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Cyclodextrin Inclusion Complex Formation and Solid-State Characterization of the Natural Antioxidants α-Tocopherol and Quercetin

JOHN L. KOONTZ, JOSEPH E. MARCY,* SEAN F. O'KEEFE, AND SUSAN E. DUNCAN

Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Cyclodextrin (CD) complexation procedures are relatively simple processes, but these techniques often require very specific conditions for each individual guest molecule. Variations of the coprecipitation from aqueous solution technique were optimized for the CD complexation of the natural antioxidants α-tocopherol and quercetin. Solid inclusion complex products of α-tocopherol/β-CD and quercetin/γ-CD had molar ratios of 1.7:1, which were equivalent to 18.1% (w/w) α-tocopherol and 13.0% (w/w) quercetin. The molar reactant ratios of CD/antioxidant were optimized at 8:1 to improve the yield of complexation. The product yields of α-tocopherol/β-CD and quercetin/γ-CD complexes from their individual reactants were calculated as 24 and 21% (w/w), respectively. ATR/FT-IR, ^{13}C CP/MAS NMR, TGA, and DSC provided evidence of antioxidant interaction with CD at the molecular level, which indicated true CD inclusion complexation in the solid state. Natural antioxidant/CD inclusion complexes may serve as novel additives in controlled-release active packaging to extend the oxidative stability of foods.

KEYWORDS: Cyclodextrin; inclusion complex; α-tocopherol; quercetin; natural antioxidant; coprecipitation; complexation

INTRODUCTION

The preparation of cyclodextrin (CD) inclusion complexes is often a relatively simple procedure; however, in most cases the reaction conditions have to be customized for the specific guest molecule. The majority of CD complexation reactions occur in aqueous solution or at least in the presence of water. The natural antioxidants α-tocopherol and quercetin (**Figure 1**) were selected as the specific guest molecules in this study. Tocopherols are the most important natural antioxidants in vegetable oils and protect these products from lipid peroxidation. Quercetin, found in onions and apples, is among the most effective of the flavonoid antioxidants and the most active in its own flavonol class (*1*). The complexation procedures of α-tocopherol and quercetin with the native CDs that are described in literature are deficient. These specific complexation methods proposed for each of the natural antioxidants require careful review to ensure that true inclusion complexes are indeed being formed.

Solid β-CD/α-tocopherol complexes have been prepared using kneading (*2*), coevaporation (*2*), and freeze-drying methods (*2*, *3*). Both the kneading and coevaporation methods used 1:1 and 2:1 molar ratios of β-CD/α-tocopherol mixed with different respective volumes of 50% aqueous methanol. Organic solvents usually decrease the association constants of CD complexes relative to pure water. The most commonly proposed idea for this behavior is that increasing the organic content of the aqueous mixture decreases the hydrophobic driving force, which is a major contributor to the stability of the complex in water (*4*). Methanol and ethanol are often used as cosolvents to aid the solubilization of very hydrophobic guest molecules, but not at the high percentages of methanol reported. CD complexes are typically dissolved in 50% aqueous ethanol followed by dilution with pure ethanol as part of the dissociation procedure for guest content determination (*5*).

Inclusion complexes of quercetin with β-CD have been prepared by a freeze-drying method from aqueous solution with equimolar ratios of components under strongly alkaline pH conditions (*6*, *7*). The use of ammonia to dissolve quercetin raises the pH to alkaline levels, which allows for the formation of the ionized species of quercetin. The ionized quercetin is susceptible to degradation at pH levels >5 (*8*, *9*). This quercetin anion also exhibits much weaker binding with two β-CD derivatives compared to the un-ionized species due to its hydrophilic character (*8*). Preparation of solid inclusion complexes of quercetin with α- and β-CD has also been reported by kneading, coevaporation, and thin layer methods (*10*). Kneaded products were obtained by wetting equimolar physical mixtures with a minimum volume of 50% aqueous methanol. Coevaporated products were obtained from 33.3% methanol in

* Author to whom correspondence should be addressed [telephone (540) 231-7850; fax (540) 231-9293; e-mail jmarcy@vt.edu.

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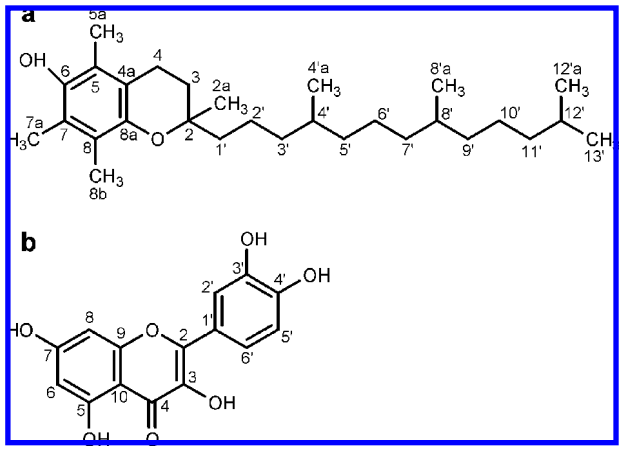

Figure 1. Structures and carbon numberings of the natural antioxidants (**a**) α-tocopherol and (**b**) quercetin.

Table 1. Characteristics of Reactants and Products in the Preparation of Solid Natural Antioxidant/CD Inclusion Complexes

inclusion complex	production batch	reactants CD/antioxidant molar ratio	product antioxidant content wt %[a]	product CD/antioxidant molar ratio
α-tocopherol/β-CD	I	8.0	18.1 ± 0.4	1.7
	II	8.0	19.6 ± 0.1	1.6
	III	8.0	20.2 ± 0.1	1.5
quercetin/γ-CD	I	7.2[b]	13.0 ± 0.3	1.7
	II	8.0	15.4 ± 0.1	1.4
	III	8.0	14.4 ± 0.3	1.6

[a] Values were reported as mean ± standard error ($n = 2$). [b] 21.6 mM γ-CD: 3.0 mM quercetin.

aqueous solution containing equimolar amounts of quercetin and CD. Guest−host associations are prevented with the addition of a high percentage of alcohol for the short-chain alcohols, such as methanol and ethanol (*11*). Longer chain alcohols, such as pentanol or even greater length, are able to cancel this association with only 1% alcohol. Similar to the α-tocopherol methods reported in the literature, the use of relatively high percentages of methanol cosolvent may result in either very weak associations or no complexation at all.

