over a period of 7 days. The solutions are then evaluated by UV/Vis to determine the amount of compound present in solution. The solutions are filtered before each evaluation to ensure that anything coming out of solution is removed. The dissociation of the complex is achieved by diluting the solutions to a final concentration of 50% v/v aqueous ethanol. A time-course plot of remaining compound (%) is produced for each method of storage for comparison. The amount of compound present is quantified by using linear regression against a standard curve. The standards are prepared by dissolving the compounds in ethanol and serially diluting them in acidified water.

References

Adams, R.P. 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th Edition. Allured Publishing Corp. Carol Stream, IL.

Ajisaka, N. Hara, K. Mikuni, K. Hara, K. Hashimoto, H. 2000. Effects of Branched Cyclodextrins on the Solubility and Stability of Terpenes. *Biosci. Biotechnol. Biochem.*, 64 (4), 731-734

ASTM. American Society for Testing and Materials. Standard Test Method for Measurements of Aqueous Solubility. Designation E 1148 – 02

Bauer, K., Garbe, D., Surburg, H., 2001. Common Fragrance and Flavor Materials: Preparation, Properties and Uses. Wiley-VCH, Weinheim, p. 293.

Boyle, W., 1955. Spices and Essential Oils as Preservatives. The American Perfumer and Essential Oil Review 66, 25– 28.

Burt, S. 2004. Essential Oils: Their Antibacterial Properties and Potential Applications in Foods – a review. *International Journal of Food Microbiology*, 94, 223–253.

Connors KA. 1997. The Stability of Cyclodextrin Complexes in Solution. Chemical Reviews. 97 (5)**:**1325-1357.

Corbo, M.R., Bevilacqua, A., Campaniello, D., D'Amato, D., Speranza, B., Sinigalglia, M., 2009. Prolonging Microbial Shelf Life of Foods Through the Use of Natural Compounds and Non-thermal Approaches- a review. *International Journal of Food Science and Technology.* 44, p.223-241

Cosentino, S., Tuberoso, C.I.G., Pisano, B., Satta, M., Mascia, V., Arzedi, E., Palmas, F., 1999. In Vitro Antimicrobial Activity and Chemical Composition of Sardinian Thymus Essential Oils. Letters in Applied Microbiology 29, 130– 135.

Daferera, D.J., Ziogas, B.N., Polissiou, M.G., 2000. GC-MS Analysis of Essential Oils from some Greek Aromatic Plants and their Fungitoxicity on Penicillium digitatum. Journal of Agricultural and Food Chemistry 48, 2576–2581.

Davidson, M.P. 2005. Parabens. In *Antimicrobials in Food 3rd Ed.* Davidson, P.M., Sofos, J.N., Branen, A.L. Taylor and Francis Group. Boca Raton, FL. P.291-304

Deans, S.G., Ritchie, G. 1987. Antibacterial Properties of Plant Essential Oils. *International Journal of Food Microbiology.* 5:165.

Delaquis, P.J., Stanich, K., Girard, B., Mazza, G., 2002. Antimicrobial Activity of Individual and Mixed Fractions of Dill, Cilantro, Coriander and Eucalyptus Essential Oils. International Journal of Food Microbiology 74, 101–109.

Del Valle E.M. 2004. Cyclodextrin and Their Uses: a review. *Process Biochemistry.* 39 (2004): 1033-1046

EAFUS. U.S Food and Drug Administration. Everything Added to Food in the U.S.: A Food Additive Database. http://www.foodsafety.gov/~dms/eafus.html. Consulted on 3/31/09.

Echezarreta-Lopez M, Torres-Labandeira JJ, Castineiras-Seijo L, Santana-Penin L and Vila-Jato JL. 2000. Complexation of the Interferon Inducer, Bropirimine, with Hydroxypropyl-beta-cyclodextrin. European Journal of Pharmaceutical Science 9(4):381-386.

Food and Drug Administration (FDA). 2006. Data on Benzene in Soft Drinks and Other Beverages. Office of Food Additive Safety, CFSAN, Food and Drug Administration. Received October 2007 from http://www.cfsan.fda.gov/~dms/benzdata.html.

Food and Drug Administration (FDA). 2001. Agency Response Letter GRAS Notice No. GRN 000074. http://www.cfsan.fda.gov/~rdb/opa-g074.html. Washington, DC.

Food and Drug Administration (FDA). 2004. Agency Response Letter GRAS Notice No. GRN 000155. http://www.cfsan.fda.gov/~rdb/opa-g155.html. Washington, DC.

Freese, E., Sheu, C.W., Galliers, E.1973. Function of Lipophilic Acids as Antimicrobial Food Additives. *Nature* p. 241-321.

Fujimura, K., Ueda, T., Kitagawa, M., Takayanagi, H., Ando, T. 1986 Reversed-phase Retention Behavior of Aromatic Compounds Involving Beta-Cyclodextrin Inclusion Complex Formation in the Mobile Phase. *Analytical Chemistry.* 58:2668

Furr, J.R., Russell, A.D. 1972. Some Factors Influencing the Activity of Esters of p-Hydroxybenzoic Acid Against *Serratia marcescens. Microbios* 5:189.

Gardner K. L.; Lawrence G. D. 1993. Benzene Production from Decarboxylation of Benzoic Acid in the Presence of Ascorbic Acid and a Transition-Metal Catalyst. *J. Agric. Food Chem.* 41(5): 693-695

Hedges AR. 1998. Industrial Applications of Cyclodextrins. *Chemical Reviews*. 98 (5)**:**2035-2044.

Helander, I.M., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M., Von Wright, A., 1998. Characterization of the Action of Selected Essential Oil Components on Gram-negative Bacteria. Journal of Agricultural and Food Chemistry 46, 3590–3595.

Higuchi TC, K.A. 1971. Phase-Solubility Diagrams. Advances in Analytical Chemistry and Instrumentation. John Wiley and Sons Inc. p. 117-212.

Juliano, C., Mattana, A., Usai, M., 2000. Composition and in Vitro Antimicrobial Activity of the Essential Oil of Thymus herba-barona loisel Growing Wild in Sardinia. Journal of Essential Oil Research 12, 516–522.

Kim, J., Marshall, M.R., Wei, C.-I., 1995a. Antibacterial Activity of Some Essential Oil Components Against Five Foodborne Pathogens. Journal of Agricultural and Food Chemistry 43, 2839– 2845.

Koontz J. L. 2003. Improved Properties of Natamycin Upon Formation of Cyclodextrin Inclusion Complexes. M.S. Thesis, Virginia Polytechnic Institute and State University, Blacksburg. p. 32.

Mielcarek, J. 1997. Photochemical Stability of the Inclusion Complexes Formed by Modified 1, 4-dihydropyridine Derivatives with β-cyclodextrin. *Journal of pharmaceutical and biomedical analysis*. 15 (6):681

Onawunmi, G.O., 1989. Evaluation of the Antimicrobial Activity of Citral. Letters in Applied Microbiology 9, 105– 108.

Pauli, A., Knobloch, K. 1987. Inhibitory Effects of Essential Oil Components on Growth of Food-Contaminating Fungi. *Zeitschrift für Lebensmitteluntersuchung und - Forschung A.*185(1): 10-13.

Roller, S.2003. Natural Antimicrobials for the Minimal Processing of Foods. CRC Press. Boca Raton, FL

Saenger W. 1984. Structural Aspects of Cyclodextrins and their Inclusion Complexes. Inclusion Compounds 2. London: Academic Press. p. 231-259.

Salzer, U.-J., 1977. The Analysis of Essential Oils and Extracts (oleoresins) from Seasonings—a critical review. Critical Reviews in Food Science and Nutrition 9 (4), 345– 373.

Schultes, R.E. 1978. *The kingdom of plants.* In W.A.R Thomson, Medicines from the Earth. McGraw-Hill Book Co., New York, NY. p. 208.

Smid, E.J., Gorris, L.G.M. 1999. Natural Antimicrobials for Food Preservation. In *Handbook of Food Preservation.* Rahman, M.S. Marcell Deker Inc. New York, NY. p. 285.

Szente L. 1996a. Analytical Methods for Cyclodextrins, Cyclodextrin derivatives, and Cyclodextrin complexes. In: J Szejtli and T Osa, editors. Cyclodextrins. First ed. vol. 3. Elsevier Science, Ltd. p. 253-278.

Szejtli J. 1988. Cyclodextrin Technology. ed. vol. Dordrecht: Kluwer Academic Publishers.

Szejtli J. 1998. Introduction and General Overview of Cyclodextrin Chemistry. Chemical Reviews. 98 (5)**:**1743-1753.

Vigil, A.L.M., Palou, E., Alzamora, S.M. 2005. Naturally Ocurring Compounds- Plant Sources. In *Antimicrobials in Food 3rd Ed.* Davidson, P.M., Sofos, J.N., Branen, A.L. Taylor and Francis Group. Boca Raton, FL p.453-506

Van de Braak, S.A.A.J., Leijten, G.C.J.J. 1999. Essential Oils and Oleoresisns: A Survery in the Netheralands and other Major Markets in the European Union. CBI, Centre for the Promotion of Imports from Developing Countries, Roterdam, p. 116

Wilkins, C., Madsen, J.O., 1991. Oregano Headspace Constituents. Zeitschrift fu¨ r Lebensmittel-Untersuchung und -Forschung 192, 214– 219.

Tables and Figures

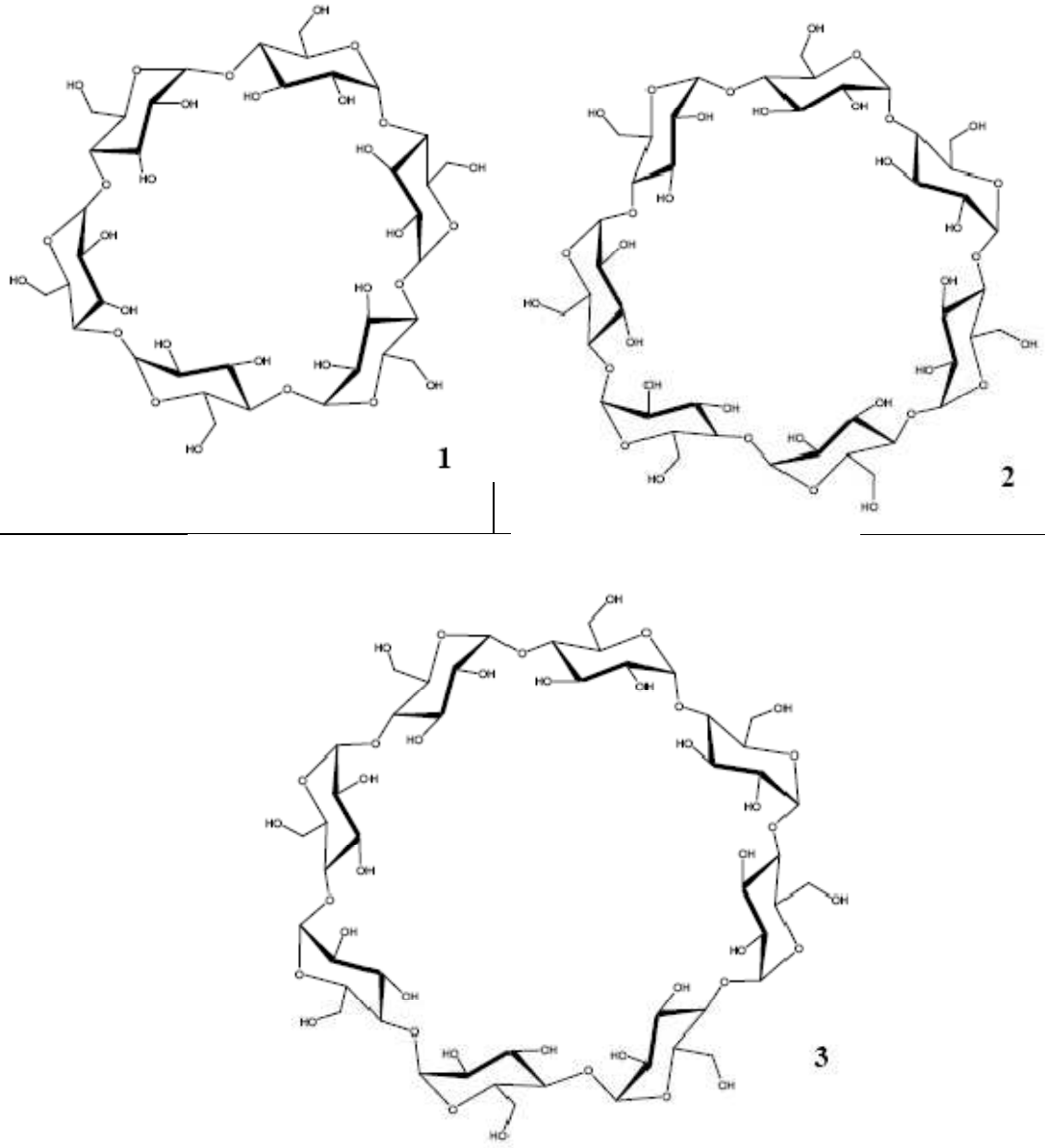

Figure 2.1 Chemical structure of parent CDs. (1) α-CD, (2) β-CD, (3) γ-CD (Koontz, 2003)

Table 2.1 Physical properties of parent cyclodextrins (CD) (Del Valle, 2004)

Property	α-CD	β-CD	γ-CD
Number of glucopyranose units	6	7	8
Molecular weight (g/mol)	972	1135	1297
Solubility in water at 25^{o}C (%, w/v)	14.5	1.85	23.2
Outer diameter (Å)	14.6	15.4	17.5
Cavity diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (Å)	7.9	7.9	7.9
Cavity volume (Å^{3})	174	262	427

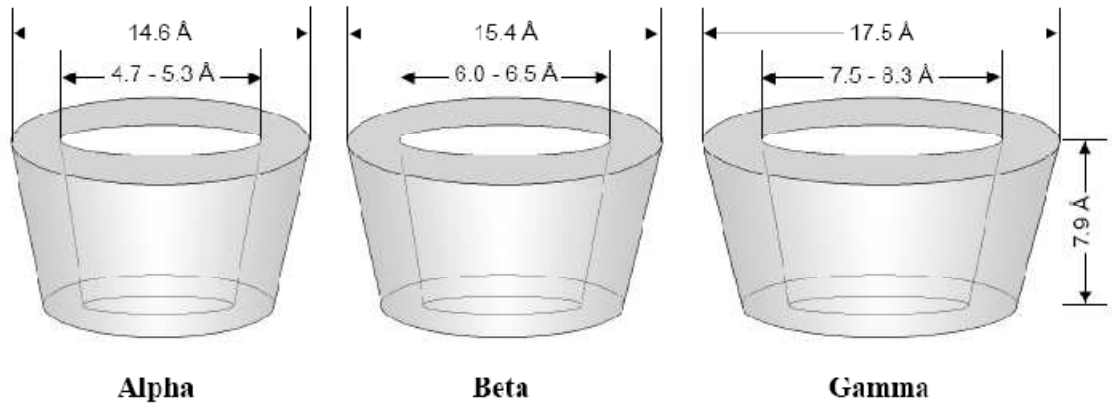

Figure 2.2 Volumetric representation and molecular dimensions of parent cyclodextrins (Koontz, 2003)

Table 2.2 Individual compounds found in essential oils evaluated in the present study. Additionally the latin name of a plant source. GC/MS retention time and Kovats retention index (KI) as reported by Adams, 2007.

Compound (Common name)	**Source**[1,4] (Latin name)	**Retention Time**[2,4] (min)	**KI**[3,4]
Cinnamaldehyde	*Cinnamomum cassia*	18.74	1270
Decanal (Caprinaldehyde; Decyl aldehyde)	*Polygonum minus*	15.83	1201
1-Decanol (n-Decyl Alcohol)	*Mentha x gentillis*	18.73	1269
o-Methoxycinnamaldehyde	*Cinnamomum cassia*	31.38	1564
Methyl nonanoate (Methyl pelargonate; Nonanoic acid methyl ester)	*Humulus lupulus*	16.83	1226
Trans, trans, 2,4 decadienal	*Nicotiana forgetiana*	20.87	1316
Nonanoic acid (Pelargonic acid)	*Sideritis amasiaca*	18.77	1270
Nonanoic lactone (Gamma-nonalactone)	*Polianthes tuberosa*	22.78	1361
Methyl trans cinnamylaldehyde (alpha-methyl-trans-cinnamaldehyde)	*Cinnamomum cassia*	21.00	1318
Perillaldehyde	*Perilla frutescens*	18.83	1271
Thymol	*Organum vulgare*	19.71	1290
Cinnamic acid benzyl ester (Benzyl Cinnamate)	*Myroxylon periferum*	50.22	2092
Cinnamic acid (trans-3-Phenylacrylic acid)	*Cinnamomum cassia*	26.80	1454
Pulegone (R-) (+)	*Acinos suaveolens*	17.27	1237
Citronellol (3,7-Dimethyl-6-octen-1-ol)	*Rosa rugosa*	16.80	1225
E-Citral (3,7-Dimethyl-2,6-octadienal)(Geranial and Neral mixture)	*Cymbopogon citratus*	18.62	1267
Eugenol	*Eugenica caryophyllus*	22.70	1359
(R) - limonene	*Citrus sinensis*	8.69	1029
β - pinene	*Ferula galbaniflua*	7.04	979
Undecalactone (Undecanoic Gamma-Lactone)	*Narcissus tazetta*	31.65	1570
Undecanal	*Machilus bombycina*	20.45	1306
Sorbic acid (2,4-hexadienoic acid)	-	11.20	1093
Eugenol methyl ester (4-Allyl-1,2-dimethoxybenzene)	-	-	-
Propyl benzoate	-	-	-

[1] Plant source not exclusive (idividual compounds are common of many species with varying concentrations in each)

[2] Mass spectra obtained on a HP 5970b MSD mass spectrometer, coupled to an HP 5890 gas chromatograph with a J&W DB-5 column

[3] Kovats index on DB-5 in reference to n-alkanes

[4] Data collected from Adams, 2007

Table 2.3 Selection of reported minimum inhibitory concentrations (MIC) of essential oil components

Compound	**Bacterial Species**	**MIC** (µl/ ml)	**References**
Citral[1]	*Bacillus cereus*	0.1875-0.9	Consentino and others, 1999
	Escherichia coli	0.5	Kim and others, 1995
	Salmonella typhimurium	0.5	Kim and others, 1995
	Staphylococcus aureus	0.5	Onawani, 1989
	Listeria monocytogenes	5.0	Kim and others, 1995
Eugenol	*Escherichia coli*	1.0	Kim and others, 1995
	Salmonella typhimurium	0.5	Kim and others, 1995
	Listeria monocytogenes	>1.0	Kim and others, 1995
Thymol	*Escherichia coli*	0.225-0.45	Consentino and others, 1999
	Salmonella typhimurium	0.056	Consentino and others, 1999
	Staphylococcus aureus	0.140-0.225	Consentino and others, 1999
	Listeria monocytogenes	0.45	Consentino and others, 1999
	Bacillus cereus	0.45	Consentino and others, 1999
Perillaldehyde	*Escherichia coli*	0.5	Kim and others, 1995
	Salmonella typhimurium	0.5	Kim and others, 1996
	Listeria monocytogenes	1.0	Kim and others, 1997
Cinnmaldehyde	*Salmonella typhimurium*	0.05	Helander and others, 1998

[1]Geranial and neral mixture

Table 2.4 Parabens with reported solubility (Davidson, 2005)[1]

Compound	**Solubility**[2] (mg/L)
Methyl paraben (methyl p-Hydroxybenzoate)	2500
Ethyl paraben (ethyl 4-Hydroxybenzoate)	1700
Propyl paraben (propyl 4-Hydroxybenzoate)	500
Butyl paraben (butyl 4-Hydroxybenzoate)	200
Benzyl paraben (Benzyl-4-hydroxbenzoate)	-[3]

[1]Adapted from Davidson, 2005
[2]Reported solubility at 25^{o}C
[3] Not reported

Table 2.5 Minimum inhibitory concentration (MIC) of parabens (experimental parameters vary)[1,2] (Davidson, 2005)[3]

Microorganism	Methyl Paraben	Ethyl Paraben	Propyl Paraben	Butyl Paraben
	Concentration µg/ml			
Gram-Positive				
Bacillus cereus	1000-2000	830-1000	125-400	63-400
Bacillus megaterium	1000	-	320	100
Bacillus subtilis	1980-2130	1000-1330	250-450	63-115
Clostridium botulinum	100-1200	800-1000	200-400	200
Lactococcus lactis	-	-	400	-
Listeria monocytogenes	1430-1600	-	512	-
Micrococcus sp.	-	60-110	10-100	-
Sarcina lutea	4000	1000	400-500	125
Staphylococcus aureus	1670-4000	1000-2500	350-540	120-200
Streptococcus faecalis	-	130	40	-
Gram-Negative				
Aeromonas hydrophila	550	-	100	-
Enterobacter aerogenes	2000	1000	1000	4000
Escherichia coli	1200-2000	1000-2000	400-1000	100
Klebsiella pneumoniae	1000	500	250	125
Pseudomonas sp	450-4000	400-4000	250-8000	100-8000
Salmonella	2000	1000	1000	1000
Salmonella typhimurium	-	-	180-300	-
Vibrio parahaemolyticus	-	-	50-100	-
Yersinia enterocolitica	350	-	-	-
Fungi				
Alternaria sp	-	-	100	-
Aspergillus flavus	-	-	200	-
Aspergillus niger	1000	400-500	200-250	125-200
Byssochlamys fulva	-	-	200	-
Candida albicans	1000	500-1000	125-250	125
Debarymyces hanseii	-	400	-	-
Penicillium digitatum	500	250	63	<32
Penicillium chrysogenum	500	250	125-200	63
Rhizopus nigricans	500	250	125	63
Saccharomyces bayanus	930	-	220	-
Saccharomyces cerevisiae	1000	500	125-200	32-200
Torula utilis	-	-	200	-
Toluraspora delbruekii	-	700	-	-
Zygosaccharomyces bailii	-	900	-	-
Zygosaccharomyces bisporus	-	400	-	-
Zygosaccharomyces rouxii	-	700	-	-

[1] Benzyl paraben not reported
[2] pH, incubation temperature and time vary
[3] Table adapted from Davidson, 2005

Table 2.6 List of chemical compounds evaluated with CAS number and Sigma-Aldrich catalog number.

CAS #	Common Name	Sigma-Aldrich Catalog #	Molecular Weight
94-18-8	Benzyl-4-hydroxbenzoate (Benzyl paraben)	54670	228.24
104-55-2	Cinnamaldehyde	W228605	132.16
110861-66-0	Cyclohexanebutyric acid (Calcium Cyclohexanebutyrate)	228141	378.56
112-31-2	Decanal (Caprinaldehyde; Decyl aldehyde)	W236209	156.27
1504-74-1	o-Methoxycinnamylaldehyde	W318108	162.19
1731-84-6	Methyl nonanoate (Methyl pelargonate; Nonanoic acid methyl ester)	W272418	172.26
25152-84-5	Trans, trans, 2,4 decadienal	W313505	152.23
104-61-0	Nonanoic lactone (Gamma-nonalactone)	W278106	156.22
2315-68-6	Propyl benzoate	307009	164.20
104-67-6	Undecalactone (Undecanoic Gamma-Lactone)	U806	184.28
112-44-7	Undecanal	W309206	170.29
101-39-3	Methyl trans cinnamylaldehyde (alpha-methyl-trans-cinnamaldehyde)	112275	146.19
18031-40-8	Perillaldehyde	W355704	150.22
89-83-8	Thymol	W306606	150.22
103-41-3	Cinnamic acid benzyl ester (Benzyl Cinnamate)	W214205	238.28
140-10-3	Cinnamic acid (trans-3-Phenylacrylic acid)	W228818	148.16
110-44-1	Sorbic acid (2 4 hexadienoic acid)	W392103	112.13
99-76-3	Methyl paraben (methyl p-Hydroxybenzoate)	W271004	152.15
94-13-3	Propyl paraben (propyl 4-Hydroxybenzoate)	W295101	180.20
89-82-7	Pulegone (R)-(+)	W296309	152.23
106-22-9	Citronellol (3,7-Dimethyl-6-octen-1-ol)	W230901	156.27
5392-40-5	Citral (3,7-Dimethyl-2,6-octadienal)(Geranial and neral mixture)	W230308	152.23
97-53-0	Eugenol	W246700	164.20
94-26-8	Butyl paraben (butyl 4-Hydroxybenzoate)	W220302	194.23
120-47-8	Ethyl paraben (ethyl 4-Hydroxybenzoate)	54660	166.17
93-15-2	Eugenol methyl ester (4-Allyl-1,2-dimethoxybenzene)	46110	178.23
5989-27-5	(R) - Limonene	W263303	136.23
18172-67-3	β-pinene	402753	136.23

CHAPTER 3

Determination of Aqueous Solubility of Natural Antimicrobial Compounds and Parabens

Abstract

Twenty eight compounds known to have antimicrobial activity were evaluated to accurately quantify their solubility. Twenty-three of the compounds evaluated are components of plant essential oils. The remaining five compounds are alkyl esters of *para*-hydroxybenzoic acid. The test compounds were evaluated for aqueous solubility as well as their solubility in an apple juice-based beverage mixture. UV/Vis spectophotometry techniques were used to quantify the solubility values. All of the compounds were found to be practically insoluble (< 100mg/L), very slightly soluble (100mg/L – 1,000mg/L) or slightly soluble (1,000mg/L to 10,000 mg/L). The comparison of the compounds' aqueous solubility against its solubility in the apple juice-based beverage mixture resulted in a general trend of decreased solubility in the beverage mixture.

Introduction

In November 2005, the Food and Drug Administration (FDA) received a small study that a private laboratory conducted indicating that low levels of benzene were present in a small number of soft drinks that contained benzoate salts and ascorbic acid (FDA, 2006). Benzene, a known human carcinogen and neurotoxin, was being produced by sodium benzoate reacting with ascorbic acid to produce free benzene (Fleming-Jones and Smith, 2003). The details of the reaction mechanism are described by Gardner and Lawrence, 1993. Sodium benzoate is used as an antimicrobial additive in a wide variety of foods and beverages including carbonated and still beverages.

In the task of finding a natural preservative suitable to replace sodium benzoate in beverages, 28 compounds known to have antimicrobial activity were evaluated to determine their aqueous solubility. In addition, all of the compounds were evaluated to determine their solubility in an apple juice-based beverage mixture.

Twenty-three of the compounds evaluated are components of plant essential oils (Table 3.1). Essential oils (EOs) are aromatic oily liquids obtained from plants and plant products such as fruits, seeds, and leaves. EOs have exhibited antiviral, antibacterial, antimycotic, antitoxigenic, antiparasitic, and insecticidal properties (Burt, 2004; Corbo and others, 2009).

The remaining five compounds included in this study are a group of alkyl esters of *para*-hydroxybenzoic acid commonly referred to as parabens (Table 3.1). Methyl paraben and propyl paraben are directly added to commercial food systems as antimicrobial agents (Davidson, 2005). The interest in these compounds in the

development of a beverage preservative arises because some are already in commercial use as food preservatives.

The objective of this study is to accurately determine the test compounds' aqueous solubility and their solubility in an apple juice-based beverage.

Materials and Methods

Materials. All compounds evaluated were supplied by Sigma-Aldrich (Table 3.1) (St. Louis, Missouri, USA). Ethyl alcohol, absolute, 99.5%, A.C.S. reagent was supplied by Arcos Organics (Geel, Belgium), Puradisc 25PP disposable filter device, 0.45 μm microcellulose was supplied by Whatman, Schleicher & Schuell (Florham Park, New Jersey, USA). Latex free 10 mL syringes were supplied by Becton Dickinson & Co. (Franklin Lakes, New Jersey, USA). A UV-Vis spectrophotometer, UV-2101PC was supplied by Shimadzu (Kyoto, Japan). Quartz cuvettes were supplied by Fisher Scientific. A refrigerated Shaker, Innova 4230, was supplied by New Burnswick Scientific (Edison, New Jersey, USA). Premium 100% pure apple juice, preservative free, pressed from fresh apples, not from concentrate, 64 Fl.oz (1/2 gallon) 1.89 L, pasteurized was supplied by Motts (Rye Brook, New York, USA). Alpha-D (+)-glucose, anhydrous 99+%, was supplied by Acros Organics (Geel, Belgium). D-Fructose, reagent grade (crystal) was supplied by Fisher Scientific (Fair Lawn, New Jersey, USA). Sucrose (α-D-glucopyranosyl; β-D-fructofuranoside; saccharose; cane sugar) was supplied by Sigma Chemical Company (St. Louis, Missouri, USA). Malic acid, food grade, powder was supplied by Sigma-Aldrich Chemical Company (St. Louis, Missouri, USA). Filtration products, 45 μm, filter unit- 500 mL, were supplied by Nalgene (Rochester, New York, USA).