Several researchers have also reported solubility increases of quercetin during phase solubility studies with β-CD from its intrinsic water solubility of 0.44 mg/L (*8*) to approximately 2−25 mg/L (*6*, *8*, *12*), although an increase in the water solubility of the flavonols has been reported in the case of physical mixtures and some kneaded preparations when no inclusion complexes were in fact formed (*13*). The limited solubility increase of quercetin in the presence of β-CD, therefore, provides only weak evidence of an association, whereas quercetin remains practically insoluble.

The purpose of this research was to optimize the complexation methods of the natural antioxidants α-tocopherol and quercetin and to characterize their cyclodextrin inclusion complexes. Solid-state techniques, including ATR/FT-IR, ^{13}C CP/MAS NMR, TGA, and DSC, provided a clear indication of guest interactions with their CD hosts at the molecular level. Molecular encapsulation with CDs can function to protect against oxidation and heat-induced degradation of guest molecules. CD complexation can provide oxidative protection to flavors [vanillin (*14*), thymol, and geraniol (*15*)] and antioxidants [ferulic acid (*16*)]. The oxidative sensitivity of α-tocopherol (*17*−*20*) and quercetin (*9*) has been observed during extended thermal processes. CD inclusion complexes of the natural antioxidants were formed for their potential thermal and oxidative stability and controlled-release properties for the future incorporation of these antioxidants into active food packaging.

Table 2. Colorimetric Analyses in the CIE $L^*a^*b^*$ Color Space

sample	L^*	a^*	b^*	ΔE^*_{ab}
α-tocopherol	27.1	+1.2	+12.7	67.9
β-CD	94.2	+0.2	+2.1	0.0
α-tocopherol and β-CD physical mixture	72.1	−0.6	+12.0	24.2
α-tocopherol/β-CD complex	94.2	−0.4	+3.6	1.6
quercetin dihydrate	83.4	−9.0	+48.8	46.4
γ-CD	91.1	+0.4	+4.0	0.0
quercetin and γ-CD physical mixture	87.6	−8.1	+39.3	36.4
quercetin/γ-CD complex	89.1	−5.3	+21.8	18.8

MATERIALS AND METHODS

Materials. (±)-α-Tocopherol of 98% purity and quercetin dihydrate of 99% purity were supplied by Sigma-Aldrich (St. Louis, MO). Food-grade α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), and γ-cyclodextrin (γ-CD) were kindly donated by Wacker Fine Chemicals (Adrian, MI). Potassium phosphate monobasic (KH_2PO_4), methanol, water of HPLC grade, and 99.5% ethanol of ACS reagent grade were obtained from Fisher Scientific (Pittsburgh, PA).

Preparation of α-Tocopherol/β-CD Complex. Solid inclusion complexes of α-tocopherol with β-CD were obtained by coprecipitation from aqueous solution. A 16.0 mM β-CD aqueous solution was prepared followed by the addition of 2.0 mM α-tocopherol. These concentrations were chosen for their relatively high complexation yield based on preliminary studies. The dispersion of α-tocopherol in the aqueous β-CD solution was protected from light and mechanically shaken at 25 °C and 250 rpm in an Innova 4230 refrigerated incubator shaker (New Brunswick Scientific, Edison, NJ) for 24 h to achieve equilibrium of the complexation reaction. Samples were allowed to settle gravimetrically, and the majority of supernatant was decanted. The remaining coprecipitate was collected by filtering the remaining supernatant through a 0.2 μm nylon membrane. These solid complexes were frozen at −20 °C and then lyophilized in a laboratory freeze-dryer (Virtis, Gardiner, NY). CD complexes of α-tocopherol were dissociated in 99.5% ethanol for a period of 24 h at 25 °C and 250 rpm. Guest content of each CD complex was quantified by UV spectrophotometry. Solid α-tocopherol/β-CD complexes were produced in triplicate, and complexes with 18.1% (w/w) α-tocopherol content were used for all characterization tests.

Preparation of Quercetin/γ-CD Complex. Solid inclusion complexes of quercetin with γ-CD were obtained by coprecipitation from aqueous solution with 10% ethanol as cosolvent. A solution of 26.7 mM γ-CD was prepared in 0.05 M potassium phosphate buffer at pH 3.0 (*8*). A 30.0 mM quercetin dihydrate solution in ethanol was prepared and added to the γ-CD solution to give final concentrations of 24.0 mM γ-CD and 3.0 mM quercetin in an aqueous solution with 10% ethanol. This final solution of γ-CD and quercetin was protected from light and mechanically shaken at 25 °C and 250 rpm in an incubator shaker for 24 h to achieve equilibrium of the complexation reaction. Samples were allowed to settle gravimetrically, and the majority of supernatant was decanted. The coprecipitate was collected by filtering the remaining supernatant through a 0.2 μm cellulose nitrate membrane. Quercetin solutions were observed to adsorb to nylon membranes. These solid complexes were frozen at −20 °C and then lyophilized in a laboratory freeze-dryer. CD complexes of quercetin were dissociated in 50% aqueous ethanol for a period of 24 h at 25 °C and 250 rpm. Guest content of each CD complex was quantified by UV spectrophotometry. Solid quercetin/γ-CD complexes were produced in triplicate/and complexes with 13.0% (w/w) quercetin content were used for all characterization tests.

Preparation of Physical Mixtures. Physical mixtures of α-tocopherol and β-CD were prepared with the identical α-tocopherol content

of 18.1% (w/w) as in its solid CD inclusion complex by thoroughly mixing the two components. Physical mixtures of quercetin dihydrate and γ-CD were prepared with the identical quercetin dihydrate content of 13.0% (w/w) as in its solid CD inclusion complex in the same manner.

UV Absorption Spectrophotometry. Spectrophotometry was performed with a UV-2101PC UV−vis scanning spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) to quantify α-tocopherol and quercetin in their free and CD-complexed forms ($n = 2$). Standard curves of α-tocopherol and quercetin were prepared in 99.5% ethanol, accounting for the sample purity of each natural antioxidant. CD complexes of α-tocopherol and quercetin were dissociated in 99.5% ethanol and 50% aqueous ethanol, respectively, for 24 h at 25 °C. Complete spectrophotometric scans between 250 and 400 nm were performed to monitor any changes in the UV spectra of the natural antioxidants. The absorbance maxima of α-tocopherol and quercetin were 292 and 375 nm, respectively, to quantify each antioxidant concentration.

Colorimetric Analysis. A Chroma Meter CR-200 tristimulus color analyzer (Minolta, Ramsey, NJ) was calibrated with a standard white plate (L 97.29, a −0.18, b +3.75) and used for measuring reflective colors of surfaces. Solid CD inclusion complexes, physical mixtures, guest antioxidants, and CDs were measured with the color analyzer and quantified using the CIE $L^*a^*b^*$ color model. ΔE^*_{ab} was used to quantify the magnitude of the total color difference as a single numerical value defined by the equation: $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$.