Methods. Saturated solutions of the compounds in water were prepared by adding 200 mg of compound into 250 mL conical Erlenmeyer flasks with 100 mL of distilled water for a concentration of 2000 mg/L. Flasks with the concentrated solutions were capped with plastic stoppers and mechanically shaken for 24 hrs at 25^{o}C and 250 rpm. The solutions were then taken up into 10mL latex-free syringes, and filtered through 0.45 μm micro-cellulose filters into 20 mL test tubes to await analysis. Tubes were stored in a cabinet protected from light and were analyzed within 2 days.

Standard curves were prepared for all compounds by dissolving them in ethanol and serially diluting them with distilled water. UV absorption spectophotometry was performed to quantify the content of compound in solution with a Shimadzu UV-2101PC UV/VIS Scanning Spectrophotometer. Samples were placed in quartz cuvettes and ran on the UV/VIS. Settings for the UV/VIS included wavelength range from 190-400 nm. Distilled water was used as a reference and for a baseline scan. A portion of the samples were placed in quartz cuvettes and ran on the UV/VIS. The maximum absorbance of the spectra was recorded for each compound. Linear regression was then used to calculate the concentration of test compound in water. The solubility of methyl paraben was greater than 2000 mg/L, so the same procedure was followed with the exception that the saturated solution was concentrated to 3000 mg/L. The procedure was done in triplicate.

The preparation of the apple juice-based beverage mixture consisted of combining 100 mL of preservative free apple juice (Motts brand), 46.8g of glucose, 59.4g of fructose, 1.8g of sucrose in a 1L beaker. The mixture was then brought up to 1L with distilled water. The mixture was stirred at room temperature until all the ingredients were incorporated. The pH of the mixture was adjusted to 3.4 with 1M malic acid. The brix of

the mixture was verified to be between 12^{o} and 13^{o} (Abbe 3L refractometer). The mixture was filtered using 0.45 μm microcellulose filter units.

The evaluation of the test compounds' solubility in the apple juice-based beverage mixture was performed using the same procedure described for the aqueous solubility determination with the exception that the distilled water was replaced with the apple juice-based beverage. Standard curves were prepared for all compounds by dissolving them in ethanol and serially diluting them with the apple juice-based beverage.

Results and Discussion

Table 3.2 lists the maximum absorbance wavelength at which each test compound was evaluated. The standard curves prepared were analyzed by the method of least squares yielding correlation coefficients, R^2. The standard curves of thymol and trans,trans-2,4-decadienal showed the lowest R^2 values with 0.8502 and 0.8949 respectively. The remaining 26 compounds' standard curves had R^2 ranging form 0.9788 to 1.00, having excellent linearity (Table 3.2).

The aqueous solubility calculated for each of the compounds evaluated is shown in Table 3.3. The results are organized from the lowest to the highest water solubility observed. It can be noted that the values range from 1.6 mg/L for γ-nonalactone to 2460.6 mg/L for methyl paraben. Using the United States Pharmacopeia (USP # 24 NF 19) solubility descriptors, we can classify the compounds evaluated in three categories: practically insoluble, very slightly soluble, and slightly soluble. Table 3.4 depicts the USP solubility descriptive terms converted to milligrams of compound per liter of solvent. With aqueous solubility of less than 100mg/L, the following group of compounds can be described as practically insoluble in water: nonanoic lactone, β-pinene, benzyl cinnamate,

R-limonene, cyclohexanebutyric acid, methyl nonanoate, benzyl paraben, propyl benzoate, and trans, trans-2, 4-decadienal. The group of very slightly (100 mg/L to 1000 mg/L) water soluble compounds tested include: perillaldehyde, butyl paraben, undecalactone, citronellol, eugenol methyl ester, citral, methyl,trans-cinnamaldehyde, propyl paraben, undecanal, cinnamic acid, cinnamaldehyde, o-methoxycinnamaldehyde, decanal, pulegone, and ethyl paraben. Finally, the group of slightly soluble test compounds (1,000 mg/L to 10,000 mg/L) include: thymol, eugenol, sorbic acid, and methyl paraben.

The aqueous solubilities of parabens were reported by Davidson, 2005. The results of this study found similar values and confirmed the trend that the longer the alkyl chain of the paraben the lower its water solubility.

The results of solubility evaluations of the test compounds dissolved in the apple juice-based beverage mixture are shown in Table 3.5. The beverage mixture interfered with the maximum absorbance wavelengths of decanal, eugenol methyl ester, limonene and pinene. For that reason, the solubility of those compounds dissolved in the beverage mixture could not be determined using this particular method. The apple juice-based beverage mixture is intended to mimic a commercial beverage and the two most influential factors are soluble solids content and pH. A direct comparison of the test compounds' solubility in water and beverage mixture is shown in Table 3.6. It can be noted that although the solubility values are fairly similar to the aqueous solubility values, there are some general trends that can be differentiated. Of the 24 compounds evaluated, 18 compounds showed a decrease in maximum solubility compared to their water solubility. This is consistent with the study by Terrance and LeMaguer (1980) which

found that all the terpenic essential oil components included in their study exhibited reduced aqueous solubility as the concentration of soluble solids was increased, “irrespective of the nature of the solid”.

It is well known that the presence of soluble solids may reduce the solubility of organic compounds in water because the water molecules that hydrate the soluble solids become no longer available to dissolve the other compounds; in this case the test compounds (Terrance, 1980). The only compound that showed a significant increase of solubility in the beverage mixture was propyl benzoate with an increase of 53%. However, the increase in solubility was only from practically insoluble in water (53.9 mg/L) to slightly soluble in the beverage mixture (116.6 mg/L). γ-nonalactone exhibited an increase in solubility from 1.6mg/L in water to 2.7mg/L in the beverage mixture however the compound remained practically insoluble. Citral, thymol, undecanal, and citronellol showed very slight increases in solubility in the beverage mixture ranging between 1.3% and 6.1%. Nevertheless, considering the average of standard deviations of the two analyses which range between 9.5% and 30.5% it can be concluded that the solubility values for these four compounds remained constant in both mediums.

References

Burt, S. 2004. Essential Oils: Their Antibacterial Properties and Potential Applications in Foods – a review. *International Journal of Food Microbiology*, 94, 223–253.

Corbo, M.R., Bevilacqua, A., Campaniello, D., D'Amato, D., Speranza, B., Sinigalglia, M., 2009. Prolonging Microbial Shelf Life of Foods Through the Use of Natural Compounds and Non-thermal Approaches- a review. *International Journal of Food Science and Technology.* 44, p.223-241

Davidson, M.P. 2005. Parabens. In *Antimicrobials in Food 3rd Ed.* Davidson, P.M., Sofos, J.N., Branen, A.L. Taylor and Francis Group. Boca Raton, FL. P.291-304

Food and Drug Administration (FDA). 2006. Data on Benzene in Soft Drinks and Other Beverages. Office of Food Additive Safety, CFSAN, Food and Drug Administration. Received October 2007 from http://www.cfsan.fda.gov/~dms/benzdata.html.

Fleming-Jones M, Smith R. 2003. Volatile Organic Compounds in Foods: A Five Year Study. *J. Agric. Food Chem.* 51: 8120-8127.

Gardner K. L.; Lawrence G. D. 1993. Benzene Production from Decarboxylation of Benzoic Acid in the Presence of Ascorbic Acid and a Transition-Metal Catalyst. *J. Agric. Food Chem.* 41(5): 693-695

Terrance, G. Smyrl; LeMaguer, Marc. 1980. Solubilities of Terpenic Essential Oil Components in Aqueous Solutions. *J. Chem. Eng. Data.* 25: 150-152

United States Pharmacopeia. Description and Solubility. USP #24 NF 19.*United States Pharmacopeial Convention, Inc.,* Rockville, MD

Tables and Figures

Table 3.1 List of chemical compounds evaluated with CAS number and Sigma –Aldrich Catalog number

CAS #	Common Name	Sigma-Aldrich Catalog #	Molecular Weight
94-18-8	Benzyl-4-hydroxbenzoate (Benzyl paraben)	54670	228.24
104-55-2	Cinnamaldehyde	W228605	132.16
110861-66-0	Cyclohexanebutyric acid (Calcium Cyclohexanebutyrate)	228141	378.56
112-31-2	Decanal (Caprinaldehyde; Decyl aldehyde)	W236209	156.27
1504-74-1	o-Methoxycinnamylaldehyde	W318108	162.19
1731-84-6	Methyl nonanoate (Methyl pelargonate; Nonanoic acid methyl ester)	W272418	172.26
25152-84-5	Trans, trans, 2,4 decadienal	W313505	152.23
104-61-0	Nonanoic lactone (Gamma-nonalactone)	W278106	156.22
2315-68-6	Propyl benzoate	307009	164.20
104-67-6	Undecalactone (Undecanoic Gamma-Lactone)	U806	184.28
112-44-7	Undecanal	W309206	170.29
101-39-3	Methyl trans cinnamylaldehyde (alpha-methyl-trans-cinnamaldehyde)	112275	146.19
18031-40-8	Perillaldehyde	W355704	150.22
89-83-8	Thymol	W306606	150.22
103-41-3	Cinnamic acid benzyl ester (Benzyl Cinnamate)	W214205	238.28
140-10-3	Cinnamic acid (trans-3-Phenylacrylic acid)	W228818	148.16
110-44-1	Sorbic acid (2 4 hexadienoic acid)	W392103	112.13
99-76-3	Methyl paraben (methyl p-Hydroxybenzoate)	W271004	152.15
94-13-3	Propyl paraben (propyl 4-Hydroxybenzoate)	W295101	180.20
89-82-7	Pulegone (R)-(+)	W296309	152.23
106-22-9	Citronellol (3,7-Dimethyl-6-octen-1-ol)	W230901	156.27
5392-40-5	Citral (3,7-Dimethyl-2,6-octadienal)(Geranial and neral mixture)	W230308	152.23
97-53-0	Eugenol	W246700	164.20
94-26-8	Butyl paraben (butyl 4-Hydroxybenzoate)	W220302	194.23
120-47-8	Ethyl paraben (ethyl 4-Hydroxybenzoate)	54660	166.17
93-15-2	Eugenol methyl ester (4-Allyl-1,2-dimethoxybenzene)	46110	178.23
5989-27-5	(R) - Limonene	W263303	136.23
18172-67-3	β-pinene	402753	136.23

Table 3.2 Maximum absorbance wavelength at which compounds were evaluated and correlation coefficient of standard curves

Test Compound	Maximum Absorbance Wavelenght nm	Correlation Coefficient of Standard Curves R^2
Benzyl-4-hydroxbenzoate (Benzyl paraben)	253	0.9997
Cinnamaldehyde	289	0.9869
Cyclohexanebutyric acid (Calcium Cyclohexanebutyrate)	193	1.000
Decanal (Caprinaldehyde; Decyl aldehyde)	289	0.9973
o-Methoxycinnamylaldehyde	287	0.9983
Methyl nonanoate (Methyl pelargonate; Nonanoic acid methyl ester)	210	0.9997
Trans, trans, 2,4 decadienal	278	0.8949
Nonanoic lactone (Gamma-nonalactone)	218	0.9825
Propyl benzoate	230	0.9979
Undecalactone (Undecanoic Gamma-Lactone)	211	0.9857
Undecanal	204	0.9943
Methyl trans cinnamylaldehyde (alpha-methyl-trans-cinnamaldehyde)	286	0.9999
Perillaldehyde	235	0.9903
Thymol	197	0.8502
Cinnamic acid benzyl ester (Benzyl Cinnamate)	278	0.9962
Cinnamic acid (trans-3-Phenylacrylic acid)	274	0.9998
Sorbic acid (2 4 hexadienoic acid)	256	0.9995
Methyl paraben (methyl p-Hydroxybenzoate)	255	0.9994
Propyl paraben (propyl 4-Hydroxybenzoate)	256	0.9988
Pulegone (R-) (+)	260	0.9924
Citronellol (3,7-Dimethyl-6-octen-1-ol)	197	0.9902
Citral (3,7-Dimethyl-2,6-octadienal)(Geranial and neral mixture)	244	0.9987
Eugenol	197	0.9996
Butyl paraben (butyl 4-Hydroxybenzoate)	255	0.9997
Ethyl paraben (ethyl 4-Hydroxybenzoate)	255	0.9991
Eugenol methyl ester (4-Allyl-1,2-dimethoxybenzene)	199	0.9995
(R) - Limonene	195	0.9921
β-pinene	197	0.9788

Table 3.3 Results of aqueous solubility evaluations

Test Compound	Water Solubility n = 3	
	mg/L	s.d.
Nonanoic lactone (Gamma-nonalactone)	1.6	0.8
β-pinene	4.5	1.0
Cinnamic acid benzyl ester (Benzyl Cinnamate)	14.1	4.5
(R) - Limonene	33.3	5.4
Cyclohexanebutyric acid (Calcium Cyclohexanebutyrate)	43.9	0.3
Methyl nonanoate (Methyl pelargonate; Nonanoic acid methyl ester)	48.5	18.2
Benzyl-4-hydroxbenzoate (Benzyl paraben)	49.9	14.9
Propyl benzoate	53.9	3.8
Trans, trans, 2,4 decadienal	55.7	0.96
Perillaldehyde	134.6	4.4
Butyl paraben (butyl 4-Hydroxybenzoate)	147.1	10.5
Undecalactone (Undecanoic Gamma-Lactone)	173.9	45.0
Eugenol methyl ester (4-Allyl-1,2-dimethoxybenzene)	282.9	46.1
Citronellol (3,7-Dimethyl-6-octen-1-ol)	294.1	83.7
Citral (3,7-Dimethyl-2,6-octadienal)(Geranial and neral mixture)	362.6	7.8
Methyl trans cinnamylaldehyde (alpha-methyl-trans-cinnamaldehyde)	388.8	46.5
Propyl paraben (propyl 4-Hydroxybenzoate)	426.0	39.7
Undecanal	566.0	193.5
Cinnamic acid (trans-3-Phenylacrylic acid)	573.1	40.3
Cinnamaldehyde	589.9	49.1
o-Methoxycinnamaldehyde	602.9	2.7
Decanal (Caprinaldehyde; Decyl aldehyde)	640.5	82.5
Pulegone (R-) (+)	808.7	130.1
Ethyl paraben (ethyl 4-Hydroxybenzoate)	940.9	280.6
Thymol	1097.0	117.7
Eugenol	1282.2	241.9
Sorbic acid (2 4 hexadienoic acid)	1514.5	179.9
Methyl paraben (methyl p-Hydroxybenzoate)	2460.6	65.1

Table 3.4 United States Pharmacopeia (USP#24 NF 19) descriptive terms for solubility values of chemical compounds

Descriptive Term	mg/L	
	From	To
Very soluble	>1,000,000	>1,000,000
Freely soluble	1,000,000	100,000
Soluble	100,000	33,000
Sparingly soluble	33,000	10,000
Slightly soluble	10,000	1,000
Very slightly soluble	1,000	100
Practically insoluble, or Insoluble	100	< 100

Table 3.5 Results of solubility evaluations of test compounds dissolved in apple juice-based beverage mixture (ABM) [1]

Test Compound	Solubility in ABM n = 3	
	mg/L	s.d.
Cinnamic acid benzyl ester (Benzyl Cinnamate)	2.16	0.24
Nonanoic lactone (Gamma-nonalactone)	2.65	0.70
Methyl nonanoate (Methyl pelargonate; Nonanoic acid methyl ester)	31.25	1.06
Benzyl-4-hydroxbenzoate (Benzyl paraben)	39.21	8.19
Cyclohexanebutyric acid (Calcium Cyclohexanebutyrate)	41.37	11.28
Trans, trans, 2,4 decadienal	54.28	0.80
Perillaldehyde	113.67	5.02
Propyl benzoate	116.61	13.93
Undecalactone (Undecanoic Gamma-Lactone)	153.70	21.92
Butyl paraben (butyl 4-Hydroxybenzoate)	148.09	12.10
Citronellol (3,7-Dimethyl-6-octen-1-ol)	298.08	38.46
Methyl trans cinnamylaldehyde (alpha-methyl-trans-cinnamaldehyde)	332.35	61.01
Propyl paraban (propyl 4-Hydroxybenzoate)	367.51	30.39
Citral (3,7-Dimethyl-2,6-octadienal)(Geranial and neral mixture)	386.52	230.16
Cinnamaldehyde	406.79	92.50
Cinnamic acid (trans-3-Phenylacrylic acid)	414.50	17.68
o-Methoxycinnamaldehyde	498.30	72.06
Undecanal	575.90	245.37
Pluegone (R-) (+)	670.84	15.40
Ethyl paraben (ethyl 4-Hydroxybenzoate)	758.05	101.14
Eugenol	1211.27	289.46
Thymol	1119.78	94.35
Sorbic acid (2 4 hexadienoic acid)	1274.57	195.80
Methyl paraben (methyl p-Hydroxybenzoate)	1992.77	206.13
Decanal (Caprinaldehyde; Decyl aldehyde)	*	
Eugenol methyl ester (4-Allyl-1,2-dimethoxybenzene)	*	
(R) - Limonene	*	
β-pinene	*	

[1]Apple juice-based beverage mixture: Apple juice with soluble solids between 12^{o} and 13^{o} brix, acidified with malic acid to pH of 3.4

*Components of the beverage mixture interfered with the compounds maximum absorbance wavelength.

Table 3.6 Comparison of water solubility and solubility in beverage mixture of compounds evaluated

Test Compound	Water Solubility n = 3 mg/L	ABM[1] Solubility n = 3 mg/L	Solubility Change (%)
Cinnamic acid benzyl ester (Benzyl Cinnamate)	14.1	2.2	-84.69
Propyl benzoate[2]	53.9	116.6	53.80
Nonanoic lactone (Gamma-nonalactone)[2]	1.6	2.65	38.81
Methyl nonanoate (Methyl pelargonate; Nonanoic acid methyl ester)	48.5	31.3	-35.57
Cinnamaldehyde	589.9	406.8	-31.05
Cinnamic acid (trans-3-Phenylacrylic acid)	573.1	414.5	-27.68
Benzyl-4-hydroxbenzoate (Benzyl paraben)	49.9	39.2	-21.35
Ethyl paraben (ethyl 4-Hydroxybenzoate)	940.9	758.1	-19.43
Methyl paraben (methyl p-Hydroxybenzoate)	2460.6	1992.8	-19.01
o-Methoxycinnamaldehyde	602.9	498.3	-17.35
Pulegone (R-) (+)	808.7	670.8	-17.05
Sorbic acid (2 4 hexadienoic acid)	1514.5	1274.6	-15.84
Perillaldehyde	134.6	113.7	-15.55
Methyl trans cinnamylaldehyde (alpha-methyl-trans-cinnamaldehyde)	388.8	332.4	-14.52
Propyl paraben (propyl 4-Hydroxybenzoate)	426.0	367.5	-13.74
Undecalactone (Undecanoic Gamma-Lactone)	173.9	153.7	-11.60
Citral (3,7-Dimethyl-2,6-octadienal)(Geraniol and neral mixture)[2]	362.6	386.5	6.18
Cyclohexanebutyric acid (Calcium Cyclohexanebutyrate)	43.9	41.4	-5.76
Eugenol	1282.2	1211.3	-5.53
Trans, trans, 2,4 decadienal	55.7	54.28	-2.49
Thymol[2]	1097.0	1119.8	2.03
Undecanal[2]	566.0	575.9	1.71
Citronellol (3,7-Dimethyl-6-octen-1-ol)[2]	294.1	298.1	1.32
Butyl paraben (butyl 4-Hydroxybenzoate)	147.1	148.1	0.65
(R) - Limonene	33.3	*	
Decanal (Caprinaldehyde; Decyl aldehyde)	640.5	*	
β-pinene	4.5	*	
Eugenol methyl ester (4-Allyl-1,2-dimethoxybenzene)	282.9	*	

[1]Apple juice-based beverage mixture: Apple juice with soluble solids between 12° and 13° brix, acidified with malic acid to pH of 3.4

[2]Compounds' solubility increased in ABM

*Components of the beverage mixture interfered with the compounds maximum absorbance wavelength.

CHAPTER 4

Formation and characterization of α- and β-cyclodextrin complexes with natural antimicrobial compounds

Abstract

o-Methoxycinnamaldehyde, *trans,trans*-2,4-decadienal, cinnamic acid, and citronellol were complexed with α- and β- cyclodextrin and evaluated through phase solubility analyses. The complex formation showed improved aqueous solubility for all compounds. The maximum concentration of guest compound that α-CD complexed resulted in 3697.4 mg/L of *o*-methoxycinnamaldehyde, 281.3mg/L of *trans,*trans-2,4-decadienal, 3411.7 mg/L of cinnamic acid, and 2437.6 mg/L of citronellol. In addition, β-CD complexed 811.8 mg/L of *o*-methoxycinnamaldehyde, 235.3mg/L of *trans,*trans-2,4-decadienal, 905.9 mg/L of cinnamic acid, and 449.4 mg/L of citronellol. α-CD complexes showed larger solubility increases than β-CD complexes. Solid inclusion complexes were prepared and analyzed for the four test compounds' α-CD complexes. Weight percentages attributed to the test compounds were determined yielding 3.7% of *o*-methoxycinnamaldehyde, 6.7% of *trans,*trans-2,4-decadienal, 8.9% of cinnamic acid, and 7.2% of citronellol.

Introduction

In April 2006 The Food and Drug Administration released a study concerning levels of benzene in commercial beverages (FDA, 2006). Sodium benzoate, a common preservative, was reacting with ascorbic acid to produce free benzene (FDA, 2006). Due to its broad availability and low cost, sodium benzoate, is used in many products including carbonated and still beverages. The mechanism by which benzene, a known human carcinogen and neurotoxin, is produced is described by Gardner and Lawrence, 1993. The study suggests that transition metals present in tap water catalyze a one-electron reduction of oxygen by ascorbic acid to produce the superoxide anion radical "which undergoes spontaneous disproportionation to produce hydrogen peroxide" (Gardner and Lawrence, 1993). The reduction of hydrogen peroxide by ascorbic acid generates a hydroxyl radical. Finally, the hydroxyl radical generated by the metal-catalyzed reduction of oxygen and hydrogen peroxide by ascorbic acid attacks benzoic acid to produce benzene under conditions prevalent in many food systems (Gardner and Lawrence, 1993). The concentration of benzene production increases with exposure to elevated temperatures (Kyoung and others, 2008). Consequently, soft drinks and beverages with ascorbic acid and added sodium benzoate that are subjected to intense heat can be susceptible for the production of benzene.

With a need to replace sodium benzoate, four natural compounds known to have antimicrobial activity but with limited aqueous solubilities were chosen to form molecular inclusion complexes with α-cyclodextrin (α-CD) and β-cyclodextrin (β-CD). The natural antimicrobial compounds included in this study were: *o*-methoxycinnamaldehyde, *trans, trans*-2,4-decadienal, cinnamic acid, and citronellol.

The objectives are to study the formation of α- and β-CD complexes with the test compounds through phase solubility analyses. In addition phase solubility analyses will be used to determine if the compounds' complexation with α-CD and β-CD show improvements in aqueous solubility and to determine the maximum amount of guest compound that could be complexed. Finally, solid inclusion complexes were formed to obtain a physical complex and characterize they weight percent attributable to the test compounds.

Materials and Methods

Materials. *o*-Methoxycinnamaldehyde ≥ 96%, Kosher, food grade (FG), *trans,trans*-2,4-decadienal, Kosher, FG, *trans*-cinnamic acid, Kosher, FG, citronellol FCC, ≥95%, supplied by Sigma-Aldrich (St. Louis, Missouri, USA). α-Cyclodextrin, CAVAMAX ® W6 and β-cyclodextrin, CAVAMAX ® W7 supplied by Wacker Fine Chemicals (Munich, Germany). Ethyl alcohol, absolute, 99.5%, A.C.S. reagent was supplied by Arcos Organics (Geel, Belgium). Puradisc 25PP disposable filter devices, 0.45 μm microcellulose membranes, were supplied by Whatman, Schleicher & Schuell (Florham Park, New Jersey, USA). Latex free 10 mL syringes, were supplied by Becton Dickinson & Co. (Franklin Lakes, New Jersey, USA). A UV-Vis spectrophotometer, UV-2101PC, was supplied by Shimadzu (Kyoto, Japan). Quartz cuvettes were supplied by Fisher Scientific. A refrigerated shaker, Innova 4230, was supplied by New Burnswick Scientific (Edison, New Jersey, USA).A Sentry Freezemobile (12SL) freeze dryer was supplied by Virtis (Gardiner, New York, USA).

Phase Solubility Analysis. Phase solubility studies were performed on citronellol, *o*-methoxycinnamaldehyde, *trans,trans*-2,4-decadienal and *trans*-cinnamic acid

complexed with α and β-CDs. 10, 40, 70, 100, and 130 mmol/L of α-CD were added to five 50 mL polypropylene conical tubes and respectively labeled. A 0 mmol/L α-CD sample was prepared to reference the solubility of the test compound by itself. The tubes were filled to a volume of 20 mL with distilled water. Tubes were tightly capped and mechanically shaken for 24 hrs at 25^{o}C and 250 rpm (Innova 4230, refrigerated shaker). After 24 hrs the tubes were taken out of the shaker and each test compound was added to the aqueous-CD solution in excess. One hundred mg each test compound were added to the 20 mL of aqueous cyclodextrin solution for a concentration of 5000 mg/L. The tubes were re-capped and mechanically shaken for 48 hrs at 25^{o}C and 250 rpm. After 48 hrs the shaker was turned off and the tubes were left stationary for 24 hrs at 25^{o}C to allow any excess test compound to settle out of solution. The solution was extracted using a 10 mL syringe and filtered with a 0.45 μm micro-cellulose filter tip and placed into glass vials. One mL of sample was diluted with 1 mL of 100% ethanol in order to disassociate the complex. UV absorption spectophotometry was performed with a Shimadzu UV-2101PC UV-VIS scanning spectrophotometer to quantify the content of compound in solution. The diluted sample was then transferred to a quartz cuvette and analyzed. A 50% aqueous ethanol solution was used as a blank. Standard curves were prepared for each test compound by mixing the compound with ethanol and diluting it in 50% aqueous ethanol. Linear regression analysis was used to quantify the concentration of test compound in each solution.

For phase solubility analysis with β-cyclodextrin, the same procedure was followed with the exception that the concentrations of β-CD added were different. β-CD was added to four tubes labeled: 4 mmol/L, 8 mmol/L, 12 mmol/L and 16 mmol/L. β-CD

was added to each tube appropriately and then filled with distilled water up to a volume of 20 mL. A 0 mmol/L β-CD sample was prepared to reference the solubility of the test compound by itself. The rest of the procedure was carried out as described above. The procedure was done in triplicate therefore values reported are averages of n =3.

The molar ratios of the complexes in solution were calculated at the highest solubility increase shown by the phase solubility curves. The calculated moles of guest compound at the highest point of the phase solubility chart were divided by the moles of α- or β-CD at that same point. The result is a guest to host molar ratio expressed in moles of guest [G] to moles of host [H]; [G]:[H].

Solid Inclusion Complex. Solid inclusion complexes were prepared for *o*-methoxycinnamaldehyde, *trans,trans*-2,4-decadienal, cinnamic acid, and citronellol complexed with α-CD. The concentration of CD which showed the largest increase in solubility for each compound in the phase solubility analysis was added to 1 L of distilled water. The aqueous-CD solution was shaken for 24 hrs at 25^{o}C and 250 rpm (Innova 4230, refrigerated shaker). An excess of 5000mg of test compound was added to the solution and placed back on the shaker for 48 hrs at 25^{o}C and 250 rpm. After 48 hrs the solution was filtered and placed in wide shallow dishes covered with plastic wrap and frozen until solid in a freezer set to -18^{o}C. The samples were freeze dried over four days (Sentry Freezemobile, Virtis,). The solid samples were stored in closed bottles inside a desiccator until further analysis.

Characterization. UV absorption spectophotometry was used to determine the percentage of test compound found in the solid complex. A concentration 1000mg/L of solid complex were diluted in distilled water. One mL of the complex solution was

diluted in one mL of ethanol to dissociate the complex and have a final 50% aqueous ethanol solution. UV/Vis was used to determine the concentration of test compound in solution. The maximum absorbance at each compounds' specific wavelength was plotted against a standard curve prepared in the same 50% aqueous ethanol solution. The weight percent (%) was calculated by dividing the concentration of guest by concentration of complex added to solution.