CD Sample Equilibration at Ambient Relative Humidity. Uncomplexed CD, physical mixtures, and inclusion complexes of 1.0 g sample weight in 20 mL glass vials were left open in a sealed glass desiccator with distilled water filled across the base of the desiccator for an equilibration period of 120 min. This 100% relative humidity environment completely hydrated the CD-containing samples through the vapor phase on the minute time scale as reported in the literature (*21*, *22*) and confirmed in a preliminary study. The guest molecules, α-tocopherol and quercetin dihydrate, were not placed in the 100% relative humidity environment. The same CD-containing samples were then left open to ambient relative humidity for an equilibration period of 120 min. This hydration and dehydration step was to allow the CD inclusion complexes of both α-tocopherol and quercetin to be at equivalent hydration states as the uncomplexed CD and physical mixtures because during their production the complexes were lyophilized. Characterization techniques of ATR/FT-IR, ^{13}C CP/MAS NMR, TGA, and DSC were performed on these samples equilibrated at ambient relative humidity.

ATR/FT-IR Spectroscopy. Attenuated total reflectance/Fourier transform-infrared (ATR/FT-IR) spectroscopy was performed on a Spectrum One FT-IR spectrometer (Perkin-Elmer, Waltham, MA). A Miracle single-reflection ATR sampling accessory (PIKE Technologies, Madison, WI) was used with a ZnSe crystal plate and a micrometric, low-pressure clamp. Background scans were recorded between 4000 and 600 cm^{-1} with 164 scans. Samples were scanned 64 times at a resolution of 4.00 cm^{-1}. Absorbance spectra were processed by the ATR correction feature of the Spectrum v. 5.0.1 (Perkin-Elmer) software package with a contact factor of 0 to correct for the variation in effective path length.

^{13}C CP/MAS NMR Spectroscopy. ^{13}C cross polarization/magic angle spinning (CP/MAS) NMR of hydrated sample powders was performed on a Bruker Avance II 300 operating at 75.48 MHz for ^{13}C and equipped with a MAS probehead using 4 mm ZrO_2 rotors. Chemical shifts were calibrated with an external standard of 1-glycine at 176.4 ppm. Samples were spun at 5 kHz at room temperature. Spectra were acquired with a proton 90° pulse length of 5 μs and a ^{13}C−^{1}H contact time of 2 ms. The repetition delay time was 2 s, and the spectral width was 25 kHz. Free induction decays were accumulated with a time domain size of 1K data points. A square-shaped pulse was used during the cross-polarization, and a TPPM decoupling pulse sequence with a phase angle of 15° was used during the acquisition. Each sample spectrum was obtained with 2048 scans and processed with 20 Hz line broadening. ^{13}C NMR of α-tocopherol was performed using a single 90° pulse for 5 μs without CP/MAS because it exists as an oily liquid at room temperature. ^{13}C resonances were assigned in spectra based on reported data of *d*-α-tocopherol (*23*), quercetin (*24*, *25*), and β-CD and γ-CD (*26*).

Thermogravimetric Analysis. A TGA Q500 thermobalance (TA Instruments, New Castle, DE) was used to measure sample weight loss to determine water content and thermal decomposition temperature. The thermobalance was calibrated with an alumel alloy and nickel for temperature settings and with a 100 mg standard for weight accuracy. Sample (5.5 ± 0.5 mg) was placed on a tared aluminum balance pan and transferred to the furnace at room temperature, where the exact sample weight was determined. The temperature program increased the temperature at a rate of 5 °C/min from 30 to 500 °C under an air atmosphere. Universal Analysis 2000 (TA Instruments, New Castle, DE) software was used to determine decomposition temperatures using the maximum of the derivative thermogravimetric curve.

Differential Scanning Calorimetry. *Enthalpies of Dehydration and Vaporization.* A DSC Q1000 instrument (TA Instruments) was used to determine the dehydration and vaporization enthalpies of samples. A standard temperature ramp was used from equilibration at 0 °C followed by an increase to 250 °C at a rate of 5 °C/min. A N_2 sample purge flow was used at 50 mL/min. A hermetic aluminum sample pan and lid with a laser-drilled 75 μm pinhole (TA Instruments) was used to hold samples with weights of 2.3 ± 0.2 mg. TA Universal Analysis 2000 software was used to process data.

T_g of α-Tocopherol. The glass transition temperatures (T_g) of α-tocopherol, α-tocopherol and β-CD physical mixture, and α-tocopherol/β-CD inclusion complex were determined by DSC. A standard temperature ramp was used from equilibration at −90 °C followed by an increase to 0 °C at a rate of 5 °C/min. A N_2 sample purge flow was used at 50 mL/min. A hermetic aluminum sample pan and lid without pinhole was used to hold samples with weights of 5.1 mg. TA Universal Analysis 2000 software was used to perform T_g analysis and calculate the midpoint T_g.

RESULTS AND DISCUSSION

Preparation of Solid CD Inclusion Complexes. *Preliminary Studies.* Various different complexation techniques were explored for the natural antioxidants with the native CDs. The complexation in aqueous solution technique (*27*) used previously was not effective for 16 mM α-CD, β-CD, or γ-CD in the preparation of water-soluble inclusion complexes with excess (2 mg/mL) α-tocopherol or quercetin dihydrate. Preparation of CD complexes by the coevaporation method with methanol as cosolvent (33.3%, v/v) in aqueous solution using 2:1 molar ratio of α-CD, β-CD, or γ-CD to guest antioxidant was not successful as had been previously reported with equimolar host/guest ratios (*10*). The association constant of complexes decreases with a corresponding increase in the hydrophobicity of the medium, which may be due to an increasing percentage of a certain alcohol or an increasing chain length of a certain alcohol (*11*). The decrease in association constants is steeper as the number of carbons in the alcohol chain increases. Addition of methanol to aqueous solutions of guest and β-CD results in a nonlinear decrease of association constants that has a greater slope at lower and less slope at higher methanol concentrations of 20% (v/v) (*28*).

Neutral compounds generally have larger complex stability constants than the corresponding protonated or ionized species (*29*). The pK_a of α-tocopherol was reported as ≥11.0 (*30*), whereas quercetin had pK_{a1} at 7.0 and pK_{a2} at 9.2 (*31*). α-Tocopherol exists predominantly in the un-ionized form at neutral pH; therefore, unbuffered water was used as the complexation medium. The presence of quercetin in its ionized form at neutral pH may hinder complexation; therefore, quercetin was complexed in 0.05 M potassium phosphate buffer at pH 3.0.

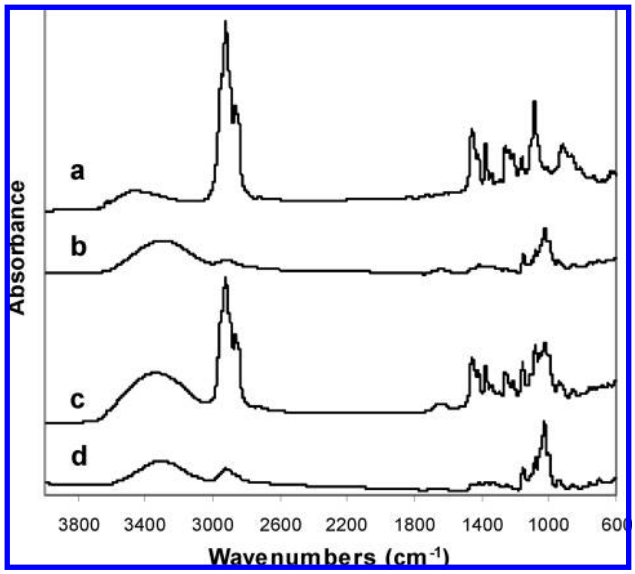

Figure 2. ATR/FT-IR spectra of (**a**) free α-tocopherol, (**b**) β-CD, (**c**) α-tocopherol and β-CD physical mixture, and (**d**) α-tocopherol/β-CD inclusion complex.

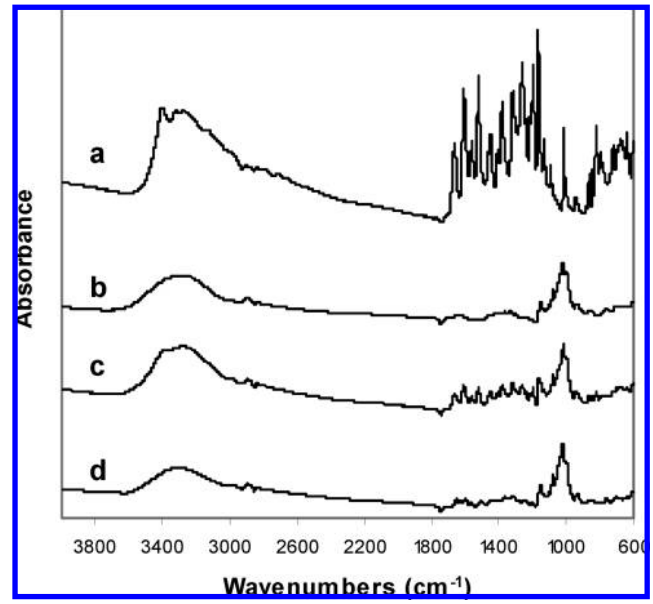

Figure 3. ATR/FT-IR spectra of (**a**) quercetin dihydrate, (**b**) γ-CD, (**c**) quercetin and γ-CD physical mixture, and (**d**) quercetin/γ-CD inclusion complex.

α-*Tocopherol.* An equilibration time study of α-tocopherol behavior in aqueous solutions of β-CD was performed over a 168 h period. At 24 h, increasing the β-CD/α-tocopherol ratio from 1:1, 2:1, 4:1, 8:1, to 16:1 resulted in complex precipitates of similar yield and α-tocopherol content. Generally, a true inclusion complex would be expected to have a molar ratio of CD/guest of >1, or at least one host CD site for each guest molecule. However, visual observation clearly shows that 16 mM α-tocopherol and 16 mM β-CD interacted to form an insoluble precipitate with the CD/guest ratio of the product dropping rapidly by 120 h to a ratio of about 0.8 at 168 h. α-Tocopherol is hypothesized to have interacted with the β-CD molecule to form a higher order complex or other association in a sequential process, but no structural experiments were conducted to prove this hypothesis.

Quercetin. After an equilibration time of 24 h, concentrations of γ-CD were increased in the presence of 3 mM quercetin in 0.05 M potassium phosphate buffer at pH 3 with 10% ethanol. CD/quercetin reactant ratios of 1:1 and 2:1 were observed as free quercetin without CD complexation, which was evidenced by CD/quercetin product ratios of <1. CD/quercetin reactant ratios of 4:1, 8:1, and 16:1 appeared as an inclusion complex as indicated by a precipitate of much lighter yellow intensity and greater density than free quercetin. The CD/quercetin product ratios of these precipitates were additionally >1.

Solution complexation techniques in most cases require a water-miscible cosolvent that efficiently dissolves the guest. The direct addition of 3.0 mM quercetin as a solid to a 38.6 mM (5%, w/w) γ-CD aqueous solution without cosolvent resulted in a CD/guest product ratio of about 0.3 with no indication of association observed. Very hydrophobic guests, such as quercetin, may also require a cosolvent. Methanol and ethanol were both evaluated, but quercetin was observed to have a greater solubility in ethanol. The addition of 10% (v/v) ethanol as a cosolvent to the system above resulted in CD/guest product ratios of about 1.7 and a precipitate of lighter yellow intensity, which indicated CD complex formation. β-CD complexation efficiency in aqueous solution has been reported to increase with increasing concentrations of ethanol up to a maximum, beyond which the efficiency decreases (*32*). The presence of ethanol changes the solvophobic characteristics of the medium, which may affect the affinity of a nonpolar guest in binding CD (*33*). Ethanol is a commonly applied cosolvent in the preparation of CD complexes, and as a result inclusion products may contain about 0.01−0.5% ethanol, which cannot be removed without potentially disrupting the formed CD complexes (*34*). Ethanol may play a space-regulating role in CD complexes by facilitating the formation of a stronger complex by filling the void inside the CD cavity (*35*, *36*).

Coprecipitation Methods. A large molar excess of host CD is required to initiate the inclusion complexation reaction of some hydrophobic guests, such as quercetin. An excess of one component is necessary to drive the equilibrium because the concentration of reactants is low and the association constant of CD inclusion complexes is also low (*37*−*39*). The molar ratios of CD/antioxidant were optimized at 8:1 to improve the yield of complexation. **Table 1** lists the ratios of reactants used in complex preparation and the antioxidant content of complexes in terms of weight percent and CD/antioxidant molar ratio. The stoichiometries of these complexes were unable to be determined. The calculated CD/antioxidant complex molar ratios were not integers, so it may be assumed that a 1:1 complex was present as a mixture with either excess, uncomplexed CD or a higher order 2:1 complex. The product yields of α-tocopherol/β-CD and quercetin/γ-CD complexes from their individual reactants were calculated as 24 and 21% (w/w), respectively. Nearly complete reaction of each natural antioxidant is observed, and the majority of yield losses are due to uncomplexed CD remaining in aqueous solution. Uncomplexed CD could easily be collected by lyophilization or reused directly in additional complexation reactions as part of a continuous production process.