Results and Discussion

Phase Solubility Analyses. Standard curves for all 4 test compounds were prepared in 50% aqueous ethanol the same solvent that was used to dissolve the aqueous complex solutions before UV/Vis analysis. Table 3.1 lists the maximum absorbance wavelength at which each test compound was evaluated. The standard curves prepared were analyzed by the method of least squares yielding correlation coefficients, R^2, from 0.9847 to 0.9995 depicting excellent linearity (Table 4.1).

The phase solubility diagrams of the four compounds evaluated are shown in Figures 4.1 through 4.4. Table 4.2 shows a summary of the guest molecule:cyclodextrin ratios. Molar ratios were calculated at the highest point of the phase solubility curves.

Figure 4.1 depicts the phase solubility diagrams of *o*-methoxycinnamaldehyde-CD complexes. The curve shown in Figure 4.1a shows the maximum amount of *o*-methoxycinnamaldehyde complexed by α-CD as 5133 mg/L. The optimum uptake was at a concentration of 0.13 M α-CD aqueous solution. At the highest point of the solubility isotherm the complex formation increased the solubility of *o*-methoxycinnamaldehyde from 505 mg/L to 5133 mg/L for a 10 fold increase. The moles of *o*-methoxycinnamaldehyde divided by the moles of α-CD denote a molar ratio of 0.24 or

approximately 1:4 guest to host. An important observation is that *o*-methoxycinnamaldehyde was added to the system in excess at a concentration of 5000 mg/L. The average calculated guest content of 5133 mg/L is higher than the excess concentration added with a standard deviation of ± 1074 mg/L giving insight that nearly all or all the compound present was complexed at a concentration of 0.13 M α-CD aqueous solution. This suggests that if more compound was present in the system the reaction could yield a more efficient complex than what was observed in this study. It can be noted that the correlation of *o*-methoxycinnamaldehyde concentration with that of α-CD is very linear (R^2=0.91). This linearity depicts a behavior in which *o*-methoxycinnamaldehyde presents an unlimited increase in solubility as the concentration of CD is increased. When this correlation is strictly linear, a complex of constant stoichiometry is formed (Szejtli, 1988).

Figure 4.1b depicts the phase solubility diagram of *o*-methoxycinnamaldehyde-β-CD complex. The solubility increase limit of the complex was observed at a concentration of 0.004 M β-CD aqueous solution. At this point the concentration of *o*-methoxycinnamaldehyde was 811 mg/L. From its initial solubility of 505 mg/L, the complex formation showed an increase in solubility of 1.6 fold. At a concentration of 0.004 M β-CD the molar ratio was 1.25 which is higher than 1:1 in favor of the guest. When the correlation of guest's concentration and CD concentration is not linear, the solubility increase deviates upward or downward. This happens when the solubility increases faster or slower than the concentration of CD. In these cases the guest to host ratio is not constant, thus it increases or decreases. The solubility limit of the complex can be observed when the curve reaches the end of the linearly increasing section which

results in a plateau. At this point increasing CD's concentration results in no further increase in the guest's solubility. When the concentration of guest in the system begins to fall, it signals that the maximum amount of guest present in the system is complexed, so the apparent solubility begins to decrease. The plateau's height signals the solubility of the complex and its length its stoichiometry (Szejtli, 1988).

Figure 4.2 shows the phase solubility diagrams of *trans,trans*-2,4-decadienal-CD complexes. The phase solubility diagram of the compound complexed with α-CD is shown in figure 4.2a. It can be noted that the solubility increase limit was seen at 0.01 M α-CD aqueous solution. The maximum concentration of *trans,trans*-2,4-decadienal complexed by α-CD was of 281.3 mg/L in aqueous solution. From an initial solubility of 86.85 mg/L there was a solubility increase of 3.2 fold. The molar ratio of α-CD *trans,trans*-2,4-decadienal complex was 0.18, approximately 1:5 guest to host. Figure 4.2b shows the phase solubility diagram of *trans,trans*-2,4-decadienal complexed with β-CD. The solubility increase limit was seen at 0.008 M β-CD aqueous solution. From the initial solubility of 86.85 mg/L the complex formation increased the compound's solubility to a maximum of 235.28 mg/L for a 3.1 fold increase. The molar ratio of β-CD *trans,trans*-2,4-decadienal complex was 0.19 or approximately 1:5 guest to host. Both solubility isotherms showed non-linear correlations.

The phase solubility diagrams of cinnamic acid-CD complexes are shown in figure 4.3. Figure 4.3a depicting the complex of cinnamic acid and α-CD shows the solubility increase limit at 0.04 M α-CD aqueous solution. The maximum concentration of cinnamic acid complexed by α-CD was 3411 mg/L aqueous solution. From a solubility of 527.13 mg/L there was a 6.3 fold increase when complexed with 0.04 M α-CD. A

molar ration of 0.58 was calculated. The 1:2 molar ratio has been observed in other studies involving α-CD and cinnamic acid (Romano, 2008; Truong, 2007). In addition literature shows results of 2518 mg/L for a cinnamate ion-α-cyclodextrin complex, although co-solvents were used to aid complex formation (Connors and Rosanske, 1980). The phase solubility diagram for β-CD cinnamic acid complex is shown in figure 4.3b. The solubility increase limit was seen 0.004 M of β-CD with a concentration of 905.99 mg/L of cinnamic acid aqueous solution. The solubility increase from 527.13 mg/L was of 1.7 fold. The molar ratio calculated was approximately 2:1 (1.53). Both of the molar ratios calculated for α- and β-CD-cinnamic acid complexes are comparable to values found in literature (Connors and Rosanske, 1980, Dodziuk and others 1999).

The phase solubility charts for α- and β-CD-citronellol complexes are shown in figure 4.4. Figure 4.4a illustrates the limit of solubility increase for α-CD-citronellol complex at concentration 0.04 M α-CD. The maximum concentration of citronellol complexed was 2437.6 mg/L. The solubility of citronellol by itself was calculated to 300.03 mg/L so the maximum amount complexed represents an 8 fold increase in solubility. At the highest point of the phase solubility curve the molar ratio was calculated to 0.39 approximately 1:3 guest to host. Figure 4.4b displays the phase solubility diagram of the β-CD-citronellol complex. The maximum concentration of citronellol complexed by β-CD was 449.43 mg/L. The optimal uptake of citronellol was at 0.004 M of β-CD. The formation of this complex increased the solubility of citronellol by 1.6 times. The average molar ratio calculated was close to 1:1 (0.72).

An increase in solubility of a poorly soluble substance as CD concentration is increased in aqueous solution indicates complex formation (Szejtli, 1988). In theory the

stoichiometry of CD complexes is characterized by constant guest:host ratios. However, in practice there are factors that modify the composition of the complexes. For instance, in solution the association/dissociation equilibrium and the size and shape of the guest allow a variety of guest to host ratios of complexes to co-exist. The general rule is that for aqueous solutions the 1:1 complex is predominant (Szejtli, 1988). According to theoretical and past experimental observations, the fact that the molar ratios calculated in this study show α- and β-CD complexes with values lower than 1:1 is more likely to indicate that not all of the CD molecules present in the system were forming inclusion complexes as opposed to the idea that more than one cyclodextrin molecule was needed to fully complex the guest compound. Nevertheless, further characterization of the inclusion complexes would be needed.

In all instances the larger cavity of β-CD produced higher molar ratios, favoring the guest, than α-CD complexes. Moreover, α-CD was more effective for the purpose of increasing the aqueous solubility of the host despite the less efficient molar ratios. The higher solubility of α-CD allowed less efficient complexes to still have larger increases in aqueous solubilities of the guests.

Solid Inclusion Complexes. Given that α-CD complexes showed better results for the purpose of increasing the aqueous solubility of the test compounds, solid inclusion complexes were prepared for the four test compounds and α-CD complexes. A solid inclusion complex allows physical characterization and the determination of weight percentage (%) of host. The resulting complex of α-CD-*o*-methoxycinnamaldehyde resulted in a lightweight, flaky white powder with very different physical appearance and color than the α-CD, *o*-methoxycinnamaldehyde physical mixture. The resulting complex

of α-CD-*trans,trans*-2,4-decadienal was a lightweight powder with a uniform but very slight hint of yellow. α-CD-cinnamic acid and α-CD-citronellol complexes resulted both in lightweight white powders.

The calculation of weight percentage (%) attributed to the guest compound in the complex, was done by dividing the concentration of compound calculated , by the total concentration of complex added to the system and multiplied by 100.

The weight percentage calculation for α-CD-*o*-methoxycinnamaldehyde complex resulted in a 3.7% of the complex's weight attributable to the compound. A low weight percentage is expected since the molar ratio indicated a 1:4 guest to host. For the α-CD-*trans,trans*-2,4-decadienal complex a weight percentage of 6.7% resulted. Again a low weight percentage confirms a molar ratio with a higher proportion of host than guest. The α-CD-cinnamic acid complex showed a weight percent of 8.9% of cinnamic acid. This value was confirmed with past work on α-CD-cinnamic acid complexes by Truong, 2007 and Romano, 2008. Finally, the α-CD-citronellol complex resulted in 7.3% of weight attributable to citronellol.

References

Connors, K., and T. Rosanske. 1980. trans-Cinnamic Acid-a-Cyclodextrin System as Studied by Solubility, Spectral, and Potentiometric Techniques. *Journal of Pharmaceutical Sciences*. 69:173-9.

Dodziuk H, Ejchart A, Lukin O and Vysotsky MO. 1999. 1H and (13)C NMR and Molecular Dynamics Study of Chiral Recognition of Camphor Enantiomers by alpha-Cyclodextrin. The Journal of Organic Chemistry 64(5):1503-1507.

Food and Drug Administration (FDA). 2006. Data on Benzene in Soft Drinks and Other Beverages. Office of Food Additive Safety, CFSAN, Food and Drug Administration. Received October 2007 from http://www.cfsan.fda.gov/~dms/benzdata.html.

Gardner K. L.; Lawrence G. D. 1993. Benzene Production from Decarboxylation of Benzoic Acid in the Presence of Ascorbic Acid and a Transition-Metal Catalyst. *J. Agric. Food Chem*. 41(5): 693-695

Kyoung Hyun Ju, Jeong Hill Park, Sung Won Kwon. 2008. Evaluation of Headspace-Gas Chromatography/Mass Spectrometry for the Analysis of Benzene in Vitamin C Drinks; Pitfalls of Headspace in Benzene Detection. *Biomedical Chromatography*. 22 (8): 900-905

Romano, L. D. 2008. Characterization of α-Cyclodextrin Inclusion Complexes with *trans*-Cinnamic Acid in an Acid-Based Beverage System**.** M.S. Thesis, Virginia Polytechnic Institute and State University, Blacksburg.

Szejtli J. 1988. Cyclodextrin Technology. ed. vol. Dordrecht: Kluwer Academic Publishers.

Truong, T. T. 2008. Effect of Cinnamic Acid-Cyclodextrin Inclusion Complexes on Populations of *Escherichia coli* O157:H7 and *Salmonella enterica* in Fruit Juices M.S. Thesis, Virginia Polytechnic Institute and State University, Blacksburg.

Tables and Figures

Table 4.1 Maximum absorbance wavelength and correlation coefficients for standard curves

Test Compound	Maximum Absorbance Wavelenght nm	Correlation Coefficient of Standard Curves R^2
o-methoxycinnamylaldehyde	287	0.9995
Trans, trans, 2,4 decadienal	278	0.9987
Cinnamic acid (trans-3-Phenylacrylic acid)	274	0.997
Citronellol (3,7-Dimethyl-6-octen-1-ol)	197	0.9847

Table 4.2 Guest to host molar ratios of cyclodextrin complexes

	Guest molecule:cyclodextrin ratio			
Cyclodextrin	*o*- methoxycinnamaldehyde	*trans,trans* -2,4-decadienal	cinnamic acid	citronellol
α-	0.24	0.18	0.58	0.39
β-	1.25	0.19	1.53	0.72

Figure 4.1 Phase solubility diagrams of o-methoxyxinnamaldehyde-cyclodextrin complexes

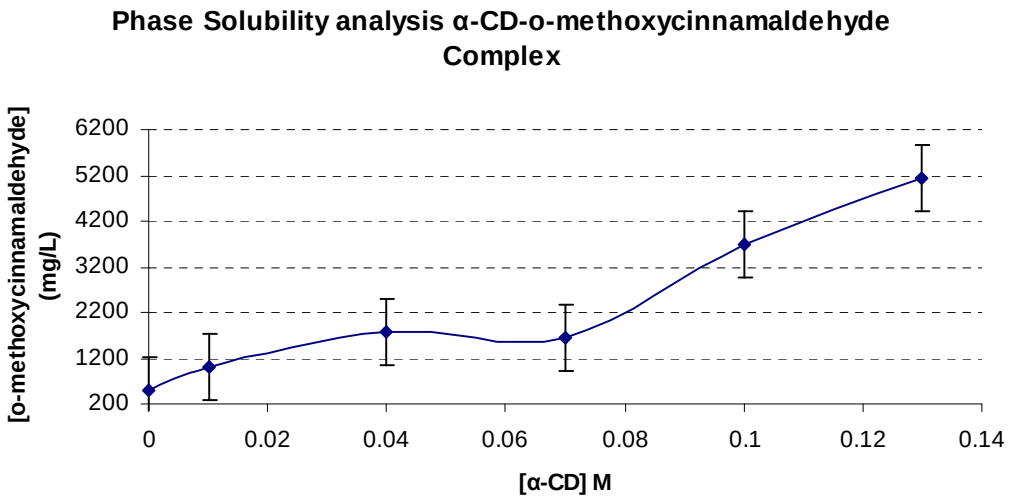

Figure 4.1a Phase solubility diagram of α-cyclodextrin-*o*-methoxycinnamaldehyde complex plotting α-cyclodextrin concentration (M) against *o*-methoxycinnamaldehyde concentration (mg/L) in solution.