Properties of Solid CD Inclusion Complexes. The guest α-tocopherol exists as an oily liquid at room temperature, and this oily character is retained upon physical mixing with β-CD. The formation of its β-CD inclusion complex results in a solid, dry powder. The more solid character of α-tocopherol within the β-CD cavity was later confirmed by ^{13}C CP/MAS NMR spectroscopy.

Solid CD inclusion complexes of natural antioxidants assume more of the physical appearance of their host CD compared to

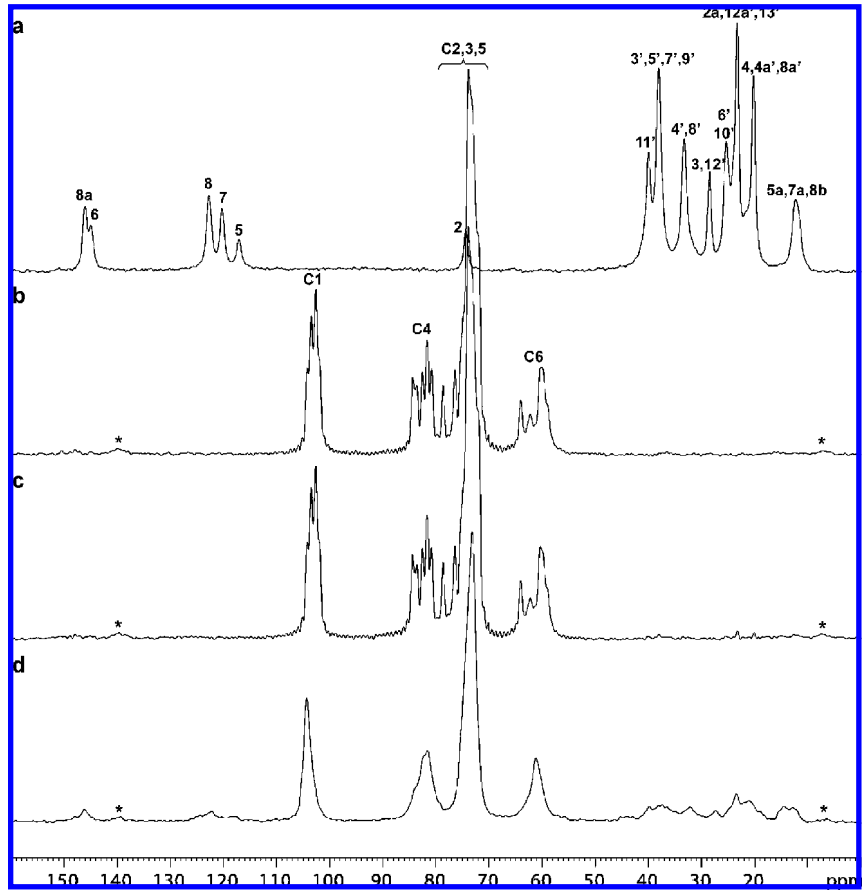

Figure 4. ^{13}C NMR spectrum of (**a**) α-tocopherol and ^{13}C CP/MAS NMR spectra of (**b**) *β*-CD, (**c**) α-tocopherol and *β*-CD physical mixture, and (**d**) α-tocopherol/*β*-CD inclusion complex. Asterisks indicate spinning sidebands.

their physical mixtures. In **Table 2**, the magnitude of the total color difference (ΔE^*_{ab}) from the host CD is shown for each antioxidant, physical mixture, and inclusion complex. The α-tocopherol/*β*-CD complex is a white powder that is almost indistinguishable from its host *β*-CD, giving it a ΔE^*_{ab} of 1.6. The physical mixture of α-tocopherol and *β*-CD retains more of the color of α-tocopherol with a ΔE^*_{ab} of 24.2. The quercetin/*γ*-CD complex is a light yellow powder with a ΔE^*_{ab} of 18.8, whereas its physical mixture retains more of the bright yellow color of quercetin with a ΔE^*_{ab} of 36.4. These observations of decoloration agree with the expectation that when a colored guest molecule is included within the CD cavity, the inclusion complex produced has lower color intensity (*40*−*42*). In this manner, colored guest molecules often provide the benefit of allowing visual observation as the first evidence of inclusion complex formation.

CDs form practically water-insoluble complexes with very hydrophobic guests, such as α-tocopherol and quercetin. The α-tocopherol/*β*-CD complex was unexpectedly observed to dissociate in water as the *β*-CD host appears to resolubilize and α-tocopherol accumulates at the water surface as a dispersion. Quercetin/*γ*-CD complexes were not observed to exhibit this unique behavior of water dissociation. Investigations of these natural antioxidant/CD complexes are complicated by their lack of water solubility and their dissociation in most organic solvents, which restricts inclusion complex characterization to solid-state techniques.

ATR/FT-IR Spectroscopy. The characteristic absorption bands of both natural antioxidants are in the spectral region where CD absorption is limited, which allows the detection of guest interactions within solid CD inclusion complexes. In **Figure 2**, the α-tocopherol spectrum shows intense bands at 2924 and 2867 cm^{-1} for asymmetrical methylene and symmetrical methyl stretching vibrations, respectively (*43*, *44*). These two intense bands are clearly present in the physical mixture of α-tocopherol and *β*-CD; however, these bands are no longer apparent upon complexation within *β*-CD. Characteristic absorption bands of α-tocopherol at 1460 cm^{-1} for the phenyl skeletal and the overlap of asymmetrical methyl bending and methylene scissoring vibration and at 1377 cm^{-1} for symmetrical methyl bending (*43*, *44*) are similarly not present in the *β*-CD complex. It is proposed that a tight fitting of α-tocopherol within the *β*-CD cavity would hinder these molecular vibrations, consequently diminishing the intensities of their absorption bands. The spectrum of the α-tocopherol/*β*-CD complex appears to be very similar to that of its *β*-CD host.