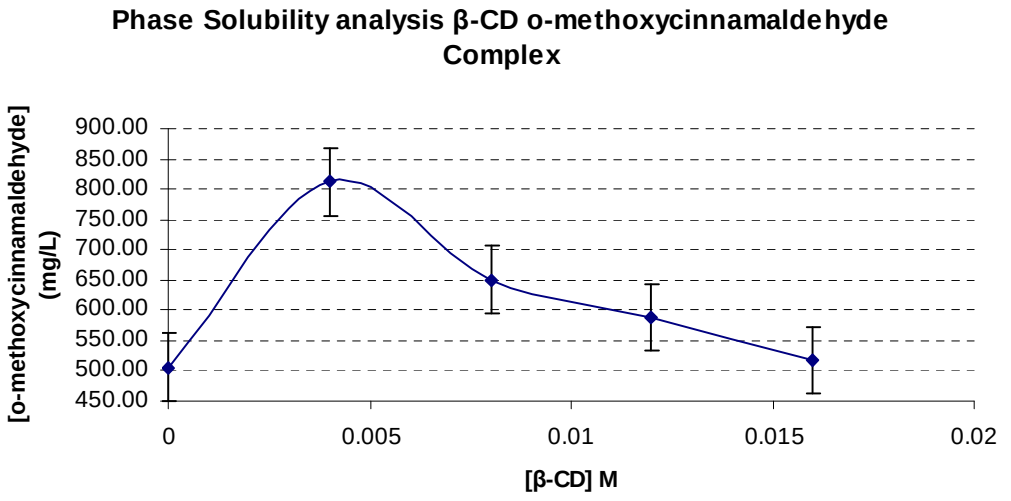

Figure 4.1b Phase solubility diagram of β-cyclodextrin-*o*-methoxycinnamaldehyde complex plotting β-cyclodextrin concentration (M) against *o*-methoxycinnamaldehyde concentration (mg/L) in solution.

Figure 4.2 Phase solubility diagrams of *trans,trans*-2,4-decadienal-cyclodextrin complexes

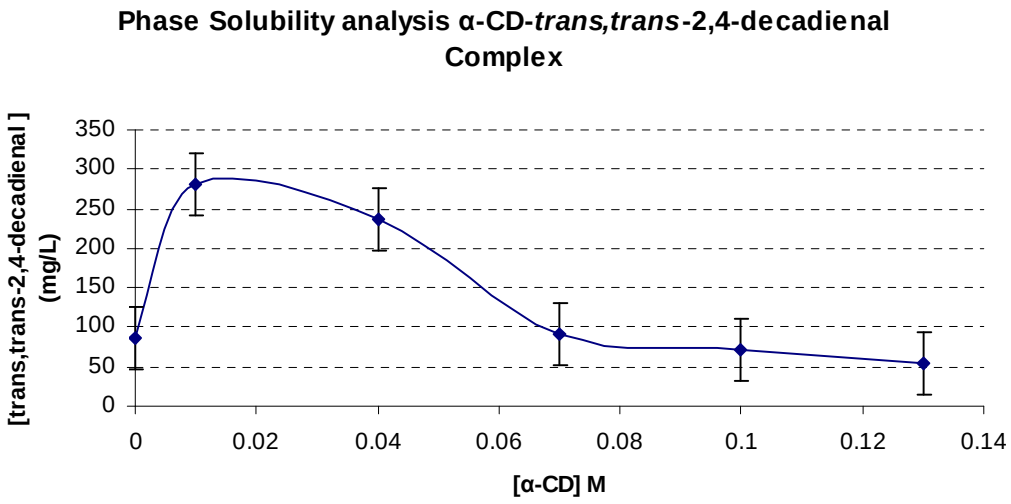

Figure 4.2a Phase solubility diagram of α-cyclodextrin-*trans,trans*-2,4-decadienal complex plotting α-cyclodextrin concentration (M) against *trans,trans*-2,4-decadienal concentration (mg/L) in solution.

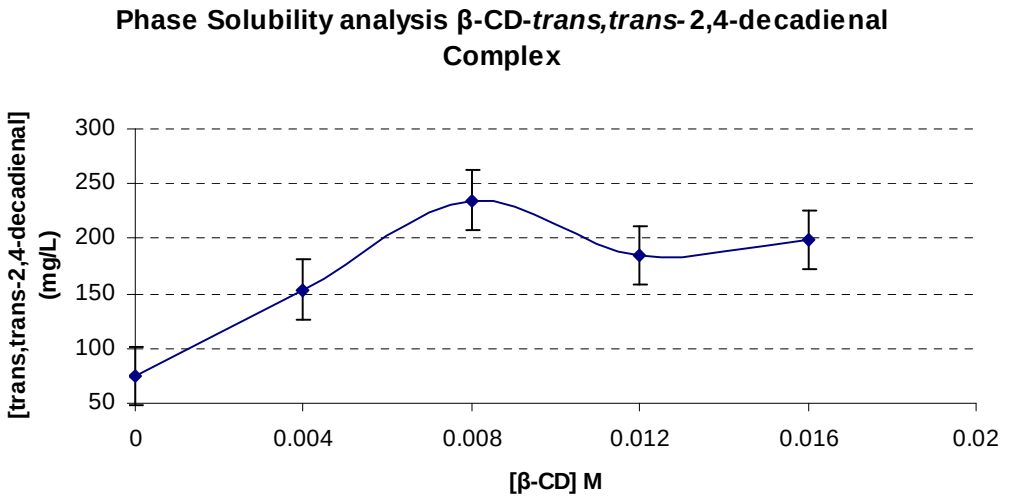

Figure 4.2b Phase solubility diagram of β-cyclodextrin- *trans,trans*-2,4-decadienal complex plotting β-cyclodextrin concentration (M) against *trans,trans*-2,4-decadienal concentration (mg/L) in solution.

Figure 4.3 Phase solubility diagrams of cinnamic acid-cyclodextrin complexes

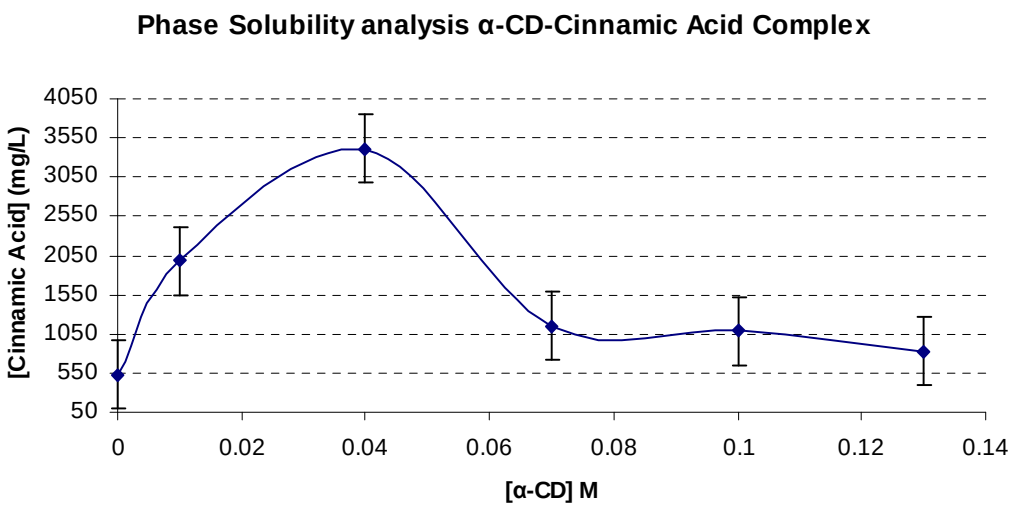

Figure 4.3a Phase solubility diagram of α-cyclodextrin-cinnamic acid complex plotting α-cyclodextrin concentration (M) against cinnamic acid concentration (mg/L) in solution.

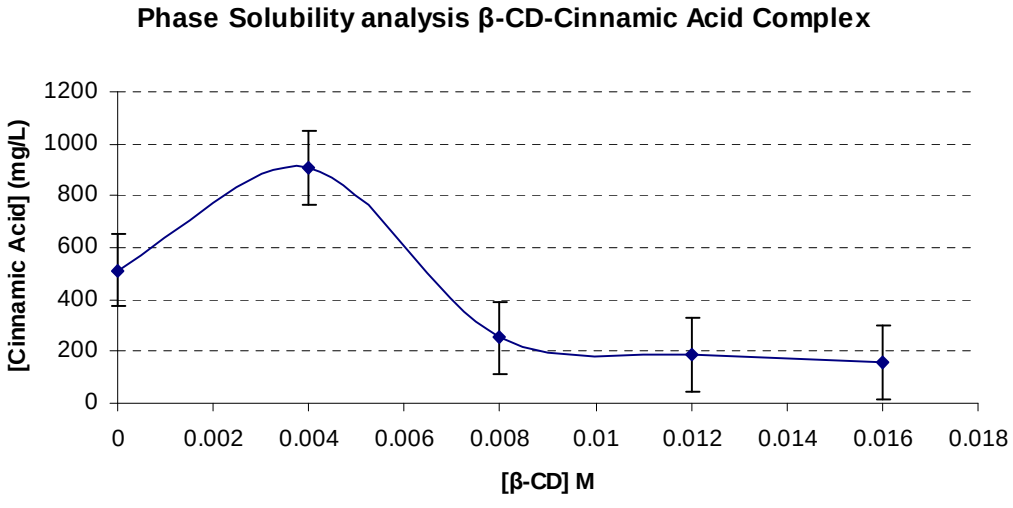

Figure 4.2b Phase solubility diagram of β-cyclodextrin cinnamic acid complex plotting β-cyclodextrin concentration (M) against cinnamic acid concentration (mg/L) in solution.

Figure 4.4 Phase solubility diagrams of citronellol-cyclodextrin complexes

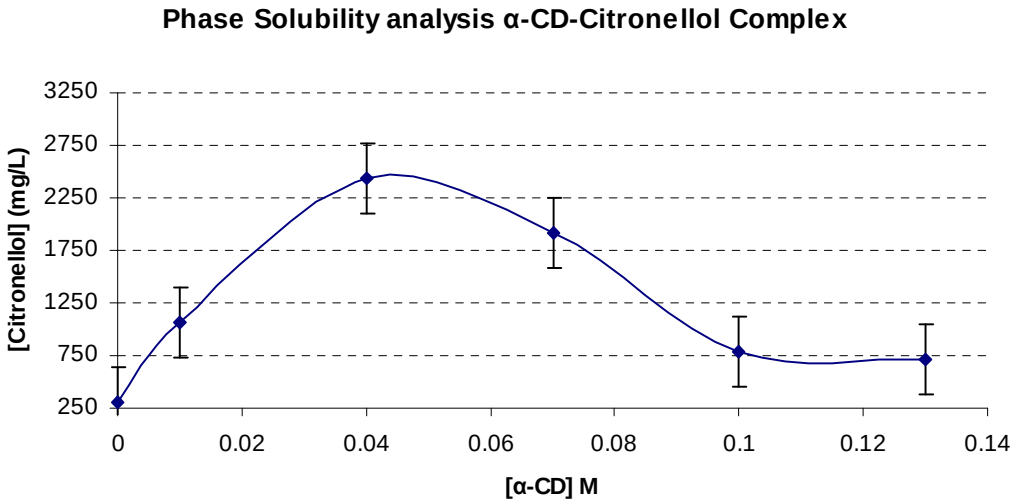

Figure 4.4a Phase solubility diagram of α-cyclodextrin-citronellol complex plotting α-cyclodextrin concentration (M) against citronellol concentration (mg/L) in solution.

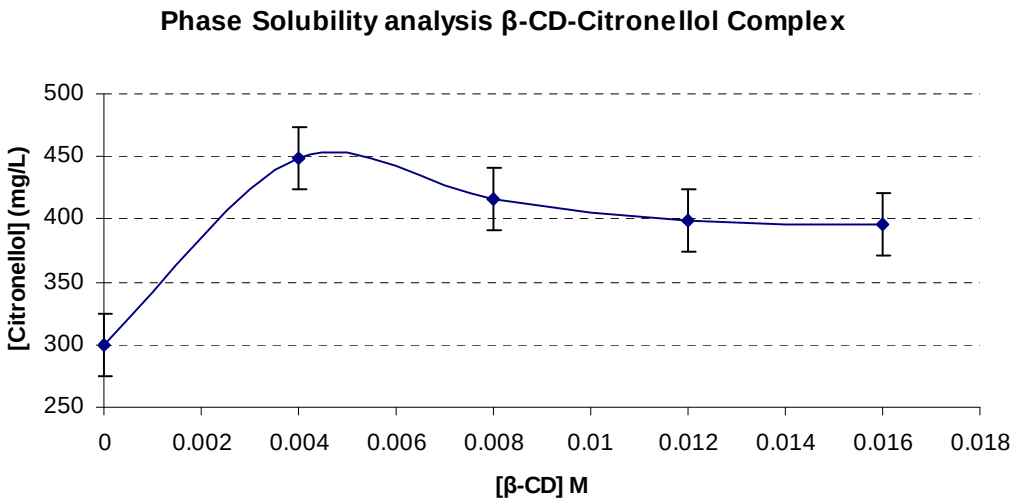

Figure 4.4b Phase solubility diagram of β-cyclodextrin citronellol complex plotting β-cyclodextrin concentration (M) against citronellol concentration (mg/L) in solution.

CHAPTER 5

Storage stability of natural antimicrobial-α-cyclodextrin complexes

Abstract

The storage stability of α-CD-*o*-methoxycinnamaldehyde, α-CD-*trans,trans*-2,4-decadienal, and α-CD-citronellol were evaluated for a period of 7 days in an apple juice-based beverage solution. Additionally, a comparison by UV/Vis spectophotometry of the storage stability of the complexes, in an acidic aqueous solution, stored in glass and PET containers was performed. Before analysis, the complexes were dissociated by diluting them in a 50% aqueous ethanol solution. The complexes in dissociated form were quantified by SPME GC-MS and UV/Vis spectophotometry.

Throughout the duration of the study (7 days) the concentration of *o*-methoxycinnamaldehyde detected by SPME GC-MS decreased by 61.7%. Similarly, the concentration of *trans,trans*-2,4-decadienal and that of citronellol decreased by 62.7% and 43% respectively. The storage stability comparison of the complexes, in an acidic aqueous solution, stored in containers of glass and PET proved that permeation through the PET polymer membrane did not occur.

Introduction

With the release of a Food and Drug Administration (FDA) study in April 2006 concerning low levels of benzene found in commercial beverages, the need for a natural antimicrobial arises (FDA, 2006). Benzene, a known human carcinogen and neurotoxin, was produced by the breakdown of sodium benzoate, a common preservative. The reaction mechanism involves metal ions present in tap water and ascorbic acid (Gardner and Lawrence, 1993). Furthermore, elevated temperatures and exposure to light accelerate the reaction (Kyoung and others, 2008). In the task of finding a replacement for sodium benzoate, three compounds known to have antimicrobial activity but limited aqueous solubilities were chosen to form inclusion complexes with α-cyclodextrin (α-CD). The compounds included in this study were citronellol, *o*-methoxycinnamaldehyde, and *trans,trans*-2,4-decadienal. α-CD was chosen as the host molecule due to its smaller interior diameter, of approximately 5 Å and its solubility of 149 mg/L in a aqueous solution (Hashimoto, 1996). Solid molecular inclusion complexes of α-CD and each of the test compounds were prepared from prior research.