In **Figure 3**, the spectrum of the quercetin/*γ*-CD complex shows that the band intensity within the 1700−1200 cm^{-1} range, where stretching modes are observed, is suppressed compared to its physical mixture. The carbonyl absorption band of quercetin and its physical mixture is observed at 1665 cm^{-1}, in agreement with the literature (*45*, *46*). In the quercetin/*γ*-CD complex, this carbonyl band of quercetin is shifted 10 cm^{-1} to the longer wavelength of 1655 cm^{-1}, which is an indication of further hydrogen bonding (*47*−*49*). Quercetin is involved in intramolecular hydrogen bonding of its carbonyl oxygen at C4 with the two hydroxyl groups at C3 and C5 (*45*). In the inclusion complex, the observed shift to 1655 cm^{-1} suggests that intermolecular hydrogen bonding occurs between the quercetin

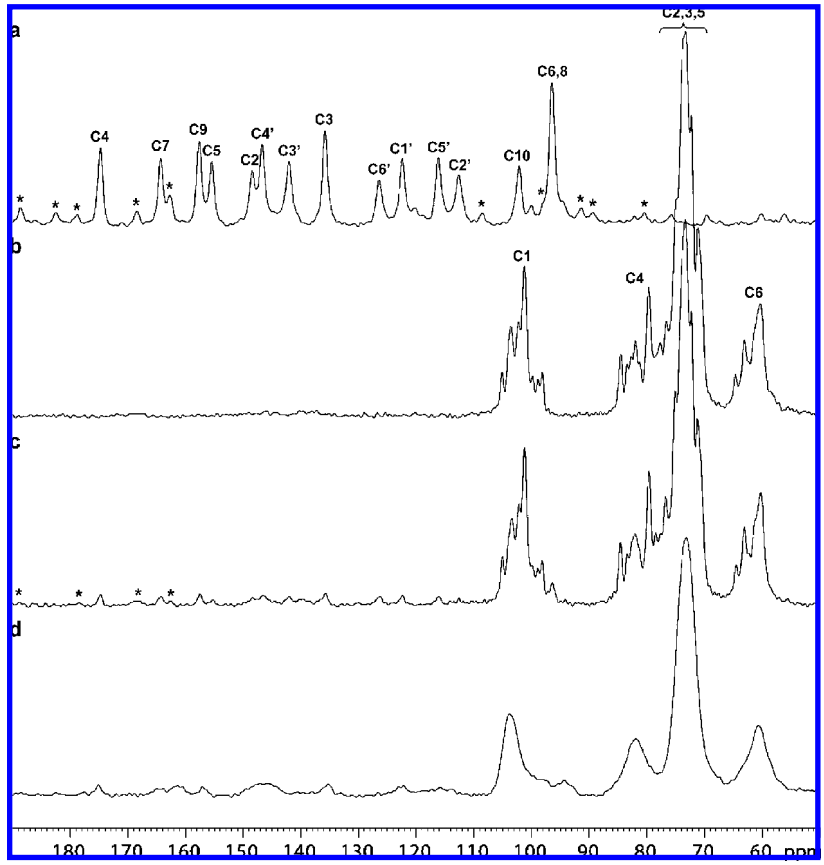

Figure 5. ^{13}C CP/MAS NMR spectra for (**a**) quercetin dihydrate, (**b**) γ-CD, (**c**) quercetin and γ-CD physical mixture, and (**d**) quercetin/γ-CD inclusion complex. Asterisks indicate spinning sidebands.

Table 3. Water Content and Thermal Decomposition Temperature (T_d) in Air Atmosphere

		first stage		second stage	
sample	H_2O wt loss (%)	T_d (°C)	wt loss (%)	T_d (°C)	wt loss (%)
α-tocopherol	0.0	280.2	65.8		
β-CD	13.7	308.8	72.7		
α-tocopherol and β-CD physical mixture	11.8	236.9	7.0	307.6	69.5
α-tocopherol/β-CD complex	7.3	203.1	1.6	297.5	74.8
quercetin dihydrate	9.2	329.2	27.2		
γ-CD	9.3	306.5	78.1		
quercetin and γ-CD physical mixture	9.0	297.4	63.7		
quercetin/γ-CD complex	7.5	163.0	2.2	283.4	47.4

carbonyl and the γ-CD hydroxyls. The spectrum of the quercetin/γ-CD complex also appears to be very similar to that of its γ-CD host.

^{13}C CP/MAS NMR Spectroscopy. The ^{13}C CP/MAS NMR spectrum of free β-CD in **Figure 4s**hows splitting of several of the carbon resonances, which indicates a rigid, nonsymmetric conformation of the CD crystals. The spectrum of the inclusion complex shows all of the major resonances of α-tocopherol and β-CD in **Figure 4**. With the presence of α-tocopherol in the β-CD cavity of the crystal structure, the resonances of the β-CDs show reduced splitting and broadening, which indicates the crystals have adopted more symmetric and possibly dynamic conformations. The simple observation of resonances due to α-tocopherol in the inclusion complex provides additional evidence for complex formation. In **Figure 4**, the spectrum of the physical mixture of β-CD and α-tocopherol shows identical resonances with free β-CD, but a near absence of resonances due to α-tocopherol. This behavior has been attributed to the long T_1 relaxation times of the guest protons in the physical mixture, whereas the interaction between guest and CD in the inclusion complex shortens this proton T_1, and the signals from the guest carbons can be observed (*50*). In addition, the α-tocopherol signals in the physical mixture with β-CD appear with much less signal intensity due to a lower degree of cross-polarization. The dipolar coupling was expected to be reduced by the molecular motion of α-tocopherol existing as a liquid when only physically mixed with β-CD. The cross-polarization rate indicates that α-tocopherol exists in a more rigid environment having more solid characteristics in the inclusion complex compared to the physical mixture.

The ^{13}C CP/MAS NMR spectrum of free γ-CD in **Figure 5** shows a similar behavior of carbon resonance splitting as in β-CD with a rigid, nonsymmetric conformation of the CD crystals. The physical mixture of quercetin dihydrate and γ-CD appears to have an equivalent spectrum as the individual component spectra, which shows the expected lack of interaction in the physical mixture at the molecular level. The spectrum of the inclusion complex shows most of the major resonances of quercetin and γ-CD in **Figure 5**. With the presence of quercetin in the γ-CD cavity of the crystal structure, the resonances of the γ-CDs show reduced splitting and broadening, which indicates the crystals have adopted more symmetric and possibly dynamic conformations. The resonances of the quercetin signals are considerably broadened relative to the spectra of pure quercetin, which suggests that quercetin molecules are in a less ordered, more amorphous environment, which is consistent with true inclusion complex formation. Different crystal lattices of quercetin have been reported between quercetin dihydrate and

Table 4. Enthalpy of the Measured Effect (ΔH_{meas}) and Peak Temperature during Dehydration and Vaporization of Water

sample	H_2O mol[a]	peak T,[b] °C 1	peak T,[b] °C 2	ΔH_{meas}[b] (J/g)	ΔH_{meas}[b] (kJ/mol of H_2O)
α-tocopherol	0.0			0.0 ± 0.0	0.0 ± 0.0
β-CD	10.0	127.8 ± 1.1		381.1 ± 15.1	50.1 ± 2.0
α-tocopherol and β-CD physical mixture	10.6	120.4 ± 1.4		351.8 ± 12.3	44.0 ± 1.5
α-tocopherol/β-CD complex	6.1	118.6 ± 2.4	147.5 ± 2.3	291.3 ± 7.0	59.5 ± 1.4
quercetin dihydrate	1.7	120.3 ± 1.4		278.8 ± 7.9	54.6 ± 1.3
γ-CD	7.4	101.9 ± 3.1		245.7 ± 4.6	47.5 ± 0.9
quercetin and γ-CD physical mixture		111.8 ± 1.6	122.0 ± 0.1	300.7 ± 15.1	
quercetin/γ-CD complex		112.1 ± 2.1		274.1 ± 8.5	

[a] Number of moles of H_2O was calculated from measured weight loss by TGA. [b] Values are reported as mean ± standard error ($n = 3$).

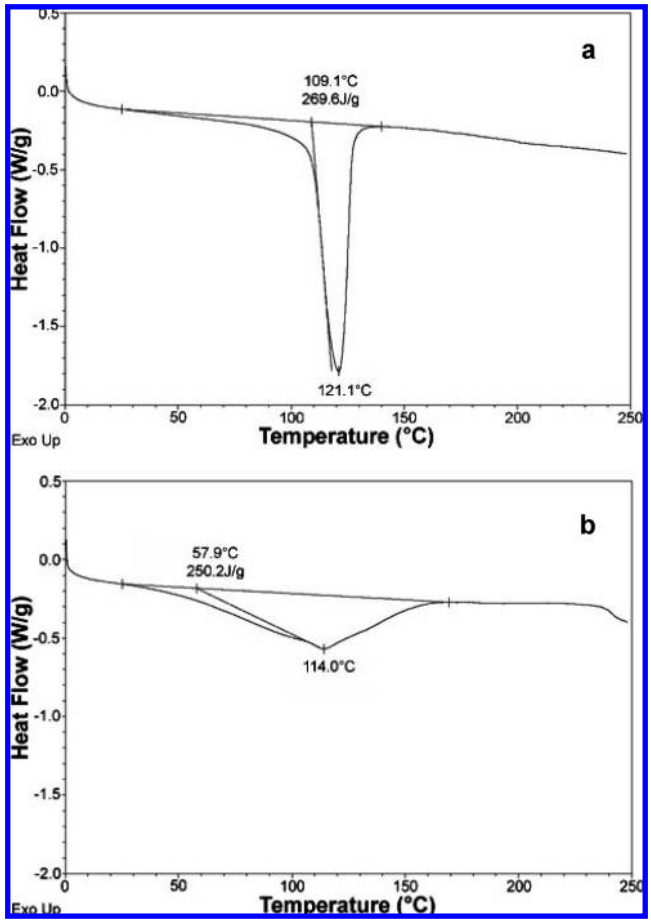

Figure 6. DSC curves of quercetin in its (**a**) free form and (**b**) γ-CD complex showing the enthalpy of the measured effect (ΔH_{meas}) during dehydration and vaporization of water under N_2 at a temperature rate of 5 °C/min.

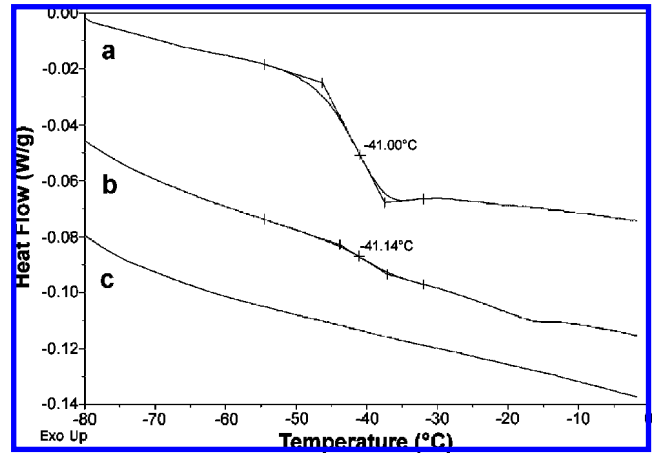

Figure 7. DSC curves showing the glass transition of α-tocopherol in its (**a**) free α-tocopherol form, (**b**) α-tocopherol and β-CD physical mixture, and (**c**) α-tocopherol in β-CD inclusion complex under N_2 at a temperature rate of 5 °C/min.

unhydrated quercetin, which exhibit themselves in ^{13}C CP/MAS NMR spectra as a broadening of resonances with the release of water from the lattice (*25*). The observed broadening of the quercetin ^{13}C resonances in the inclusion complex may be due to the loss of bound crystallization water from the lattice or a change in the conformation of quercetin to a less ordered state.

Thermogravimetric (TG) Analysis. Thermal analysis has mainly been applied to demonstrate the different behavior of an inclusion compound relative to its physical mixture of component compounds. **Table 3** confirms that the natural antioxidants α-tocopherol and quercetin have relatively high thermal decomposition temperatures of 280 and 329 °C, respectively. It is important to note that TG data provide only weight loss information, and oxidative reactions can occur in a sample without observed weight loss (*51*). The loss of hydration water of each sample was calculated from room temperature to 125 °C. The respective hydration states of β-CD·10.0H_2O and γ-CD·7.4H_2O at ambient humidity are in agreement with the reported water vapor sorption isotherms for β- and γ-CD at a relative humidity of approximately 30% (*52*).

Because most CD complexes exist as three-component systems of host/guest/water, it would be expected that upon inclusion of a guest molecule, there would be some degree of displacement of water molecules from the host CD dependent upon the structural fit inside the CD cavity. The 7.3% water content of α-tocopherol/β-CD complex was less than its α-tocopherol and β-CD physical mixture with 11.8%. Quercetin/γ-CD complexes had a water content of 7.5%, which was less than its quercetin and γ-CD physical mixture with 9.0%. These TG data are consistent with the inclusion of each guest antioxidant displacing a portion of the original crystalline water molecules present in the CD cavity. The thermograms of both physical mixtures and inclusion complexes were qualitatively different; however, their thermal decomposition temperature did not vary to a great extent. The inclusion complexes of each natural antioxidant were not observed to have substantially higher thermal decomposition temperatures as is often observed during CD complexation of volatile guests.