The purpose of this study was to determine the percentage of guest compound in each of the complexes remaining in an apple juice-based beverage mixture over a period of 7 days. In addition, a study was carried out to compare the storage stability of the complexes, in aqueous solutions, stored in glass and polyethylene terephthalate (PET).

Materials and Methods

Materials. *o*-methoxycinnamaldehyde ≥ 96%, Kosher, FG, *trans,trans*-2,4-decadienal, Kosher FG, citronellol FCC, ≥95%, were supplied by Sigma-Aldrich (St. Louis, Missouri, USA). α-cyclodextrin, CAVAMAX ® W6 was supplied by Wacker Fine

Chemicals (Munich, Germany). Solid inclusion complexes of α-CD-*o*-methoxycinnamaldehyde, α-CD-*trans,trans*-2,4-decadienal, and α-CD-citronellol were prepared as previously stated (Chapter 4). Ethyl alcohol, absolute, 99.5%, A.C.S. reagent was supplied by Arcos Organics (Geel, Belgium), Puradisc 25PP disposable filter devices, 0.45 μm microcellulose membrane, were supplied by Whatman, Schleicher & Schuell (Florham Park, New Jersey, USA). Latex free 10 mL syringes were supplied by Becton Dickinson & Co. (Franklin Lakes, New Jersey, USA). A UV-Vis spectrophotometer, UV-2101PC, was supplied by Shimadzu (Kyoto, Japan). Quartz cuvettes were supplied by Fisher Scientific. Premium 100% pure apple juice, preserevative free, pressed from fresh apples, not from concentrate, 64 Fl.oz (1/2 gallon) 1.89 L, pasteurized was supplied by Motts (Rye Brook, New York, USA). Alpha-D (+)-glucose, anhydrous 99+% was supplied by Acros Organics (Geel, Belgium). D-Fructose, reagent grade (crystal) was supplied by Fisher Scientific (Fair Lawn, New Jersey, USA). Sucrose (α-D-glucopyranosyl; β-D-fructofuranoside; saccharose; cane sugar) was supplied by Sigma Chemical Company (St. Louis, Missouri, USA). Ascorbic acid, food grade, powder supplied by Sigma-Aldrich Chemical Company (St. Louis, Missouri, USA). Filtration products, 45 μm, filter units- 500 mL were supplied by Nalgene (Rochester, New York, USA). HP 5980 gas chromatograph, HP-5-MS crosslinked (5%-Phenyl)-methylpolysiloxane column (30m x 0.25mm x 0.25μm), HP 5972 series mass selective detector, HP Enhanced ChemStation software version B.01.00 were supplied by Hewlett Packard (Palo Alto, California, USA). SPME fiber, 100 μm PDMS, 23 gauge needle was supplied by Supelco (Bellefonte, Pennsylvania, USA). A Leap Technologies Combi Pal autosampler was supplied by Leap Technologies (Carrboro, North Carolina, USA).

Preparation of apple juice-based beverage mixture. One hundred mL of preservative free apple juice (Motts brand) were combined with 46.8g of glucose, 59.4g of fructose, 1.8g of sucrose in a 1L beaker. The mixture was then brought up to 1L of volume with distilled water. The mixture was stirred at room temperature until all the ingredients were incorporated. The pH of the mixture was adjusted with ascorbic acid until it reached 3.4. The brix of the mixture was verified to be between 12^{o} and13^{o} (Abbe 3L refractometer). The mixture was filtered using 0.45 μm microcellulose filter units.

Once the beverage mixture was prepared 300 mg of each complex were added to 200 mL of beverage mixture in 250 mL Erlenmeyer flasks. The mixtures were stirred for 30 min in a stir plate and tightly capped with glass stoppers. Samples were stored in the bench top of a laboratory exposed to regular daylight and lighting.

Sample preparation. Five mL of each sample were syringed and filtered using 0.45 μm microcellulose filter tips into 10 mL test tubes. Next, 3 mL of the filtered samples were transferred into another test tube and 3 mL of 100% ethanol were added. Finally, 4 mL of the resulting samples diluted to a final concentration of 50% ethanol were transferred into 10 mL clear vials and tightly capped with AlumiTin, 20mm caps with polytetrafluoroethylene (PTFE) gaskets. Samples from each complex were produced in triplicate. This proceudre was performed at day 0, day 2, day 4 and day 7.

Preparation of standards. An *o*-methoxycinnamaldehyde stock solution was prepared by dissolving 10 mg of compound into 50 mL of 100% ethanol for a concentration of 200 mg/L. Stock solutions for *trans,trans*-2,4-decadienal and citronellol were prepared in the same manner. Each stock solution was serially diluted with the same apple juice-based beverage mixture prepared for the samples ensuring that the final

concentration contained 50% ethanol matching the preparation of the samples. Vials with 100 mg/L, 50 mg/L, 40 mg/L, and 25 mg/L of test compound were prepared. Standards from each compound were produced in triplicate.

SPME Analysis. Analysis of the samples' headspace was done using a solid phase micro extraction (SPME) poly(dimethylsiloxane) fiber (PDMS; 100 μm). Vials containing sample were placed in the sample tray of a COMBI PAL autosampler. Each sample was automatically transferred into a heating block set at 80^{o}C. The SPME fiber was inserted into the headspace above the sample and timed for 5 minutes of adsorption.

GC-MS Parameters and Analysis. The SPME fiber was desorbed at 250^{o}C for 10 minutes in the injection port of an HP5890/HP5972 gas chromatograph mass spectrometer (GC-MS). The injection port was operated in splitless mode. The initial oven temperature was 35^{o}C, ramped at a rate of 10^{o}C per minute to 240^{o}C held for 1 min. The HP5972 quadrupole mass spectrometer was operated in electron ionization mode with a source temperature of 250^{o}C, quadrupole temperature of 260^{o}C, and an interface temperature of 250^{o}C, performing a continuous scan from *m/z* 50 to 550 at a scan rate of 4.5 sec^{-1}.

Positive identification of each guest compound was achieved on the basis of mass spectra from the HP ChemStation mass spectral database. Samples and standards were run in triplicate. Standard curves were prepared with the averaged integrated areas of each compound's peak. The procedure was performed at day 0 of storage and replicated at day 2, day 4, and day 7. Linear regression analysis was used to quantify the content of each test compound.

Stability of complexes in different packaging materials. An acidic aqueous solution was prepared by acidifying distilled water with ascorbic acid until a pH of 3.4 was reached. Solutions of each complex in acidified water were prepared by adding 100 mg of complex to 100 mL of acidified water for a concentration of 1000 mg/L. Each solution was divided into two equal parts. Fifty mL of each solution were stored in glass erlenmeyer flasks and the other 50 mL were stored in PET containers. Samples were stored in the bench top of a laboratory exposed to regular daylight and lighting. Before analysis, each sample was filtered through a 0.45 μm microcellulose filter tip and diluted for a final concentration of 50% ethanol. Standard curves were prepared by dissolving the guest compounds in ethanol and serially diluting them with distilled acidified water. UV absorption spectophotometry was performed with a Shimadzu UV-2101PC UV-VIS scanning spectrophotometer. Settings for the UV/VIS included wavelength range from 190 - 400 nm. A 50% ethanol-50% acidified water solution was used as a reference and for a baseline scan. A portion of the samples were placed in quartz cuvettes and ran on the UV/VIS. The maximum absorbance of the spectra was recorded for each compound. Linear regression analysis was used to calculate the concentration of test compound in solution. The procedure was performed at day 0 of storage and replicated at day 2, day 4, and day 7.

The samples were stored in the bench top of a laboratory exposed to regular daylight and lighting.

Results and discussion

Identification of the guest compounds analyzed by SPME GC-MS was based on retention time and mass spectra of their particular standards. Table 5.1 summarizes the

retention time at which each compound was evaluated. Each compound was identified using a mass spectral library and every standard showed excellent match quality between 96% and 98% (Table 5.1). Standard curves prepared to quantify the presence of each guest compound showed excellent linearity with correlation coefficients, R^2, between 0.9818 and 0.997 (Table 5.1). Before analysis the solutions containing each complex were diluted to a final concentration containing 50% ethanol to achieve the complete dissociation of the complex (Szente, 1996).

Figure 5.1 illustrates the storage stability results for α-CD-*o*-methoxycinnamaldehyde complex. Figure 5.1a depicts how the detected concentration of *o*-methoxycinnamaldehyde decreased from 86.16 mg/L at day 0 to 47.15 mg/L at day 2. From day 2 to day 7 the concentration detected remained fairly constant. At the end of the analysis period, day 7, the detected concentration of *o*-methoxycinnamaldehyde dropped by 61.7%. Figure 5.1b shows the storage stability comparison of the complex in an acidified aqueous solution stored in a glass container compared to the same stored in a PET container. The initial concentration (day 0) of compound detected by this method was 40.3 mg/L. It can be noted that the solution stored in glass had a more constant decline in concentration of compound. The concentration of *o*-methoxycinnamaldehyde was higher in the solution stored in PET up until day 4. However, at day 7 the concentration of *o*-methoxycinnamaldehyde detected was very close it both containers with 18.0mg/L in glass and 20.8 mg/L in PET. At the end of the analysis period, day 7, the concentration of *o*-methoxycinnamaldehyde had dropped by 55.3% in the solution stored in the glass container and by 48.3% in the solution stored in the PET container.

The storage stability results for α-CD-*trans,trans*-2,4-decadienal complex are shown in figure 5.2. Figure 5.2a illustrates the concentration of *trans,trans*-2,4-decadienal in the apple juice-based beverage mixture as detected by SPME GC-MS over a period of 7 days. At day 0 the concentration of guest compound detected was 123.9 mg/L. By day 2 the concentration had decreased by 14.3 % to 106.3 mg/L. At day 4 the concentration detected decreased by another 31.0% to 67.8 mg/L. At the end of the analysis period, day 7, the concentration of guest compound had decreased by 62.7% to 46.2 mg/L. Additionally, figure 5.2b shows the storage stability comparison of the complex in acidified aqueous solution stored in glass and PET containers. The initial concentration was calculated to 84.6 mg/L. Both samples, showed a steady decline with similar trend lines. By the end of the study the concentration of guest compound declined by 42.4% in the sample stored in glass and by 40.7% in the sample stored in PET.

Figure 5.3 shows the results for the α-CD-citronellol complex. Figure 5.3a shows the time course plot of the guest compound remaining in the apple juice-based beverage mixture after 7 days. The initial concentration of citronellol detected was 141.1 mg/L. The concentration of compound found in the system decreased by 26.6% to 103.6 mg/L by day 2. From day 2 to day 4 the concentration of compound was maintained at 102 mg/L. Finally, by the end of the analysis period, day 7, the detected concentration of citronellol in the system had declined by 43.4% to 79.8 mg/L. Moreover, Figure 5.3b depicts the analysis to compare the stability of the complex stored in glass and PET containers. The initial concentration of compound found at day 0 was 117.24 mg/L. The concentration of compound decreased with very similar trend lines in both samples. By day 7 the concentration of citronellol found in the sample stored in glass had decreased

by 49.9% to 58.7 mg/L. Similarly, the sample stored in PET had a decrease of 48.3% to 60.6 mg/L.

The concentration of guest compound of all the complexes in the apple juice-based beverage mixture showed a decrease in concentration ranging from 44%-63%. A similar study conducted by Ajisaka and others (2000) which evaluated the stability of cyclodextrin-terpene complexes including β-CD-citronellol found comparable results. However, their complex solutions were stored in open beakers. Literature reports analysis of aromatic compounds complexed with cyclodextrin and packed under vacuum to only have lost 25%-30% of their active ingredient even after being exposed to elevated temperatures of 150^{o}C for 24 hrs (Szejtli, 1988).

Aldehydes and dienaldehydes such as *o*-methoxycinnalamdehyde and *trans*,*trans*-2,4-decadienal are frequently involved in self-condensation polymerization reactions which can be catalyzed by acid. In addition, these aldehydes as well as unsaturated monoterpenoids, such as citronellol, can be susceptible to autoxidation reactions initiated by exposure to light or air (NTP, 1992). Degradation of the test compounds causes the inverse relationship between concentration of test compound and storage time.

The results of the storage stability analysis of the samples stored in glass and PET packages did not show any remarkable differences. It is well known that thermoplastic polymers, including PET, have varying degrees of permeability to small molecules such as volatile organic compounds. In beverages, sorption (also called scalping) is a common phenomena of permeation where molecules from the product are taken up into (but not through) the package (Robertson, 2006). The concentration of *d*-limonene in citrus juices was used by Mannheim and others (1988) to demonstrate the absorption capacity of

polyethylene showing that after 14 days it was 25% lower than the same samples stored in glass containers. Conversely, in this case, none of the complexes studied exhibited a behavior in which permeation into the polymer film could be attributed to its decrease in concentration since the decrease in guests' concentration was very close in both methods of storage. It is possible that the complexation of the guest compounds make up a molecule too large to be adsorbed by the polymer film.

References

Ajisaka, N. Hara, K. Mikuni, K. Hara, K. Hashimoto, H. 2000. Effects of Branched Cyclodextrins on the Solubility and Stability of Terpenes. *Biosci. Biotechnol. Biochem.*, 64 (4), 731-734

Food and Drug Administration (FDA). 2006. Data on Benzene in Soft Drinks and Other Beverages. Office of Food Additive Safety, CFSAN, Food and Drug Administration. Received October 2007 from http://www.cfsan.fda.gov/~dms/benzdata.html.

Gardner K. L.; Lawrence G. D. 1993. Benzene Production from Decarboxylation of Benzoic Acid in the Presence of Ascorbic Acid and a Transition-Metal Catalyst. *J. Agric. Food Chem*. 41(5): 693-695

Hashimoto H. 1996. Cyclodextrins in Foods, Cosmetics, and Toiletries. In: Atwood, J. D., JED; MacNicol, DD; Vogtle, F, editor. Comprehensive Supramolecular Chemistry. Yokohama: Pergamon. p. 483-502.