Differential Scanning Calorimetry (DSC). *Enthalpies of Dehydration and Vaporization.* Large increases in resolution of endothermic peaks were obtained by using the pinhole DSC lid compared to an open container for the measurement of the vaporization of water (*53, 54*). The β- and γ-CD hydrates show

very broad endothermic peaks from about 25 to 160 °C, representing loss of water. Two overlapping peaks were observed in the α-tocopherol/β-CD complex and the physical mixture of quercetin and γ-CD; however, the entire peak area was used to calculate the enthalpy of measured effect (ΔH_{meas}) in each case. CD dehydration has an endothermic enthalpic effect, which should be considered in the study of CD inclusion processes in the solid state. The dehydration step involves breaking the bonds between the CD and water, which is then followed by the vaporization of the freed water molecules.

$$\beta\text{CD}\cdot n\text{H}_2\text{O(s)} \xrightarrow{\Delta H_{meas}} \beta\text{CD(s)} + n\text{H}_2\text{O(g)}$$

$$\Delta H_{meas} = 50.1 \text{ kJ/mol}$$

This reaction can be considered as a two-step process:

$$\text{dehydration} \quad \beta\text{CD}\cdot n\text{H}_2\text{O(s)} \rightarrow \beta\text{CD(s)} + n\text{H}_2\text{O(l)}$$

$$\Delta \text{H}_{dehyd} = 9.4 \text{kJ/mol}$$

$$\text{vaporization of water} \quad n\text{H}_2\text{O(l)} \rightarrow n\text{H}_2\text{O(g)}$$

$$\Delta H_{vap} = 40.7 \text{kJ/mol}$$

In **Table 4**, the ΔH_{meas} values are presented with physical mixtures and inclusion complexes adjusted on a weight basis for 1 g of CD and by amount for 1 mol of water due to their respective antioxidant contents. The value for the enthalpy of vaporization (ΔH_{vap}) of water at 100 °C of 40.66 kJ/mol was acquired from handbook data (*55*). The enthalpy of dehydration (ΔH_{dehyd}) of β-CD obtained from the two-step reaction scheme is 9.4 kJ/mol H_2O. This result is in agreement with the ΔH_{dehyd} of 9.6 kJ/mol H_2O reported by Bilal et al. (*56*) and the ΔH value of 10.5 kJ/mol H_2O obtained with dissolution enthalpy measurements at 25 °C. An energy input on the order of 10 kJ is required to remove 1 mol of water from 1 mol of β-CD.

If the same reaction scheme is applied to β-CD in its physical mixture and inclusion complex with α-tocopherol, the ΔH_{dehyd} values obtained are 3.3 and 18.8 kJ/mol H_2O, respectively. The ΔH_{dehyd} of β-CD in the physical mixture was expected to be equivalent to that of β-CD because a host−guest interaction is not present. However, the ΔH_{dehyd} of 18.8 kJ/mol H_2O in the α-tocopherol/β-CD inclusion complex appears to have a sizable difference from the ΔH_{dehyd} of the free β-CD cavity. This larger ΔH_{dehyd} may be the result of some of the water molecules included within the CD cavity forming hydrogen bonds with the guest molecule (*57*).

The ΔH_{dehyd} of γ-CD obtained is 6.8 kJ/mol H_2O, which is less than the ΔH_{dehyd} of β-CD due to γ-CD having weaker intramolecular hydrogen bonding to hold water molecules. In the CD cavity, the C2 hydroxy group of one glucose unit can form a hydrogen bond with the C3 hydroxy group of the adjacent glucose unit (*58*). β-CD has a rather rigid structure due to its forming a complete secondary belt of these hydrogen bonds, whereas γ-CD has a noncoplanar, more flexible structure. The strength of hydrogen bonding increases as the CD ring size becomes smaller. This is also evidenced in the DSC curves with β-CD having a considerably higher endothermic peak temperature of 127.8 ± 1.1 °C compared to γ-CD with 101.9 ± 3.1 °C, which was near the boiling point of water.

The guest quercetin likely exists as a mixture of dihydrate and monohydrate forms at ambient humidity with a calculated 1.7 mol of H_2O per mole of quercetin. The DSC profile of quercetin dihydrate in **Figure 6** shows a strong endothermic peak at 120.3 ± 1.4 °C for the release of water from the crystal lattice. This temperature is much higher than the boiling point of water, which indicates that the water molecules are strongly held by quercetin through hydrogen bonding (*25*). The ΔH_{dehyd} of quercetin is 13.9 kJ/mol H_2O. Because quercetin holds bound water in its structure, it was not possible to differentiate the enthalpies of dehydration and vaporization of quercetin from that of its γ-CD host as shown in **Figure 6**.

T_g of α-Tocopherol. α-Tocopherol, in the form of a vitamin E preparation, has been observed to exhibit a glass transition (T_g) at approximately −63 °C, which was associated with the change from a glassy state to a supercooled liquid (*59*). In **Figure 7**, DSC curves of α-tocopherol and its β-CD physical mixture both show an endothermic transition characteristic of a T_g at −41 °C. This T_g of α-tocopherol was not observed in its complexed form with β-CD. The absence of thermal events of a guest molecule in a CD complex is generally taken as evidence of true inclusion complexation.

CD complexes of the natural antioxidants α-tocopherol and quercetin were formed by the coprecipitation technique, which was optimized for complexation yield. ATR/FT-IR, ^{13}C CP/MAS NMR, TGA, and DSC are powerful and complementary tools for providing evidence of true CD inclusion complexation in the solid state, in addition to observed changes in physical appearance. Natural antioxidant/CD inclusion complexes did not show increased thermal stability, but may provide increases in oxidative stability. CD complexes of α-tocopherol and quercetin may serve as novel additives in controlled-release active packaging to extend the oxidative stability of foods.

ABBREVIATIONS USED

CD, cyclodextrin; β-CD, β-cyclodextrin; γ-CD, γ-cyclodextrin; ATR/FT-IR, attenuated total reflectance/Fourier transform-infrared; CP/MAS, cross-polarization/magic angle spinning; T_g, glass transition temperature; TG, thermogravimetric; ΔH_{dehyd}, enthalpy of dehydration; ΔH_{meas}, enthalpy of measured effect; ΔH_{vap}, enthalpy of vaporization; DSC, differential scanning calorimetry.

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