Kyoung Hyun Ju, Jeong Hill Park, Sung Won Kwon. 2008. Evaluation of Headspace-Gas Chromatography/Mass Spectrometry for the Analysis of Benzene in Vitamin C Drinks; Pitfalls of Headspace in Benzene Detection. *Biomedical Chromatography.* 22 (8): 900-905

Mannheim, C.H., Miltz, J., Passy, N.1988. Interaction Between Aseptically Filled Citrus Products and Laminated Structures. *In* Food and Packaging Interactions. ACS Symposium Series #365, Hotchkiss J. H. Ed., American Chemical Society, Washington, DC. Ch. 8.

NTP. 1992. National Toxicology Program, Institute of Environmental Health Sciences, National Institutes of Health (NTP). 1992. National Toxicology Program Chemical Repository Database. Research Triangle Park, North Carolina: NTP.

Robertson, G. L. 2006. Permeability of Thermoplastic Polymers. *In* Food packaging, principles and practice. CRC Press Taylor and Francis Group. Boca Raton, FL. P. 55-78

Szente L. 1996a. Analytical Methods for Cyclodextrins, Cyclodextrin Derivatives, and Cyclodextrin Complexes. In: J Szejtli and T Osa, editors. Cyclodextrins. First ed. vol. 3. Elsevier Science, Ltd. p. 253-278

Szejtli J. 1988. Cyclodextrin Technology. ed. vol. Dordrecht: Kluwer Academic Publishers.

Figures and Tables

Table 5.1 Evaluation of guest compounds' standards by SPME GC-MS.

RT[1] (min)	Compound	Match quality[2] (%)	Correlation Coefficient of Standard Curves R^2
15.65	o-methoxycinnamaldehyde	97	0.9818
12.61	trans,trans-2,4-decadienal	98	0.9889
11.22	citronellol	96	0.997

[1]Retention time of compound obtained with an HP5890/HP5972 GC-MS with HP-5-MS column
[2]Match quality of mass spectra of each compound identified positively with the HP ChemStation mass spectral database

Table 5.2 Maximum absorbance wavelength and correlation coefficients for standard curves

Test Compound	Maximum Absorbance Wavelenght nm	Correlation Coefficient of Standard Curves R^2
o-methoxycinnamylaldehyde	287	0.9995
Trans, trans, 2,4 decadienal	278	0.9987
Citronellol (3,7-Dimethyl-6-octen-1-ol)	197	0.9847

Figure 5.1 Results of stability studies of α-CD-*o*-methoxycinnamaldehyde complex

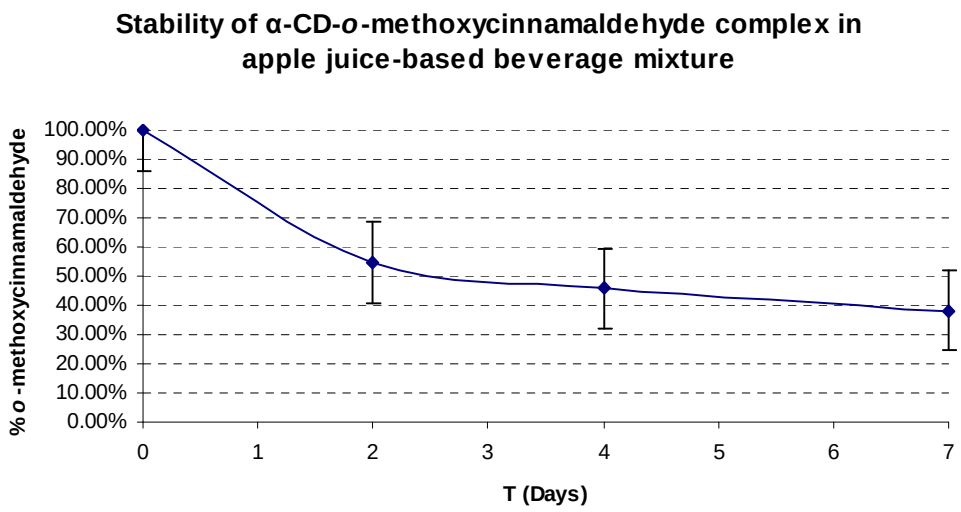

Figure 5.1a o-methoxycinnamaldehyde detected by GC/MS throughout a period of 7 days. Percentage change relative to concentration detected at day 0.

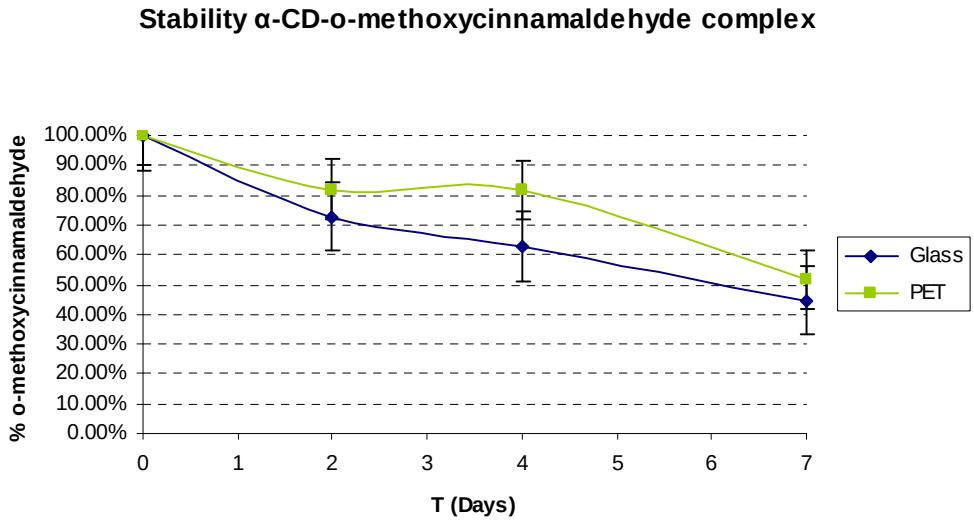

Figure 5.1b o-methoxycinnamaldehyde detected by UV/Vis spectophotometry in acidified aqueous solution throughout a period of 7 days. Comparison of two different packaging materials.

Figure 5.2 Results of stability studies of α-CD-*trans,trans*-2,4-decadienal complex

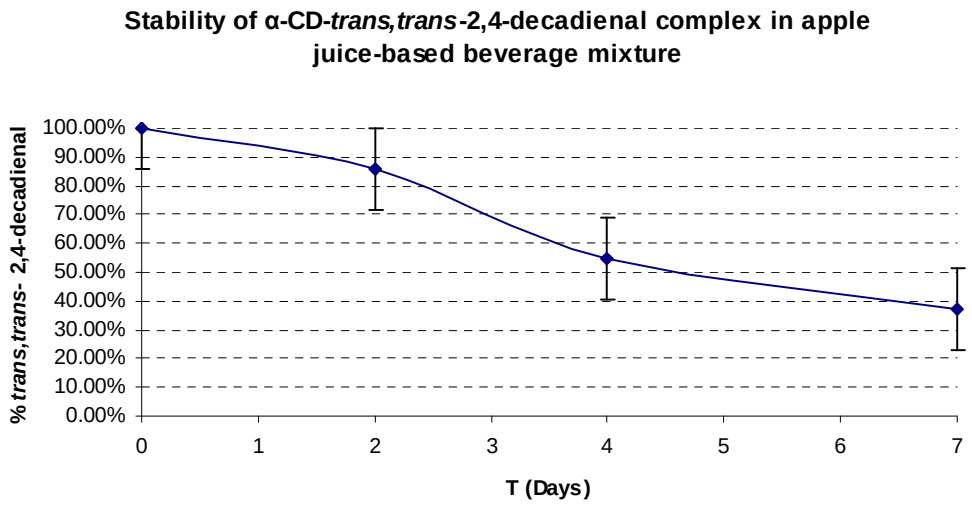

Figure 5.2a *trans,trans*-2,4-decadienal detected by GC/MS throughout a period of 7 days. Percentage change relative to concentration detected at day 0.

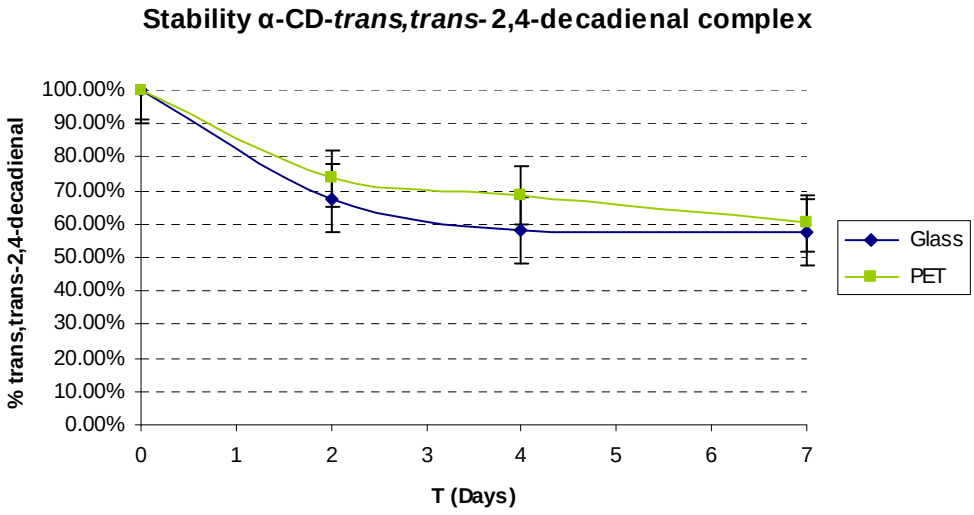

Figure 5.2b *trans,trans*-2,4-decadienal detected by UV/Vis spectophotometry in acidified aqueous solution throughout a period of 7 days. Comparison of two different packaging materials.

Figure 5.3 Results of stability studies of α-CD-citronellol complex

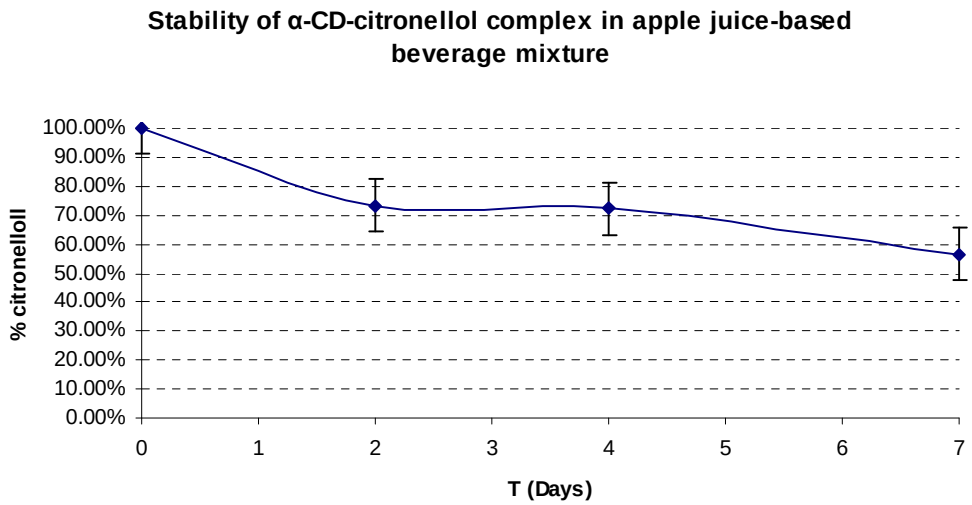

Figure 5.3a Citronellol detected by GC/MS throughout a period of 7 days. Percentage change relative to concentration detected at day 0.

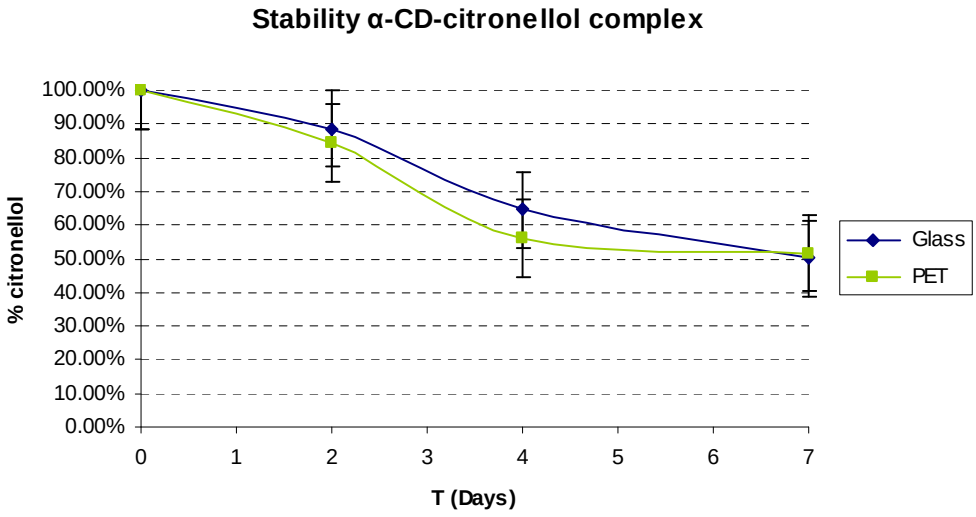

Figure 5.3b Citronellol detected by UV/Vis spectophotometry in acidified aqueous solution throughout a period of 7 days. Comparison of two different packaging materials.

Summary and Conclusions

As a response to the need for a natural antimicrobial to replace sodium benzoate's use in beverages, twenty-eight compounds were evaluated to quantify their solubility in water and in an apple juice-based beverage. Four compounds were then chosen to form molecular inclusion complexes with α- and β-cyclodextrin. The complexes were studied through phase solubility analyzes. In addition, solid inclusion complexes were prepared for the α-CD complexes of three test compounds. The storage stability of these complexes was studied in an apple juice-based beverage. Finally, the storage stability of the complexes stored in glass containers was compared to the same stored in PET containers.

In conclusion it can be stated that the solubilities of the test compounds range from practically insoluble (< 100 mg/L) to just slightly soluble (1,000 to 10,000 mg/L) in both water and beverage. The solubility of the compounds in the apple juice-based beverage showed a general trend of decreased solubility as compared to their water solubility. Furthermore, using cyclodextrin technology proved to be a viable way of improving the solubility of natural antimicrobial compounds. α-CD was more effective at increasing the solubility of the compounds than β-CD. As far as the storage stability of the complexes studied, in beverages systems, they showed fairly poor storage stability with decreases in detected levels between 43% and 62.7% of guest compound after 7 days of storage. On the other hand, none of the complexes studied exhibited a behavior in which adsorption into the PET polymer film could be attributed to its decrease in concentration.